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Abstract: Walnuts are classified as an important allergenic ingredient that can cause severe reactions in sensitized individuals. To prevent unintended exposure to products containing walnut, food manufacturers have the responsibility to declare its presence in packaged foods.

Immunochemical methods are widely used to detect walnut proteins. However, available immunoassays rely on the use of antibodies raised in animals. In this work, an affinity probe for walnut proteins has been isolated from the Tomlinson I library, and further engineered in Pichia pastoris to produce the in vivo Juglans regia Biotinylated Soluble Fragment-single chain and multimeric antibody (JrBSF-scFv). The multimeric scFv has been used to develop a direct enzyme-linked immunosorbent assay (ELISA), allowing detection of walnut in a food matrix with a limit of detection (LOD) of 1616 mg kg-1. This is the first recombinant antibody available for detection of walnut proteins. The assay is specific, only cross-reacting to some extent (2.25 %) to pecan, thus being useful as a screening tool for detection of walnut in raw or baked food matrices. Multimerization of the scFv with different avidin derivates could be of interest to improve sensitivity of the assay.

TITLE PAGE

ORIGINAL RESEARCH ARTICLE

Multimeric recombinant antibody (scFv) for ELISA detection of allergenic walnut. An alternative to animal antibodies

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Highlights

- WA walnut specific phage-scFv has been was isolated by phage display from the Tomlinson I library
- *In vivo* biotinylated scFv (JrBSF-scFv) has been produced in *Pichia pastoris*
- BThe biotinylated scFv was multimerized with ExtrAvidin-Peroxidase and used in ELISA
- LOD of direct ELISA for walnut with the multimerized JrBSF-scFv was 1616 mg kg^{-1}
- This is the first recombinant antibody available for walnut detection

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Abstract

Walnuts are classified as an important allergenic ingredient that can cause severe reactions in sensitized individuals. To prevent unintended exposure to products containing walnut, food manufacturers have the responsibility to declare its presence in packaged foods. Immunochemical methods are widely used to detect walnut proteins. However, available immunoassays rely on the use of antibodies raised in animals. In this work, an affinity probe for walnut proteins has been isolated from the Tomlinson I library, and further engineered in *Pichia pastoris* to produce the *in vivo* *Juglans regia* Biotinylated Soluble Fragment-single chain and~~biotinylated and multimeric~~ multimeric antibody (JrBSF-scFv). The multimeric scFv has been used to develop a direct enzyme-linked immunosorbent assay (ELISA), allowing detection of walnut in a food matrix with a limit of detection (LOD) of 1616 mg kg^{-1}. This is the first recombinant antibody available for detection of walnut proteins. The assay is specific, only cross-reacting to some extent (2.25 %) to pecan, thus being useful as a screening tool for detection of walnut in raw or baked food matrices. Multimerization of the scFv with different avidin derivates could be of interest to improve sensitivity of the assay.

Keywords

Phage display; *Pichia pastoris; In vivo* biotinylation; m~~M~~ultimeric scFv; ELISA; walnut detection; recombinant antibodies; food allergens; food analysis; food composition.

1. Introduction

Walnuts are amongst the most widely consumed of all commercially grown tree nuts in the world. Member of *Juglandaceae* family and seeds of *Juglans regia* L., walnuts are a highly nutritious food. The regular consumption of walnuts has been associated with decreased risk of cardiovascular disease, coronary heart disease and type II diabetes, while lessening aged related symptoms (Kris-Etherton, 2014; Rock et al., 2017). Accordingly, they are included as ingredient in many foodstuffs such as bakery products to enhance their nutrition value (Hayes et al., 2015; Mao et al., 2014; Wang et al., 2014). However, food-induced allergies are an emergent problem of public health. Among food allergens, walnut is classified as an important allergenic ingredient and frequent cause of adverse food reactions in allergic patients. Even small amounts of walnut can cause severe reactions in sensitized individuals, being a real problem of allergen management (Clark and Ewan, 2003). Food processing has the potential to alter walnut immunoreactivity due to modifications of specific epitopes in the walnut allergens. Nevertheless, boiling and roasting treatments do not affect the antigenicity of walnut proteins, while a slight decrease has been described after frying in vegetable oil at 191 ºC for 1 minute (Su et al., 2004). Only harsh conditions of pressure and temperature, like autoclaving at 2.8 atm (37 psi), 138 ºC for 15 or 30 minutes, lead to the fragmentation of proteins accompanied by a reduction of the IgE binding (Cabanillas and Novak, 2017).

The walnut, within the group of tree nuts, is a product set by the European Union that causes allergy or intolerance. To protect consumers, and in accordance with Regulation 1169/2011, it is mandatory to indicate this ingredient when it is used in the manufacture or preparation of a food and still present in the finished product, even if in an altered form (The European Parliament and the Council of the European Union,

2011). Therefore, food manufactures have the responsibility to declare the presence of walnut on packaged foods even when trace residues may be present from the use of shared equipment or the adventitious contamination of ingredients (Niemann et al., 2009; Van Hengel, 2007).

There are several methods available for the detection of walnut allergens in food products. However, immunochemical assays such as enzyme-linked immunosorbent assay (ELISA) are by far the most widely used to detect and quantify walnut allergens or proteins, due to their direct assessment of the allergen or marker protein, low set-up cost, moderate running time and no special requirements for expertise knowledge (Costa et al., 2014). One of the drawbacks of available immunoassays for walnut is that they rely on the use of polyclonal or monoclonal antibodies raised in animals, while current trends in animal welfare (European Union, 2010) encourage avoiding the use of live animals when possible.

The phage display technology allows production of recombinant antibodies of defined specificity and constant amino acid sequence without animal immunization. This method uses libraries of recombinant bacteriophages that expose functional antibody binding sites in their surface, like the single-chain variable fragments (scFv). Isolation of phage-antibody fragments of the desired specificity is achieved by an iterative biopanning procedure with the immobilized antigen (Hoogenboom et al., 1998). The use of prokaryotic expression systems for production of antibody fragments can result in unstable proteins, leading to low scFv yields (Arbabi-ghahroudi et al., 2005; Miller et al., 2005). In this sense, the use of *Pichia pastoris* as alternative to *Escherichia coli*, provides appropriate post-translational modifications and is highly productive (Cregg et al., 2000).

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In this work we describe the selection of a walnut-specific scFv from the synthetic Tomlinson I library, followed by the production and *in vivo* biotinylation of the scFv in *Pichia pastoris*. After tetramerization of the biotinylated probe with ExtrAvidin-peroxidase, a direct ELISA has been developed for detection of walnut protein in experimental food mixtures.

2. Material and methods

2.1. Materials and chemicals

The human scFv library Tomlinson I, M13 K07 helper phage and *Escherichia coli* TG1 strain (K12Δ (*lac-proAB) supE thi hsdD5/F' traD36 proA+B laclq lacZΔM15*) were obtained from Source BioScience (Nottingham, UK). The Tomlinson I library is constructed in the ampicillin resistant phagemid vector pIT2 (HIS myc tag) with a size of 1.47 $x10^8$. This repertory is based on a single human VH framework (V3-23/D47 and JH4b), paired with a single Vk (O12/O2/DPK9 and JK1). The repertory has been designed to contain short complementarity-determining region 3 (CDR3) ~~CDR3~~ of the heavy chains while maintaining good antigen binding properties, and has been displayed as a fusion with the terminal phage gene III protein.

Walnuts, other tree nuts, heterologous products, and commercial food products were acquired from local retailers and delicatessen stores in Madrid (Spain).

~~HRP~~Horseadish peroxidase/anti-M13 monoclonal mouse antibody was purchased from GE Healthcare (Little Chalfont, ~~UK~~United Kingdom). Phosphate-buffered saline (PBS) composition is 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4. Milk phosphate-buffered saline (MPBS) contains 1 % skimmed milk powder in PBS. Tris-buffered saline (TBS) composition is 0.05 M Tris-Cl and 150 mM NaCl, pH 7.6. TBST is TBS containing 0.05 % Tween

20. The protein extraction buffer consisted of 0.035 M phosphate solution containing 1 M NaCl, pH 7.5. Tryptone, yeast extract and European Bacteriological agar were purchased from Laboratorios Conda (Madrid, Spain). 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.

Low salt Luria-Bertani (LB) agar is 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl, 15 g L^{-1} agar, pH 7.5. Buffered Glycerol-complex Medium (BMGY) is 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 100 mL of 100 mM potassium phosphate, pH 6.0, 100 mL 1.34 % Yeast Nitrogen Base (YNB), 2 mL of 4×10^{-5} % biotin and 100 mL 1 % glycerol. Buffered Methanol-complex Medium (BMMY) is BMGY but adding 100 ml 0.5 % methanol instead of glycerol. Yeast Extract Peptone Dextrose Medium (YPD) is 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} dextrose and 20 g L^{-1} agar. Yeast Extract Peptone Dextrose Medium with Sorbitol (YPDS) is YPD with 1 M sorbitol.

Selection antibiotic Zeocin was purchased from Invitrogen (Carlsbad, CA, USA~~United States~~), and Blasticidin from InvivoGen (Toulouse, France). *E. coli* XL1-Blue Chemically Competent Cells (Agilent Technologies, Santa Clara, CA, USA) were employed for the propagation of plasmids, and *P. pastoris* X-33 strain (Invitrogen) was used for scFv and biotin ligase (BirA) enzyme expression. *P. pastoris* expression vectors pPICZαB and pPIC6αA were purchased from Invitrogen. Restriction enzymes *PstI*, *NotI*, *XbaI* and *SacI*, calf intestinal alkaline phosphatase, T4 DNA ligase, and GoTaq DNA Flexi Polymerase were purchased from Promega (Madison, WI, USA). Plasmid purification kit (QIAGEN Plasmid Midi Kit), PCR product purification kit (QIAquick PCR Purification Kit) and gel extraction kit (QIAquick Gel Extraction Kit) were purchased from Qiagen (Hilden, Germany).

