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Detection of hidden hazelnut protein in food by IgY-based indirect competitive enzyme-immunoassay

E. Drs [a], S. Baumgartner [a,*], M. Bremer [b], A. Kemmers-Voncken [b], N. Smits [b], W. Haasnoot [b], J. Banks [c], P. Reece [c], C. Danks [c], V. Tomkies [c], U. Immer [d], K. Schmitt [d], R. Krska [a]

[a] *IFA-Tulln Center for Analytical Chemistry, Institute for Agrobiotechnology, Konrad Lorenz Strasse 20, A-3430 Tulln, Austria*
[b] *RIKILT, Bornsesteeg 45, 6708 PD Wageningen, The Netherlands*
[c] *Central Science Laboratory, Sand Hutton, YO41 1LZ York, UK*
[d] *r-Biopharm, Landwehrstr. 54, 64293 Darmstadt, Germany*

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Abstract

The development of an indirect competitive enzyme-immunoassay for the detection of hidden hazelnut protein in complex food matrices is described. A sensitive and selective polyclonal antibody was raised by immunisation of laying hens with protein extracts from roasted hazelnuts. In contrast to traditional antibody generation in mammals, the antibody was not isolated from the blood of immunised mammals but from the egg yolk of immunised chickens. A standard calibration curve was optimised using immunoaffinity purified antibody extract and a coating antigen concentration of 10 $\mu g\,ml^{-1}$. One percent skim milk powder was chosen for blocking. The assay has a minimum detection limit of 10 $\mu g\,l^{-1}$, with an IC_{50} of 618 $\mu g\,l^{-1}$ when a 50 mM phosphate buffer at pH 7.5 and 10 mM sodium chloride is used as assay buffer. The cross reactivity testing shows a high specificity for hazelnut proteins and various foods and food additives were found to be non reactive except beans, sunflower seed or poppy seed.
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1. Introduction

Allergic reactions are very common in industrialised countries. The part of food allergies is highly underestimated with a relatively broad range of 1–8% of the population [1,2]. The most common elicitors of allergic reactions in the sector of food allergy are mainly cow's milk, egg, fish, crustacea, nuts, peanut, soybean, and wheat [3,4]. Those foods can elicit severe anaphylactic reactions in sensitised patients. Because their severe reaction and that allergies against peanut and hazelnut occur quite frequent throughout the population, peanut and hazelnut allergies are of particular interest [1,5,6]. At the moment there are no therapies available and the medication is not always sufficient. So the contact with all possibly contaminated food has to be avoided [3]. Quite often the allergy eliciting substances are not identifiable in the food and a not known consumption is the result (hidden allergens). The reasons therefore are a partly incomplete declaration or the contamination during the processing if there are no "allergen-free" production lines. There are nearly no rapid analytical detection methods, so a lot of possible contamination stay unknown for the producer. Additionally there is no, e.g. EU-wide declaration rule for allergens at the moment. As a consequence, the development of accurate and sensitive analytical methods for monitoring of traces of hazelnut proteins in complex food matrices is demanded. Immunoassays offer the best possibilities in this field. They are cheap, easy to perform, an automation is possible and because of the high sensitivity no time consuming steps like extraction, back-extraction or preconcentration are necessary. Many enzyme-linked immunosorbent assays (ELISAs) based on antibodies raised in mammals or monoclonal antibodies were already published [7]. But usually the concentration of antibodies in the blood

* Corresponding author. Tel.: +43 2272 66280413; fax: +43 2272 66280403.
E-mail address: baum31@ifa-tulln.ac.at (S. Baumgartner).

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of the rabbits is low and frequent bleeding of the animals is necessary. Chickens are a possible alternative to the rabbits because of their ability to accumulate a high antibody concentration in their eggs and the injection of the immunogen is better tolerated.

In this work chicken eggs after the third immunisation with Tris-buffered saline (TBS) extractable hazelnut protein were used to obtain antibodies for the development of an indirect competitive hazelnut ELISA. Thereby, immunoabsorption was used as a further clean-up step to obtain specific antibodies from the polyclonal antibody extract [8,9].

