



Analytical Methods

Development of a sandwich ELISA-type system for the detection and quantification of hazelnut in model chocolates

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ARTICLE INFO

Article history:

Received 11 December 2013

Received in revised form 26 September 2014

Accepted 4 October 2014

Available online 13 October 2014

Keywords:

Immunoassay

Hazelnut allergens

Corylus avellana

Food allergy

Protein-based method

ABSTRACT

Hazelnut is one of the most appreciated nuts being virtually found in a wide range of processed foods. The simple presence of trace amounts of hazelnut in foods can represent a potential risk for eliciting allergic reactions in sensitised individuals. The correct labelling of processed foods is mandatory to avoid adverse reactions. Therefore, adequate methodology evaluating the presence of offending foods is of great importance. Thus, the aim of this study was to develop a highly specific and sensitive sandwich enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of hazelnut in complex food matrices. Using in-house produced antibodies, an ELISA system was developed capable to detect hazelnut down to 1 mg kg^{-1} and quantify this nut down to 50 mg kg^{-1} in chocolates spiked with known amounts of hazelnut. These results highlight and reinforce the value of ELISA as rapid and reliable tool for the detection of allergens in foods.

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1. Introduction

Hazelnut (*Corylus avellana* L.) is one of the most appreciated edible nuts. This seed is consumed either raw or roasted in snacks and it is used as an ingredient in a wide variety of processed foods (cakes, creams, chocolates and confectionary products) (Alasalvar & Shahidi, 2008). As a consequence, hazelnuts along with other tree nuts play an important role in economy since they are a significant part of the human food supply (Costa, Mafra, Carrapatoso, & Oliveira, 2012a). In 2012, among the worldwide production of tree nuts, hazelnuts represented the sixth most relevant culture and occupied the fourth place, in terms of global trade, just behind pistachios, almonds and cashew nuts (FAOSTAT., 2014). Therefore, hazelnuts and other nuts are consumed all over the world by the majority of the individuals in a large variety of forms, which are more or less related to population habits and/or their geographical availability (Costa et al., 2012a). Although hazelnuts are considered as a safe food, a small percentage of global population can develop adverse immunological reactions after its consumption.

Presently, hazelnut allergy is estimated to have an overall incidence of 7.2%, being this value determined according to a recent multicountry study involving research centres from USA, Australia

and eleven countries from Europe (Burney et al., 2010). Allergy induced by this nut often triggers mild to severe abnormal immunological responses (anaphylactic shocks) that are frequently related to birch pollinosis (Roux, Teuber, & Sathe, 2003). Considering the severity and the frequency of cases involving hazelnut allergy, in addition with the low levels (1 mg of allergen) responsible for eliciting observable symptoms upon ingestion (Eller, Hansen, & Bindslev-Jensen, 2012), the sensitised/allergic individuals are obliged to completely avoid products susceptible of containing hazelnut as ingredient.

Thus, in order to safeguard those individuals, the EU created specific legislation establishing the mandatory labelling of all products containing tree nuts and other ingredients that were included in a list comprising 14 groups of foods considered to be potentially allergenic (Directive 2007/68/EC). To verify the compliance with mandatory labelling, the development of new and more sensitive methodologies for food allergen monitoring is of utmost importance. However, until now no official method was recommended and reference materials are still lacking for the analysis of allergens in foods.

For the detection and quantification of hazelnut in foods, several techniques have been developed, either directly targeting the allergen or other marker proteins (Ansari, Stoppacher, & Baumgartner, 2012; Cucu et al., 2010; Garber & Perry, 2010; Platteau, De Loose, De Meulenaer, & Taverniers, 2011a; Trashin, Cucu, Devreese, Adriaens, & De Meulenaer, 2011) or indirectly via

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DNA analysis (Arlorio, Cereti, Coisson, Travaglia, & Martelli, 2007; Costa, Mafra, Kuchta, & Oliveira, 2012b; D'Andrea et al., 2011; Platteau, De Loose, De Meulenaer, & Taverniers, 2011b; Platteau et al., 2011a). In the field of protein-based methods, the enzyme-linked immunosorbent assays (ELISA) are among the most used approaches for allergen detection (Schubert-Ullrich et al., 2009). They are analytical platforms that use antibodies (immunoglobulins) as detecting agents, which specifically bind to the antigen. These tests are highly sensitive, specific and versatile, being able to detect proteins/allergens and other tri-dimensional structures from complex mixtures (Diaz-Amigo, 2010). Immunoassays are more cost-effective than alternative protein mass spectrometry techniques, being adequate for qualitative and quantitative food allergen analysis. However, the development of a reliable ELISA system involves several parameters, namely the definition of the target protein(s), the selection of antibodies (monoclonal vs. polyclonal), the choice of animal species for raising the antibodies, the type of ELISA format (competitive vs. non-competitive assays) and the type of labels to be used (Diaz-Amigo, 2010). From all the available immunoassays, the non-competitive sandwich ELISA format is one of the most commonly used. In this format, antibodies are fixed onto a microtitre plate, which specifically bind to the target proteins (allergens). After the recognition of the target molecules/allergens by the fixed antibody, a second antibody is used to attach to this complex. For signal acquisition, a third antibody (usually labelled with an enzyme, a fluorophore or biotin) is normally used. Although ELISA commercial kits have been developed targeting either hazelnut allergens or species-specific protein markers, most of them still reveal some issues of cross-reactivity with the other species such as sunflower or other tree nuts (walnut and pecan nut).

This work intended to evaluate the adequacy of a sandwich ELISA using in-house produced anti-hazelnut polyclonal antibody from rabbit and anti-hazelnut monoclonal antibody from mouse to trace and quantify hazelnut from model chocolates. These antibodies were characterised regarding possible cross-reactivity and their specificity was approved (Data not shown). Because of the complexity of the chocolate matrix, the detection and quantification of allergens using ELISA systems is usually considered as a hard task. In this sense, model chocolates were the elected matrices for the development of the proposed ELISA system aiming at detecting and quantifying traces of hazelnut in chocolates.

