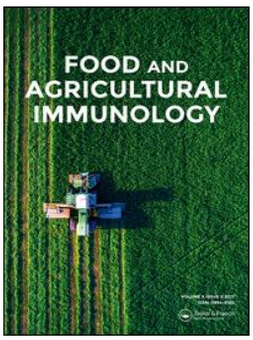

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Goji berries superfood – contributions for the characterisation of proteome and IgE-binding proteins

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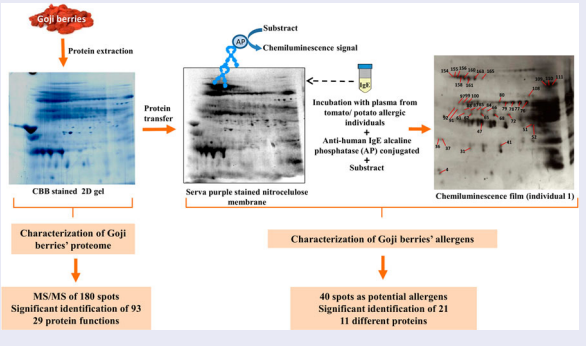
ABSTRACT

Goji berries' bioactive compounds, which allowed classifying them as superfruits, led to an enormous increase of its consumption in western countries. However, the potential risk of allergy is a concern. In this study, we aimed to characterise the proteome of goji berries (*Lycium barbarum*) and identify proteins with putative role in the allergic reaction (IgE-binding proteins). We firstly used two-dimensional (2D) gel electrophoresis followed by mass spectrometry (MS) to characterise goji berries' proteome, and then Immunoblot reactivity with plasma from tomato and potato (same botanical family, *Solanaceae*) allergic individuals was assessed to characterise goji berries IgE-binding proteins. An inhibition assay was further performed to evaluate cross-reactivity among potato, tomato and goji berries. We significantly identified 93 out of the 180 MS analysed spots, corresponding to 29 protein functions. From these, 11 could be identified as goji berries IgE-binding proteins. We further demonstrated cross-reactivity between goji berries, tomato and potato.



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1. Introduction

The fruits of *Lycium barbarum*, also known as goji berries, or wolfberries, have been consumed throughout Asia, and used in Chinese traditional medicine, for at least 2000 years. In the food field, the continuous alteration in consumers' demands, with an increasing number of people searching for traditional and exotic foods, brought goji berries to western countries, where they are now significantly used.

There are several studies reporting the health-promoting properties of goji berries, namely hypoglycemic (Cui, Jing, Feng, Xiao, & Putheti, 2010; Luo, Cai, Yan, Sun, & Corke, 2004; Ming, Guanhua, Zhanhai, Guang, & Xuan, 2009); hypolipidemic (Ming et al., 2009; Pai et al., 2013); protective at retina cells level (Chiu et al., 2010; Hu, Lee, Colitz, Chang, & Lin, 2012; Tang et al., 2011); immunostimulatory (Chen, Kwong Huat Tan, & Chan, 2008; Deng et al., 2018; Gan, Zhang, Liu, & Xu, 2003); anticancer (Mao et al., 2011; Tang et al., 2012) and antioxidative (Amagase, Sun, & Borek, 2009; Bucheli et al., 2011; Xiao et al., 2012). The health-promoting properties have been mostly attributed to an elevated concentration of nutrients with high biological activity, such as polysaccharide complexes, carotenoids, and phenylpropanoids (Qian, Zhao, Yang, & Huang, 2017). Goji berries, further contain several other important nutrients such as vitamins C, B1 and B2, and at least 16 different amino acids (Guo et al., 2015). These fruits are still rich in minerals such as potassium, sodium, phosphorous, magnesium, and calcium (Llorent-Martínez, de Córdova ML, Ortega-Barrales, & Ruiz-Medina, 2013) and, although in smaller quantities, also contain copper and iron (Nascimento, Silvestre, Leme, Nomura, & Naozuka, 2015). The reported health-promoting components of goji berries justify their qualification as "superfruit". However, goji berries may also have risks because of potential interactions with anticoagulation therapy (Lam, Elmer, & Mohutsky, 2001; Leung, Hung, Hui, & Chan, 2008; Rivera, Ferro, Bursua, & Gerber, 2012; Zhang, Tian, & Xie, 2015), hepatotoxicity (Arroyo-Martinez, Sáenz, Arqüelles Arias, & Acosta M, 2011), systemic photosensitivity (Gómez-Bernal et al., 2011), and allergenicity (Ballarín S, López-Matas, Sáenz Abad, Pérez-Cinto, & Carnés, 2011). Although allergenicity studies on goji berries are scarce, some authors already reported positive reactions after skin prick tests with goji berries, cross-reactivity with plasma antibodies from individuals with allergic reactions to peach, tomato, tobacco, a mixture of nuts, and *Artemisia sp.* pollen, and have described a lipid transfer protein (LTP) as the major allergen involved in sensitisation and cross-reactivity (Carnés et al., 2013; Larramendi et al., 2012). Our study aimed not only to characterise the berry proteome of goji (*L. barbarum)* but also to contribute to a better understanding of the proteins involved in the allergic reaction.

2. Materials and methods

2.1. Plant materials

Prepacked dried goji berries (*L. barbarum*) of two different brands were purchased in a local supermarket and stored in the dark, at room temperature. Raw and fresh tomatoes and potatoes (undefined varieties), to be used as positive controls in Western assays, were purchased in a local supermarket and then washed, peeled, lyophilised and frozen at −80°C.

Table 1. Individuals tested.

Patients	Age	Sex	Tomato and potato specific IgE (kU/l)	Other reported food allergies	Allergy symptoms
1	41	F	Tomato: 33.2 (class 4) Potato: 30.8 (class 4)	Wheat products, nuts (peanut, cashew, pecan), beans (soybean, white bean) seeds (corn, rice, sesame), fruits (apple, coconut, orange), crab	Hives, stomach cramping, vomiting, diarrhoea, anaphylaxis
2	63	F	Tomato: 34 (class 4) Potato: Not reported	Milk (73.9 kU/l), wheat, vegetables (broccoli, squash, cabbage, cauliflower, broccoli, peas, beans), fruits (bananas, blackberries, blueberries, kiwifruit), chicken meat, fish, shellfish, egg	Tingling lips, itching throat, itching mouth, trouble breathing
3	31	M	Tomato: 21.3 (class 4) Potato: 21.1 (class 4)	Fruits (watermelon, avocado, kiwifruit)	Itching eyes and throat, shortness of breath
4	55	M	Tomato: 14.1 (class 3) Potato: 14.6 (class 3)	–	Sneezing, watery eyes, nose running
Control	24	M	<0.35 (class 0)	–	–

2.2. Plasmas

Plasmas from individuals with a documented history of allergy to goji berries are not commercially available. Therefore, we have used plasmas from individuals who are allergic to foods belonging to the same botanical family (*Solanaceae*). Plasmas were purchased from PlasmaLab International (Everett, Washington, USA) and obtained from four individuals with a documented history of tomato and/or potato allergy, and with positive specific IgE values equal or higher than class 3 (Table 1).