2. Materials and methods

2.1. Reagents and instrumentation

Hazelnuts were provided by MasterFoods (Breitenbrunn Austria). Any other samples were bought from a local food store. Derco brown laying hens were supplied from Heindl (Vienna, Austria). Immunisation and animal housing were performed at the Institute for Molecular Genetics, Biocenter of the University of Vienna (Vienna, Austria). The peroxidase-conjugated rabbit anti-chicken immunoglobulin Y (IgY), skim milk powder, perhydrol, dimethylsulfoxide and all salts or solvents were purchased by VWR (Vienna, Austria). Tween 20, and both Freund's complete and incomplete adjuvant as well as polyethyleneglycol (average molecular weight 8000) were obtained from Sigma (Vienna, Austria). Tetramethylbenzidine was obtained from Roche Diagnostics (Vienna). Water was of Milli-Q Plus quality (Millipore, Vienna) and all chemicals were of analytical grade or as specified. The 96-well flat bottom high binding certified microtiter plates (MTP) were from Costar (Szabo Scandic, Vienna). Washing of microtiter plates was done using an eight channel automatic Elisa washer. Both the washer and the ELISA reader (SLT Spectra Reader) controlled by Biolise 2.0 for Windows software were purchased from Tecan SLT Laboratory Instruments (Gröding, Austria). CNBr-activated Sepharose 4 fast flow and HiTrap IgY purification column were operated with a fast protein liquid chromatography system (FPLC) consisting of an LKB Pump P-500, an LCC-501 Plus Controller, a fraction collector Frac-100, all by Amersham Biosciences (Vienna, Austria). The eluent was monitored at 280 nm by an LKB control unit UV-1, data was recorded by the FPLCdirector, also Amersham Biosciences. BCA(Bicinchoninic acid) protein assay kit for the determination of the protein content was purchased from Pierce. Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) was performed in a XCell SureLockTM Mini Cell with 12% NuPAGE Novex Bis-Tris-Gels, Seeblue Plus2 (Pre-Stained Standard) and NuPAGE LDS Sample Buffer. Running buffer (pH 7.3) contained 1 M 2-(*N*-morpholino)ethanesulfonic acid (MES), 1 M Tris Base, 69.3 mM SDS and 20.5 mM EDTA. The gels were stained with Simply Blue SafeStain. All the materials for the SDS-PAGE were obtained from Invitrogen.

2.2. Preparation of immunogen

Hazelnuts were frozen with liquid nitrogen and ground with a commercial hand blender. The nuts were mixed with 2.5 l (10 ml g^{-1}) icy acetone for about 5 min at 4 °C with an analytical mill (Ultra-Turrax T50). Acetone was removed with a glass filter funnel (pore size: 16–40 μm) and the residue washed twice with acetone. After evaporating acetone overnight the nut powder was covered with 1-butanol and crushed for about 5 min in a mortar. Subsequently, butanol was removed and the residue washed four times with acetone. Again, acetone was evaporated overnight before the nut powder was extracted with 10 ml TBS at pH 7.4 [20 mM tris(hydroxymethyl)aminomethane (Tris) buffer containing 1 M NaCl] per gram nut for 4 h at 4 °C. The suspension was centrifuged (10 °C, 14,000 × *g*, 20 min) and the supernatant filtered through a piece of gauze. The amount of protein in the extract was determined using a BCA protein assay kit. Hazelnut immunogen extract was stable at −20 °C for several months.

2.3. Immunisation of chickens

Specific antibodies were raised in laying hens. For the first immunisation 250 μl of the immunogen mixture (hazelnut protein dissolved in TBS, pH 7.4) were emulsified with 250 μl Freund's complete adjuvant (FCA) and injected into the breast muscle of the chicken. Subsequent (booster) injections were performed after 6, 12 and 18 weeks to increase the antibody yield using 250 μl Freund's incomplete adjuvant (FIA) instead of FCA. The applied concentration of the immunogen in TBS, pH 7.4 was 1 mg ml^{-1}. Starting 1 week after the third immunisation eggs were collected and stored at 4 °C until further processing.

2.4. Isolation of antibodies from the egg yolk

The antibody fraction was isolated from the egg yolk in sufficient purity by means of polyethylene glycol (PEG) precipitation based on a modification of Polson's method [10]. Eggs were usually used within 2–3 weeks after their collection, but they sustain longer storage periods at 4 °C. For antibody preparation batches of five subsequently laid eggs are pooled. The yolks were carefully separated from the white and washed with distilled water. Then the vitalline membrane was removed and the yolk content was mixed with an equal volume of IgY preparation buffer (0.1 M phosphate buffer pH 7.5 containing 1 M NaCl) and 3.5% (w/v) PEG. After stirring for 15 min the mixture was shaken for further 15 min to improve the precipitation. Then the precipitate was centrifuged at 15,000 × *g* and 10 °C for 15 min. The nearly clear supernatant was filtered through a piece of gauze at −20 °C until further use.