HiTrap Protein L Column was purchased from GE Healthcare. Methanol was purchased from Fisher Scientific (Loughborough, UK). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of protein extracts

All food samples (5 g) were ground using an IKA A11 analytical mill (IKA®, Staufen, Germany), and stored in screw-capped vials at - 20 ˚ C. The sample (200 mg) was mixed with 1200 µL of protein extraction buffer, and the mixture was shaken for 10 min at room temperature in a vertical rotator (HulaMixer Sample Mixer, Invitrogen) to extract soluble proteins. The slurry was centrifuged at 10,000 g for 10 min at 4˚ C, and the supernatant was filtered through a 0.45 mm syringe filter (Sartorius, ~~Göttingen~~ Gottingen, Germany). Bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc., IL, USA) was employed to determine protein concentration. Protein extracts were stored at - 20 ˚ C until further use.

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2.3. Selection of scFv against walnut by phage display

Preparation of the Tomlinson I phage display library for biopanning procedure was performed as described in the manufacturer's protocol. Following amplification of the library and poly-ethylene glycol (PEG)/NaCl phage precipitation, phages were tittered, and kept at 4 ºC for short term storage or at – 80 ºC in 15 % glycerol for longer term storage.

Polystyrene paddles and magnetic beads were alternately used for target immobilization to avoid the isolation of unspecific phages which would produce false-positive results. For the first and third rounds of selection, polystyrene paddles (Nunc, Denmark) with a surface area of 5.2 cm^2 were coated with 1 mL of 100 µg

180 mL^{-1} walnut extract (positive screening) or pecan nut extract (negative screening) in
181 PBS, and incubated overnight at 4 ˚C. Then, paddles were washed three times with
182 PBS and blocked with 3 % bovine serum albumin (BSA) at 37 ˚C for 1 h.
183 For the second round of selection, Dynabeads M-280 Tosylactivated (Invitrogen)
184 were used to bind the target proteins following manufacturer's instructions. Briefly,
185 5 mg of Dynabeads were coated with 100 µg of walnut proteins (positive panning) in
186 0.1 M Na-phosphate buffer, pH 7.4, to a final volume of 150 µL and then, 100 µL of
187 3 M ammonium sulphate in Na-phosphate buffer was added. Coupling procedure was
188 performed on a vertical rotator at 37 ˚C overnight. Next day, Dynabeads were blocked
189 with 1 mL of 0.5 % BSA in PBS for 1 h at 37 ˚C with rotation. The same procedure
190 was performed with the Dynabeads used for negative panning, but employing a pecan
191 nut protein extract as the ligand.
192 Three rounds of biopanning were performed for selection of walnut-specific phage-
193 scFv, as previously described (Madrid et al., 2017) with the following modifications:
194 approximately 10^{12} phage particles from Tomlinson I library were resuspended in 2
195 mL of 3 % BSA in PBS and added to the pecan nut-coated polystyrene paddle. The
196 mixture was incubated at 25 ˚C for 60 min on a rotator to capture phage-scFv
197 recognizing pecan nut (negative panning). The supernatant containing unbound phage
198 particles was added to the walnut coated paddle (positive panning) and incubated at
199 25 ˚C for 60 min with rotation, and for further 60 min without rotation. After positive
200 panning, unbound phages were removed by washing 10 times with PBS, and phages
201 specifically bound to walnut proteins were eluted by adding 500 µL of trypsin
202 solution (1 g L^{-1} trypsin in PBS) for 10 min at room temperature with rotation. A total
203 of 250 µL of the eluted phages was used to infect 1.75 mL of a TG1 cell culture at an
204 OD_{600} of 0.4, and incubated 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 10 g L^{-1} glucose, and grown overnight at 37 ˚ C. Titre of eluted phage was also determined. Following overnight incubation, *E. coli* colonies were scraped into 2 mL of 2xTY containing 15 % glycerol and stored at -80 ˚ C (labelled as first round stock). To amplify the phages for the second round of selection, 50 µL of recovered bacteria from the first panning experiment were inoculated into 50 mL of 2xTY containing 100 µg mL^{-1} ampicillin and 10 g L^{-1} glucose, and incubated at 37 ˚ C until reaching an OD_{600} of 0.4. Then, 10 mL of the culture was infected with 5×10^{10} particles of helper phage, and incubated at 37 º C for 30 min. Bacterial cells were pelleted and resuspended in 100 mL 2xTY containing 100 µg mL^{-1} ampicillin, 50 µg mL^{-1} kanamycin and 0.1 % glucose, and incubated overnight at 30 º C. Next day, phage particles from the supernatant were PEG/NaCl precipitated, and resuspended in 1 mL of PBS, and tittered before being used for the second round of selection. A second round of selection was performed like the first one, but employing 2.5 mg of Dynabeads instead of polystyrene paddles, and increasing the number of washes to 20. The third round of selection was carried out exactly like the first one.

2.4. Indirect Phage Enzyme-Linked Immunosorbent Assay (ELISA)

Polyclonal phage- ELISA was used to assess enrichment of the phage display library with walnut binding phages after each round of selection, while monoclonal phage ELISA was used for analysis of individual clones.

Flat-bottom polystyrene microtiter plates (F96 MaxiSorp Nunc immuno plates, Nunc, Denmark) were coated with the appropriate dilutions of the protein extracts assayed (walnut, heterologous species or experimental mixtures) in PBS for 16 h at 4 º C. Then, the plates were washed 3 times and blocked with 200 µL of MPBS for 1 h at

230 37 ˚C. After washing 3 times, 1 µL of precipitated phages (containing approximately
231 10^{12} phage particles) was added to each well, diluted in 100 µL of MPBS, and plates
232 were incubated for 1 h at room temperature. After washing 10 times, plates were
233 incubated at room temperature for 1 h with 150 µL of HRP/anti-M13 monoclonal
234 mouse antibody diluted 1:5000 in MPBS. Finally, plates were washed 5 times, and
235 100 µL of tetramethylbenzidine substrate solution was added to each well, and plates
236 were incubated with shaking in the dark. Colour development was performed for 10
237 min at room temperature before addition of 50 µL 1 M sulphuric acid to stop reaction.
238 OD_{450} was measured with an iEMS Reader MF (Labsystems, Helsinki, Finland). All
239 washing steps were performed with PBS. All experiments were performed in
240 triplicate.
241 Monoclonal walnut phage ELISA was used to assess the ability of single clones to
242 recognize walnut proteins. With that purpose, 95 individual colonies from the second
243 and third rounds of selection were randomly picked and inoculated in separate wells
244 of cell culture microplates (Nunc, Denmark) containing 200 µL 2xTY with 100 µg
245 mL^{-1} ampicillin and 10 g L^{-1} glucose. Plates were grown for about 2 h at 37 ˚C with
246 shaking (250 rpm). One hundred microlitres from each well was transferred to a
247 second microplate, and 25 µL 2xTY, with 100 µg mL^{-1} ampicillin and 10 g L^{-1}
248 glucose containing 10^9 particles of helper phage were added to each well. After 1 h
249 incubation at 37 ˚C, the plates were centrifuged at 1800 g for 10 min at 4 ˚C.
250 Supernatants were discarded, and bacterial pellets were resuspended in 200 µL 2xTY
251 containing 100 µg mL^{-1} ampicillin, 50 µg mL^{-1} kanamycin and 1 g L^{-1} glucose, and
252 incubated overnight at 30 ˚C. Next day, plates were centrifuged at 1800 g for 10 min,
253 and 50 µL of the phage supernatants diluted in 50 µL MPBS were employed in
254 monoclonal phage ELISA as described above, instead of precipitated phage particles.

255

256 2.5. Sequence analysis

257 Polymerase Chain Reaction (PCR) amplification of walnut-recognizing clones was
258 carried out from single colonies to check for the presence of full length V_H and V_k
259 inserts using My Taq Mix 2x (Bioline Reagents Limited, London, UK) and primers
260 LMB3 and pHENseq (Table 1). The following PCR program was used: 95 ˚C for 9
261 min, then, 95 ˚C for 30 s, 62 ˚C for 30 s, 72 ˚C for 30 s for 30 cycles, and final
262 extension at 72 ˚C for 7 min. PCR products were examined by electrophoresis on
263 1 % agarose gel.
264 Sequencing of phagemid DNA from the clones that presented a complete VH + Vk
265 fragment was performed as previously described (de la Cruz et al., 2015).
266 Nucleotide sequences were compared using European Molecular Biology Open
267 Software Suite (Emboss software), and then analysed with Ig BLAST to determine
268 framework and complementary determining regions (CDR) of the VH and Vk chains.
269 Amino acid sequences were deduced from the nucleotide sequences by Expasy
270 website (www.expasy.org).

271

272 2.6. Vectors construction

273 Vector pMJA186 was derived from pPICZαB with the following modifications: the
274 nucleotide sequence encoding the walnut-specific scFv (JR35) was amplified from the
275 corresponding phagemid pIT2 using a high fidelity DNA polymerase with primers
276 MJA254 and MJA253 (Table 1). The purified PCR product was digested with *Pst*I
277 and *Not*I and cloned between the *Pst*I and *Not*I sites in the pPICZαB plasmid.
278 Moreover, sequence encoding the biotin-accepting domain (BAD) was obtained by
279 enforcing hybridization of primers MJA257 and MJA258. Hybridized BAD

nucleotide sequence was then digested with *Not*I and *Xba*I, and ligated into the *Not*I and *Xba*I sites of the vector. Correct orientation of the insert (scFv + BAD) was assessed by DNA sequencing with primers MJA254 and MJA259 at the Genomics unit of Universidad Complutense de Madrid.

Vector pMJA180 (de la Cruz et al., 2016) contains the nucleotide sequence codifying Bir A enzyme (GenBank accession no. P06709) ligated between *Eco*RI and *Sac*II sites of pPIC6αA plasmid.

2.7. Transformation of *E. coli*

Competent *E. coli* XL1-Blue cells were transformed according to manufacturer's protocol. Once transformed, cells were spread on prewarmed low salt Luria-Bertani agar plates containing the selective antibiotic (25 μg mL^{-1} Zeocin for plasmid pMJA186, and 100 μg mL^{-1} Blasticidin for plasmid pMJA180). Plates were incubated overnight at 37 ˚C.