As negative control, we used plasma from a non-allergic individual with tomato and potato specific IgE values <0.35 (class 0) (Table 1).

Human plasma products were collected in PlasmaLab International under informed consent. Each plasma unit had been tested, and confirmed negative, for Human immunodeficiency virus (HIV), Hepatitis C virus (HCV) and Surface antigen of the hepatitis B virus (HBsAg) by Food and Drug Administration (FDA) licensed test kits. PlasmaLab International is a company licensed by the United States FDA. All applicable FDA, Good Manufacturing Practices, HIPAA, and Clinical Laboratory Improvement Act regulations are strictly followed.

2.3. Protein extraction

Protein extraction was performed with Trichloroacetic acid (TCA)/acetone precipitation (Méchin, Damerval, & Zivy, 2007) followed by a method based on phenol extraction (Faurobert, Pelpoir, & Chaïb, 2007). In short, about 5 grams of a mixture of the samples of the two goji berries brands were ground in a mortar with liquid nitrogen and incubated with 10% (w/v) TCA, 60 mM Dithiothreitol (DTT), in cold acetone, at −20°C, overnight. After centrifugation at 11,000 ×g, for 15 min, at 4°C, pellets were washed twice by incubation with 60 mM DTT in acetone (cooled to −20°C) for 1 h and then centrifuged at 11,000 ×g, for 15 min, at 4°C. Pellets were then dissolved in phenol extraction buffer [0.7 M sucrose; 0.1

M KCl; 0.5 M Tris-HCl, pH 8.5; 50 mM Ethylenediamine tetraacetic acid (EDTA); 1% (w/v) insoluble (Polyvinylpolypyrrolidone) PVPP; 40 mM DTT] on vortex. Thereafter, an equal volume of a phenol solution (Tris-HCl saturated, pH 6.6/7.9) was added, the mixture was vortexed and, after centrifugation at 11,000 ×g, for 15 min, at 4°C, the phenolic upper phase was collected. This phenol phase was then back-extracted with phenol extraction buffer. The sample was vortexed, the centrifugation was repeated for phase separation, and the phenolic upper phase carefully recovered and poured into a new tube. Five volumes of precipitation solution (0.1 M ammonium acetate in cooled methanol) were added, the tube was shaken by inversion, and the mixture was incubated, at −20°C, overnight. Proteins were finally pelleted by centrifugation (15 min, 11,000 ×g, at 4°C). After centrifugation, the pellet was washed two times with cooled methanol and then two times with a solution of 20 mM DTT in cooled acetone. The obtained pellet was dried, dissolved in Lysis Buffer [30 mM Tris-HCl (pH 8.5); 7 M urea; 2 M thiourea; 4% 3-[(3-Cholamidopropyl)dimethylammonio]−1-propanesulfonate (CHAPS) (w/v); 60 mM DTT] and purified using the 2D clean-up kit (GE Healthcare) according to manufacturer's instructions. Finally, the pellet was dried, and stored at −20°C until further analysis.

2.4. One-dimensional (1D) and two-dimensional (2D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Pellets, obtained after protein extraction, were dissolved in Lysis Buffer and the protein was measured according to Ramagli (1998) using albumin from chicken egg white (Sigma) as standard.

For 1D SDS-PAGE, samples (containing 50 µg of protein and run in duplicate) were diluted 1:1 in Sample Buffer [0.125 M Tris–HCl; 4% SDS; 20% (v/v) glycerol; 0.2 M DTT; 0.02% (w/v) bromophenol blue, pH 6.8] and boiled for 5 min before electrophoresis.

For 2D SDS-PAGE, samples were diluted in Rehydration Buffer [2 M thiourea; 7 M urea; 4% (w/v) CHAPS; 1% (v/v) IPG buffer; 60 mM DTT], and IPG strips passive hydration performed, overnight, at room temperature. For the characterisation of goji berries proteome, 18 cm-long IPG strips (GE Healthcare) with a non-linear pH gradient range of 3–10, and 1500 µg of protein, were used. Thirteen cm-long IPG strips (GE Healthcare) with a non-linear pH gradient range of 3–11 and 600 µg of protein were used to identify the IgE-reacting proteins of the goji berries. After rehydration, focusing was done with the following programmes: 2 h at 30 V; 1 h at 250 V; 1 h 30 min at 500 V; 1 h 30 min at 1,000 V; 1 h at 2,500 V; 24 min of a linear gradient to 8,000 V (for 13 cm-long IPG strips) or 3 h of a linear gradient to 10,000 V (for 18 cm-long IPG strips) and 3 h at 8000 V (for 13 cm-long IPG strips) or 45 min at 10,000 V (for 18 cm-long strips).

Prior to the second dimension on SDS-PAGE, the IPG strips were equilibrated at room temperature for 15 min in Equilibration Buffer [50 mM Tris-HCl, pH 8.8; 6 M Urea; 30% (v/v) glycerol; 2% (w/v) SDS] plus 1% (w/v) DTT. Proteins were subsequently alkylated with 2.5% (w/v) iodoacetamide in the equilibration buffer, for 15 min.

SDS-PAGE was performed on 11% acrylamide/bisacrylamide 19:1 (5% C) gels in a Hoefer SE600 system (Amersham Biosciences) and the gels run, at 15°C, with a constant current of 15 mA/gel, in the first 15 min, and 30 mA/gel after that. To calibrate proteins migration we used 10 µl of Spectra TM Multicolor Broad Range Protein Ladder (ThermoFisher Scientific).

All gels were stained with Coomassie Brilliant Blue G (CBB) (Candiano et al., 2004).

2.5. Protein identification and prediction of potential allergenicity

From the gels performed to characterise goji berries proteome, all the protein spots simultaneously present in the three CBB-stained 2D electrophoresis ($n = 180$) were excised. In-gel protein digestion was then performed with trypsin as previously described (Luıs, Alexandre, Oliveira, & Abreu, 2016). After digestion, peptides were resuspended in 0.1% (v/v) trifluoroacetic acid (TFA, Sigma), desalted in C18 Zip-Tips (EMD Millipore), according to manufacturer's instructions, and directly spotted on the MALDI plate.

Direct spotting was accomplished through the elution of peptides from the C18 tips using approximately 2 µL of 2.5 g/L α-cyano-4-hydroxycinnamic acid matrix (CHCA, Sigma) prepared in an aqueous solution containing 50% (v/v) acetonitrile and 0.1% (v/v) TFA. Additionally, 1 µL of Peptide Mix 1 (Laser Biolabs), prepared in the CHCA matrix solution described above, was added to the internal calibration spots in the MALDI plate. All spots were allowed to air dry before mass spectrometry analysis.