2.5. Egg yolk antibody clean-up

2.5.1. Affinity column preparation

Typically, a protein affinity resin is prepared by coupling the protein of interest via its carboxy- or amino-terminal residue to agarose beads through chemical conjugation of free amino, sulfhydryl, phenolic or carboxylic acid groups. In the present study we coupled cross reacting protein (walnut, coconut, brazil nut, sunflower seed, poppy seed, bean, wheat, rye, barley, raisins and cocoa) via its amino-terminal residue to CNBr-activated Sepharose 4 fast flow, as recommended by the manufacturer's protocol. Ten milligrams of protein were dissolved in 5 ml of coupling buffer (0.1 M carbonate buffer pH 8.3 containing 0.5 M NaCl) and incubated with 300 mg (corresponding to 1 ml) of pre-activated matrix. The suspension was incubated overnight at 4 °C. After coupling, non-reacted groups of the medium were blocked. Therefore, the medium was transferred to 1 M ethanolamine pH 8.0 and incubated overnight at 4 °C. To prevent coupled medium from microbial growth it was stored in 20% ethanol at 4 °C.

2.5.2. Affinity purification

Pre purification on HiTrap IgY purification column was performed according to the manufacturer's protocol. Five millilitres of egg yolk antibody extract (corresponding to 1/4 of an egg yolk) were loaded onto the column equilibrated with binding buffer (20 mM sodium phosphate buffer pH 7.5 containing 0.5 M potassium sulfate). After elution of unbound material, the eluent was changed to elution buffer (20 mM sodium phosphate buffer pH 7.5), to elute bound IgY and finally the resin was washed with cleaning buffer (20 mM sodium phosphate buffer pH 7.5 containing 30% isopropanol) to remove the non-specific adsorbed material. For immunoaffinity clean-up samples, corresponding to 2 mg of IgY, were loaded onto the column equilibrated at a flow rate of 1 ml min^{-1} with binding buffer (100 mM potassium phosphate buffer pH 7.5). After elution of unbound IgY, retained antibodies were eluted by lowering the pH with 100 mM glycine buffer pH 2.5. The unbound fraction (or break through) was immediately characterised by enzyme-immunoassay, SDS-PAGE and BCA protein assay kit, to evaluate the immunoreactivity, purity and the amount of recovered immunoglobulins. IgY fraction was stable at 4 °C for several weeks or could be stored at −20 °C for a longer period.

2.6. Preparation of hazelnut standards

2.6.1. Hazelnut protein stock

Frozen hazelnuts were ground with a commercial hand blender before 10 ml pre-heated TBS at pH 8.2 (20 mM Tris buffer containing 0.3 M NaCl; 60 °C) per gram nut were added. This suspension was vortexed for a few seconds before the solution was rotated end-over-end for 30 min at 37 °C. Afterwards the samples were centrifuged at room temperature for 10 min at 5000 × *g* and the supernatants are filtered over syringe filters with pore sizes of 5, 1.2 and 0.45 μm. The amount of protein in the hazelnut protein stock was determined by BCA protein assay kit. Hazelnut protein stock extract was stable at −20 °C for several months.

2.6.2. Hazelnut protein standards

First hazelnut protein standard (100,000 μg l^{-1}) was derived from the hazelnut protein stock by dilution with extraction buffer. Further standards were achieved by a factor 5 dilution of the first standard, covering a concentration between 6.4 and 20,000 μg l^{-1}. Diluted hazelnut protein standards were not stable at −20 °C and had to be freshly prepared prior to use.

2.7. Indirect competitive immunoassay

2.7.1. Stock solutions and buffers

Coating buffer (pH 9.6) contained 50 mM sodium carbonate and 1.5 mM NaN_3. Blocking buffer (pH 7.5) was prepared by dissolving 1% (w/v) skim milk powder in 50 mM phosphate buffer containing 90 M NaCl. Assay buffer (pH 7.5) consisted of 50 mM sodium phosphate and 0.1% polyethylene-sorbitan monolaurate (Tween 20). Substrate solution was achieved by mixing 25 ml substrate buffer, 5 μl 30% H_2O_2 and 250 μl TMB stock solution. It is reasonable to perform the substrate preparation just before use in order to avoid an undesirable colour reaction caused by light influence. Substrate buffer (pH 4.0) contained 0.2 M citric acid and 0.01% (w/v) sorbic acid potassium. The pH has to be adjusted with concentrated sodium hydroxide solution. TMB stock solution was achieved by dissolving 375 mg tetramethylbenzidine in 5 ml dimethylsulfoxide and 25 ml methanol. The washing buffer consisted of 50 mM phosphate, 90 mM NaCl and 0.02% Tween 20.

