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Selection of Recombinant Antibodies by Phage Display Technology and Application for Detection of Allergenic Brazil Nut (*Bertholletia excelsa*) in Processed Foods

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ABSTRACT: Current immunological methods for detection of Brazil nut allergens in foods are based on polyclonal antibodies raised in animals. Phage display technology allows the procurement of high-affinity antibodies avoiding animal immunization steps and therefore attaining the principle of replacement supported by animal welfare guidelines. In this study, we screened Tomlinson I and J libraries for specific binders against Brazil nut by employing a Brazil nut protein extract and a purified Brazil nut 2S globulin, and we successfully isolated a phage single chain variable fragment (named BE95) that specifically recognizes Brazil nut proteins. The selected phage scFv was further used as affinity probe to develop an indirect phage-ELISA for detection of Brazil nut in experimental binary mixtures and in commercial food products, with a limit of detection of 5 mg g^{-1}. This study describes for the first time the isolation of recombinant antibody fragments specific for an allergenic tree nut protein from a naïve library and paves the way to develop new immunoassays for food analysis based on probes that can be produced in vitro when required and do not rely on animal immunization.

KEYWORDS: *Brazil nut, Bertollethia excelsa, phage display, scFv, ELISA*

INTRODUCTION

Brazil nuts (*Bertholletia excelsa*, family Lecythidaceae) are a valuable source of selenium.[1] Their 2S albumin protein fraction (named Ber e 1) is unusually rich in sulfur amino acids and comprises around 30% of the total protein.[2] For these reasons, Brazil nuts are regarded as a value added ingredient and therefore used in the preparation of different foods, including breakfast cereals, baked goods, and confectionery. Nevertheless, Ber e 1 is also the major allergen of Brazil nut, responsible for triggering allergic reactions in sensitized individuals,[3] making Brazil nut, which also includes the allergen Ber e 2, one of the best characterized allergenic foods.[4,5]

Thus far no effective treatment for food allergy is described, so strict avoidance of Brazil nut in the diet is the only possible measure to prevent life-threatening reactions in sensitized individuals.[6] Nonetheless, accidental exposure to products containing Brazil nut might happen because of mislabeling of packaged foods, cross-contamination due to the use of shared processing equipment, or the presence of undeclared Brazil nut in ingredients.[7] Since adequate information concerning substances causing allergies is crucial for consumer protection, several countries enforce labeling requirements for allergenic food ingredients.[8−11] Enforcement of regulations regarding allergen labeling depends on the availability of appropriate analytical methods. The enzyme-linked immunosorbent assay (ELISA) is an accessible technique that allows sensitive and specific detection of protein residues in foods. Several ELISA assays for the detection of Brazil nut in foodstuffs have been developed over the past few years.[12−15] Although performed in different assay formats, all of them have in common the use of polyclonal antibodies, either raised in rabbits or produced in egg yolk.

With increased international awareness of welfare issues regarding animal use for scientific purposes, new regulatory policies have encouraged replacement and reduction of the use of animals in procedures and promotion of alternative approaches as soon as it is scientifically possible to do so.[16]

Recombinant DNA methods using phage display technology provide an alternative way of producing antibodies with desirable characteristics in large amounts and with little or no variability between batches. This approach has the potential to reduce the cost and effort of the traditional methods associated with polyclonal and monoclonal antibody production, while avoiding the use of live animals.[17−19]

Phage display is the technology for exposing exogenous peptides on the surface of a bacteriophage that contains the gene encoding the displayed protein, thereby physically linking the genotype and the phenotype. Phage-displayed single chain variable fragment (scFv) libraries are combinatorial libraries that consist of a wide diversity of variable heavy (VH) and variable light (Vκ) domains of an antibody, connected by a flexible glycine-serine linker. The antibody fragment is fused to the phage minor coat protein pIII, encoded by gene III, and

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displayed externally as a scFv.[20,21] In the recent years, scFv antibodies against toxins like *Bacillus thuringiensis* toxins Cry1C and Cry1B[22,23] and palytoxin[24] have been isolated and characterized, but so far scFv antibodies have not been used for detection and identification of food allergens.

Here, we report the isolation of recombinant antibodies against Brazil nut from the naïve human scFv libraries Tomlinson I + J, by an iterative affinity selection procedure instead of animal immunization. We also describe an indirect phage-ELISA that allows the rapid, easy, and economical detection of Brazil nut in commercial food products by employing the affinity probe developed against a Brazil nut protein extract.

■ MATERIALS AND METHODS

Materials and Chemicals. The human single-fold scFv libraries I + J (Tomlinson I + J), KM13 protease cleavable helper phage, and *Escherichia coli* TG1 strain (K12 Δ *(lac-proAB) supE thi hsdD5/F′ traD36 proA*$^+$*B lacI*^{q} *lacZ*Δ*M15*) were obtained from Source BioScience (Nottingham, UK). Both libraries are based on a single human framework for VH (V3-23/DP-47 and JH4b) and Vκ (O12/O2/DPK9 and J_K1) with side chain diversity incorporated at positions in the antigen binding site that make contacts to antigen in known structures and are highly diverse in the mature repertoire. The size of the Tomlinson I library is 1.47×10^8, and the size of the Tomlinson J library is 1.37×10^8. The scFv is displayed as a fusion with the terminal phage gene III protein, cloned in the ampicilin resistant phagemid vector pIT2.

Brazil nut kernels and other tree nuts were purchased from local retailers in Madrid, Spain. They were cleaned and shelled separately to avoid cross-species contamination and stored at −20 °C until used. Plant and animal species analyzed for specificity purposes (Table 1) were acquired in different local markets in Madrid. Finally, a total of 92 commercial food products were purchased from various retail markets and delicatessen stores (Spain).

Experimental binary mixtures of Brazil nut in a wheat flour matrix (from 100 to 0.1 mg g^{-1}) were prepared to evaluate the test sensitivity. First, 50 g of finely ground Brazil nut were added to 450 g of wheat flour, and the mixture was homogenized at high speed using a food processor (Thermomix, Vorwerk, Germany). Then, 50 g of the former mixture was added to 450 g of wheat flour to obtain a concentration of 10 mg g^{-1}. Remaining concentrations (up to 0.1 mg g^{-1}) were made following the same procedure. Three additional mixtures of 50, 25, and 5 mg g^{-1} of Brazil nut in wheat flour were also prepared by mixing 250 g of wheat flour with 250 of the mixtures containing 100, 50, and 10 mg g^{-1}, respectively.

HRP/anti-M13 monoclonal mouse antibody was purchased from GE Healthcare (München, Germany). Tryptone, yeast extract, and European bacteriological agar were purchased from Pronadisa (Madrid, Spain). Acetone and acetonitrile were purchased to Panreac (Barcelona, Spain). All others reagents were purchased from Sigma-Aldrich (St. Louis, MO).