2. Materials and methods

2.1. Reagents and solutions

For ELISA and immunoblotting analysis the reagents were of analytical grade. The water used for the preparation of all 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, 2-(N-Morpholino)ethanesulfonic acid (MES, PUFFERAN[®] 99%), sodium dodecyl sulphate (SDS, $\geq 99\%$), 1,4-dithiothreitol (DTT, $\geq 99\%$), acetonitrile (HPLC gradient grade), Ficoll-400 and milk powder were purchased from Carl Roth GmbH (Karlsruhe, Germany). Bicine, 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, $\geq 98\%$), gelatine from cold water fish skin, albumin bovine serum (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 concentration determination was purchased from Thermo Scientific (Rockford, IL, USA). NUPAGE[®] Novex 4–12% Bis-Tris Gels (1.0-mm thick, 15 well and 1.5-mm thick, 10 well), NUPAGE[®] LDS sample buffer, Magicmark[™] XP western standard 20–220 kDa, SeeBlue[®] Plus2 prestained standard 4–250 kDa and SimplyBlue[™] SafeStain were obtained from Invitrogen Corporation, (Carlsbad, CA, USA). α -Casein and β -casein standards were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation of buffers and solutions

For ELISA system, several solutions were previously prepared. The phosphate-buffered saline (PBS, pH 7.5) 0.2 mol L⁻¹ was made in 1000 mL distilled water using 32.22 g of Na₂HPO₄, 2.62 g of NaH₂PO₄ and 21.18 g of NaCl. The PBST1 solution used for washing was done by adding 0.1% of Tween 20 in PBS 0.01 mol L⁻¹. The coating solution (pH 9.6) and the assay buffer (pH 7.5) were prepared by adding 1.22 g of Na₂CO₃, 3.25 g of NaHCO₃ and 0.1 g of NaN₃ in 1000 mL of distilled water and 100 mL of PBS 0.2 mol L⁻¹, 400 μ L of Tween 20 in 400 mL of distilled water, respectively. The substrate buffer (pH 4.0) was prepared in 1000 mL of distilled water by adding 42 g of citric acid and 100 mg of potassium-sorbate. The substrate solution used in each ELISA was prepared immediately before use, by adding 12.5 mL of substrate buffer (pH 4.0), 2.5 μ L of H₂O₂ 30% and 100 μ L of TMB stock solution (1.6 mmol L⁻¹ of TMB, 0.070 mol L⁻¹ of DMSO in 25 mL of methanol).

To prepare the NUPAGE[®] MES SDS running buffer (20 $\times$) for SDS–PAGE electrophoresis performance, 60.6 g of Trizma[®] base, 97.6 g of MES, 10 g of SDS and 3.0 g of EDTA in 500 mL were used. Prior to gel running, the stock solution of NUPAGE[®] MES SDS was diluted 20-fold with distilled water. For western blot, the Tris-Bicine transfer buffer was made just before performing electrophoresis by adding 4.08 g of bicine, 5.24 g of Bis-Tris, 0.3 g of EDTA and 100 mL of methanol to 900 mL of distilled water. The PBST2 solution used for washing steps during immunoblotting was prepared by adding 0.1% of Tween 20 in PBS 0.2 mol L⁻¹. The substrate solution for it was made with 24 mg of TMB, 80 mg of DONS, 10 mL of ethanol and 30 mL of citrate buffer (0.15 mol L⁻¹, pH 5.0). Immediately before incubation with substrate solution, 5 μ L of H₂O₂ was added per each 10 mL of substrate buffer.

2.3. Model chocolate preparation

Chocolate with 41% of cocoa and hazelnuts used for model chocolates were purchased at local markets in Portugal. Hazelnuts kernels were grounded to a fine powder in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany). Model chocolates containing 50%, 10%, 8%, 5%, 4%, 2.5%, 1%, 0.5%, 0.25%, 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 subsequent binary mixtures were prepared by serial 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.

Other chocolates and hazelnut samples used in this study were purchased at local markets in Austria. A standard reference material of baking chocolate (SRM 2384) acquired from the National Institute of Standards and Technology (Gaithersburg, MD, USA) was also used herein.

2.4. Protein extraction

Grounded hazelnut, model hazelnut chocolates and chocolates samples were extracted with two different buffers and in the presence/absence of milk powder (MP) or gelatine from cold water fish (GCF). As these reagents are frequently used to increase the extraction of protein from different matrices, they were added to ensure a better assessment of the proteins present in chocolates. For comparison purposes, samples were also extracted without addition of any of these two reagents. Briefly, 1 g of sample was weighted (with or without addition of 1 g of milk powder or gelatine from cold water fish to it) and 10 mL of 0.2 mol L⁻¹ of PBS or 0.1 mol L⁻¹ of NH₄HCO₃ were added as the extraction buffers. All mixtures were incubated at 60 °C for 30 min with occasional mixing. After incubation, they were centrifuged for 15 min (9400×g, 4 °C) and the supernatant transferred to a new 15 mL falcon tube. If needed, supernatant was further filtrated using a fibre glass filter MN 85/90 BFØ 45 mm (Macherey–Nagel GmbH & Co., Düren, Germany) and/or 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 estimated with the commercial BCA protein assay, according to manufacturer's instructions.

2.5. ELISA system

The anti-hazelnut antibodies (polyclonal from rabbit and monoclonal from mouse) were produced in-house. The third antibody, anti-mouse-IgG labelled with horseradish peroxidase (anti-mouse-IgG-HRP) used for ELISA and immunoblotting assays were acquired from Sigma–Aldrich (Steinheim, Germany).

2.5.1. Production of anti-hazelnut polyclonal antibodies in rabbit

The polyclonal anti-hazelnut antibodies in rabbit were produced as described earlier (Rudolf et al., 2009). The immunogen mixture was used with a protein concentration of 1 mg mL⁻¹ of hazelnut extract.