2.7.2. Coating of microtiter plate

The microplate (MTP) was washed three times with Milli-Q water with an automatic plate washer. Then 200 μl of hazelnut protein stock diluted with coating buffer (10000 μg l^{-1} final concentration) were added to each cavity of the plate and left to incubate overnight at 4 °C. Afterwards the coated MTP was washed three times with washing buffer before 300 μl of blocking buffer were added to each cavity and the plate was incubated for 60 min at 37 °C. Then the plate was washed with washing buffer again and stored at −20 °C until further use. The microplates prepared and stored as mentioned were stable up to 3 months and could therefore be laid on a stock.

2.7.3. Assay procedure

One hundred and fifty microlitres standard or sample were added to each cavity and immediately 50 μl of the antibody solution (1:2000 in assay buffer) were added. The sealed plate was shaken at room temperature for 30 min and then washed three times with washing buffer. Two hundred

microlitres of peroxidase labelled rabbit anti-IgY IgG solution (1:2000 in assay buffer) were added to each cavity and the plate was shaken for another 30 min at room temperature. After washing the plates 200 μl of the substrate solution were added to the cavities and the plate was shaken for 15 min. As the substrate solution is light sensitive the microplate must be covered. Finally, the enzyme reaction was stopped by adding 50 μl of 1 M sulfuric acid to each cavity. The absorbance was measured at 450 nm with an ELISA reader. All measurements were performed in triplicates. The competition curves were fitted by using the four parameter logistic equation according to Rodbard [11].

2.8. Cross-reactivity studies

Various nuts and stonefruits (cashew, almond, walnut, pecan, coconut, chestnut, pistachio, Brazil nut), legumes (peanut, soybean, chick pea, green pea, lentil, bean), seeds and cereals (sunflower seed, sesame seed, poppy seed, wheat, rye, corn, rolled oats, barley, rice) as well as other ingredients (raisins, cocoa powder, carob, yeast, skim-milk powder, egg white) were used in this study. The extraction of proteins was conducted using the hazelnut protein stock extraction procedure described above. The extracts were tested by enzyme-immunoassay using a 1:10 dilution in TBS buffer pH 8.2 simulating a proportion of 100% of the food. Extracts leading to an inhibition of more than 10% compared to the inhibition of pure hazelnut protein stock were used as protein source for immunoaffinity column.

2.9. Matrix effect

Ten grams of blank cookie crumb were extracted according to the hazelnut protein stock extraction. The extract was filtered over a folded filter paper and used either directly, or after storage at 4 °C. Hazelnut free cookie matrix was spiked with hazelnut protein stock at levels within the working range of the indirect competitive enzyme-immunoassay. The amount of hazelnut protein was determined by ELISA and the recovery was calculated as indicated:

$$\text{recovery (\%)} = \frac{\text{amount of hazelnut protein determined } (\mu g\, l^{-1})}{\text{amount of hazelnut protein added } (\mu g\, l^{-1})} \times 100$$

3. Results and discussion

A simple extraction with TBS buffer at 4 °C was chosen to isolate protein from defatted hazelnut powder. This protein was used as an immunogen to generate polyclonal antibodies in derco brown laying hens. The hens showed good immunological reaction and compatibility to the antigen. In contrast to traditional antibody generation in mammals, the antibody was not isolated from the blood but from the egg yolk. Therefore, the lipid fraction of the yolk was removed by precipitation with 3.5% (w/v) PEG. Thiophilic interaction chromatography (HiTrap IgY purification column) was applied in order to separate IgY from matrix that do not show any thiophilic properties. HiTrap IgY purification removes 90–95% of matrix protein. A typical chromatogram and a SDS-PAGE of unreduced crude and purified antibody extract is shown in Fig. 1. Around 1.5 g of total IgY antibodies were produced per hen and month. The IgY extracts prior and subsequent to immunoaffinity cleanup procedure were tested for specificity and immunoreactivity in