The protein extraction solution consisted of 0.035 M phosphate buffer containing 1 M NaCl, pH 7.5.[25] 2xTY broth is 16 g L^{-1} tryptone, 10 g L^{-1} yeast extract, and 5 g L^{-1} NaCl. TYE agar is 15 g L^{-1} Bacto Agar, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 8 g L^{-1} NaCl.

Preparation of Protein Extracts. Food samples were ground to fine flour using a mortar and pestle and stored at −20 °C in plastic screw-capped vials until further use. An aliquot of 200 mg was mixed with 1800 μL of extraction solution, by continuous mixing using a vertical rotator (HulaMixer Sample Mixer, Life Technologies, Carlsbad, CA) for 10 min at room temperature (rt) to solubilize proteins. The slurry was centrifuged at 10 000*g* for 10 min at 4 °C, and the supernatant was filtered through a 0.45 μm syringe filter (Sartorius, Göttingen, Germany). Protein content was determined by the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc.) using bovine serum albumin (BSA) as the standard protein. Aliquots were stored at −20 °C until needed. The quality of the Brazil nut extract was verified by SDS−PAGE electrophoresis (see below).

Table 1. List of Species Showing No Cross-Reactivity with Brazil Nut in the Indirect Phage-ELISA

Species		
Nuts		
almond (*Prunus dulcis*)	macadamia (*Macadamia integrifolia*)	pine nut (*Pinus pinea*)
cashew nut (*Anacardium occidentale*)	peanut (*Arachis hypogaea*)	pistachio (*Pistacia vera*)
hazelnut (*Corylus avellana*)	pecan nut (*Carya illinoinensis*)	walnut (*Juglans regia*)
Vegetal Species		
anise (*Pimpinella anisum*)	lentil (*Lens culinaris*)	potato (*Solanum tuberosum*)
apple (*Malus domestica*)	lupine (*Lupinus albus*)	prune (*Prunus domestica*)
barley (*Hordeum vulgare*)	maize (*Zea mays*)	pumpkin seed (*Cucurbita maxima*)
carrot (*Daucus carota*)	mandarin orange (*Citrus retticulata*)	rice (*Oriza sativa*)
chickpea (*Cicer arietinum*)	mung bean (*Vigna radiata*)	rye (*Secale cereale*)
chufa sedge (*Cyperus esculentus*)	oats (*Avena sativa*)	sesame (*Sesamum indicum*)
cinnamon (*Cinnamomum verum*)	olive (*Olea europaea*)	soy (*Glycine max*)
cocoa (*Theobroma cacao*)	onion (*Allium cepa*)	sunflower (*Helianthus annuus*)
dry bean (*Phaseolus vulgaris*)	orange (*Citrus sinensis*)	tomato (*Solanum lycopersicum*)
flax (*Linum usitatissimum*)	paprika (*Capsicum annuum*)	vanilla (*Vanilla planifolia*)
garlic (*Allium sativum*)	pea (*Pisum sativum*)	wheat (*Triticum aestivum*)
grape (*Vitis vinifera*)	pear (*Pyrus communis*)	zucchini (*Cucurbita pepo*)
kiwifruit (*Actinidia deliciosa*)		
Animal Species		
cattle (*Bos taurus*)	poultry (*Gallus gallus domesticus*)	swine (*Sus scrofa domestica*)
fish (*Salmo salar*)		
Others		
brown sugar	milk	

Purification of Brazil Nut 2S Albumin (Ber e 1). Defatted Brazil nut flour was prepared as described by Doi et al.[26] with some modifications. Briefly, shelled Brazil nuts were ground and defatted by adding acetone (1:10 w/v), vortexing for 1 min, and then centrifuging at 10 000*g* for 30 min at 4 °C. This procedure was repeated three times before Brazil nut flour was left to dry overnight at 25 °C. The powder was stored in screw-capped plastic vials at −20 °C until further use.

Purification of Ber e 1 was performed according to the method of Sharma et al.[25] with several modifications. Two grams of defatted Brazil nut flour was extracted with 20 mL of extraction solution, by continuous mixing for 1 h at 25 °C. The slurry was centrifuged at 10 000*g* for 30 min at 4 °C, and the supernatant was filtered through a 0.20 μm syringe filter. Brazil nut extract was subjected to size-exclusion chromatography on a HiPrep 16/60 Sephacryl S-200 HR column (GE, Healthcare UK Ltd., Buckinghamshire, UK) equilibrated with extraction solution. The flow rate was maintained at 0.8 mL min^{-1}. The 2S albumin fraction obtained was purified via preparative HPLC (Varian Dynamax 21.4 × 250 mm Microsorb 5 μm 300 Å C_8 reverse-phase column) using an Agilent 1200 HPLC system (Agilent Technologies) with a quaternary pump, a column thermostat, an autosampler, and a photodiode-array multiple-wavelength UV detection. The chromatographic conditions for this HPLC system were set as follows: column temperature was maintained at 40 °C,

elution was performed at a flow rate of 1.0 mL min^{-1}, with simultaneous UV detection at 254 and 280 nm. Mobile phase was a gradient prepared from 0.01% formic acid in water (component A) and 0.01% formic acid in acetonitrile (component B). The program used for elution was 0−5 min of 100% A and then a linear gradient from 100% A to 100% B over 100 min. The collected fraction containing Ber e 1 protein was immediately neutralized with ammonium hydroxide and thereafter was dialyzed against Milli-Q water, for 24 h with three water changes (4 L per change), using a dialysis cassette (Slide-A-Lyzer Dialysis Cassettes, 3.5K MWCO, Thermo Scientific, Rockford, IL). The dialysate was lyophilized and stored in glass vials at −20 °C until further use. Soluble protein content was determined using the BCA assay. In order to characterize the purified protein, SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out. Samples were diluted 1:1 in sample buffer [0.5 MTris-HCl buffer, pH 6.8, 10% (w/v) SDS, 20% v/v glycerol, 0.5% (w/v) bromophenol blue as the tracking dye, 5% (v/v) β-mercaptoethanol], boiled for 10 min, loaded onto a 4% monomer acrylamide/bis stacking gel, and separated in a 13% monomer acrylamide/bis resolving gel. Electrophoresis was run at a constant voltage (100 V) for 3 h using a Miniprotean TetraCell (Bio-Rad). ColorBurst Electrophoresis Marker (Sigma-Aldrich, St. Louis, MO) was used as the molecular weight standard. The gel was stained with Coomassie Brilliant Blue R-250, and the band was cut out with a sterile scalpel and immersed in 5% acetic acid solution. Protein sequencing was performed using the 4800 Plus MALDI TOF/TOF Analyzer mass spectrometer (AB SCIEX), at the Unidad de Proteómica at Parque Científico de Madrid (Madrid, Spain). Ion spectra of the peptides obtained were searched in the MASCOT search engine (http://www.matrixscience.com) using the SwissProt database. The following search parameters were used: trypsin enzymatic cleavage, allowing one possible missed cleavage; peptide mass tolerance of 80 ppm; fragment mass tolerance of 0.3 Da; peptides were assumed to be monoisotopic; carbamidomethyl (C) fixed modification; and methionine oxidation variable modification.