Mass Spectrometry (MS) analysis was performed at the iBET/ITQB NOVA MS unit (UniMS) on a 4800plus MALDI-TOF/TOF (AB Sciex) operated in reflector and positive ion modes. Upon full-scan MS (MS^1) data acquisition, the top-20 most abundant ions in the spectra were selected for tandem MS (MS^2) analysis. Proteins were identified by searching the MS^1 and MS^2 data against NCBI database (downloaded on December 01, 2015) with a taxonomic restriction for *Viridiplantae* (1,543,981 sequences). Database searches were performed with ProteinPilot V4.5 (AB Sciex) using Mascot (Matrix Science). All searches were conducted with the following parameters: MS^2 fragment mass tolerance set to 0.3 Da, peptide mass tolerance set to 50 ppm, two missed cleavages allowed, carbamidomethylation of the Cysteine residues (C) set as fixed modification and oxidation of Methionine (M) and deamination of Asparagine and Glutamine residues (NQ) set as variable modifications. Proteins were identified with a minimum of two MS^2 spectrum matching the database sequence and a protein score above 74 ($p < 0.05$).

Potential allergenicity of all identified proteins was predicted using AlgPred software hybrid approach (http://crdd.osdd.net/raghava/algpred/).

2.6. IgE immunoblot reactivity assay of plasma from tomato and potato-allergic subjects

Because there are no plasmas from individuals allergic to goji berries commercially available, a Western blot was performed, after 1D SDS-PAGE, to check if plasmas from tomato and potato allergic individuals would also react with goji berries proteins. After this confirmation, a Western blot was performed, after 2D SDS-PAGE, to identify goji berries IgE-binding proteins.

IgE reactivity in the patient's plasma was evaluated after 1D or 2D SDS-PAGE, followed by protein transfer onto Hybond ECL nitrocellulose membranes (Amersham Biosciences) of 50 µg/lane (1D SDS-PAGE) or 600 µg total protein (2D SDS-PAGE). Protein transfer was performed at 15°C by wet transfer in 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol, overnight, at 20 V.

After protein transfer, and before blocking the blots, protein staining in the nitrocellulose membranes was performed using Serva Purple stain (Serva Electrophoresis GmbH) according to the manufactureŕs instructions, and the images were acquired with a FUJI

FLA-5100 scanner. The 2D IgE Immunoblot Reactivity Assay was performed in duplicate for all individuals.

Blots were blocked at 4°C, overnight with PBS-T [50 mM Na_2HPO_4; 17 mM $NaH_2PO_4 \cdot H_2O$; 68 mM NaCl; 0.2% (v/v) tween 20] and 5% (w/v) skimmed milk powder, and washed with PBS-T prior to incubation for 90 min at room temperature, in plasma diluted 1:10 in the blocking solution (first antibody incubation). After washing in PBS-T, the membranes were incubated at room temperature for 1 h with alkaline phosphatase-conjugated monoclonal anti-human IgE (Southern Biotechnology Associates) diluted 1:1,000 in blocking solution (second antibody incubation).

After second antibody incubation, the membranes were washed with PBS-T and assay buffer and subsequently incubated for 5 min with CDP-Star solution and Nitro-Block II enhancer (Tropix Western-Star immunodetection system). The signals were visualised after blot exposure to a high-performance chemiluminescence Hyperfilm ECL (Amersham Biosciences). For optimal signal intensity, the blots were exposed between 5 s and 5 min.

2.6.1. Image analysis and spot identification

For image analysis, each chemiluminescence film was overlaid to the corresponding total protein Serva Purple stained nitrocellulose membrane image, enabling the correlation of each spot on the 2D gel with the immunoreactive protein. For each tested individual two immunoblots were obtained. The Allergome aligner tool was used to check if identified IgE-reactive proteins were already reported as allergens in the Allergome database (http://www.allergome.org/).

2.6.2. Cross-reactivity evaluation- inhibition assay

To evaluate if the reaction of tomato and potato allergic individuals to goji berries protein extracts occurred due to the similarity of allergens among these foods an Immunoblot inhibition assay was performed.

In this experiment, we have used plasma from individual 1 in 1D SDS-PAGE followed by Western blot, as described above. However, in this case, we used 80 µg of goji berries protein/lane and pre-incubated the plasma (before first antibody incubation) with 1 mg protein extract of goji berries, tomato or potato. We have also performed a negative control where the plasma of individual 1 was pre-incubated with Lysis buffer.

3. Results

3.1. Characterisation of goji berries proteome

Both 1D and 2D goji berries SDS-PAGE analyses show protein profiles widespread amongst the molecular weight range analysed. However, most intense bands/spots concentrate mostly in the 20, 34, 42–48 and 54–70 kDa regions (Figure 1).

From the 180 trypsin-digested spots analysed, 93 showed significant protein matches, 68 of which have more than one positive identification and 25 have only one (Table 2; Supplementary Table 1).

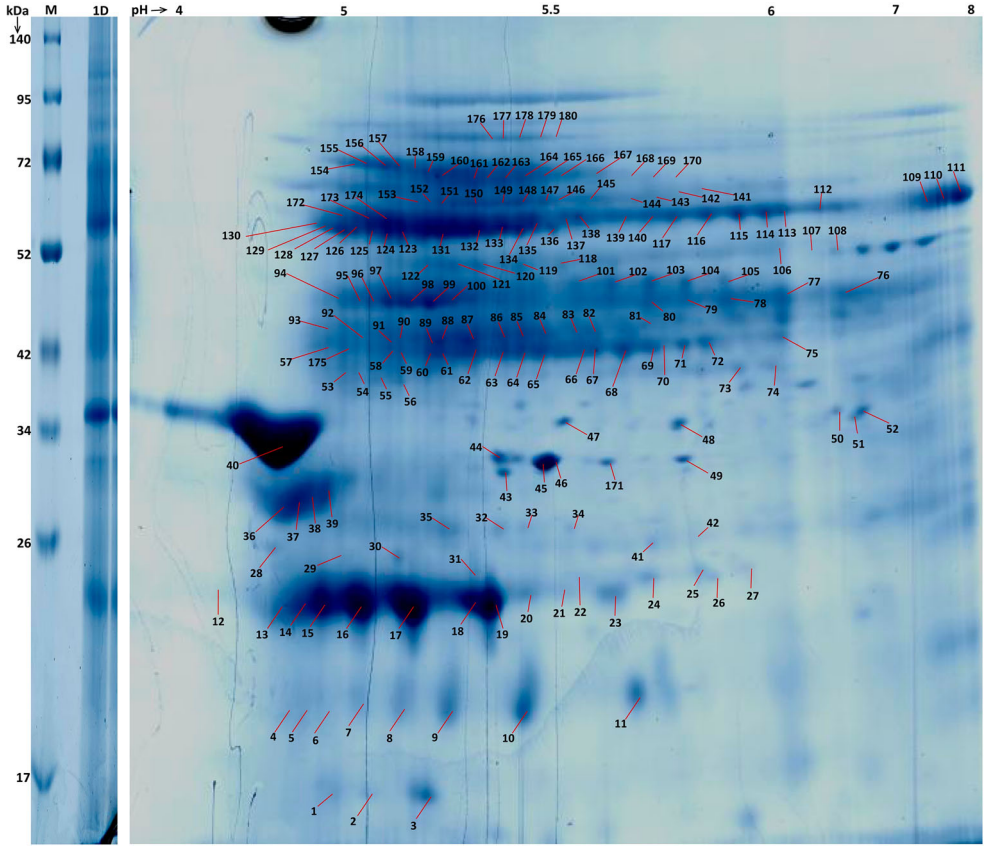

Figure 1. 1D and 2D goji berries SDS- PAGE with the identifications of the spots that were extracted from the gel and submitted to MS/MS. kDa- Kilodalton; M- Molecular weight marker; 1D- One-dimensional SDS-PAGE.