2.5.2. Production of anti-hazelnut monoclonal antibody

The 6–8 weeks old mice (Balb/c) were immunised with 100 µL immunogen mixture (0.1 mg mL⁻¹) and 100 µL TiterMax. Five subsequent boosters were performed at 2-week intervals with 100 µL adjuvant. After 4 days, a blood sample was taken and screened with indirect competitive ELISA. The last booster was done after 1 week with immunogen mixture (1 mg mL⁻¹) without adjuvant; spleen cell isolation followed 4 days afterwards. Spleen lymphocytes were fused with SP2/0-AG14 mouse myeloma cells (DSMZ, German Collection of Microorganisms and Cell Cultures) using a standard procedure. Further selection and confirmation of monoclonal antibodies were also performed by indirect competitive ELISA. The cells were centrifuged at 400×g for 5 min. The supernatant was filtered through 0.45 µm sterile filter (stericcup-HA filter system, Millipore, MA, USA).

The purification of monoclonal antibodies was performed as described by Rudolf et al. (2009) with the affinity chromatographic pre-packed HiTrap Protein G HP columns (Amersham Biosciences, Uppsala, Sweden) and the FPLC system from Pharmacia (Sweden).

2.6. Optimisation of ELISA procedure

ELISA was carried out using a non-competitive assay, namely sandwich-type. Each assay was performed in high-binding plates (Greiner bio-one GmbH, Frickenhausen, Germany) coated overnight at 4 °C with 1 µg mL⁻¹ of anti-hazelnut polyclonal antibody from rabbit in coating buffer. Before using, the ELISA plates were blocked with 300 µL of 1% or 2% of Ficoll-400 or 2% of milk powder in coating buffer for 2 h at 37 °C. Between coating and blocking, the plates were washed once with PBST1 solution. This step was crucial to attain higher homogeneity and reproducibility among wells. The plates were then incubated with the hazelnut standard dilutions and the samples with different concentrations. For checking possible matrix effects, several plates were prepared using hazelnut diluted serially 1:5 in different blank matrices (commercial chocolate and SRM 2384), which were previously prepared with different extraction buffers and with/without addition of milk powder or gelatine from cold water fish. For testing the model mixtures, a calibration curve was used with hazelnut extract serially diluted in blank matrix (SRM 2384 and chocolate extracted with 0.1 mol L⁻¹ of NH₄HCO₃ 10-fold diluted) to get 5 different concentrations ranging from 10,000 µg mL⁻¹ to 0.13 µg mL⁻¹. The model chocolates were diluted to a concentration that allowed the samples to be read against the linear range of the sigmoid calibration curve. For the evaluation of blank matrices (SRM 2384 and chocolate samples), no sample dilution or 1/10 dilution was used. Protein extracts (100 µL) were incubated in the ELISA plate for 1 h at room temperature with continuous agitation. After incubation, the plate was washed twice with PBST1 solution; then 100 µL of anti-hazelnut monoclonal antibody (1.0 µg mL⁻¹) in assay buffer were added to each well and the plate was again incubated for 1 h with continuous agitation. In order to obtain a colour reaction by the end of the assay, a third antibody marked with an enzyme (HRP) was used; for this purpose, after washing the plate once with PBST1, 100 µL of anti-mouse-IgG labelled with HRP (1.0 µg mL⁻¹) in assay buffer were added to each well and incubated for 1 h with continuous agitation. After washing again once with PBST1, the plates were incubated with 100 µL of substrate solution for approximately 2–3 min with continuous agitation under dark conditions, until the formation of a consistent blue colour. The reaction was stopped with 30 µL of 1 mol L⁻¹ of H₂SO₄ which lead to the formation of yellow colour in the wells.

2.6.1. Calculations of the calibration curves

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 Megallan5 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 – IC₅₀ (µg L⁻¹), D the minimum absorbance, X the analyte concentration (µg L⁻¹). The calibration curves were further normalised according to the equation:

$$B_{\text{normalised}} = \frac{B}{B_0} \quad (2)$$

where B is the absorbance of each hazelnut protein dilution and B_0 the absorbance of the highest hazelnut concentration.

2.7. SDS-PAGE, Western-blot and immunoblotting

The performance of the anti-hazelnut monoclonal antibody was also checked with western blot. SDS-PAGE was performed on NUPAGE® 4–12% Bis-Tris gels and MES SDS running buffer, as recommended by the manufacturer's instructions, using 2 µg of protein. The samples were run under both reduced and non-reduced conditions. The electrophoresis under reducing conditions was carried out by adding 50 mmol L⁻¹ of DTT during the denaturing heating step, following manufacturer's instructions. The proteins were either visualised with staining the gels using Coomassie (Simply-Blue™ SafeStain) or blotted into a nitrocellulose membrane (western blot). Two different molecular weight markers, namely SeeBlue® Plus2 Prestained standard (4–250 kDa) and Magicmark™ XP western standard (20–220 kDa) were used for staining and blotting, respectively.

To proceed with immunoblotting, at first, the proteins were transferred to a Protran® 0.45 µm nitrocellulose membrane (Schleicher & Schuell Bioscience, Dassel, Germany) using the XCell II™ Blot Module (Invitrogen Corporation, Carlsbad, CA, USA) and Tris-Bicine transfer buffer. The nitrocellulose membranes were blocked with 2% of BSA in 0.05 mol L⁻¹ of PBS overnight at 4 °C. After carefully washing the membrane 3 × (10 min each) with PBST2 solution, they were incubated with anti-hazelnut monoclonal antibody (0.05 mg mL⁻¹). The same antibody used in ELISA (anti-mouse-IgG labelled with HRP, 1/1000 dilution) was also used here. Both incubations were performed for 1 h at room temperature, washing the membranes for 10 min (×3) with PBST2 solution between incubations. The final steps included the addition of 0.01 mol L⁻¹ of Tris-HCl (pH 6.0) for 1 min followed by incubation the membrane in substrate buffer for 3–6 min at room temperature and in dark until the development of blue colour. The membranes were then washed with distilled water and scanned.