Preparation of Phage Libraries for Selection Procedure. Tomlinson phage libraries I + J were prepared separately for selection procedure as depicted by the MRC protocol (http://www.lifesciences.sourcebioscience.com/media/143421/tomilinsonij.pdf). Briefly, 500 μL of the library stock was inoculated into 200 mL of 2xTY broth containing 100 μg mL^{-1} ampicillin and 1% (w/v) glucose and incubated with shaking at 37 °C until the optical density at 600 nm (OD_{600}) reached 0.4. Then, 2×10^{11} particles of KM13 helper phage were added to 50 mL of the bacterial culture and incubated without shaking in a 37 °C water bath for 30 min. Subsequently, infected cells were centrifuged at 3000*g* for 10 min at 4 °C; resuspended in 100 mL of 2xTY containing 100 μg mL^{-1} ampicillin, 50 μg mL^{-1} kanamycin, and 0.1% (w/v) glucose; and incubated with shaking at 30 °C overnight. Phage particles were precipitated with 20 mL of ice-cold 20% polyethylene glycol 6000 in 2.5 M NaCl and pelleted by centrifugation at 3300*g* for 30 min at 4 °C. After pouring away supernatant, the phage pellet was resuspended in 4 mL of PB, and centrifuged at 11 600*g* for 10 min to remove bacterial debris. Phages were stored at 4 °C for short-term storage or at −80 °C in 15% glycerol for longer term storage. In order to estimate the titer of phage particles, 10 μL of the stock solution was employed to produce a 10-fold dilution series from 10^2 to 10^{12} in PBS. Then, 10 μL of each dilution was added to an Eppendorf tube containing 200 μL of a TG1 culture at an OD_{600} of 0.4 and incubated at 37 °C for 30 min to allow infection. Finally 10 μL of each dilution was spotted on a TYE agar plate containing 100 μg mL^{-1} ampicillin and 1% (w/v) glucose and incubated overnight at 37 °C.

Target Immobilization. For the first round of selection of scFv clones against Brazil nut, polystyrene paddles (Nunc, Denmark), with a surface area of 5.2 cm^2, were coated by incubation overnight at 4 °C with 1 mL of Brazil nut extract in PBS (positive screening) or with 1 mL of peanut extract in PBS (negative screening), both with a final concentration of 100 μg mL^{-1} of protein. The protein-coated polystyrene paddles were washed three times with PBS and blocked with 3% BSA at 37 °C for 1 h.

For the second round of selection, Dynabeads M-280 Tosylactivated (Invitrogen, Life Technologies, Carlsbad, CA) were employed. These magnetic beads bind proteins physically and chemically through primary amino or sulfhydryl groups. Coupling of the antigen was carried out as recommended by the manufacturer's instructions. Briefly, 5 mg of Dynabeads was coupled with 100 μg of Brazil nut proteins (positive panning) in 0.1 M borate buffer pH 9.5 to a final volume of 150 μL and then 100 μL of 3 M ammonium sulfate in borate buffer was added. Coupling was performed on a vertical rotator (HulaMixer) at 37 °C overnight. After overnight coupling, supernatant was removed with the aid of a magnet, and Dynabeads were blocked with 1 mL of 0.5% (w/v) BSA in PBS, at 37 °C for 1 h on a rotator. Subsequently, supernatant was discarded, and the Brazil nut coupled beads were stored in 240 μL of 0.1% (w/v) BSA in PBS at 4 °C until use. The same procedure was performed with 5 mg of new Dynabeads but employing a peanut protein extract as the ligand (negative panning). Both coating surfaces were used alternately throughout the complete selection procedure. The aim of employing different coating surfaces was to overcome the "target-unrelated phages" problem, which might produce false-positive results[27] if selected phages showed affinity for coating surfaces.

For selecting scFv clones against Ber e 1, the procedure was performed as described above, but employing 100 μg mL^{-1} of HPLC-purified Ber e 1 for coating polystyrene paddles and 100 μg of the same protein for coating magnetic beads.

Panning Procedure. *1. Selection of scFv Clones against Brazil Nut.* Approximately 10^{12} phage particles from each library were added to paddles coated with peanut protein extract in 2 mL of 3% (w/v) BSA in PBS and incubated at 25 °C for 60 min on a rotator. After performing the negative panning, supernatants containing phage particles not bound to peanut proteins were added to polystyrene paddles coated with Brazil nut extract and then incubated at 25 °C for 60 min with rotation and a further 60 min without rotation. Unbound phage particles were removed by washing 10 times with PBS, and then, specifically bound phage scFvs were eluted from the paddle by adding 500 μL of trypsin solution (1 mg mL^{-1} trypsin in PBS) for 10 min at rt with rotation. Supernatant was collected, and 250 μL of the eluted phages was transferred to a tube containing 1.75 mL of TG1 cell culture at an OD_{600} of 0.4. After incubating for 30 min at 37 °C in a water bath, infected cells were spread on a TYE agar plate containing 100 μg mL^{-1} ampicillin and 1% (w/v) glucose and grown overnight at 37 °C. The titer of eluted phage was also determined. The next day, all colonies of the TYE agar plate were scraped into 2 mL of 2xTY containing 15% glycerol and stored at −80 °C (first round stock). In order to produce new phages for a second round of selection, 50 μL of the first round stock was inoculated into 50 mL of 2xTY containing 100 μg mL^{-1} ampicillin and 1% (w/v) glucose and incubated with shaking at 37 °C until an OD_{600} of 0.4 was reached. Then, 10 mL of the former culture was infected with 5×10^{10} particles of helper phage. Phage particles were rescued from the culture as described above. Selection was repeated one more time, employing 2.5 mg of Dynabeads instead of paddles, and increasing the washing step to 20 times to remove unspecifically bound phage scFvs

2. Selection of scFv Clones against Ber e 1. The screening rounds were performed as previously described, but the number of selection rounds was increased to four, maintaining the alternation of polystyrene paddles with magnetic beads.