An analysis of the 93 significantly identified spots for the most probable protein match, resulted in the assignment of 29 different protein functions.

AlgPred analysis of the 93 significantly identified spots, predicted that 37 of them are potential allergens and that 56 correspond to non-allergens (Table 2).

3.2. Characterisation of goji berries allergome- Western blot assays

3.2.1. 1D Western blotting assay

Protein extracts of goji berries, tomato and potato were separated by 1D SDS-PAGE (Figure 2) and analysed by Western blot with the plasma from the tested individuals (Figure 2). Except for individual 2, we could see that all tested individuals that have a documented history of tomato and/or potato allergy, revealed IgE reaction against proteins from these foods, here used as positive controls. Individual 2, although with a history of tomato allergy and presenting IgE (34 kU/L) against this food, did not show a positive reaction on the Western blot. In fact, it was difficult to optimise the exposure time for the blot of this individual, due to the presence of a large background signal. The fact

Table 2. Mass spectrometry results- Significant identifications.

Spot #	Protein ID	Mass Theo./ Exp. (Da)	pI Theo./ Exp.	Protein Score	Ion Score	Coverage %	Queries matched	Most probable identification (source)
11	gi|508786804	18024/ 21000	5.57/5.7	110	100	15%	5	**HSP20-like chaperones superfamily protein** (*Theobroma cacao*)
21	gi|38605724	22982/ 25000	6.11/5.5	194	180	40%	9	**NADH-ubiquinone oxidoreductase 27 kDa subunit** (*Solanum tuberosum*)
23	gi|823163882	22626/ 25000	5.95/ 5.6	191	171	28%	10	**Proteasome subunit beta type**
26	gi|802581360	22295/ 25000	5.72/ 5.9	236	214	27%	12	(*Gossypium raimondii*)
31	gi|922516433	25332/ 25000	5.18/ 5.3	156	145	29%	7	(*Jatropha curcas*) (*Brassica oleracea var. oleracea*)
24	gi|75264210	21782/ 25000	5.96/5.7	196	178	43%	9	**Flavodoxin-like quinone reductase 1** (*Arabidopsis thaliana*)
27	gi|557555337	17601/ 25000	6.06/5.9	160	148	28%	7	**NADPH-dependent FMN reductase region** (*Citrus clementina*)
30	gi|116342	28014/26000	5.08/5.1	118	111	12%	5	**Acidic endochitinase Q** (*Nicotiana tabacum*)
36	gi|22261807	35671/ 29000	5.26/ 4.5	172	161	22%	9	**Light-induced protein, chloroplastic; Drought-induced stress protein CDSP-34** (*Solanum tuberosum*)
37	gi|22261807	35671/29000	5.26/ 4.6	174	164	16%	8	
38	gi|22261807	35671/29000	5.26/ 4.7	122	112	15%	8	
39	gi|22261807	35671/ 29000	5.26/4.8	182	165	26%	12	
40	gi|22261807	35671/ 33000	5.26/4.5	169	158	22%	8	
53	gi|550320244	44151/ 40000	8.65/4.9	206	183	36%	16	**Malate dehydrogenase** (*Populus trichocarpa*) (*Cicer arietinum*) (*Nicotiana tabacum*)
54	gi|550320244	44151/ 40000	8.65/ 5.0	206	193	22%	12	
55	gi|502152034	43441/ 40000	7.56/5.1	349	337	26%	14	
66	gi|75172323	35727/ 43000	5.91/5.5	163	153	28%	9	
67	gi|75172323	35727/ 43000	5.91/5.6	117	109	28%	11	
68	gi|75172323	35727/ 43000	5.91/5.6	246	228	36%	15	
69	gi|75172323	35727/ 43000	5.91/5.7	146	138	29%	14	
71	gi|75172323	35727/ 43000	5.91/5.8	251	242	27%	12	
61	gi|951067672	43152/ 43000	7.63/ 5.2	92	64	45%	15	**Fructose-bisphosphate aldolase**, (*Vigna radiata var. radiata*) (*Erythranthe guttata*)
72	gi|848892151	42998/ 43000	8.45/5.8	146	133	27%	9	
59	gi|34582499	42146/ 43000	5.71/5.1	128	115	21%	17	**Alpha-1,4-glucan-protein synthase [UDP-forming]** (*Solanum tuberosum*) (*Medicago truncatula*)
60	gi|34582499	42146/ 43000	5.71/5.2	80	65	28%	16	
62	gi|34582499	42146/ 43000	5.71/ 5.3	93	79	28%	12	
63	gi|34582499	42146/ 43000	5.71/ 5.4	130	105	29%	20	
82	gi|34582499	42146/ 44000	5.71/5.6	151	122	35%	23	
84	gi|34582499	42146/ 44000	5.71/5.5	224	187	35%	27	
85	gi|922341433	41185/ 44000	5.66/5.4	109	93	21%	13	
87	gi|922341433	41185/ 44000	5.66/5.3	128	118	25%	15	

(*Continued*)

Table 2. Continued.