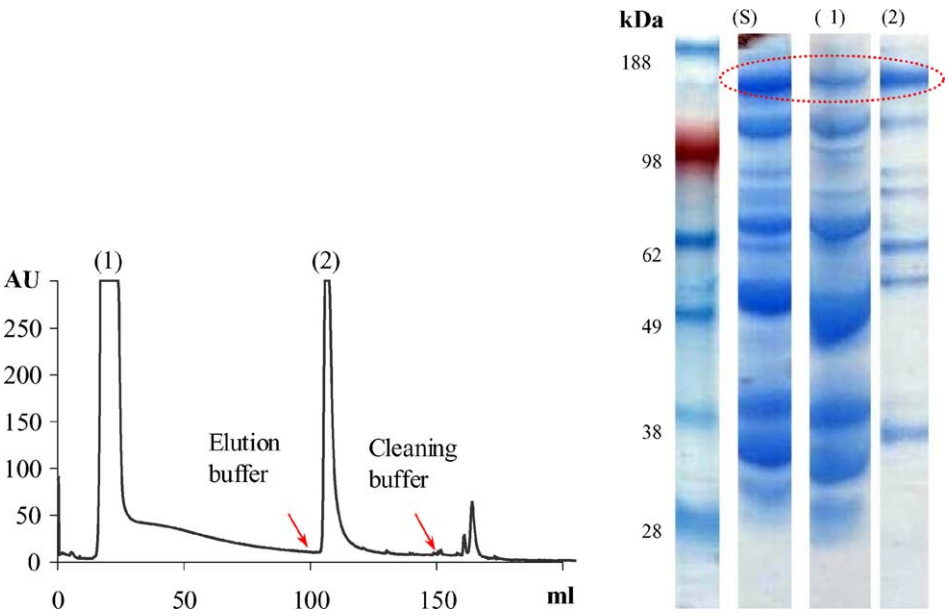

Fig. 1. Chromatogram of IgY purification on a HiTrap IgY purification column and SDS-PAGE of unreduced antibody sample (S) and fractions (1 or 2), using NuPAGE gel 12%, Coomassie staining. Au: absorption units; kDa: kilodalton.

Table 1
Cross reactivity of the immunoaffinity purified anti-hazelnut-antibody compared to the crude anti-hazelnut-antibody extract determined by indirect competitive ELISA

Commercial products	Cross reactivity (%)	
	Purified IgY	Initial IgY
Cashew nut	–	8.42
Almonds	–	–
Walnut	–	19.68
Pecan	–	7.85
Coconut	–	24.87
Chestnut	0.60	2.23
Pistacchio	–	–
Brazil nut	2.10	11.75
Peanut	–	2.82
Soy bean	–	5.89
Chickpea	3.42	4.91
Green pea	3.09	5.66
Lentil	–	–
Bean	8.09	13.61
Sunflower seed	6.26	16.02
Sesame seed	1.72	–
Poppy seed	5.36	14.74
Wheat	2.21	33.04
Rye	–	26.11
Corn	0.30	2.63
Rolled oats	1.34	1.35
Barley	2.32	12.81
Rice	–	7.51
Raisins	–	15.84
Cocoa powder	–	11.22
Carob	–	5.04
Yeast	1.42	2.89
Milk powder	–	1.84
Egg white	–	6.31

–, Not detectable.