Brazil Nut Indirect Phage-ELISA. The ability of selected phage scFvs to recognize target proteins was assessed by polyclonal phage-ELISA. With that purpose, flat-bottom polystyrene microtiter plates (F96 MaxiSorp Nunc immuno plates, Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 100 μL of target solution (25 μg mL^{-1} of Brazil nut protein extract or a 100 μg mL^{-1} solution of purified Ber e 1), a peanut protein extract (25 μg mL^{-1}), or a BSA solution (100 μg mL^{-1}), in PBS. Plates were washed three times (all washing steps were performed with PBS) and blocked to prevent nonspecific binding with 0.1% gelatin in PBS for 1 h at 37 °C. After washing three times, 10 μL of precipitated phages from each round of selection was added to 100 μL of 1% ovalbumin in PBS. Plates were incubated for 1 h at rt, washed 10 times prior to adding 100 μL per well of a 1:5000

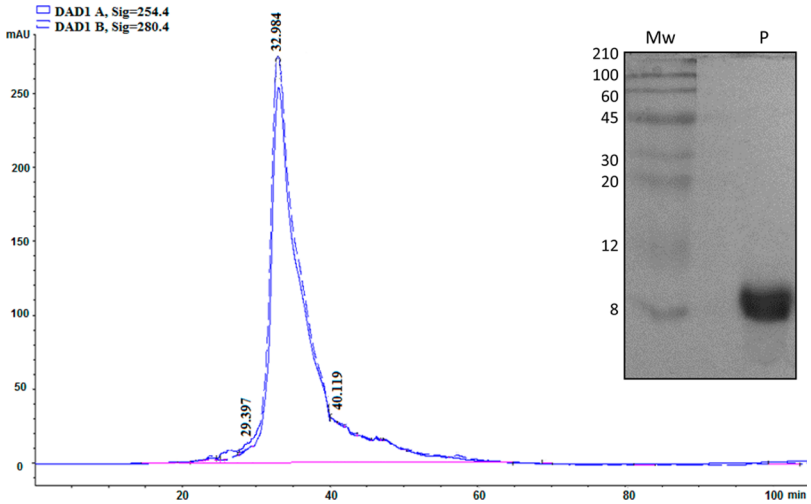

Figure 1. Elution profile of Brazil nut Ber e 1 protein obtained from preparative HPLC column. (Inset) SDS−PAGE electrophoresis (under reducing conditions) analysis of fraction eluted off the column. M_w, protein marker; P, protein eluted off the column. The molecular mass of each standard (kDa) is indicated in the left margin of the inset.

dilution of a HRP/anti-M13 monoclonal mouse antibody, and incubated again at rt for 1 h. Finally, plates were washed five times, and then 100 μL of tetramethylbenzidine substrate solution was added to each well, and the plates were incubated in the dark. Color development was performed for 10 min at rt and stopped with 50 μL per well of 1 M sulfuric acid. OD_{450} was measured with an iEMS Reader MF (Labsystems, Helsinki, Finland). All analyses were performed in triplicate.

In order to confirm the antigen recognition ability of single clones, 95 individual colonies from the last rounds of selection were randomly picked from the TYE agar plate with 100 μg mL^{-1} ampicillin and 1% (w/v) glucose. Bacteria were inoculated into 100 μL of 2xTY containing 100 μg mL^{-1} ampicillin and 1% glucose in 96-well plates (Nirco, Barcelona, Spain), and were grown with shaking (250 rpm) overnight at 37 °C. The next day, 5 μL from each well was used to infect a second 96-well plate containing 200 μL 2xTY, with 100 μg mL^{-1} ampicillin and 1% glucose. Plates were incubated for 2 h more at 37 °C before rescuing phages by adding 10^9 particles of helper phage to each well. After incubation for 1 h at 37 °C, the plates were centrifuged at 1800*g* for 10 min at 4 °C. Supernatant was discarded, and pellets were resuspended in 200 μL of 2xTY containing 100 μg mL^{-1} ampicillin, 50 μg mL^{-1} kanamycin, and 0.1% glucose and incubated overnight at 30 °C. The following day, after spinning the plate at 1800*g* for 10 min, 50 μL of the supernatant was employed in monoclonal phage ELISA.

Multiwell plates were coated overnight with 25 μg mL^{-1} of Brazil nut protein extract (positive control) or with 25 μg mL^{-1} of peanut protein extract (negative control). The ELISA technique was performed as described above, but using 50 μL of phage supernatant from the overnight culture, diluted in 100 μL of 1% ovalbumin, instead of using precipitated phages.

Sequence Analysis. Phagemid DNA from positive clones was amplified by PCR using GoTaq Green Master Mix (Promega, Madison, WI). Polymerase chain reaction (PCR) of colonies (95 °C for 9 min and then 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s for 30 cycles and the final extension at 72 °C for 7 min) was carried out to check individual clones for the presence of full length VH and Vκ inserts. Primers used were LMB3 (5′-CAG GAA ACA GCT ATG AC-3′) and pHEN seq (5′-CTA TGC GGC CCC ATT CA-3′) for the whole scFv fragment. LMB3 and link seq new (5′-CGA CCC GCC ACC GCC GCT G-3′) were used to amplify just the VH fragment, and DPK9 FR1 seq (5′-CAT CTG TAG GAG ACA GAG TC-3′) and pHEN seq were used to amplify only the Vκ fragment. PCR products were examined by electrophoresis on 2% agarose gel. Phagemid DNA from positive clones was purified with a High Pure Plasmid Isolation Kit (Roche Applied Science, Mannheim, Germany) and sequenced at Sistemas Genómicos (Valencia, Spain), using primers LMB3 and pHEN seq. The sequenced clones were compared using the European Molecular Biology Open Software Suite (Emboss software) and then analyzed with IgBLAST to determine framework and complementary determining regions (CDR) of the VH and Vκ chains. Amino acid sequences were deduced from the nucleotide sequences by the Expasy Web site (www.expasy.org).

Assay Validation. Specificity of the Brazil nut indirect phage-ELISA was assessed by challenging protein extracts obtained from nine nuts and also from several plant and animal species (Table 1). Extracts were obtained according to the protein extraction protocol and diluted at 1:200 in PBS to be used in the ELISA assay. Each sample was analyzed in triplicate.

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to the guidelines of the International Union of Pure and Applied Chemistry (IUPAC).[28] The LOD for the ELISA was calculated by considering the minimum concentration of the target protein that is larger than the mean absorbance value of the blank (wells with PBS) plus 3 times the standard deviation (SD) after 20 experiments, whereas LOQ was determined considering the mean absorbance value of the blank plus 10 times the SD. Additionally, the LOD and LOQ for binary mixtures of a wheat flour matrix spiked with decreasing concentrations of raw Brazil nut were determined in a similar fashion, but using wells coated with wheat flour extract as blank.