Spot #	Protein ID	Mass Theo./ Exp. (Da)	pI Theo./ Exp.	Protein Score	Ion Score	Coverage %	Queries matched	Most probable identification (source)
79	gi|73919692	43130/ 47000	5.85/5.8	166	150	31%	11	**GDP-mannose 3,5-epimerase**
102	gi|778666529	42888/ 50000	5.94/5.6	239	228	16%	10	(*Arabidopsis thaliana*)
103	gi|508719181	48472/ 50000	6.88/5.7	200	164	31%	22	(*Cucumis sativus*)
105	gi|901822072	43176/ 50000	5.58/5.9	268	257	23%	15	(*Theobroma cacao*)
								(*Zostera marina*)
81	gi|527504049	45805/ 45000	7.96/5.7	100	93	25%	9	**Isovaleryl-CoA dehydrogenase, mitochondrial**
								(*Solanum tuberosum*)
83	gi|901824429	39330/ 44000	6.73/5.6	148	130	24%	10	**UDP-glucuronic acid decarboxylase 5**
								(*Zostera marina*)
94	gi|3219772	37265/ 48000	5.28/4.9	337	313	35%	16	**Actin-51**
95	gi|3219772	37265/ 48000	5.28/5.0	347	312	46%	17	(*Solanum lycopersicum*)
96	gi|3219772	37265/ 48000	5.28/5.0	335	287	50%	22	
97	gi|3219772	37265/ 48000	5.28/5.1	147	142	19%	7	
98	gi|3219772	37265/ 48000	5.28/5.1	310	290	36%	16	
104	gi|550338158	42747/ 50000	6.05/5.8	409	382	33%	18	**NAD-dependent epimerase/dehydratase family protein**
								(*Populus trichocarpa*)
107	gi|571469199	44476/ 54000	8.20/6.3	194	179	35%	14	**Serine-glyoxylate aminotransferase**
108	gi|823227416	44477/ 54000	8.11/6.5	152	142	25%	9	(*Glycine max*)
								(*Gossypium raimondii*)
110	gi|482576438	17522/ 65000	8.78/7.5	144	122	44%	9	***P*-loop containing Nucleoside Triphosphate** (*Capsella rubella*)
113	gi|1705616	56928/ 60000	6.56/6.0	311	273	33%	21	**Catalase isozyme 2**
								(*Solanum tuberosum*)
114	gi|916441433	53337/ 60000	6.71/6.0	348	263	49%	30	**Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit**
115	gi|916441433	53337/ 60000	6.71/5.9	361	301	38%	25	(*Physalis virginiana*)
117	gi|827046023	53351/ 60000	6.55/5.8	485	399	52%	31	(*Iochroma tingoanum*)
139	gi|824055382	53040/ 60000	6.33/5.6	221	187	32%	22	(*Geranium robertianum*)
140	gi|817991757	49975/ 60000	6.30/5.7	111	86	25%	16	(*Calluna vulgaris*)
119	gi|50400860	47120/ 51000	5.77/5.4	163	149	22%	13	**Monodehydroascorbate reductase**
120	gi|951027526	46893/ 51000	5.59/5.3	159	149	25%	14	(*Solanum lycopersicum*)
121	gi|823136845	47324/ 51000	6.23/5.3	131	117	16%	9	(*Vigna radiata var. radiata*)
122	gi|50400860	47120/ 51000	5.77/5.2	125	111	29%	11	(*Gossypium raimondii*)
								(*Solanum lycopersicum*)
124	gi|724471834	19509/ 59000	7.03/5.1	219	180	67%	13	**ATP synthase**
137	gi|929046212	55574/ 60000	5.84/5.5	348	348	11%	7	(*Rhynchoryza subulata*)
138	gi|114408	55847/ 60000	6.23/5.6	407	388	27%	20	(*Cannabis sativa*)
172	gi|114421	59933/ 60000	5.95/4.9	663	625	37%	26	(*Oenothera biennis*)
173	gi|724471830	19383/ 60000	6.99/5.0	218	176	34%	7	(*Nicotiana plumbaginifolia*)
174	gi|114421	59933/ 60000	5.95/5.1	295	284	24%	13	(*Zizania aquatica*)
								(*Nicotiana plumbaginifolia*)

125	gi|937546689	53623/ 59000	5.04/5.0	369	344	36%	19	**AtpB (chloroplast)**
126	gi|937546689	53623/ 59000	5.04/5.0	357	316	41%	25	*(Capsicum frutescens)*
127	gi|937546689	53623/ 59000	5.04/4.9	392	351	42%	25	
128	gi|923921519	54235/ 59000	5.08/4.9	306	270	36%	24	**ATPase like**
129	gi|937923381	40041/ 59000	5.31/4.8	217	208	22%	11	*(Brassica napus)*
130	gi|923921519	54235/ 59000	5.08/4.8	553	509	41%	29	*(Oryza sativa, japonica Group)* *(Brassica napus)*
132	gi|749319792	55348/ 59000	5.89/5.3	102	79	31%	20	**Aldehyde dehydrogenase 1** *(Capsicum annuum)*
133	gi|602218966	65477/ 59000	6.50/5.4	195	186	23%	12	**Phytoene desaturase**
134	gi|602218966	65477/ 59000	6.5/5.4	121	111	19%	12	*(Lycium chinense)*
135	gi|602218966	65477/ 59000	6.5/5.4	140	131	17%	12	
141	gi|550322251	63406/ 66000	7.65/5.8	100	86	26%	15	**Delta-1-pyrroline-5-carboxylate dehydrogenase**
142	gi|357477461	61757/ 66000	6.36/5.8	176	161	22%	16	*(Populus trichocarpa)*
143	gi|502152110	61850/ 64000	6.17/5.7	203	181	24%	17	*(Medicago truncatula)*
145	gi|357477461	61757/ 63000	6.36/5.6	189	174	19%	14	*(Cicer arietinum)*
146	gi|357477461	61757/ 63000	6.36/5.5	144	135	18%	12	*(Medicago truncatula)*
154	gi|731910338	68865/ 72000	5.31/5	417	386	28%	20	**Vacuolar ATP synthase catalytic subunit**
155	gi|731910338	68865/ 72000	5.31/5	249	226	29%	17	*(Ornithogalum saundersiae)*
156	gi|937553899	69044/ 72000	5.37/5.1	253	231	26%	18	*(Prunus humilis)*
157	gi|731910338	68865/ 72000	5.31/5.1	245	226	26%	16	*(Ornithogalum saundersiae)*
148	gi|1172578	67812/ 63000	6.92/5.4	86	69	22%	22	**Polyphenol oxidase**
150	gi|1172583	66768/ 63000	6.27/5.3	88	79	17%	13	*(Solanum lycopersicum)*
159	gi|1172578	67812/ 70000	6.92/5.1	96	77	22%	23	
160	gi|1172578	67812/ 69000	6.92/5.2	81	71	16%	17	
161	gi|1172583	66768/ 68000	6.27/5.3	88	76	18%	15	
162	gi|1172583	66768/ 68000	6.27/5.0	75	62	18%	16	
163	gi|1172582	66822/ 68000	6.36/5.4	98	84	23%	16	
164	gi|1172578	67812/ 69000	6.92/5.4	101	79	31%	26	
166	gi|1172578	67812/ 69000	6.92/5.5	78	66	20%	23	
167	gi|1172582	66822/ 70000	6.36/5.6	94	75	26%	20	
168	gi|1172578	67812/ 69000	6.92/5.7	99	77	22%	24	
178	gi|870864801	80287/ 78000	5.94/5.4	81	71	9%	11	**Transketolase_C** *(Beta vulgaris subsp. vulgaris)*

Da: Dalton; **pI**: Isoelectric point; **Theo**: Theoretical; **Exp**: Experimental; **nd**: Not determined; Underlined shadowed spot numbers: more than one significant identification; Underlined protein ID: Allergen as predicted by AlgPred; Non-underlined Protein ID: Non allergen as predicted by AlgPred.