2.8. MS analysis

2.8.1. Protein preparation

After performing SDS-PAGE electrophoresis in reducing and non-reducing conditions, five bands susceptible of containing hazelnut and milk proteins (according to western blot) were cut from the gels and prepared for mass spectrometry (MS) analysis. The gel-bands were placed in 1.5 mL reaction tubes and submitted to proteolysis digestion according to the protocol described for in-gel digestion (In-Gel Digestion Protocol, M., & N. E., 2000) with minor alterations.

2.8.2. Liquid chromatography–tandem mass spectrometry (LC-MS/MS)

Proteins recovered from electrophoresis gels were submitted to MS analysis using the conditions defined by Ansari, Stoppacher, Rudolf, Schuhmacher, and Baumgartner (2011) and Ansari et al., 2012. The chromatographic separation was carried out in a 1200 series HPLC (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⁻¹. Two different eluents were used to create a gradient: eluent A (10% of acetonitrile, 5 mmol L⁻¹ of ammonium acetate) and eluent B (95% of acetonitrile, 5 mmol L⁻¹ of ammonium acetate). HPLC conditions consisted on 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 (ESI) source was used. The optimisation of the

selected reaction monitoring (SRM) parameters for profiling of selected tryptic-digested peptides were performed by directly infusing of peptide standard solutions into the mass spectrometer at a flow rate of 10 µL min⁻¹. Analysis of chromatographic and (tandem) mass spectrometric data was carried out using the Analyst™ software version 1.5.2. The 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, 150 °C for direct infusion and 535 °C for LC-MS/MS; and +4000 V of ion spray voltage. The collision gas (nitrogen) was defined to high. For each SRM transition, a dwell time of 40 ms was set.

3. Results and discussion

3.1. ELISA procedure

The development of ELISA systems for the specific detection of allergenic ingredients in foods is highly dependent on the selection of proper antibodies, which should present high affinity towards the target protein to ensure better sensitivity (Cucu et al., 2012). Although animals can be immunised against almost any molecule, large molecules such as proteins are considered good immunogens (Diaz-Amigo, 2010). In this study, for the specific development of the non-competitive sandwich ELISA for the detection and quantification of hazelnut, two sets of antibodies, poly- and monoclonal, were produced against whole hazelnut proteins using rabbit and mouse, respectively. These antibodies were of high-quality, enabling the development of an ELISA system with adequate sensitivity and specificity to detect and quantify hazelnut in processed foods, namely in binary mixtures of chocolates. Considerable contents of substances like polyphenols, saturated and monounsaturated fatty acids, carbohydrates and aromatic compounds are present in chocolate, being regarded as a very complex and difficult matrix to analyse, independently on the type of assay selected (Costa, Melo, Santos, Oliveira, & Mafra, submitted for publication).

In the first experimental attempts for the development of the ELISA system applied to model chocolates containing 50% down to 0.0001% of hazelnut, some inconsistencies were verified. Spiked chocolates with low amounts of hazelnut (≤50 mg/kg) and without hazelnut, which were used undiluted or with small dilution (5- or 10-fold), presented high values of absorbance (data not shown). This result was not expected since previous evaluation confirmed the absence of hazelnut in the commercial chocolate by DNA-based methods (Costa et al., submitted for publication). These preliminary results suggested that the anti-hazelnut antibodies could be reacting with the other chocolate component. Since the antibodies are raised in animals, their reactivity can be greatly affected by several factors, being quite frequent to cross-react with more than one food component (Cucu et al., 2012).

3.1.1. Matrix effects

To evaluate possible matrix effects of chocolate towards the ELISA system, chocolate without hazelnut (blank chocolate – BC) and a standard reference material of baking chocolate (SRM 2384) were tested. Both chocolate extracts were spiked with hazelnut extracts serially diluted 1:5 in the range of the calibration curve (10,000–0.13 µg mL⁻¹). The chocolate extracts were tested undiluted and with a 1:10 dilution (Fig. 1). For this assessment, the chocolates (BC and SRM 2384) were extracted with two different buffers (0.2 mol L⁻¹ of PBS or 0.1 mol L⁻¹ of NH₄HCO₃) with/without milk powder or gelatine from cold water fish.

For the undiluted chocolate extracts, the effect of interfering compounds was considered high since the normal slope for ELISA system could not be observed in this conditions (Fig. 1a and b). The matrix effect was more evident in the chocolate used for model mixtures (Fig. 1a) rather than with the reference material of