Brazil nut protein extract was tested in the concentration range from 0 to 25 μg mL^{-1}. The concentration−response curve was obtained by plotting the absorbance values vs the analyte concentration, and the standard curve obtained for Brazil nut dilution was fitted to the four-parameter logistic equation using Origin 8.0 software (OriginLab Corp.)

$$y = (A - D)/[1 + (x/C)^B] + D$$

where A is the maximum absorbance at infinite concentration, B is the curve slope at the inflection point, C is the x value at the inflection point, and D is the minimum absorbance for no analyte (background signal). Data were analyzed for statistical significance by one-way ANOVA and the Fisher's least significant difference (LSD) test ($p < 0.05$) using Statgraphics Centurion 15.2.14 (XV) (Statpoint Technologies, Inc., Warranton, VA).

The effect of heat treatment on the technique's ability to identify Brazil nut protein was assessed through the analysis of protein extracts from roasted Brazil nuts. With that purpose, 70 g of ground Brazil nut were processed in an oven at 160 °C for 13 min. Standard curve was obtained as for raw Brazil nut extract described above.

Table 2. Peptides Identified by MALDI-TOF/TOF Tandem Mass Spectrometry and Mascot Database Search

protein identification	accession no.	total score	ion scores[a]	peptide sequences
2S sulfur-rich seed storage protein 2	AB044391	122	48	R.KAENLLSR.C
			74	R.QREEMELQGEQMQR.I
			23	R.QREEMELQGEQMQR.I + oxidation (M)
2S sulfur-rich seed storage protein 1	X54491.1	109	41	R.LAENIPSR.C
			69	R.MQQEEMQPR.G

[a]Individual ion scores bigger than 40 indicate identity or extensive homology ($p < 0.05$).

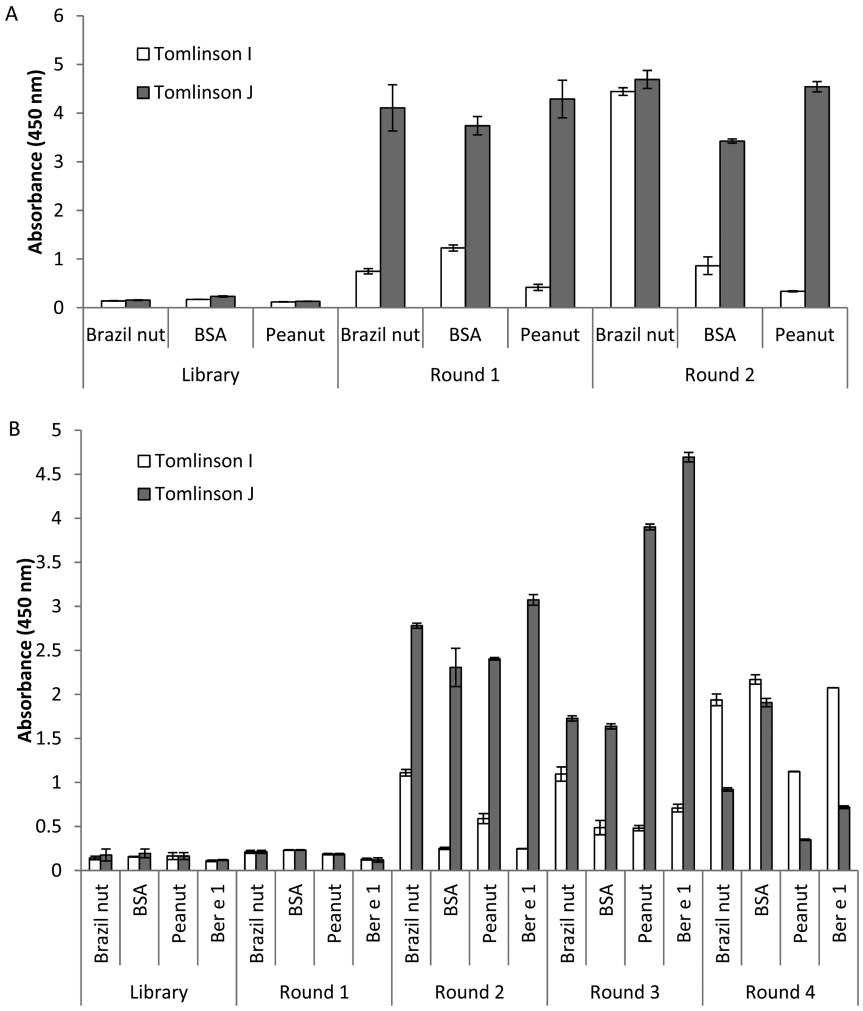

Figure 2. Indirect phage-ELISA results obtained with polyclonal phages rescued at each round of panning against Brazil nut extract (A) and purified Ber e 1 (B). Precipitated phages from each round of panning (containing approximately 10^{11} phage particles per milliliter) were analyzed by ELISA. Absorbance values are the mean of three independent determinations. Error bars show the standard deviation for each set of data.

To validate the applicability of the developed method for detection of Brazil nut protein in foodstuffs, a total of 92 commercial processed food products were analyzed. Samples including breakfast cereals, ice cream, cookies, nut beverages, chocolate, and nutritional bars were selected because they are varieties of food containing Brazil nut as one of the ingredients or might have been exposed to cross-contamination with Brazil nut. Each sample was analyzed in triplicate using the Brazil nut indirect phage-ELISA. Furthermore, in order to confirm the results, samples were also analyzed by a real-time PCR method developed in our laboratory that employs Brazil nut specific primers and probe and targets the 2S albumin gene.[29]

RESULTS AND DISCUSSION

Ber e 1 Purification. Size-exclusion chromatography of defatted Brazil nut protein extract was resolved in a protein profile with three major peaks, similar to that obtained by Sharma et al.[25] Fraction corresponding to Ber e 1 protein was further purified by passing it through a preparative HPLC column. Proteins were eluted off the column at 25−35% acetonitrile (min 30 to 40), and SDS−PAGE electrophoresis of the fraction, performed in reducing conditions, showed a single band of approximate 9 kDa (Figure 1). A proteomics approach was used to confirm the identity of purified Ber e 1 protein. The excised band was trypsinized and subsequently identified by MALDI-TOF/TOF. As shown in Table 2, two peptide isoforms of the 2S sulfur-rich seed storage protein (Ber e 1) from *B. excelsa* were identified, with 11% and 14% of sequence coverage. It is described that Ber e 1 has a polymorphic nature, because it is encoded by multiple gene sequences and,