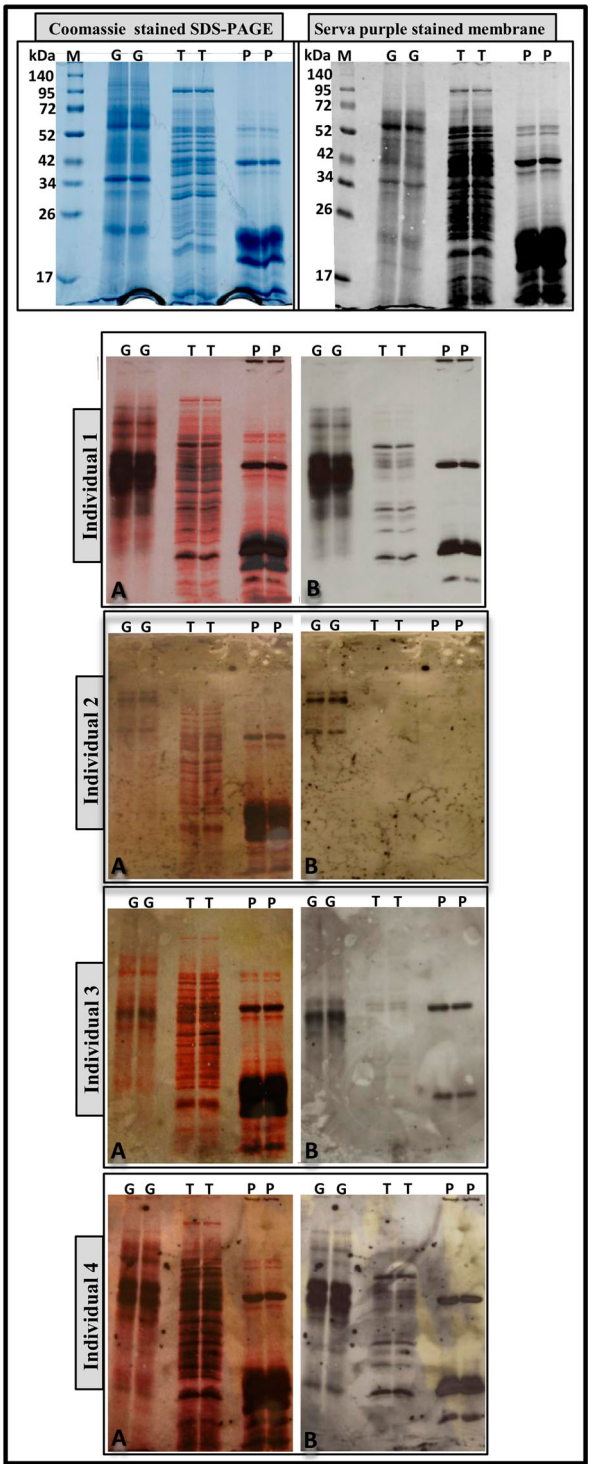

Figure 2. 1D Western blotting assay. A- Chemiluminescence film over Serva Purple stained Nitrocellulose membrane; **B**- Chemiluminescence film; **M**- Molecular weight marker; **G**- Goji berries; **T**- Tomato; **P**- Potato; **kDa**- Kilodalton; 11% polyacrylamide gels.

that this individual is also allergic to milk (73.9 kU/L), which was the blocking agent used, probably explains the high background, since the IgE present in the plasma may have bound to milk proteins blocking the membrane. The possibility of using another type of blocking solution, like casein, gelatine from cold-water fish or gelatine from porcine skin, was discarded because this individual also shows allergy to casein (83.4 kU/L), fish, and meat products.

However, plasma from all tested individuals reacted against goji berries proteins making it possible to use them in 2D Western assays for characterisation of goji berries allergome.

The comparison of the obtained chemiluminescent signals with the protein bands on 1D SDS-PAGE, allowed us to conclude that goji berries IgE binding proteins range from 20 to 95 kDa being more concentrated in the area of 30–52 kDa.

The negative-control individual presented no reaction against either tomato, potato or goji berries proteins (Supplementary Figure 1).

3.2.2. Inhibition assay

Cross-reactivity between allergens occurs when an antibody directed against one specific allergen successfully binds to another, which happens because the two allergens in question present similar three-dimensional structural regions, known as epitopes. In this assay, a high degree of cross-reactivity between goji berries allergens and plasma antibodies from individuals allergic to tomato and potato protein extracts was proven by the total inhibition of the reaction of individual 1 plasma, against goji berries extract after pre-incubation with potato and tomato protein extracts (Figure 3). As expected, pre-incubation of individual 1 plasma with goji berries protein extract also inhibited reaction against goji berries extract (auto-inhibition – positive control) while the pre-incubation with Lysis buffer did not affect the reaction of this individual plasma against goji berries extract (negative control).

3.2.3. 2D Western blotting assay

Plasma from individual 2 was not used in this assay because, as already explained, a persistent high background, probably due to individual's allergy to milk, could not be removed.

In this assay, individual 1 was the one presenting IgE originating more visible and clean chemiluminescence signals, and reacting to a higher diversity of proteins (Figure 4). For this individual, it was possible to relate the obtained chemiluminescence signals to 40 spots that were further identified by mass spectrometry, 21 showing significant identification. Regarding individual 3, it was not possible to relate any of the obtained chemiluminescence signals to the 180 spots excised from 2D SDS-PAGE. Finally, it was possible to relate chemiluminescence signals obtained for individual 4 to spots 76, 77, and 78. However, none of them had a significant identification. All three tested individuals reacted against proteins present in a part of the gel (identified with a red circle on Figure 4) not selected for analyses by mass spectrometry because of the unclear spots.

As already seen in 1D Western blotting, results obtained in this assay showed that the tested individuals reacted to goji berries proteome, which was particularly visible in the area of 30–52 kDa.