2.8. Transformation of *P. pastoris*

To direct the scFv + BAD and the BirA enzyme into the yeast secretory pathway, the codifying sequences were inserted in frame with the methanol inducible 5'-AOX1 promoter, the α-factor secretion signal and the AOX1 transcription terminator.

The *Sac* I linearized pMJA186 expression vector was precipitated by ethanol and transformed into *P. pastoris* X-33 with a BioRad MicroPulser electroporation apparatus (Bio-Rad, Hemel Hempsted, UK) using the following parameters: 2,5 V, 24 μF, 400 ohm. Transformed cells were selected on YPDS agar supplemented with 100 μg mL^{-1} Zeocin for 72 h at 30 ˚C. Ninety-five individual clones were screened for scFv production by inoculation in 200 μL YPD medium with 100 μg mL^{-1} Zeocin and

overnight growth at 30 ˚C with shaking, followed by overnight growth in 1 mL BMGY medium with 100 μg mL^{-1} Zeocin at 30 ˚C in 24-well Costar plates (Cultek, Spain). After centrifugation of the plates, the cells were resuspended in BMMY medium to induce scFv expression, and methanol (1 %) was replenished every 12 h for 72 h. Finally, plates were centrifuged (1800 g, 10 min, 4 ˚C) and the supernatant was analysed by dot-blotting in search for clones expressing and secreting the scFv, as previously described (de la Cruz et al., 2016).

Following dot-blotting analysis, a single clone was selected based on the intensity of the signals obtained. The selected clone was transformed with the second *P. pastoris* expression vector, pMJA180, and transformed cells were grown on YPDS agar plates containing 100 μg mL^{-1} Zeocin and 500 μg mL^{-1} Blasticidin for 72 h at 30 ˚C. Isolated colonies were picked from the selective agar plate and induced with methanol following the microscale induction described above. Supernatants were analysed by dot-blotting to check for the presence of biotinylated scFv using ExtrAvidin-Peroxidase (Sigma-Aldrich, SKU E2886) (1:5000 v/v) in 1% BSA for detection, and the membrane was developed with the chemiluminescent substrate Clarity Western ECL (Bio-Rad).

A single clone was selected again, based on signal intensity obtained in the dot-blotting analysis, and called JrBSF (*Juglans regia* Biotinylated Soluble Fragment). The insertion of both plasmids in the genomic DNA of the selected clone was assessed by PCR with the primer pairs MJA254/MJA259 (for scFv-BAD) and MJA255/MJA256 (for BirA).

2.9. Biotinylated scFv production and purification

The clone JrBSF was grown overnight at 30 ˚C in 10 mL of YPD with 100 µg mL^{-1} Zeocin and 500 µg mL^{-1} Blasticidin. Then, 1 mL of this culture was inoculated in 600 mL BMGY containing 100 µg mL^{-1} Zeocin and 500 µg mL^{-1} Blasticidin, and incubated for 18 h at 30 ˚C with shaking. After centrifugation at 4000 g for 15 min at 4 ˚C, cells were induced for 72 h in 600 mL BMMY, with methanol being replenished every 12 h. The culture was then centrifuged at 4000 g for 20 min at 4 ˚C to remove yeast cells.

The supernatant containing biotinylated scFv was filtered through a 0.4 µm membrane filter (Millipore, Darmstadt, Germany) and loaded onto a 1 × 1 mL HiTrap protein L column (GE Healthcare Life Sciences) attached to an ÄKTA purifier FPLC system (GE Healthcare, Sweden). Three hundred millilitres of supernatant were loaded onto the PBS equilibrated column, and the biotinylated scFv eluted with 0.1 M glycine-HCl (pH 2.7) as previously described (de la Cruz et al., 2016). Recovered fractions were pooled and dialyzed against PBS buffer employing Amicon Ultra-15 Centrifugal Filter Units (Millipore) with a pore size~~n~~ MWCO of 10 kDa. Protein concentration was measured in a Nanodrop (Thermo Scientific, Waltham, MA, USA), adjusted to 2 mg mL^{-1} of total protein, and stored in 100 µL aliquots at −80 ˚C until further use.

2.10. Multimerization of biotinylated scFv

ExtrAvidin- peroxidase (Sigma-Aldrich) was used as a core a molecule for multimerization of biotinylated scFv, following the NIH Tetramer Core Facility guidelines (http://tetramer.yerkes.emory.edu/support/protocols#10). Briefly, 0.5 µL ExtrAvidin-HRP solution (2.5 mg mL^{-1}) was added every 10 min up to a total of 10 times to an aliquot of 100 µL (200 µg) of biotinylated scFv. The reaction was carried out at room temperature in the dark, and with continuous but gentle rotation in a

sample mixer (HulaMixer Sample Mixer, Life Technologies). Multimerized scFv tubes were kept in the dark at 4 ˚C until further use.

2.11. ScFv multimerization assessment

Peptide mass fingerprinting and analytical ultracentrifugation methods were used for multimerization assessment. Multimerized scFvs were concentrated using an Amicon Ultra 50 kDa filtration unit (Merck Millipore, Darmstadt, Germany) and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 12 % in non-reducing conditions. The gel was stained with Coomassie Brilliant Blue R-250, and the bands of interest were cut out with a scalpel and immersed in a solution of 5% (v/v) acetic acid. Peptide mass fingerprinting was performed in a 4800 Plus MALDI TOF/TOF Analyzer mass spectrometer (AB SCIEX, MA, USA), at the Proteomics Unit, Universidad Complutense de Madrid (Spain).

Interpretation of the mass spectra data into protein identities was performed with the Mascot search engine software (http://www.matrixscience.com) (Matrix Science Ltd., London, UK) using the SwissProt database. Search parameters employed were: trypsin enzymatic cleavage, one possible missed cleavage allowed; peptide mass tolerance of ± 80 ppm; fragment mass tolerance of ± 0.3 Da; peptides were assumed to be monoisotopic; carbamidomethyl fixed modification; and methionine oxidation variable modification.

Ultracentrifugation analyses of the multimerized scFv were carried out at Instituto de Química-Física Rocasolano, CSIC, Madrid (Spain) as previously described (de la Cruz et al., 2016).

2.12. Preparation of binary mixtures

379 To evaluate the sensitivity of the assay, binary mixtures of raw walnut in wheat flour
380 (10^5 to 100 mg kg^{-1}) were prepared using a food processor (Thermomix, Vorwerk,
381 Germany) as follows: Concentration of 10^5 mg kg^{-1} was prepared by adding 10 g of
382 ground walnuts to 90 g of wheat flour. Then, 10 g of the former mixture was added to
383 90 g of wheat flour to obtain 10^4 mg kg^{-1}. Concentrations of 10^3 mg kg^{-1} and 100 mg
384 kg^{-1} were made in a similar way with the previous mixtures. Additional mixtures of 5
385 x 10^4, 2.5 x 10^4, 5 x10^3, and 500 mg kg^{-1} were prepared by mixing 25 g of wheat flour
386 with 25 g of the mixtures containing 10^5, 5 x 10^4, 10^4 and 10^3 mg kg^{-1} respectively.
387 To determine the effect of heat treatment on scFv's ability to identify walnut protein,
388 30 g of ground walnut were processed in an oven at 160 ˚C for 13 min. Heat treated
389 ground walnut samples were mixed in wheat flour as described above for raw walnut
390 mixtures. Protein extracts from binary mixtures were prepared following the
391 procedure described in Section 2.2.
392
393 2.13. Direct ELISA with multimerized scFv
394 The protein extracts from walnut/wheat flour binary mixtures and commercial food
395 products were diluted 1:100 in PBS to coat the wells of microtiter plates for 16 h at 4
396 ºC. Next day, the plates were washed three times with TBS and blocked with 200 µL
397 3 % BSA in TBS for 1 h at 37 ˚C. After washing 3 times, 100 µL of multimerized
398 scFv stock (2 mg mL^{-1}) diluted 1:500 (v/v) in TBST with 1 % BSA, was added to
399 each well, and plates were incubated for 2 h at room temperature with shaking in the
400 dark. After washing 10 times with TBS, 100 µL of tetramethylbenzidine substrate
401 solution was added to each well and the plates were incubated at room temperature
402 with shaking for 10 min. Fifty microliters of 1 M sulphuric acid was added to stop
403 reaction and OD_{450} was measured with an iEMS Reader MF. All experiments were

performed in triplicate. To check for non-specific reactions, different wells were coated with walnut protein extract and incubated with 2 μg mL^{-1} of monomeric scFv (without ExtrAvidin) or with 0.125 μg mL^{-1} of ExtrAvidin-HRP (without scFv). A calibration curve of different concentrations of walnut in wheat flour (10^6–100 mg kg^{-1}) was included in each plate. The concentration-response curves obtained by plotting the absorbance values vs. the log of walnut protein concentration, was fitted to the four-parameter logistic equation using Origin 8.0 software (OriginLab Crop.,USA).

2.14. Assay validation

The specificity of the assay was assessed by challenging the isolated phage-scFv clones to protein extracts obtained from different animal and plant species (Table 2) that had been previously diluted 1:200 in PBS. Each sample was analysed in triplicate. The results obtained by analysis of food samples with multimeric-scFv ELISA were compared to those obtained by a walnut-specific real time PCR method (López-Calleja et al., 2015). The limit of detection (LOD) was calculated following the guidelines of the International Union of Pure and Applied Chemistry (IUPAC) (Thompson et al., 2002). The LOD for the binary mixtures of wheat flour matrix spiked with walnut was also determined, but employing wells coated with wheat flour as blank.

Data were analysed 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).