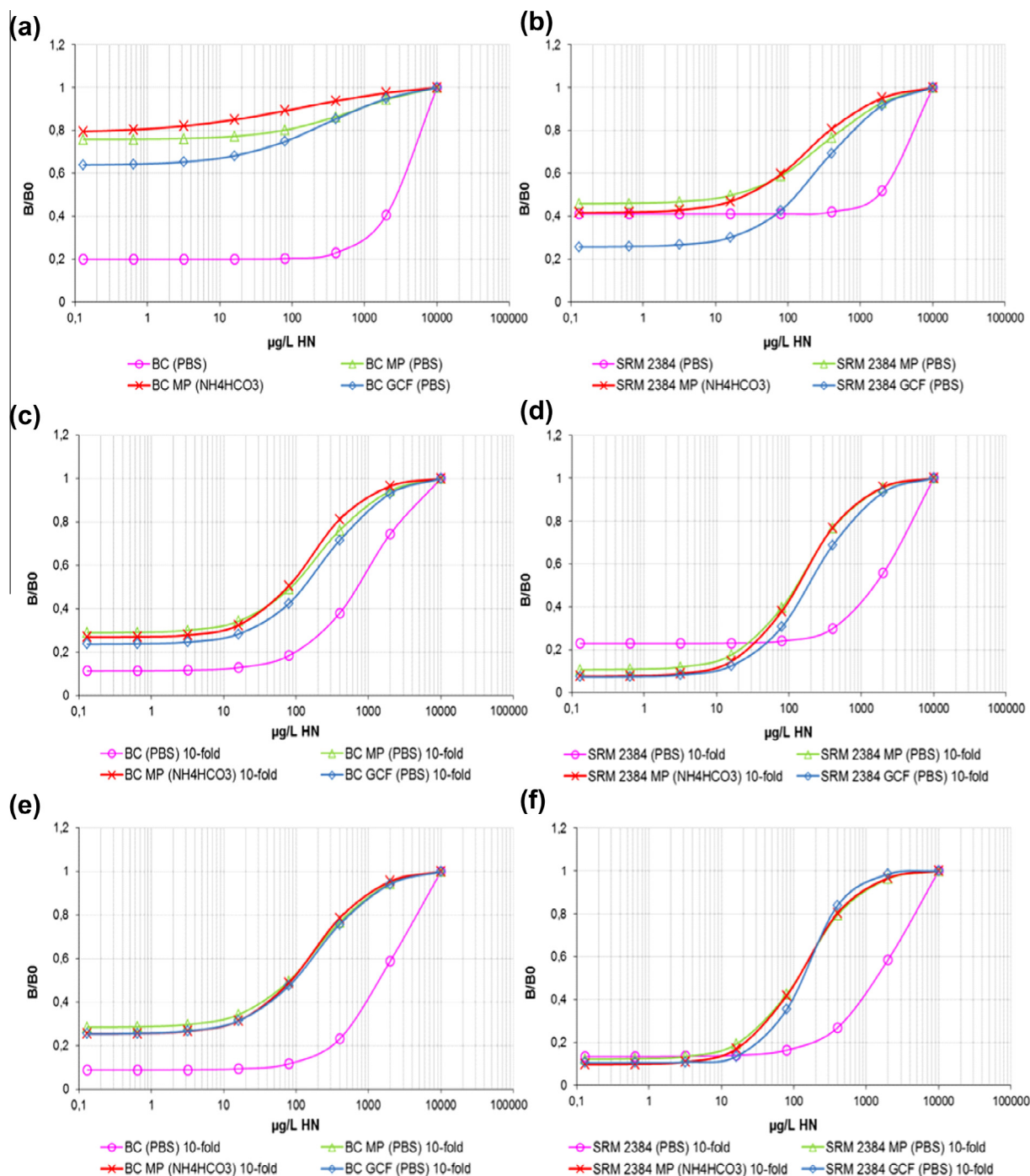


Fig. 1. Normalised curves obtained for chocolate without hazelnut (blank chocolate – BC) (a, c and e) and standard reference material of baking chocolate (SRM 2384) (b, d and f) of undiluted (a and b) and 10-fold diluted (c and d) matrices in ELISA system, using anti-hazelnut polyclonal and monoclonal antibodies. Dynamic range of the calibration curve is 0.13 to 10,000 µg/L. Plate blocking solution used 1% of Ficoll-400 (a–d) or 2% of milk powder (e and f).

chocolate (SRM 2384) (Fig. 1b). For chocolate extracts with a 10-fold dilution factor, the interfering effect of the matrix was less obvious, allowing to obtain better calibration curves for both types of chocolate (Fig. 1c and d). The use of milk powder or gelatine from cold water fish is known to facilitate the extraction of high amounts of proteins. Chocolates extracted using either of those reagents presented better normalised curves in ELISA system, in comparison with chocolate assessed only with PBS. In the last case, the sigmoidal curve in the chosen range of concentrations that is typical in ELISA system could not be observed. Consequently, the extraction of proteins from chocolates was potentiated by the

use of milk powder or gelatine from cold water fish, justifying their application in the developed ELISA system.

3.1.2. Optimisation of the immunoassay conditions

The optimisation of the assay for the detection of hazelnut from chocolates involved the use of additional strategies. Since proteins from chocolates were better assessed by the addition of milk powder, ELISA plates were also blocked with 2% of this reagent (Fig. 1e and f). The shape and aspect of the sigmoidal curves using 2% of milk powder as blocking agent appeared to be better when

compared with the ones performed in plates blocked with 1% or 2% of Ficoll.

Accordingly, several conditions were defined considering the best performance of the ELISA system for detecting hazelnut in chocolate: (i) blocking the plates with 2% of milk powder solution, (ii) washing the plates twice after the incubation of testing samples and (iii) diluting the test samples in blank chocolate, in order to reduce matrix effects.

The influence of the interfering compounds in ELISA was not expected since the monoclonal antibody should only recognise hazelnut proteins. The previously developed anti-hazelnut monoclonal antibody (D9) exhibited some cross-reactivity between proteins from hazelnut and other nuts such as pecan nut, pistachio, cashew, Brazil nut, macadamia and almond. However, the anti-hazelnut monoclonal antibody used in this work belongs to a novel lineage (D10) of antibodies, which is expected to exhibit less cross-reactivity with the other plant species.

3.1.3. Limit of detection (LOD) and limit of quantification (LOQ)

Model chocolates with different contents of hazelnut were serially diluted to establish the LOD. The model mixture containing 50% of hazelnut produced measurable signal until a dilution factor of 100,000. The system enables the detection of 4 ng mL^{-1} of hazelnut protein in assay buffer, considering that for each 1000 mg of sample (50% hazelnut chocolate) approximately 80 mg correspond to hazelnut proteins, which gives a conversion factor of 12.5. The criteria for the measurable signal was defined taking into account the linear range of the calibration curve, although higher dilution values for hazelnut could be performed. The model chocolate spiked with 0.0005% of hazelnut producing the same signal was obtained with a 1:10 dilution, thus confirming the detection of 4 ng mL^{-1} of hazelnut in assay buffer with the proposed ELISA system. This value is in good accordance with the LOD of 4 ng mL^{-1} of hazelnut protein in phosphate buffer reported by Trashin et al. (2011).