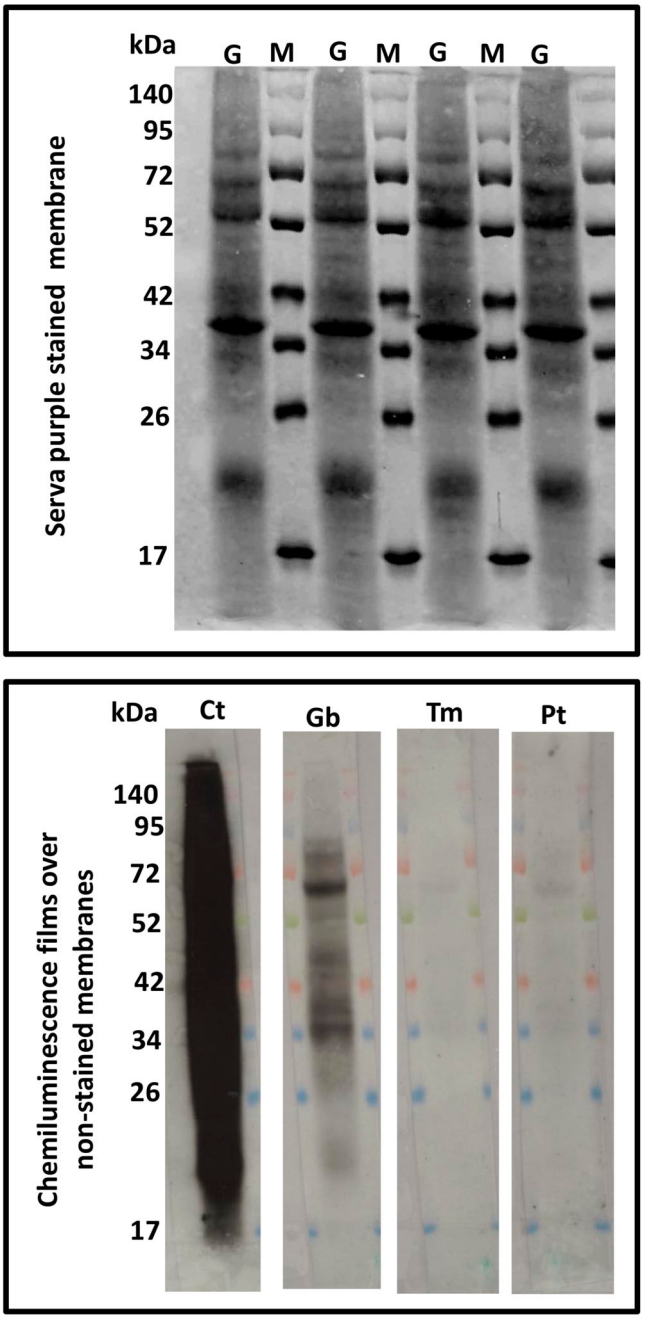

Figure 3. Western Blotting inhibition assay using plasma from individual 1 and 11% polyacrylamide gel. **M**- Molecular weight markers; **G**- Goji berries protein extract; **Ct**- no inhibition; **Gb**- Inhibition with 1 mg of goji berries protein; **Tm**- Inhibition with 1 mg of tomato protein; **Pt**- Inhibition with 1 mg of potato protein.

3.2.4. Goji berries allergens

For the 21 IgE-reactive spots with MS significant identification, the proteins found correspond to 11 different functions: Proteasome subunit; Fructose-bisphosphate aldolase;

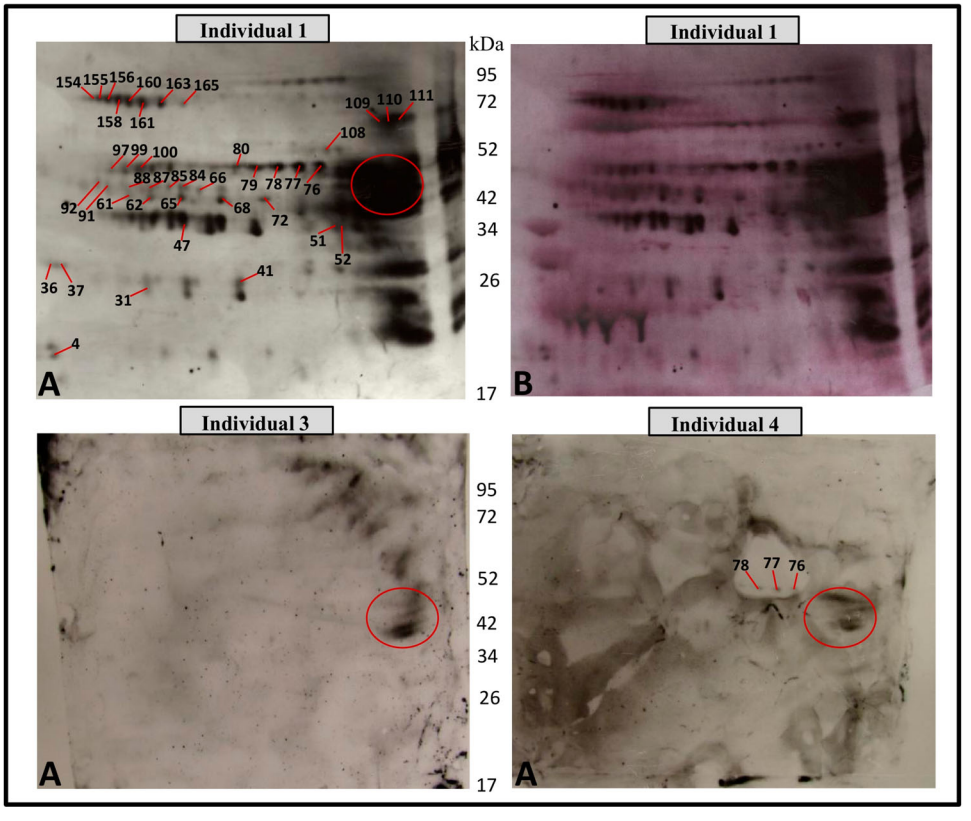

Figure 4. 2D Western blotting assay. A- Chemiluminescence film; **B-** Chemiluminescence film over Serva purple stained nitrocelulose membrane.

Alpha-1,4-glucan-protein synthase; Malate dehydrogenase; Actin; Serine-glyoxylate aminotransferase; Polyphenol oxidase; Light-induced protein; Mannose 3,5-epimerase; P-loop containing Nucleoside Triphosphate; and Vacuolar ATP synthase (Table 3).

The sequences of the 21 IgE-binding proteins, significantly identified by mass spectrometry, were then compared with the sequences present in Allergome database, using Allergome aligner tool. The obtained results were compared with those obtained with AlgPred software (Table 3).

4. Discussion

4.1. Characterisation of goji berries proteome

The one-dimensional protein profile obtained in this work for the goji berries was distinct from the ones shown by others authors. Carnés et al. (2013), for instance, obtained bands ranging from 7 to 100 kDa with the most prominent locating in the 7, 26 and 65 kDa regions. Larramendi et al. (2012) and Ballarín S et al. (2011) also showed similar results presenting protein profiles with bands also ranging from 7–9 to 100 kDa with the most intense ones in the 7–9, 25, 66 and 100 kDa regions. Differences between our results and those of these authors may be related to the protein extraction

Table 3. Identified goji berries allergens.