3. Results and discussion

3.1. Enrichment of the Tomlinson I library in walnut-specific phage-scFv clones

Phage display technology is a powerful tool for the isolation of recombinant antibody fragments. Using this technology and the Tomlinson I library, target specific phage-scFv clones were enriched through the "biopanning" process. In this work, walnut-specific clones were isolated through three rounds of selection or biopanning using as a target a protein extract from shelled and peeled crude walnut. The walnut skin or seedpod was removed because it contains tannins, polyphenols that bind and precipitate proteins, and can hinder the process of binding walnut proteins with the phage-scFv repertoire (Sze-Tao and Sathe, 2000). Enrichment in walnut recognizing phage-scFv occurred along the rounds of panning. However, the increase of the ratio between the input number of phage particles (10^{12} pfu mL^{-1}) and the phage particles recovered at the end in each round was lower than expected. The number of phage particles recovered after first biopanning was 7×10^5 pfu mL^{-1}, being of 1.75×10^6 pfu mL^{-1} after the second round, and 10^6 pfu mL^{-1}after the third round of panning. Compared to the guidelines described for phage display technology (Lee et al., 2007) and our previous experience (de la Cruz et al., 2015, 2013), the increase between rounds should be 100 times. With an input of 5×10^{12} phages, approximately 10^5- 10^7 bacterial colonies were expected after the first and second rounds of selection. In the third round the titre should rise to 10^7-10^9. Nevertheless, between the first and second rounds of selection eluted phages raised only 2.5 times, and between second and third rounds of panning the titre did not increase further. These results could indicate that methodology of negative biopanning with a closely related but non-target protein (pecan extracts) eliminates a part of the walnut reactive phages, selecting exclusively the most specific phage-scFv.

To confirm this hypothesis, a polyclonal phage -ELISA was performed with phage pools collected from the three rounds of selection. The results showed that the highest absorbance values for walnut proteins corresponded to the second and third rounds, and very low cross-reactivity was found to wells coated with bovine serum albumin (BSA), pecan and peanut (Madrid et al., 2017). Thus, according to these results, the second round of panning allowed selection of the phage population that specifically recognised walnut, and additional rounds of selection were not necessary.

3.2. Screening of individual phage-scFv clones by monoclonal phage ELISA

Monoclonal phage ELISA was performed to isolate and identify the scFvs that recognised walnut protein. Ninety five *E. coli* TG1 colonies from each the second and third rounds of panning were picked to be analysed. A total of 8 out of 95 clones (8.4 %) from the second round and 3 out of 95 clones (3 %) from the third round were considered as positive clones using the criteria of binding to walnut extract and not peanut extract, used as negative control, with a walnut/peanut ratio > 5 (absorbance values against walnut/absorbance against negative control). Precipitated phage-scFv from those 11 selected clones were also analysed in monoclonal ELISA, and only 6 clones were selected for further analysis, based on the stability of the results.

3.3. PCR and sequence analysis of the positive clones

The six positive clones selected from the previous step were amplified by PCR with primers LMB3 and pHEN, and PCR products were analysed in agarose gel to estimate the proportion of clones containing the complete V_H–V_L insert (approximately 935 bp). Only one clone (JR35) analysed showed a band with the expected size, and thus was selected for additional characterization. Plasmid DNA sequencing was

478 performed to determinate the immunoglobulin framework, linker and complementary
479 determining regions (CDRs) of the VH and VL chains of the scFv, and the amino acid
480 sequence was deduced from nucleotide sequence through Expasy web (Madrid et al.,
481 2017).
482
483 3.4. Co-transformation of into *P. pastoris* with constructed vectors
484 *Pichia pastoris* is a widely used expression system that improves the production of
485 recombinant and heterologous proteins either intracellularly or extracellularly, thanks
486 to the simplicity of techniques needed for the molecular genetic manipulation of this
487 yeast and the capability of performing many eukaryotic post-translational
488 modifications (Cereghino and Cregg, 2000). Expression of any foreign gene in *P.*
489 *pastoris* requires the insertion of the gene into a vector, transformation of *P.*
490 *pastoris* genome with the expression vector and examination of potential
491 transformants for expression of the foreign gene product. Many vectors for
492 transformation of *P. pastoris* and their DNA sequences are available
493 (http://www.invitrogen.com). In this work, the biotin-accepting domain (BAD)
494 sequence was inserted at the C-terminus of the scFv into the vector of expression
495 pPICZαB, resulting in plasmid pMJA186 (Figure 1) to create a potential biotinylation
496 site in the scFv sequence of the JR35 clone. In addition to BAD sequence, the scFv
497 expressed by the *P. pastoris* clones contained a *c-myc* epitope (EQKLISEEDL) and a
498 poly histidine tail that allow its purification and detection. The production of the
499 soluble specific scFv fragments by 95 transformed clones of *P. pastoris* was
500 confirmed by dot-blotting of the supernatants after methanol induction. This dot-blot
501 screening step is very useful to assure selection of successfully transformed clones
502 that express the protein of interest (Neophytou and Alcocer, 2017). One of the highest

503 expresser clones (named pMJA186-G2) was randomly selected to prepare competent
504 cells to proceed with the second transformation with pMJA180 vector, that codifies
505 for the biotin ligase (BirA) enzyme. In order to improve transformation, the dominant
506 antibiotic makers available for *P. pastoris* were used: *Sh ble* gene from
507 *Streptoalloteichus hindustanus* (Zeocin resistance) (Drocourt et al., 1990) and the
508 blasticidin S-deaminase gene from *Aspergillus terreus* (blasticidin resistance)(Kimura
509 et al., 1994). Blasticidin concentration was increased up to 500 µg mL^{-1} to ensure the
510 selection of cotransformed clones. DNA from the co-transformed clone named JrBSF
511 was analysed to demonstrate the presence or absence of the scFv and BirA sequences.
512 PCR with primers MJA254 and MJA259 confirmed that the clone JrBSF contained a
513 780 kb fragment consisting of the scFv linked to BAD nucleotide sequence (Figure
514 2A, lane 3) codified by plasmid pMJA186. Moreover, PCR with primers MJA255 and
515 MJA256 demonstrated the presence of a band of about 975 kb, corresponding to BirA
516 nucleotide sequence (Figure 2B, lane 3) codified by plasmid pMJA180, confirming
517 the co-transformation with the two vectors in clone JrBSF. On the contrary,
518 pMJA186-G2 clone only produced the 780 kb band, corresponding to the pMJA186
519 vector (Figure 2A, lane 2), but the band for BirA nucleotide sequence was absent
520 (Figure 2B, line 2).
521
522 3.5. Expression of biotinylated scFv by co-transformed JrBSF clone
523 Many conditions could influence heterologous protein production in *P. pastoris*.
524 Expression of foreign genes inside the methanol pathway (AOX1) is repressed by
525 glucose, glycerol and ethanol, but strongly induced by methanol, increasing
526 concentration of the soluble protein in the culture medium with cell density (Cregg et
527 al., 2000; Demain and Vaishnav, 2009).

To optimize production of biotinylated scFv, the JrBSF clone was grown in buffered media (BMGY and BMMY) as induction medium pH values of 6.5-8.0 have been found the most appropriate for scFv production (Shi et al., 2003). The BirA enzyme, also produced by JrBSF clone, would catalyse the strong binding of a biotin molecule to the acceptor peptide attached to the scFv, resulting in a straightforward production of *in vivo* biotinylated scFv. Production of the expected walnut-specific biotinylated scFv in the culture supernatants of JrBSF clone was assessed by dot-blotting analysis (Figure 3). The polyvinylidene difluoride (PVDF) membrane was coated with culture supernatants from pMJA186-G2 and JrBSF clones before and after methanol induction. When the membrane was revealed with anti-c-myc antibody (Figure 3A), scFv was detected in supernatant from both methanol induced cultures. Nevertheless, the membrane containing the same supernatants but developed with ExtrAvidin-Peroxidase (Figure 3B) demonstrated that only the *P. pastoris* co-transformed clone (JrBSF) was capable to produce biotinylated scFv. These results confirm that this unique clone (JrBSF) was effective in the co-expression of both foreign genes and production of functional BirA enzyme. In contrast with *in vitro* biotinylation methods (Li and Sousa, 2012) that require the previous production and purification of enzyme, in this work the biotinylation was performed *in vivo*. This *in vivo* biotinylation technology can be applied for protein purification, analysis of protein localization, and protein-protein interaction mainly in eukaryotic yeast cells (de la Cruz et al., 2016; Neophytou and Alcocer, 2017).

One of the advantages of the use of *P. pastoris* for production of foreign proteins is that the secreted heterologous protein comprises the vast majority of the total protein in the medium (Cregg et al., 2000).

552 An affinity chromatography column (HiTrap protein L) was used to purify the
553 biotinylated scFv from the JrBSF culture supernatant. This column consists of an
554 agarose matrix linked to protein L, which presents affinity towards the variable region
555 of the kappa light chain of immunoglobulins and immunoglobulin fragments (Lee et
556 al., 2007; Ma and O'Kennedy, 2015). The purification process rendered 6 mL of
557 biotinylated scFv (2 mg mL^{-1}) that were distributed in 100 µL aliquots of and kept
558 frozen at -80 ºC.
559
560 3.6. Production and characterization of multimeric scFv
561 Avidin is a tetrameric protein which binds one biotin molecule per subunit with a very
562 high affinity (Kd = 4 x 10^{-14} M). Due to this property, avidin and streptavidin have
563 been widely used to produce tetramers of various biotinylated ligands, including
564 antibody fragments (Kipriyanov et al., 1995). Because recombinant antibodies
565 isolated from naïve libraries lack affinity maturation undergone by antibodies raised
566 in animals, tetramerization of biotinylated scFv has been used to increase affinity for
567 the antigen, thus improving avidity and signalling in enzyme-linked immunosorbent
568 assays (Cloutier et al., 2000).
569 The walnut-specific biotinylated scFv antibodies were transformed in multivalent
570 scFv by means of ExtrAvidin-HRP to be used in ELISA. To demonstrate
571 multimerization of the scFv, a sodium dodecyl sulfate polyacrylamide gel
572 electrophoresis (SDS-PAGE) ~~SDS-PAGE~~ in non-reducing conditions of monomeric
573 and multimeric scFv was carried out (Figure 4). Electrophoretic analysis of
574 multimeric scFv showed a band with a molecular weight of about 220 kDa that was
575 not present in the monomeric scFv, and might correspond with the expected size of
576 the tetramers (≈ 230 kDa). To confirm this hypothesis, the band was excised and

trypsinized to be identified by matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-TOF/TOF). Comparison to protein database showed that the band contained a mixture of peptides identified as peroxidase from *Armoracia rusticana*, Ig heavy chain from *Homo sapiens* and a human Ig light chain variable region that shared the same CDR2 than the JrBSF scFv (Table 3). This result is consistent with the presence of a JrBSF tetramerized scFv. In addition, when the mass spectrometry (MS) results were compared to the amino acid sequence of the JrBSF, the coverage was 34% (Table 3).