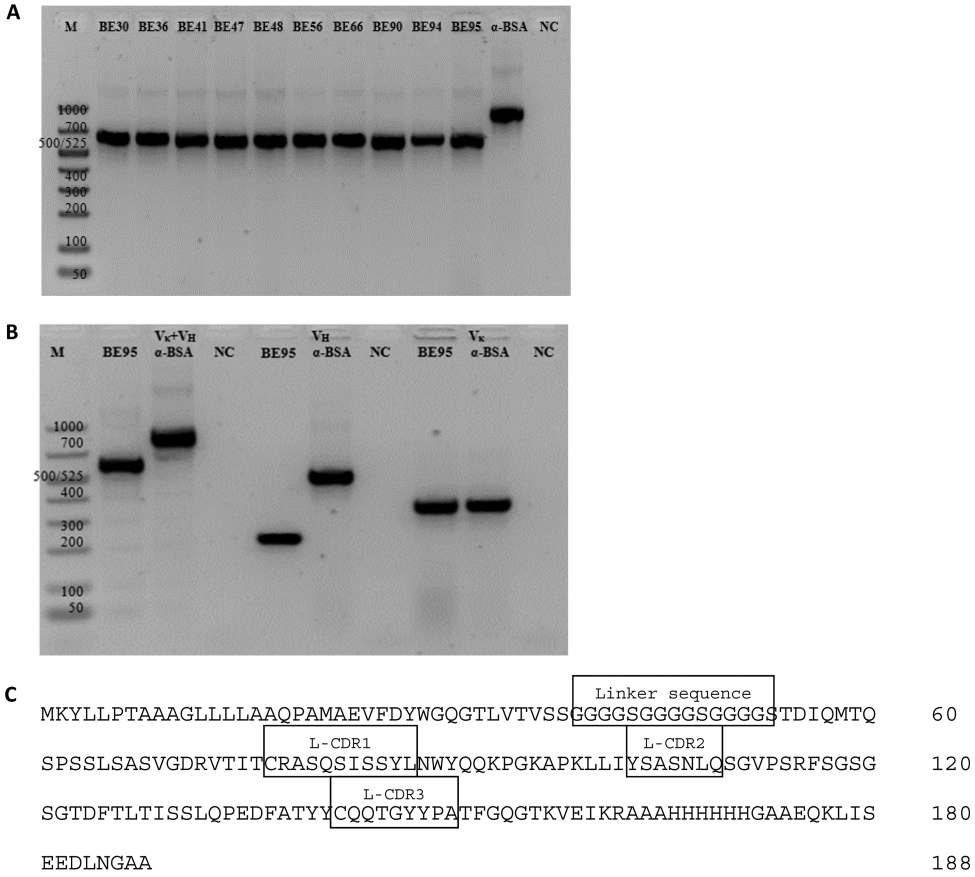

Figure 3. PCR amplification and sequencing of the selected clones. (A) PCR amplification using primers LMB3 and pHEN seq, for the complete scFv fragment. Clones with full length scFv gene should have bands at about 935 bp. ScFv clones against Brazil nut had bands at 628 bp. Lane M, DNA marker (BioMarker Low, BioVentures Inc.); lanes BE30, BE36, BE41, BE47, BE48, BE56, BE66, BE90, BE94, and BE95, different scFv clones against Brazil nut; lane α-BSA, scFv clone against BSA (positive control); lane NC, nontransduced TG1 (negative control). (B) PCR amplification using primers for the complete scFv fragment, the VH fragment, and the Vκ fragment. Lane M, DNA marker; lane BE95, scFv clone against Brazil nut; lane α-BSA, scFv clone against BSA (positive control); lane NC, nontransduced TG1 (negative control). (C) Amino acid sequences were deduced from the nucleotide sequences by the Expasy Web site. Positions of the respective complementary determining regions for the variable domains of light chain (L-CDR 1−3) are indicated.

moreover, it is subjected to post-translational modifications.[30] Thus, the results obtained confirm the identity of the purified Brazil nut 2S albumin.

Libraries Screening. Usually, selection strategies employing phage libraries are carried out on purified molecules, since this allows performing the screening on well-defined antigens. However, by selecting a complex protein extract, cumbersome protein purification steps could be avoided. Furthermore, protein extracts allow isolation of phage clones that bind to specie-specific epitopes in a wider variety of molecules. Accordingly, we have used the Tomlinson I + J libraries to isolate phage scFvs against a purified Brazil nut protein (Ber e 1) or a whole Brazil nut protein extract, to determine the efficiency of the selection procedure for both types of targets.

Approximately 10^{12} phage particles were used for each round of selection. As the selection procedure progresses, a gradual increase in phage titers is expected, and such an increase frequently indicates the enrichment of specific phage binders.[31] The clear increase in recovery values of phage particles obtained after the second round of selection for the Brazil nut extract was noteworthy, particularly regarding Tomlinson I library (2×10^3 phage particles recovered after the first round and 4×10^8 phage particles after the second round).

Polyclonal Phage-ELISA. The presence of positive binding phages after each round of selection was confirmed by polyclonal phage-ELISA using a Brazil nut protein extract or purified Ber e 1 as positive control, and a peanut extract and BSA as negative controls. As shown in Figure 2A, phages from the unselected libraries were not able to recognize both the target or negative control proteins. When selecting phages against the whole protein extract, scFv phages against Brazil nut became detectable even after the first round of selection, with a substantial increase in hypothetically specific phages at the second round of selection for library Tomlinson I ($A_{450} > 4$). On the other hand, library Tomlinson J showed high absorbance values for all the antigens assayed after both rounds of selection, therefore demonstrating no specificity for the target species. Consequently, no further analyses were performed with the Tomlinson J library.

Concerning phage antibodies raised against purified Ber e 1 (Figure 2B), no significant enrichment of the phage population specifically recognizing the Ber e 1 protein was obtained either for the I library or for the J library. After round three of selection, a generalized increase in the absorbance values for both positive and negative controls was found. Regarding the Tomlinson J library, the absorbance values for target protein dropped substantially in the polyclonal ELISA after the third