Spot number	Most probable protein function	Allergome aligner output	Other organisms where this protein function was identified as allergen
31	Proteasome subunit	Zea m 20S (UniProt – Q5XML0- Proteasome subunit); Allergome code: 7664 (*Zea mays*- seed)	*Hevea brasiliensis*- **latex**
61;72;	Fructose-bisphosphate aldolase	Sal s3 (UniProt – C0H9I1- Frutose1,6- bisphosphate aldolase); Allergome code: 10151 s3 (*Salmo salar*)	*Anisakis simplex; Candida albicans; Esox lucius; Forcipomyia taiwana; Gadus morhua; Gallus domesticus; Lates niloticus; Manihot esculenta¸ Oncorhynchus masou; Oreochromis mossambicus; Plantago lanceolata*- **pollen**; *Solenocera melantho; Scomber albacares; Vespa affinis*
62; 84; 85; 87	Alpha-1,4-glucan-protein synthase	Pho d 40kD Allergome code: 8444 (*Phoenix dactylifera*)	——
66; 68	Malate dehydrogenase	Mala f 4.0101 (UniProt – Q9Y750- Malate dehydrogenase) Allergome code: 3363 (*Malassezia furfur*)	*Aspergillus fumigatus; Aspergillus versicolor; Citrullus lanatus; Cucumis melo; Malassezia furfur; Malassezia globosa; Malassezia sympodialis; Plantago lanceolata*- **pollen**; *Senecio jacobaea*- **pollen**
97	Actin	Sal s alpha_Actin (UniProt – Q78BU2- Actin alpha 1–1) Allergome code: 10938 (*Salmo salar*)	*Dermatophagoides farinae; Bos domesticus; Chionoecetes opilio; Labrus niloticus; Pandalus borealis; Solenocera melantho*
108	Serine-glyoxylate aminotransferase	Asp f AT_V (UniProt – B0XQ69- Aminotransferase, class V); Allergome code: 8983 (*Aspergillus fumigatus*)	*Bos domesticus*
160;161;163	Polyphenol oxidase	Meg c Hemocyanin (UniProt – Q53IP9- Hemocyanin); Allergome code: 2852 (*Megathura crenulata*)	*Blattella germanica; Holocnemus pluchei; Macrobrachium; rosenbergii; Periplaneta americana; Tegenaria domestica; Megathura crenulata*
36;37	**Light-induced protein**	No match on Allergome database	——
79	**Mannose 3,5-epimerase**	No match on Allergome database	——
110	***P*-loop containing Nucleoside Triphosphate**	No match on Allergome database	——
154;155;156	**Vacuolar ATP synthase**	No match on Allergome database	

Underlined spot numbers- Allergens as predicted by AlgPred; Underlined most probable protein function- Similar allergen found on Allergome; **Bold most probable protein function**- No similar allergen found on Allergome.

method used that in their case was limited to the use of phosphate buffered saline (PBS). Instead, we used TCA/acetone precipitation followed by a phenol-based extraction method and a clean-up procedure for our extraction procedure. This methodology resulted in a clean protein profile in SDS-PAGE and high spot number in 2D gels. The goji berries 2D protein profiles, obtained in this study, showed a higher concentration of protein spots at pH 4–5.5 and similarities to the profiles presented by Carnés et al. (2013) (Figure 1). However, a rigorous comparison of the two 2D protein profiles is hard to achieve since different gel concentrations and protein extraction methods have been used (Carnés et al., 2013).

4.2. Goji berries allergens

From the 11 types of proteins identified as potential allergens in this study, only seven were similar to others already reported on the Allergome database (Table 3), while four are here reported as allergens for the first time.

For those allergens already reported on the Allergome database, we could verify that some were identified in pollen and latex, which could indicate a cross-reaction between goji berries proteins and plasma antibodies of individuals with latex allergy (latex-food syndrome) (Blanco, 2003) and /or of individuals with pollen allergy (oral-food syndrome) (Price et al., 2015). The possible cross-reaction between goji berries allergens and plasma antibodies of latex-allergic individuals was already mentioned by others (Gámez, Marchán, Miguel, Sanz, & del Pozo, 2013).

When comparing our results with those predicted by AlgPred, we see that some of the proteins found as potential allergens in our study and already reported as allergens on Allergome, were not predictably allergens using AlgPred. For example, Actin and Serine-glyoxylate aminotransferase (spots 97 and 108, respectively) could bind the IgE present in the plasma of the tested individuals and were already reported as allergens in other organisms, however, they were not predictably allergens by the AlgPred software (Table 3). Also, interestingly, not all identifications corresponding to the same putative protein function lead to the same prediction when using AlgPred. For example, most probable identifications found for spots 62, 84, 85, and 87 correspond to Alpha-1,4-glucan-protein synthase. Despite the similarity among the Mass Spectrometry identifications retrieved for these spots and the fact that they all match the same allergen when using Allergome aligner, these sequences are not all predicted as allergens by AlgPred. The same happened for Polyphenol oxidase identification (Table 3).

Regarding the protein functions that we reported here for the first time as potential allergens, we could observe that two of them [Light-induced protein (spots 36, 37) and Vacuolar ATP synthase (spots 154, 155, 156)] are predictably non-allergens by AlgPred, and that the other two [Mannose 3,5-epimerase (spot 79); and P-loop containing Nucleoside Triphosphate (spot110)] are predictably allergens by AlgPred (Table 3).

Remarkably, none of the 11 identified IgE-binding protein functions had been previously identified as allergen in *Solanaceae*. When searching the Allergome database, we could find 37 different potential allergens in this family but none with a similar protein function as those we have identified in our study. Consequently, none of the identified allergens matched those already reported for *L. barbarum* (Table 4).

Allergen Lyc ba 3 corresponds to a 7–9 kDa Lipid Transfer Protein (LTP), described as a major allergen involved in sensitisation and cross-reactivity (Carnés et al., 2013; Larramendi et al., 2012; Ballarín S et al., 2011). We hypothesised that we were not detecting LTP due to the loss of low molecular weight proteins during the SDS-PAGE separation in 11% gels, which were chosen to allow a better visualisation of spots in the molecular weight area

Table 4. *Lycium barbarum* allergens reported on Allergome database.

Lycium barbarum allergen	Protein function
Lyc ba 3; Allergome code: 10194	Lipid Transfer Protein
Lyc ba Enolase; Allergome code: 10798	Enolase
Lyc ba Glucosidase: Allergome code: 10799	Glucosidase

where allergens were more concentrated (30–52 kDa). Therefore, using plasma from individuals 1, 3 and 4, we tried to improve the visualisation of the low molecular weight protein range by using 1D Western blot assay in 15% gels. Still, we could not detect any signal in the area of 7–9 kDa (Supplementary figure 2). This shows that either the tested individuals do not react against LTP, or the protein extraction method used here was not effective to extract LTP. In conclusion, our study contributed to characterise the goji berries proteome, with the identification of 93 out of the 180 spots separated in 2D SDS-PAGE, corresponding to 29 different protein functions. Furthermore, we have identified, for the first time in the *Solanaceae* family, 11 potential allergens, four of which had never been reported in the Allergome database. We have further proved the cross-reactivity between goji berries allergens and plasma antibodies of individuals with allergic history to potato and tomato proteins.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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