Sedimentation velocity experiments were carried out to study the degree of multimerization. Ultracentrifugation analyses showed differences between the sedimentation coefficient (S) of monomeric biotinylated scFv and the scFv tetramerized with ExtrAvidin-HRP (Figure 6). Although Extravidin-HRP is not a homogeneous reagent, it presented a main peak (A) with a S value of 6.33, and an approximate Mw of 107 kDa. The value corresponding to biotinylated monomeric scFv was 2.63 S (Mw 25.1 kDa). The scFv fused to ExtrAvidin-HRP showed a different profile than their isolated components, with the appearance of a new broad peak (B) of 8.3 S and Mw_{app} 143 kDa, and a second peak (C) of 13.3 S (Mw of 290 kDa), consistent with the addition of at least two biotinylated scFv molecules to a single ExtrAvidin-peroxidase core. Even though four molecules of biotin would be able to join with an ExtrAvidin core, our results only supported that most of the ExtrAvidin molecules would join just two biotinylated scFv. The conjugation of peroxidase to avidin would hide biotin binding sites in the avidin molecule, hampering the production of complete tetramers. This fact was also observed by de la Cruz et al., 2016.

3.7. Direct ELISA with multimeric scFv

The multimerized JrBSF scFv was used to detect walnut protein by a direct ELISA. Analysis of walnut samples from different geographic origins (Spain and California) showed the same absorbance values in the direct ELISA (result not shown). Moreover, the assay was able to detect spiked walnut proteins in a wheat flour matrix in a concentration-dependent manner (Figure 7). The limit of detection (LOD) of raw walnut in the binary mixture after six triplicate experiments, performed in different days, was 1616 mg kg^{-1}. Compared to the indirect phage-ELISA results obtained for the same binary mixture using the JR35 phage-scFv (LOD 6378 mg kg^{-1}), it can be concluded that, as expected, tetramerization of the scFv substantially improved the assay sensitivity (Figure 7). Moreover, the direct ELISA performed with multimeric scFv is faster and requires less handling than phage-scFv ELISA.

The effect of heat treatments on the assay ability to detect walnut proteins has been also analysed. Baking (160 ºC / 13 min) was applied to ground walnuts to prepare experimental binary mixtures in a wheat flour matrix. Under these conditions, and performing triplicate experiments in six different days, the LOD for the baked walnut binary mixture was 2466 mg kg^{-1}. According to these results, baking may denature to some extent the epitope recognized by the multimeric scFv in the walnut protein, raising the LOD from 1616 to 2466 mg kg^{-1} in a food matrix.

The close phylogenetic relationships among walnut, pecan and tree nut species, together with the varied number of plants and animal components that can be present in different commercial food products, indicates the need to check the cross reactivity of the ELISA against a wide range of species. Specificity was assessed by analysis of protein extracts from 63 non target species (Table 2), including nine tree nuts, 48 different plant species and six animal species. Only pecan nut extract showed

absorbance values different than the blank. When raw pecan extract was analysed, the concentration in ELISA with multimerized scFv was estimated 22541 mg kg^{-1}, (2.25 % of raw walnut value, 10^6 mg kg^{-1}). Cross-reactivity with pecan nut has been frequently reported in ELISA kits and published methods for detection of walnut. Pecan nut belongs to the same botanic family (*Juglandaceae*) and presents allergenic proteins like albumins with 92 % of sequence similarity with walnut. Cross-reactivity with tree nuts (pistachio, hazelnut, Brazil nut, chestnut, pine nut) and other plant species (quinoa, sesame, buckwheat and soybean) are also referred to be frequent (Costa et al., 2014; Niemann et al., 2009; Wang et al., 2014). The cross-reactivity of 2.25 % to pecan observed with the multimeric JrBSF scFv makes this ELISA not specific enough for detection of walnut in pecan-containing products. However, this ELISA is highly specific for walnut regarding all the rest of food matrices analysed. Applicability of the direct ELISA using multimerized scFv was assessed through analysis of 30 food products (Table 4) that declared or may contain walnut in their composition. Ten of the analysed products declared walnut as ingredient, ten declared to contain tree nuts different than walnut or traces, and ten did not declare to contain tree nuts or traces. The results obtained by analysis of these food samples with multimeric-scFv ELISA were compared to those obtained by a walnut-specific real time PCR method (López-Calleja et al., 2015). Walnut was detected in 7 out of 10 processed foods that included walnut as ingredient in the label. The three samples (a chocolate, a bread stick with nuts and soy, and a yogurt) that showed negative results in ELISA, had amplifiable DNA (Positive amplification control with Cp values lower than 16). However, while the chocolate and the bread stick were also negative by real-time PCR for walnut, DNA from the yogurt sample was amplified with the walnut-specific PCR. Lack of detection of walnut protein and DNA in the chocolate and

bread samples might be due to a fraudulent substitution by other nuts or incorrect labelling, as real-time PCR is consistent with ELISA results. However, the absence of a positive ELISA result in the yogurt sample, that declared to contain 0.1 % walnuts (1000 mg kg^{-1}), can be explained by the LOD of the ELISA, that is higher (1616 mg kg^{-1}) than the walnut content declared. Regarding the ten samples that declared tree nuts different than walnut, or traces of tree nuts, walnut was detected in three breakfast cereal samples with ELISA, but only two of these samples resulted positive in walnut-PCR. The three positive samples declared pecan nut, but not walnut, so they were also analysed by pecan-specific real time PCR (López-Calleja et al., 2015). Pecan DNA was detected in all of them. According to the results obtained, two of the samples were incorrectly labelled, as they contained undeclared walnut, but the third sample only contained pecan nut as stated in the label. The positive result obtained for these samples can be explained by their high pecan content (2 % pecan, 4 % pecan + Brazil nut, and 16 % almond + hazelnut + Brazil nut + pecan). Even though cross-reactivity of the walnut-ELISA was only 2.25 % with pecan, the presence of walnut and pecan in two of the samples, and a high amount of pecan in the third one, explains the result obtained, and should be considered for analysis of commercial products containing pecan as ingredient.

Conclusion

In summary, an affinity probe for walnut proteins has been isolated from the Tomlinson I library, and engineered in *Pichia pastoris* to produce the *in vivo* biotinylated and multimeric JrBSF-scFv, allowing detection of walnut in a food matrix with a LOD of 1616 mg kg^{-1}. For the first time, recombinant antibody technology that does not rely on animal immunization has been successfully used for

production of a specific probe for detection of allergenic walnuts in food products.~~The present work describes for the first time the isolation of recombinant antibody fragments specific for walnut and its multimerization with an ExtrAvidin-HRP core, demonstrating that this procedure can be used to develop immunoassays for food allergens detection based on homogeneous probes that do not rely on animal immunization.~~ The LOD of the walnut assay develop is higher than that of other reported immunoassays (Doi et al., 2008; Niemann et al., 2009; Yang et al., 2014). However, the multimeric JrBSF scFv is specific, only cross-reacting to some extent (2.25 %) to pecan, thus being useful as a screening tool for detection of walnut in food matrices either raw or baked. Multimerization of the scFv with different avidin derivates could be of interest to improve sensitivity of the assay.

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Figure captions

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Tables

Table 1. List of primers employed in this work.

Primer	Sequence (5’→3’)
LMB3	CAG GAA ACA GCT ATG AC
pHEN seq	CTA TGC GGC CCC ATT CA
MJA253	CAGATCCTCTTCTGAGATGAGTTTTTGTTC
MJA254	AATTAACTGCAGCCGAGGTGCAGCTGTTGGAGT
MJA255	ATATTATGAATTCATGAAGGATAACACCGTGCCACTGA
MJA256	ATAATATCCGCGGTTATTTTTCTGCACTACGCAGGGATATTTC
MJA259	CACCTTCGTGCCATTCGATTTTCT
MJA257	AATTGCGGCCGCGGGTCTGAACGACATCTTCGAGGCTCAGAAAATCGAATGGCACG AAGGTGCTCTAGAAATT
MJA258	AATTTCTAGAGCACCTTCGTGCCATTCGATTTTCTGAGCCTCGAAGATGTCGTTCAG ACCCGCGGCCGCAATT

Table 2. List of heterologous species analysed in the Indirect phage enzyme-linked immunosorbent assay (ELISA)~~ELISA~~.