3.1.4. Applicability of ELISA system to model chocolates

All model chocolates spiked with known amounts of hazelnut were evaluated with the ELISA system using the adequate dilutions, according to the linear range of the calibration curve. For this purpose, all model chocolate dilutions were performed using blank chocolate as assay buffer. The ELISA results concerning the estimation of the total amount of protein, hazelnut protein and the per-

centage of hazelnut, calculated as the relative amount of hazelnut protein in the model chocolates are presented in Table 1. Comparing the true values with the estimated relative contents of hazelnut, it can be noted that they were similar. The calculation of bias, which corresponds to the difference between the average of all test results and the reference value, enabled to verify that samples until 0.005% of hazelnut in chocolate were within the suitable criteria of acceptance ($\leq 20\%$). Thus, below that proportion, the estimated ELISA values can only be interpreted for qualitative purposes since the relative errors for the proportions $\leq 0.0010\%$ were found to be very high. In the case of the three lowest concentrations of hazelnut, extracts were used undiluted or with a 1:5 dilution leading to a higher interference of the matrix and subsequently to the inaccurate quantification of hazelnut in those model chocolates. According to this, the ELISA system is considered an appropriate and reliable tool for the quantification of hazelnut proteins in chocolate down to 50 mg kg^{-1} , while below that value the positive results can only be faced as qualitative information. The method allowed detecting 1 mg kg^{-1} of hazelnut proteins in chocolates, which is in good accordance with the ELISA system developed by Rejeb, Abbott, Davies, Cl  roux, and Delahaut (2005) to trace hazelnut and other nuts in chocolates. The results presented herein are also in good agreement with those proposed by Holzhauser, Stephan, and Vieths (2002) (1 mg kg^{-1} of hazelnut protein) in processed foods like chocolates, cookies, biscuits and breakfast/bar cereals.

In order to access the accuracy and the repeatability of the method, three independent ELISA tests were performed, using four replicates of each protein extract. Mean recovery and the relative repeatability standard deviation (RSD_r) were calculated for the three assays and are represented on Table 2. According to the criteria defined in the validation of a quantitative protein-based method (CAC/GL 74-2010), recovery should be between 70–120%. The mean recovery calculated for the spiking samples of chocolates ranged from 77.3% to 111.2%, which is within the criteria of acceptance for the repeatability of the method.

3.2. Immunoblotting analysis

An important part of protein analysis is based on the gel electrophoresis since it constitutes a confirmatory tool for the separation and identification of proteins. With separation and gel staining, it is possible to obtain the profiles of protein bands from

Table 1

Quantity of hazelnut (%) determined in the model chocolates spiked with known amounts of hazelnut estimated according to ELISA determinations (mg of hazelnut protein/kg of chocolate) and the total extracted protein (mg kg^{-1}).

Model chocolates spiked with hazelnut (%)	Total protein extracted (mg kg^{-1})	ELISA determinations (mg of hazelnut protein/kg of chocolate) $\pm$ SD ^a	Estimated amount of hazelnut (%) $\pm$ SD	Bias (%)
50	159,746	$81,350 \pm 6882$	51 ± 4	1.8
10	164,853	$15,697 \pm 756$	9.5 ± 0.5	4.8
8.0	135,688	$11,330 \pm 1578$	8.3 ± 1.2	4.4
5.0	150,966	7917 ± 1096	5.2 ± 0.7	4.9
4.0	140,957	6271 ± 1444	4.4 ± 1.0	11.2
2.5	150,572	2911 ± 113	1.9 ± 0.1	22.7
1.0	160,932	1335 ± 124	0.83 ± 0.07	17.1
0.50	136,750	644 ± 294	0.47 ± 0.22	5.7
0.25	147,983	399 ± 15	0.27 ± 0.01	7.8
0.10	171,898	177 ± 11	0.10 ± 0.01	2.9
0.050	144,307	76.6 ± 2.9	0.053 ± 0.002	6.1
0.010	180,925	15.7 ± 0.3	0.0087 ± 0.0002	13.0
0.0050	151,530	7.19 ± 1.85	0.0047 ± 0.0012	5.1
0.0010	148,753	4.80 ± 0.61	0.0032 ± 0.0004	223
0.00050	151,283	3.68 ± 0.53	0.0024 ± 0.0004	387
0.00010	151,293	2.90 ± 0.46	0.0019 ± 0.0003	1820

^a Mean and standard deviation (SD) of the estimated value of hazelnut in chocolates for $n = 12$ replicates performed in three independent assays.

Table 2
Recovery (%) of hazelnut in model chocolates.

Model chocolates spiked with hazelnut (%)	Recovery (%)			Mean recovery (%) ^a	RSD _r (%) ^b
	Assay 1	Assay 2	Assay 3		
50	111.8	97.6	96.2	101.8	8.5
10	100.5	92.5	92.6	95.2	4.8
8.0	120.1	101.5	91.5	104.4	13.9
5.0	121.3	99.6	93.7	104.9	13.8
4.0	140.0	91.0	102.6	111.2	23.0
2.5	73.9	78.8	79.3	77.3	3.9
1.0	80.0	91.7	77.1	82.9	9.3
0.50	126.7	73.1	65.9	88.5	37.5
0.25	103.7	108.0	111.8	107.8	3.8
0.10	110.0	97.3	101.3	102.9	6.3
0.050	110.4	102.4	105.6	106.1	3.8
0.010	85.6	88.9	86.5	87.0	2.0
0.0050	115.1	67.9	101.7	94.9	25.7
0.0010	<LOQ ^a	<LOQ	<LOQ	–	–
0.00050	<LOQ	<LOQ	<LOQ	–	–
0.00010	<LOQ	<LOQ	<LOQ	–	–

^a Mean recovery (%).

^b Relative repeatability standard deviation (RSD_r) of the estimated value of hazelnut in chocolates for $n = 12$ replicates performed in three independent assays.

the extracts (Ansari et al., 2012). After transferring the gel into a nitrocellulose membrane and blocking the free binding sites, the proteins can be detected by the use of specific antibodies (immunoblotting). The application of this technique enables the evaluation of the antibody specificity and the occurrence of

cross-reactivity between non-target proteins and the developed antibody. Thus, to assess eventual cross-reactivity related to matrix compounds, several extracts including model chocolates spiked with hazelnut, four different commercial chocolates without hazelnut, milk powder, and proteins from milk (α -casein and β -casein) were tested in this work.