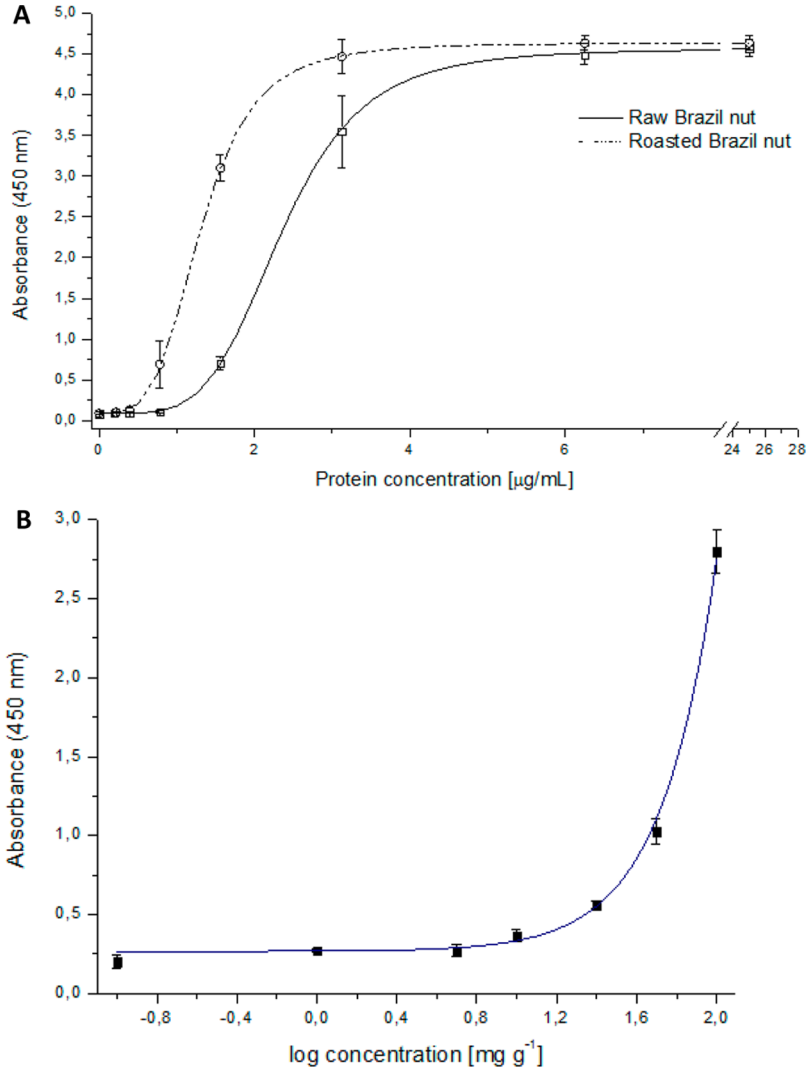

Figure 4. Representative standard curve of the developed Brazil nut indirect phage-ELISA. Each curve shows the mean value of six experiments and the standard deviation in each point of the curve. (A) Dilutions of raw and roasted Brazil nut protein extracts. (B) Protein extracts obtained from experimental Brazil nut/wheat flour binary mixtures.

round of panning, and the absorbance values for negative controls in the second and third rounds of selection were similar to those of positive controls. Therefore, after four rounds of selection with the purified Ber e 1 protein, no scFv phages that specifically recognized purified Ber e 1 were isolated using the Tomlinson I + J libraries. These results suggest that the VH−Vκ combination necessary for the specific detection of Ber e 1 are not present in these libraries and further stress the importance of a comprehensive initial library. Thus, the panning using the purified protein was discontinued.

According to these results, a successful enrichment in scFv phages with affinity for Brazil nut was achieved only when the panning procedure was carried out using a Brazil nut protein extract obtained with phosphate buffer with 1 M NaCl. Therefore, the Brazil nut protein extract was further employed to test the specificity and sensitivity of the assays and also as a reference (positive control) in the ELISA for detection of Brazil nut in food samples.

Monoclonal Phage-ELISA. Phages eluted from Tomlinson I library after the second round of selection were used to infect *E. coli* TG1. Ninety-five phage clones were randomly picked and tested against the Brazil nut protein extract. Approximately 90% of the selected clones resulted positive, with 87 clones recognizing Brazil nut extract (absorbance values against Brazil nut extract/absorbance against negative controls >5). Ten of those 87 clones were randomly selected for further analysis.

Sequence Analysis of the Positive Clones. PCR amplification (using LMB3 and pHEN seq primers) of the scFv genes encoded in all phagemids selected after the second round of selection yielded products of 628 bp (Figure 3A), indicating the scFv inserts were incomplete (a complete fragment like that of the anti-BSA control has a length of about 935 bp). Then, PCR amplifications were performed for both VH and Vκ inserts with specific primers. Figure 3B shows that clone BE95 contains a complete Vκ fragment, with an insert of 368 bp, but an amplicon length of 227 bp for VH, indicating the absence of a VH sequence. For comparison, the anti-BSA positive control clone (provided with the library) had a full length VH insert of 527 bp. The complete nucleotide sequence was determined for 10 selected clones, and the results indicated that the coding sequences of all clones were identical, confirming the absence of the VH sequence. Hence, these scFv phagemids were uniformly designated as BE95. When compared to the NCBI database, the selected positive clone

was confirmed to be a human Vκ insert. Deduced amino acid sequences of the positive clone showing the CDRs and immunoglobulin framework regions (FRs) are illustrated in Figure 3C. Although heavy and light chains are usually paired in the human immune system, VH and VL can in some circumstances exist in isolation, maintaining their ability to recognize antigens.[31] In fact, a commercially available human antibody library consisting of antibody fragments based exclusively on a single VH domain is available.[32] In our case, we have isolated a fragment consisting solely of a single Vκ domain, which has the property of recognizing and binding the target antigen. In this respect, studies about immunoglobulin free light chains (Ig-fLCs) have demonstrated that although affinity might not reach that of tetrameric antibodies, binding of Ig-fLCs to their corresponding antigens is of sufficient strength to be detected with common immunodetection methods.[33]

Assay Specificity. Specificity of the developed ELISA assay using BE95 phage scFv as affinity probe was ascertained through analysis of protein extracts from a wide variety of plant and animal species (Table 1). It should be noted that other ELISA assays described in the literature for Brazil nut detection showed interference problems with members of the Lauraceae family such as *C. verum*.[15] In our case, we found that Brazil nut extract produced an A_{450} value of 4.386 ± 0.08, whereas none of the nontarget crop protein extracts tested developed a significant signal (A_{450} value above the negative control value +3 standard deviations) in the Brazil nut indirect phage-ELISA, thus confirming the specificity of the assay.

The sensitivity of the assay (minimum quantity of Brazil nut protein detectable by the method developed in this work) was evaluated by analysis of serial dilutions of Brazil nut extract in PBS. Absorbance values at 450 nm increased in a concentration-dependent manner, until saturation levels were reached. Calibration curves showing the means and standard deviations were constructed for the Brazil nut indirect phage-ELISA (Figure 4A). LOD for Brazil nut without food matrix was found to be 0.9 μg mL^{-1} of Brazil nut protein extract (90 ng of Brazil nut protein per well), whereas LOQ was 1.2 μg mL^{-1}.