Species		
Nuts		
Almond (*Prunus dulcis*)	hazelnut (*Corylus avellana*)	pecan nut (*Carya illinoinensis*)
brazil nut (*Bertholletia excelsa*)	macadamia (*Macadamia integrifolia*)	pine nut (*Pinus pinea*)
cashew nut (*Anacardium occidentale*)	peanut (*Arachis hypogaea*)	pistachio (*Pistacia vera*)
Vegetal Species		
anise *(Pimpinella anisum*)	flaxseed (*Linum usitatissimum*)	pineapple (*Ananas comosus*)
apple (*Malus domestica*)	garlic (*Allium sativum*)	plum (*Prunus domestica*)
apricot *(Prunus armeniaca*)	kiwifruit (*Actinidia deliciosa*)	pomegranate (*Punica granatum*)
asparagus (*Asparagus officinalis*)	lentil (*Lens culinaris*)	poppy seed (*Papaver rhoeas*)
aubergine (*Solanum melongena*)	lupine (*Lupinus albus*)	pumpkin seed (*Cucurbita maxima*)
banana *(Musa acuminata*)	maize (*Zea mays*)	quinoa (*Chenopodium quinoa*)
barley *(Hordeum vulgare*)	mandarin orange (*Citrus reticulata*)	rice (*Oryza sativa*)
blackberry (*Rubus ulmifolius*)	melon (*Cucumis melo*)	rye (*Secale cereale*)
brown sugar (*Saccharum officinarum*)	oats (*Avena sativa*)	sesame (*Sesamum indicum*)
carrot (*Daucus carota*)	olive (*Olea europaea*)	soya (*Glicine max*)
cherry (*Prunus avium*)	onion (*Allium cepa*)	sunflower seed (*Helianthus annuus*)
chia (*Salvia hispánica*)	orange (*Citrus sinensis*)	tiger nut (*Cyperus esculentus*)
chickpea (*Cicer arietinum*)	paprika (*Capsicum annuum*)	tomato (*Solanum lycopersicum*)
cinnamon (*Cinnamomum verum*)	pea (*Pisum sativum*)	vanilla (*Vanilla planifolia*)
cocoa (*Theobroma cacao*)	peach (*Prunus persica*)	wheat (*Triticum aestivum*)
common bean (*Phaseolus vulgaris*)	pear (*Pyrus communis*)	zucchini (*Cucurbita pepo*)
Animal Species		
cattle (*Bos taurus*)	fish (*Salmo salar*)	poultry (*Gallus gallus domesticus*)
egg (*Gallus gallus domesticus*)	milk (*Bos taurus*)	swine (*Sus scrofa domestica*)

Table 3. Peptides identified by matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-TOF/TOF)~~MALDI-TOF/TOF Spectrometry~~.

Protein identification	Accession number	Sequence coverage	Total score	Ion scores	Peptide sequences
Peroxidase C1A (*Armoracia rusticana*)	P00433	21 %	290	43 47 92 76	R.DTIVNELR.S R.DAFGNANSAR.G R.TEKDAFGNANSAR.G R.MGNITPLTGTQGQIR.L
Ig heavy chain V-III región 23	P01764	29 % (*Homo sapiens*)	87	64	K.NTLYLQMNSLR.A
Ig light chain variable region	AAR91610	14 % (*Homo sapiens*)	133	133	K.LLIYNASSLQSGVPSR.F
pMJA186-scFv		34 %	312	103 163	K.LLIYNASSLQSGVPSR.F R.EAEAAAEVQLLESGGGLVQPGGSLR.L

Table 4. Determination of the presence of walnut in various commercial processed food products using walnut multimeric-scFv enzyme-linked immunosorbent assay (ELISA)~~ELISA~~ and real-time polymerase chain reaction (PCR)~~PCR~~.

Label statement	Product	Number of samples analysed	Multimeric scFv ELISA [a]	ITS real-time PCR [a]
Walnut declared as ingredient	biscuit	2	+ (2)	+ (2)
	nut bar	2	+ (2)	+ (2)
	breakfast cereals	1	+ (1)	+ (1)
	chocolate	1	- (1)	- (1)
	bread	3	+ (2)/- (1)	+ (2)/- (1)
	yogurt	1	- (1)	+ (1)
Contains other tree nuts or traces thereof	biscuit	2	- (2)	- (2)
	nut bar	2	- (2)	- (2)
	breakfast cereals	4	+ (3)/- (1)	+ (2)/-(2) [b]
	chocolate	2	- (2)	- (2)
Not declaring to contain nuts or traces	biscuit	3	- (3)	- (3)
	nut bar	1	- (1)	- (1)
	breakfast cereals	1	- (1)	- (1)
	chocolate	1	- (1)	- (1)
	sauce	1	- (1)	- (1)
	beverage	2	- (2)	- (2)
	ice cream	1	- (1)	- (1)

[a] A plus (+) indicates absorbance values above the LOD (1616 mg kg^{-1} for ELISA) or the presence of amplification after 35 cycles (real-time PCR), corresponding to walnut concentration lower than 10 mg kg^{-1}.

[b] The two positive samples and one of the negative samples for walnut PCR were also positive for pecan PCR. Pecan nut, but not walnut, was stated as ingredient in the labels.

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Figure1

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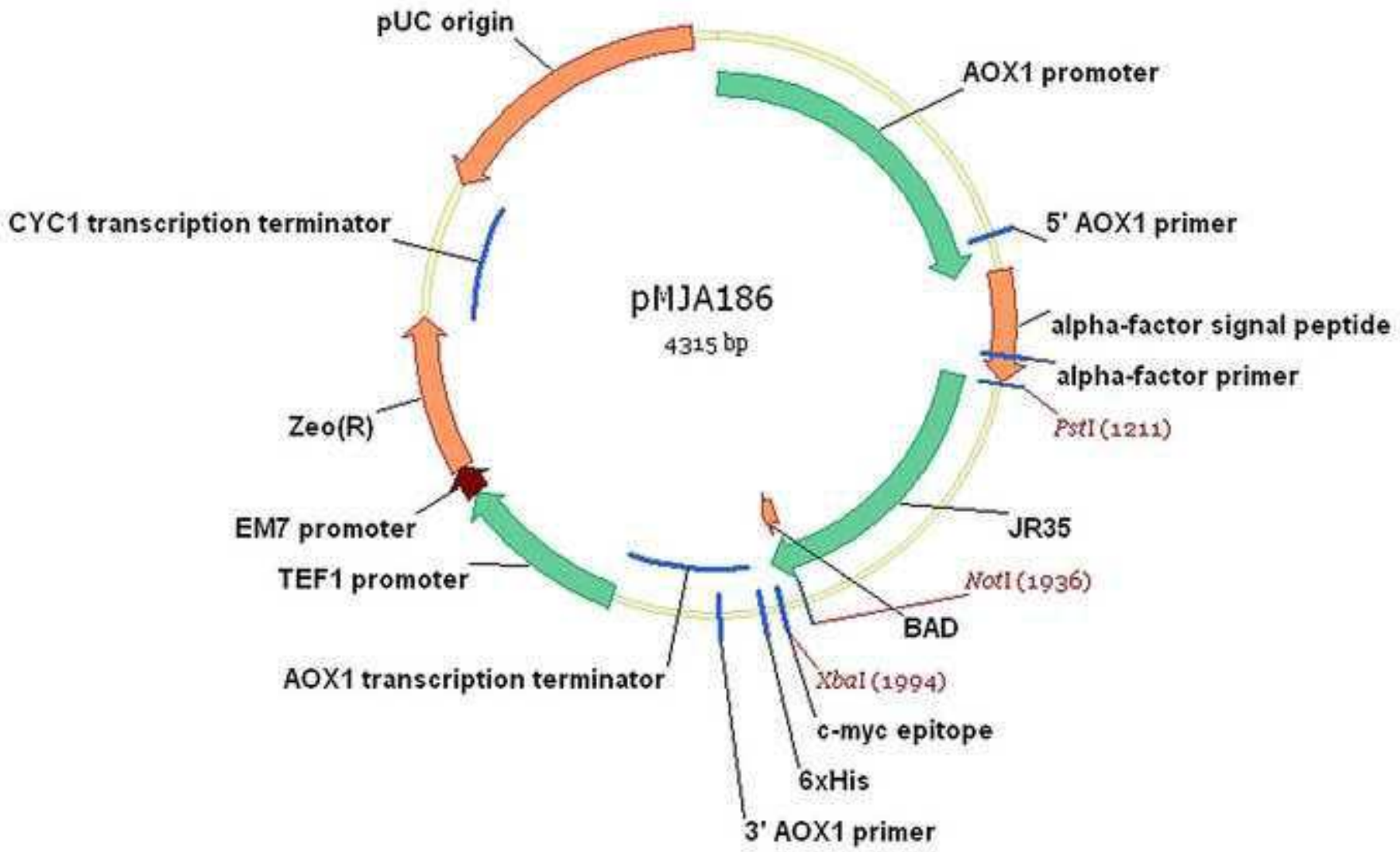

Figure2

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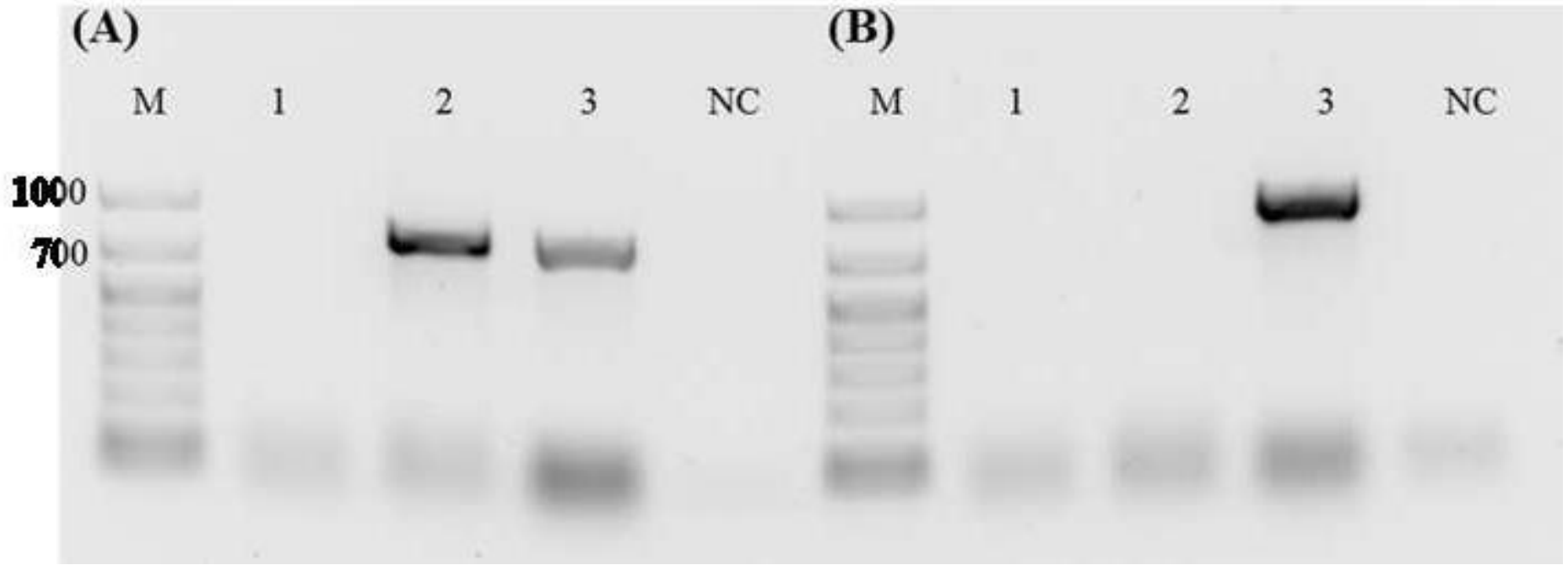