Samples of model chocolates spiked with hazelnut extracted with PBS buffer and milk powder, along with standards (α -casein and β -casein), were primarily run in gel electrophoresis (Fig. 2). After staining the western blot membranes with Ponceau Red 0.02%, the protein profiles were visualised (Fig. 2a and b). Two large sets of bands with molecular weight of approximately 30–40 kDa could be observed in all samples (both model chocolates and hazelnut) that were extracted with milk powder, which seem to correspond to α -casein and β -casein due to their similarity with protein standards and milk powder (Fig. 2a and b). This profile of bands could not be visualised only on lane 3, hazelnut extracted without milk powder.

The membranes were further incubated with anti-hazelnut monoclonal antibody (Fig. 2c and d). Model chocolates containing more than 2.5% of hazelnut exhibited a band at approximately 55 kDa with different intensity that could be attributed to the presence of Cor a 9, since it is very close to the molecular mass of 59 kDa defined for 11S globulin-like protein (Beyer, Grishina, Bardina, Grishin, & Sampson, 2002; Uniprot, 2013). A second group of bands with ~20 kDa was identified in the same model chocolates. Additionally, a clear band around 150 kDa was found with the same intensity in milk powder and all chocolates and hazelnut

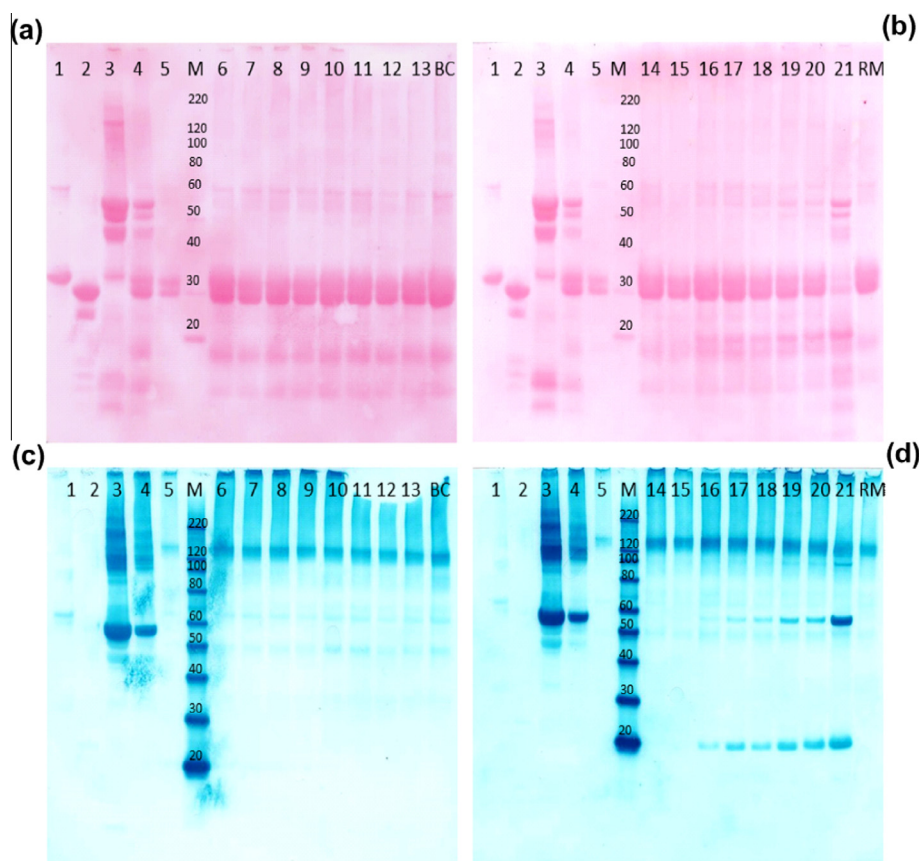


Fig. 2. Western blot membranes stained with Ponceau Red 0.02% for the visualisation of total content of proteins (a and b) and blotted with anti-hazelnut monoclonal antibody (c and d). Chocolate samples were extracted with 0.2 mol L⁻¹ of PBS and 1 g of milk powder. 1, α -casein; 2, β -casein; 3, hazelnut; 4, hazelnut with milk powder; 5, milk powder; 6–21, model chocolates spiked with 0.0001%, 0.0005%, 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.25%, 0.5%, 1%, 2.5%, 4%, 5%, 8%, 10% and 50% of hazelnut, respectively; BC, blank chocolate used for model chocolate preparation; RM, chocolate standard reference material (SRM 2384); M, molecular weight marker (Magicmark™ XP, Invitrogen).