It is well-established that food processing can lead to modification of food constituents, resulting in alteration of protein solubility or integrity of target proteins.[34] Therefore, to appraise the capability of the method for also detecting heat-treated proteins, a protein extract from roasted Brazil nut was submitted to ELISA. Under these conditions, the LOD was 0.4 μg mL^{-1} of roasted Brazil nut extract, whereas the LOQ was established at 0.6 μg mL^{-1} (Figure 4A). The observed increase in the sensitivity of protein detection could be explained by the fact that thermal processing can modify protein structures, denaturing them and making epitopes more accessible for antibody binding.[35]

Because food matrix may affect assay performance due to the presence of interfering matrix components,[36] experimental binary mixtures of Brazil nut in wheat flour were also analyzed. Wheat flour was chosen as a representative matrix to test how it may affect the ability of the assay to detect Brazil nut proteins, for most Brazil nut containing foodstuffs are cereal-based products. The practical LOD of the ELISA was ascertained by spiking wheat flour with different concentrations of Brazil nut and subjecting them to the assay (Figure 4B). Subsequently, the LOD and LOQ for the Brazil nut and wheat flour mixtures were established at 5 and 20 mg g^{-1} of Brazil nut, respectively.

Even though the threshold dose of Brazil nut protein required to elicit an allergic reaction in sensitive individuals is not known, there is scientific agreement that the detection limits for different allergenic ingredients in foodstuffs must be somewhere between 1 and 100 mg kg^{-1}, depending on the respective food.[37−39] Therefore, for an optimal performance as a screening test, the ELISA method should be able to detect allergens at about the former interval. Hence, taken together these results suggests that the present Brazil nut ELISA is highly specific but not as sensitive as the developed PCR technique[29] or other published ELISA methods using polyclonal antibodies.[12,14,15] However, the present method is particularly suitable to carry out the quality assurance of raw materials, where the sensitivity of the technique would entail a less critical role.

Determination of Brazil Nut in Commercial Food Products. The development of analytical methods to monitor food products for the presence of Brazil nut is important not only because an undeclared presence of this nut could cause a serious health problem in sensitized individuals but also because Brazil nuts are a susceptible target for economically motivated adulteration, due to their superior value when compared with other tree nuts or peanut.[40] The applicability of the developed method for detection of Brazil nut proteins in foodstuffs was assessed through analysis of 92 commercial food products (Table 3) that could contain Brazil nut in their composition. Among the samples analyzed, 18 were labeled as containing Brazil nut as an ingredient, 15 were labeled as "containing traces of Brazil nut", 43 were labeled as "may contain traces of nuts", and the remaining 16 did not declare containing nuts. Brazil nut protein was detected in 15 of the 18 processed foods including Brazil nut in the ingredient list. Regarding the three samples that did not show positive results in ELISA, real-time PCR analysis supported negative results for the chocolate samples, but amplified Brazil nut DNA was indicated from the nut bar sample. The absence of a positive signal or DNA amplification in chocolate samples that declared to contain Brazil nut might be ascribed to a fraudulent substitution with cheaper nuts. However, the absence of positive results in the nut bar could be explained because the content of Brazil nut is below the LOD of the developed ELISA. Concerning samples that declared traces of Brazil nut, none of the 15 chocolates showed positive results, in ELISA or in real-time PCR. One alternative interpretation for these results might be that the company elaborates Brazil nut containing products and products not containing Brazil nut at the same factory and prefers to warn allergic consumers about possible cross-contaminations. Finally, no positive results were found after ELISA analysis in commercial food products that declared to contain traces of tree nuts nor in commercial food products that declared not to contain nuts, and the real-time PCR analysis supported the ELISA findings.

In summary, the results obtained demonstrate that the affinity probe isolated from the commercial library Tomlinson I is specific for Brazil nut detection and can be used in an indirect phage-ELISA to detect concentrations up to 5 mg g^{-1} of Brazil nut in commercial foodstuffs. The present work describes for the first time the isolation of recombinant antibody fragments specific for an allergenic tree nut and demonstrates that they can be used to develop immunoassays for food allergen detection based on probes that can be produced in vitro when required and do not rely on animal immunization. As a genetically well-defined probe was obtained, improvement of

Table 3. Determination of the Presence of Brazil Nut Protein in Various Commercial Processed Food Products Using the Brazil Nut ELISA

	no. of samples analyzed	Brazil nut ELISA[a]	Ber e 1 (BNS) real-time PCR[a]
Commercial Food Products That Declared Brazil Nut as an Ingredient			
chocolate	5	+ (3)/− (2)	+ (3)/− (2)
food bar	6	+ (5)/− (1)	+ (6)
muesli	2	+ (2)	+ (2)
cereal	4	+ (4)	+ (4)
biscuit	1	+ (1)	+ (1)
Commercial Food Products That Declared Traces of Brazil Nut			
chocolate	15	− (15)	− (15)
Commercial Food Products That Declared To Contain Traces of Tree Nuts			
biscuit	9	− (9)	− (9)
bread	3	− (3)	− (3)
chocolate	8	− (8)	− (8)
cereal	2	− (2)	− (2)
rice cake	1	− (1)	− (1)
food bar	5	− (5)	− (5)
granola	2	− (2)	− (2)
ice cream	3	− (3)	− (3)
candy bar	1	− (1)	− (1)
sauce	1	− (1)	− (1)
cream	3	− (3)	− (3)
beverage	5	− (5)	− (5)
Commercial Food Products That Declared Not To Contain Nuts			
chocolate	1	− (1)	− (1)
bread	2	− (1)	− (1)
beverage	3	− (3)	− (3)
biscuit	6	− (6)	− (6)
precooked meal	1	− (1)	− (1)
ice cream	1	− (1)	− (1)
powdered infant cereals	2	− (2)	− (2)

[a] A plus (+) indicates absorbance values above the LOD (ELISA) or the presence of amplification after 50 cycles (real-time PCR), and a minus (−) indicates absorbance values lower that LOD or an absence of amplification after 50 cycles (real-time PCR).

the assay sensitivity by engineering enzyme ligands, affinity maturation of the phage-antibodies, or further selecting intact VH-Vκ phages is possible.

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Notes

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ABBREVIATIONS USED

BCA, bicinchoninic acid; BSA, bovine serum albumin; CDR, complementary determining regions; ELISA, enzyme-linked immunosorbent assay; FR, framework regions; Ig-fLCs, immunoglobulin free light chains; LOD, limit of detection; LOQ, limit of quantification; OD, optical density; PBS, phosphate-buffered saline; RT, room temperature; SD, standard deviation; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; scFv, single chain variable fragment; VH, variable heavy; Vκ, variable kappa light.

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