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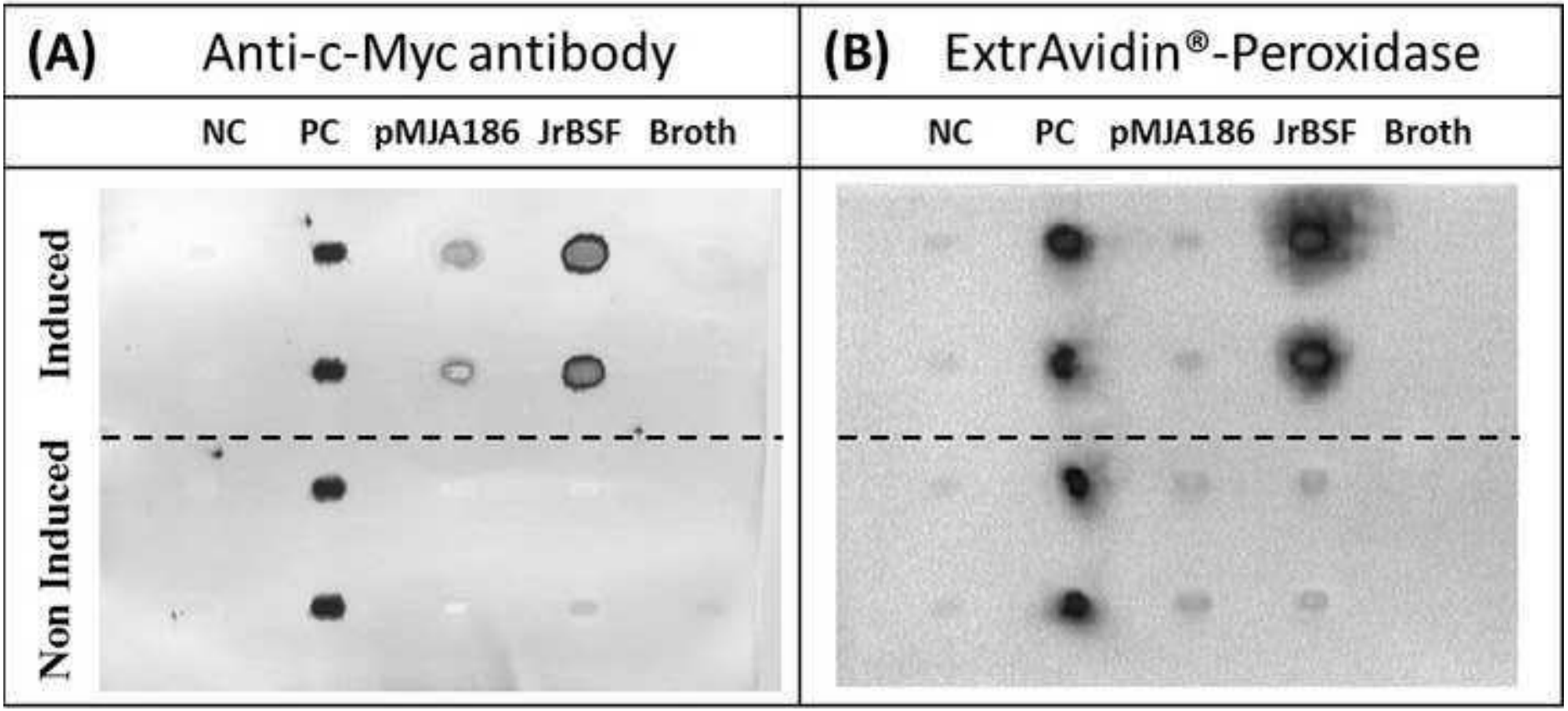

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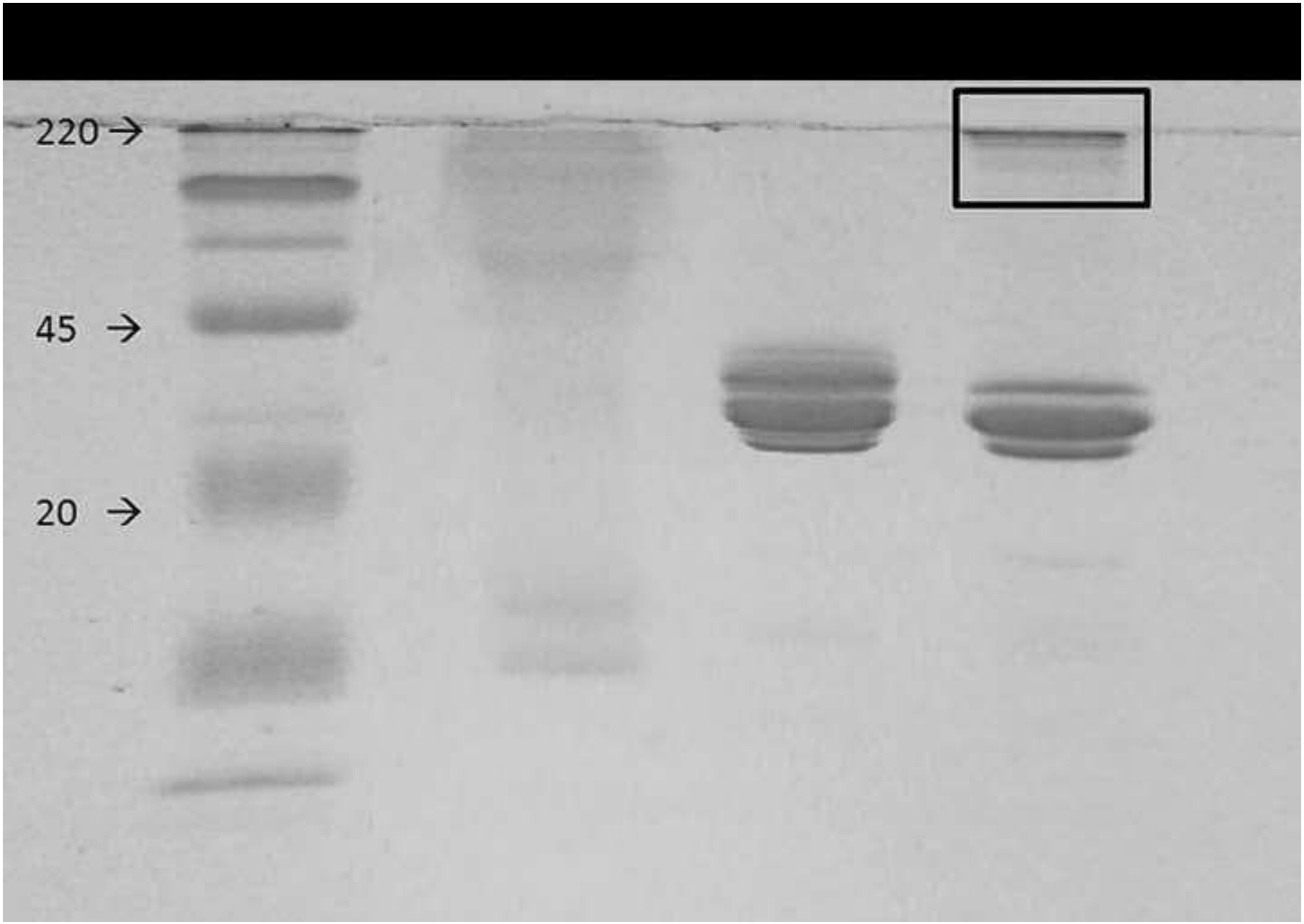