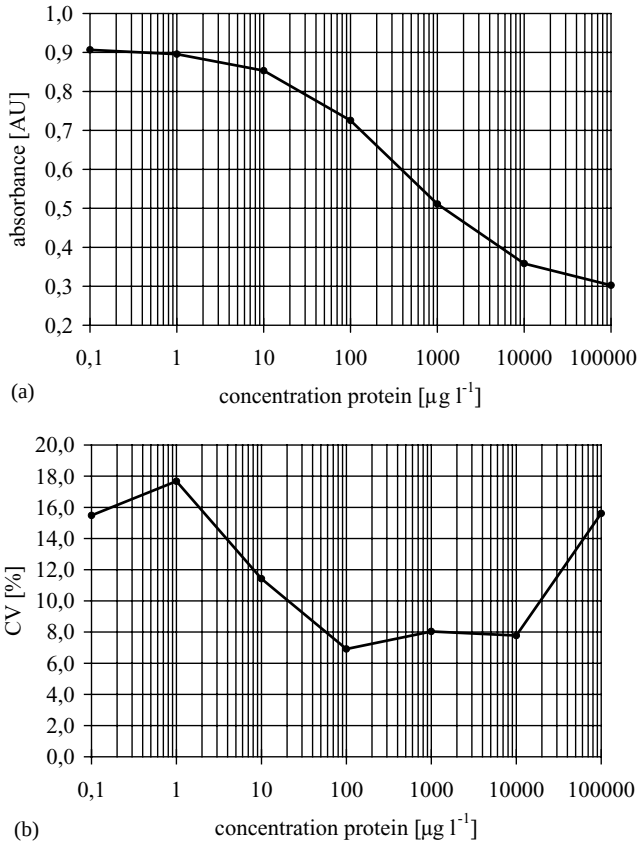

Fig. 2. (a) Standard curve of the indirect competitive ELISA. (b) Precision profile of the standard curve.

first screening investigations. The system applied was indirect competitive ELISA technique with hazelnut protein precoated to microtiter plates. Various nuts, stonefruits, seeds and cereals as well as some characteristic food ingredients were applied for cross-reactivity testing. The crude antibody extract showed extreme cross reactivity for almost all tested products. In Table 1 the percentage values of the cross reactants signal in comparison to 100% of the hazelnut signal are listed. Extracts of wheat, rye, walnut or coconut cross-reacted corresponding to 20–35% of hazelnut protein signal, when investigated at proportions of 100% of food. For that purpose an antibody clean up providing high amounts of specific anti-hazelnut-antibody was necessary. Immunoabsorption against a mix of extracts from walnut, coconut, brazil nut, sunflower seed, poppy seed, bean, wheat, rye, barley, raisins and cocoa powder leads to an enormous reduction of cross reactivity while the titer of the antibody extract remained nearly unchanged. For instance, cross reactivity for wheat could be reduced from 33 to 2%. Table 1 shows the cross reactivity of the immunoaffinity purified anti-hazelnut-antibody compared to the cross reactivity of the crude IgY extract. The specificity of the purified antibody was found to be high enough that other related food components will not cause false positives but to be still able to detect the traces of hazelnut. Therefore, immunoaffinity purified anti-hazelnut-IgY was chosen for ELISA development. A representative standard curve with the average characteristics of the standard curves from 12 different experiments is shown in Fig. 2a. The sensitivity of the immunoassay is expressed as the limit of detection (LOD) and was calculated as three times the standard deviation (3s) from the mean measurement of the blank dose signal. The limit of quantification (LOQ) that represents the level above where quantitative results can be obtained is defined by the upper limits of the linear range of the dose–response curve and was calculated as three times the limit of detection. The limit of detection and the limit of quantification were evaluated with 10 and 30 $\mu g\,l^{-1}$, when cookie matrix was used.

A precision profile of the standard curve is given in Fig. 2b. Standard precision, as expressed by the coefficient of variation (CV), is even below 8% for quantification in the range of 10000–100 $\mu g\,l^{-1}$, and still below 11% for quantification in the range of 100–10 $\mu g\,l^{-1}$.

The first approach to the analysis of samples of environmental origin was performed by using cookies for spiking experiments. This means, previously extracted negative cookie matrix was spiked with pure hazelnut protein solution

Table 2
Analytical characteristics of the hazelnut assay

Sensitivity	
LOD ($\mu g\,l^{-1}$)	10
LOQ ($\mu g\,l^{-1}$)	30
IC_{50} ($\mu g\,l^{-1}$)	618
Standard precision (CV, %)	<11
Recovery (%)	115–154

at different concentrations within the working range of the optimised ELISA. The overall recoveries were good with an average of 128% dependent from the concentration level investigated.

4. Conclusion

An indirect competitive ELISA format—based on egg yolk antibodies—for the detection of hazelnuts in food samples was developed. A standard calibration curve was optimised achieving a minimum detection limit of $10\,\mu g\,l^{-1}$ with a limit of quantification of $30\,\mu g\,l^{-1}$ and an IC_{50} of $618\,\mu g\,l^{-1}$ (Table 2). Negligible cross reactivity to several foods and food additives was observed after affinity purification and the assay displays no matrix effects in cookie samples. Future work will focus on the assay development for detection of hazelnut proteins in chocolate as the chocolate represents the more difficult matrix due to its content of polyphenols, which mask the analyte of interest.

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