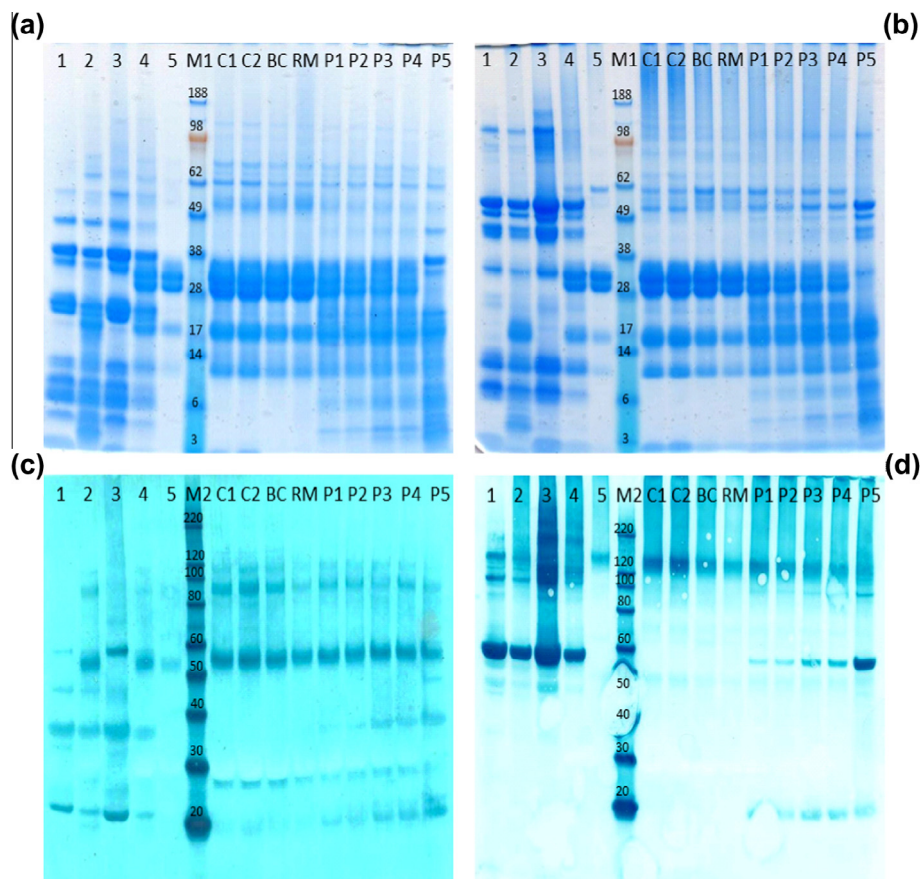


Fig. 3. Coomassie blue stained gels for the visualisation of the total content of proteins (a and b) and membranes blotted with monoclonal anti-hazelnut antibody (c and d). Chocolates were extracted with 0.2 mol L^{-1} of PBS and 1 g of milk powder. (a and c) NUPAGE Novex reduced samples, (b and d) NUPAGE Novex non reduced samples. 1, hazelnut; 2, hazelnut extracted with milk powder; 3, an older extract of hazelnut; 4, older hazelnut extracted with milk powder; 5, milk powder; C1, commercial chocolate 1; C2, commercial chocolate 2; BC, blank chocolate used for model chocolate preparation; RM, chocolate standard reference material (SRM 2384); P1–P5, model chocolates spiked with 4%, 5%, 8%, 10% and 50% of hazelnut; M1, molecular weight marker (SeeBlue Plus2, Invitrogen); M2, molecular weight marker (Magicmark™ XP, Invitrogen).

samples extracted with milk powder. Here was the question, if the specific anti-hazelnut monoclonal antibody bound also to some milk proteins.

Therefore, in order to confirm the results, a new gel electrophoresis was run using extracts in reduced (Fig. 3a) and non-reduced (Fig. 3b) conditions. Western blot membranes of those gels were also incubated with anti-hazelnut monoclonal antibody (Fig. 3c and d). Results evidenced the presence of a band at $\sim 30 \text{ kDa}$ in reduced conditions, which was not visible with non-reducing conditions. This band is only present in extracts containing chocolate. The gels using reduced and non-reduced proteins were run separately and each gel included two extracts from hazelnut of different batches (2007 and 2012) and extracts from two commercial milk chocolates. Both gels were stained with coomassie blue for the visualisation of the total protein content (Fig. 3a and b). Regarding the extracts ran in reduced and non-reduced conditions, when compared with pure hazelnut, the milk powder presented a very distinct profile of proteins (lanes 1, 3 and 5, Fig. 3a and b). As it was expected, both blank chocolates and the binary mixtures contained the same profile of proteins exhibited in the milk powder, but no protein corresponding to hazelnut profile seems to be present in milk chocolates. This evidence appears to confirm the absence of hazelnut proteins in the blank chocolates tested, including the one used for model mixtures preparation. The apparent binding between milk protein and the specific anti-hazelnut monoclonal antibody could be the explanation for the previous

ELISA findings, that indicate the interference of milk in chocolate matrix and was investigated more with MS.

3.3. LC-MS/MS analysis

For the characterisation of the profile of bands recognised by the anti-hazelnut monoclonal antibody, five sets of bands according to proteins visualised by western blot (one at ca. 150 kDa , three between $49\text{--}62 \text{ kDa}$ and one at ca. 20 kDa) were cut from the gels and submitted to proteolysis digestion to find out, if they really include milk proteins. MS analysis was performed on these in gel digested samples (data not shown) targeting seven marker peptides from milk (β -lactoglobulin, α -lactalbumin, α -casein and β -casein) (Ansari et al., 2011) and eight marker peptides for hazelnut (Cor a 8, Cor a 9 and Cor a 11) (Ansari et al., 2012).

The milk peptides either could not be found in these bands, or in very low concentration mainly in the fifth band (20 kDa) and in the samples, which should include milk, which comply with the fact that our target milk proteins are in the range of $14\text{--}25 \text{ kDa}$. However in the first band (150 kDa), which was suspected to be a milk protein detected by anti-hazelnut antibody, we could not find milk peptides. Low concentration of Cor a 9 could be found in this band only in samples of pure hazelnut and hazelnut with milk powder. An unspecific bound between membrane and antibody could also be the reason for the band visualised by antibody in western blot.

The amount of Cor a 9 was high in the third band (ca 55 kDa), which confirm this band as Cor a 9.

4. Conclusion

The development of a successful ELISA system for the detection and quantification of hazelnut in foods is difficult to accomplish. Since these systems are highly dependent on the type of antibodies developed, immunological methods such as ELISA have to be carefully evaluated. Herein, we present a sandwich ELISA system with high specificity and sensitivity to detect and quantify hazelnut in processed and complex food samples as the case of chocolates. This study also highlighted the importance of using adequate standards for the correct quantification of hazelnut in foods. With this system it was possible to establish a LOD of 1 mg kg^{-1} and a LOQ of 50 mg kg^{-1} for the assessment of hazelnut in complex matrices.

Acknowledgements

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 Romer Labs. The authors also thank Marcela Hermann, Institute of Medical Biochemistry, Department of Molecular Genetics, University Vienna, for the immunization of rabbits as well as Thomas Kolbe, University of Veterinary Medicine Vienna for the immunization of mice.

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. PEst-C/EQB/LA0006/2013 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).

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