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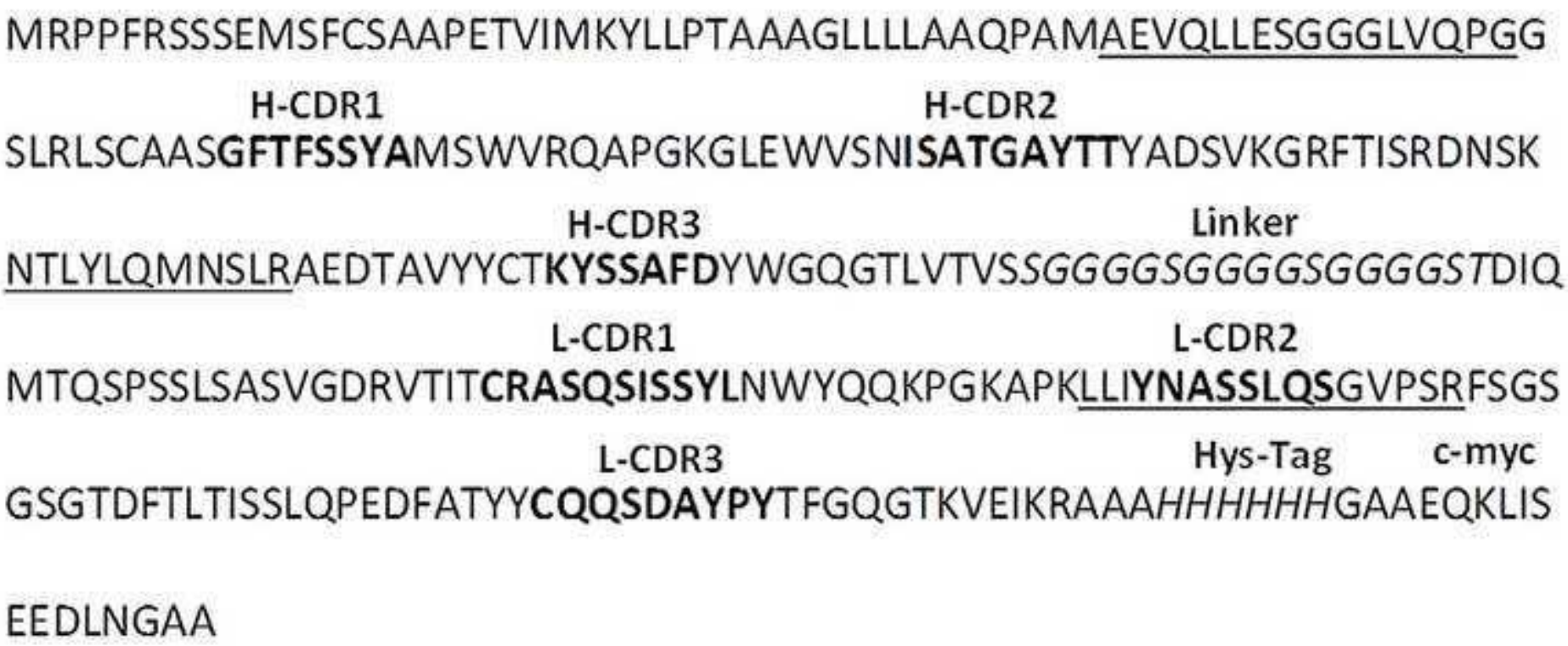

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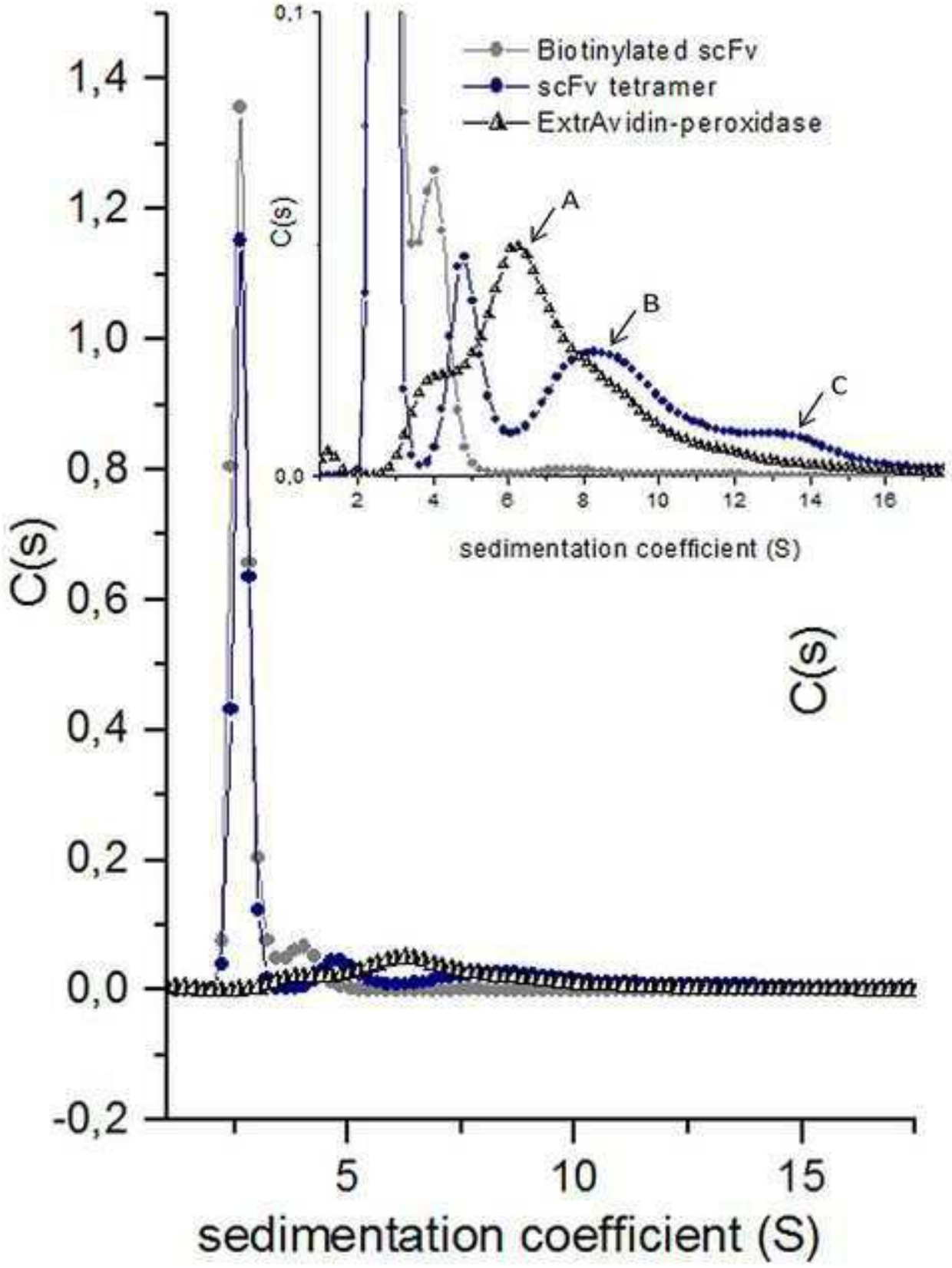

Figure7

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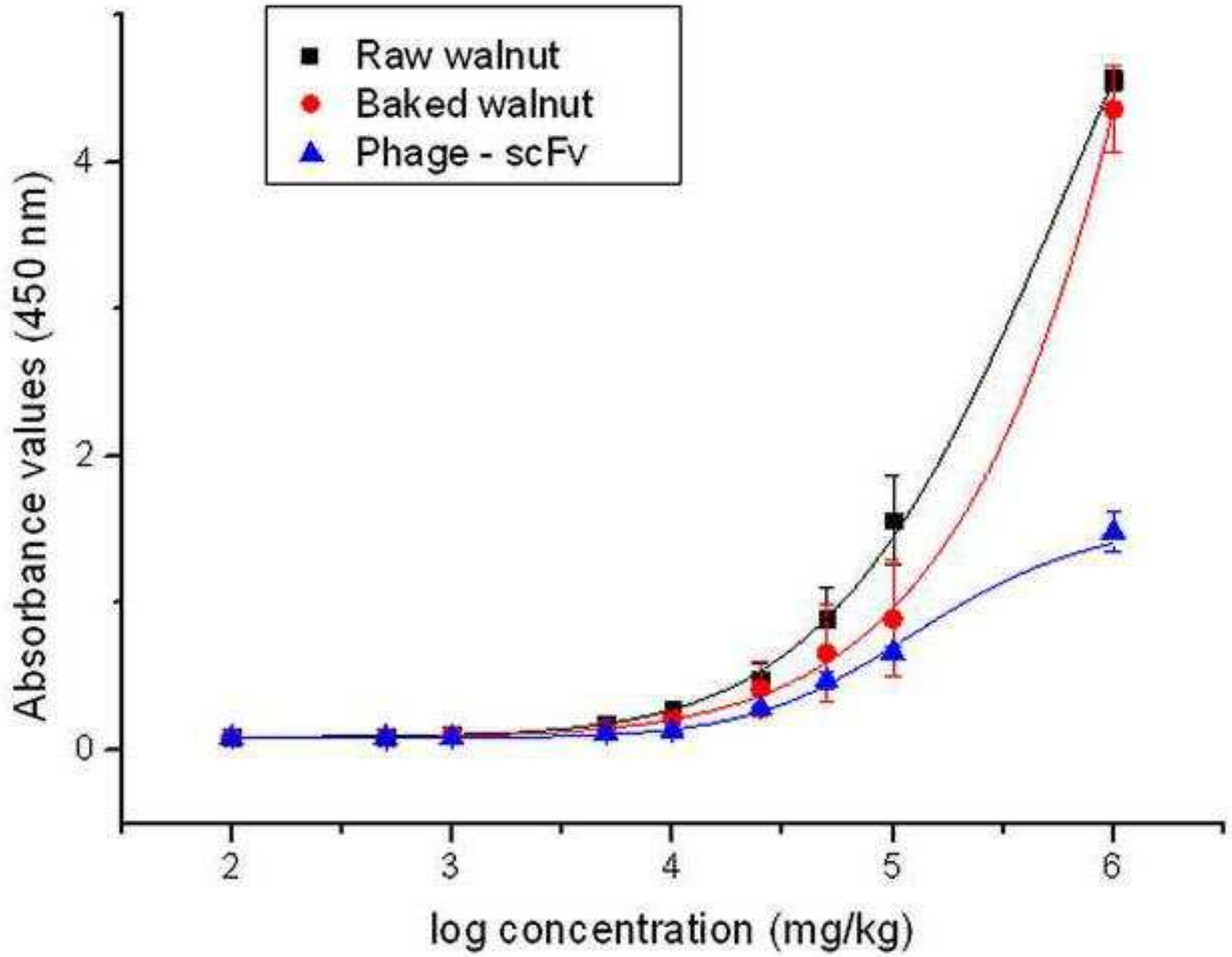

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Highlights

- ~~WA w~~walnut specific phage-scFv ~~has been~~was isolated by phage display from ~~the~~ Tomlinson I library
- *In vivo* biotinylated scFv (JrBSF-scFv) has been produced in *Pichia pastoris*
- ~~BThe b~~iotinylated scFv was multimerized with ExtrAvidin-Peroxidase and used in ELISA
- LOD of direct ELISA for walnut with the multimerized JrBSF-scFv was 1616 mg kg^{-1}
- This is the first recombinant antibody available for walnut detection

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