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# Identification of seed storage proteins as the major constituents of the extra virgin olive oil proteome

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## ABSTRACT

Proteins are minor components of extra virgin olive oil (EVOO), but the nature of the olive oil proteome is still elusive. In this paper, we have uncovered the EVOO proteome for the first time. Seed storage proteins of globulin-type were identified as the most abundant proteins in EVOO, which also contains an active 13-lipoxygenase and several potential allergenic proteins, including the “panallergen” profilin. We validated our proteomic data by Western blotting and enzyme activity assays. Our data also demonstrated that the seed is the main source of proteins in EVOO, while the contribution of the pulp is uncertain and needs further verification. The impact of EVOO proteins on its stability and quality, and on human health is discussed.

## 1. Introduction

The olive tree (*Olea europaea* L.) is a millennial crop with edible fruits that are used to produce oil and table olives intended for human consumption. Olive oil is a functional food since it provides most of the fat content of the Mediterranean diet but also promotes countless health benefits (Perona & Botham, 2013). The annual world production of olive oil has doubled from 1.45 million tones in 1991 to around 3.31 million tones nowadays, on nearly 10 million ha of land (International Olive Council, IOC, 2019). In parallel, a similar upward trend occurred in olive oil consumption, with non-IOC countries leading this increase. Therefore, the olive is a strategic crop for the agrifood industry of the Mediterranean countries.

According to the IOC, virgin olive oil (VOO) is a natural juice that is obtained from fruits solely by mechanical or other physical procedures (e.g. cold pressing) without any additional refining process. Among the different types of VOO, extra virgin olive oil (EVOO) is that of highest quality. It shows a free acidity, expressed as the oleic acid (18:1cis-Δ<sup>9</sup>) content, of less than 0.8 g per 100 g of oil (EU Commission implementing regulation No. 1348/2013). The saponifiable fraction of VOO represents around 98–99% of olive oil and consists of triacylglycerides (~94–96%), mono- and diacylglycerides (1–3%), phospholipids, sterol esters and waxes (Boskou, 2011). The olive oil is distinguishable from other vegetable oils because of its high oleic acid content (55–83%). The unsaponifiable fraction represents the remaining 1–2% and contains different biomolecules including free fatty

acids, hydrocarbons, aliphatic and aromatic alcohols, sterols, tocopherols, pigments (chlorophylls and carotenoids), phenolic compounds and volatiles (Boskou, 2011). These minor components are of great importance for the sensory (flavor) and healthy properties of VOO (García-González, Aparicio-Ruiz & Aparicio, 2008), as well as for its oxidative stability (Mateos, Domínguez, Espartero & Cert, 2003). Their quantitative analysis also determines the quality grade of olive oil and constitutes a tool to detect adulterations (Aparicio, Aparicio-Ruiz & García-González, 2007).

Among these minor components, peptides and proteins are of special interest because of their relation with the stability of vegetable edible oils (Hidalgo & Zamora, 2006). The presence of proteins in VOO was first reported by Hidalgo, Alaiz & Zamora (2001) and further verified by other authors (Martín-Hernández, Benet & Obert, 2008; Esteve, D'Amato, Marina, García & Righetti, 2013). The presence of oxidizing enzyme activities in VOO was also previously reported (Georgalaki, Bachmann, Sotiroidis, Xenakis, Porzel & Feussner, 1998a; Georgalaki, Sotiroidis & Xenakis, 1998b). These proteins are relocated from the fruit to the oil during the mechanical pressing of fruits. The protein content in olive oils is small and fluctuates between 0.07 and 2.4 mg Kg<sup>-1</sup> depending on the type of oil (EVOO, VOO, refined, etc.) and the methodology employed for protein determination (Montealegre, Esteve, García, García-Ruiz & Marina, 2014). However, despite several studies succeeded in isolating and quantifying proteins from both virgin and refined olive oil matrices, the identification of these proteins has been challenging, so the nature of the olive oil

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proteome is still elusive (Esteve et al., 2013). An insufficient quantity and/or quality of the starting material used and the lack of transcriptomic and genomic records for this species until 2015 might explain this fact. In the present work, all these restrictions have been overcome, with the objective of identifying the main protein constituents of EVOO.

## 2. Material and methods

### 2.1. Plant material and antibodies

Mature drupes were collected at maturity (210 days after anthesis, DAA) from selected olive trees (cv. Picual) grown at the Estación Experimental del Zaidín-CSIC (Granada, Spain). Olives were destoned, and seeds were extracted by mechanical breakage of the woody endocarp and stored at  $-20\text{ }^{\circ}\text{C}$  until use. Freshly bottled extra virgin olive (cv. Picual) oil of the Protected Designation of Origin (PDO) “Montes de Granada” was purchased from a local market and stored in the dark at  $15\text{--}18\text{ }^{\circ}\text{C}$  until use.

The rabbit anti-soybean 13S-lipoxygenase (LOX) polyclonal antibody was purchased from Agrisera (catalog no. AS6\_128, Vännäs, Sweden). Anti-olive 7S globulin, anti-olive 11S globulin, and anti-olive profilin (Ole e 2) primary polyclonal sera were produced by immunization of rabbits as previously described (Alché, Jiménez-López, Wang, Castro & Rodríguez-García, 2006; Morales, Jiménez-López, Castro, Rodríguez-García & Alché, 2008; Zafra, M'rani-Alaoui, Lima, Jiménez-López & Alché, 2018).

### 2.2. Protein extraction, quantification and SDS-PAGE

Mature seeds ( $\sim 0.3\text{ g}$ ) were powdered in liquid  $\text{N}_2$  and total proteins were extracted in 125 mM Tris-HCl buffer (pH 6.8) containing 0.2% SDS and 1%  $\beta$ -mercaptoethanol as previously described (Alché et al. 2006). EVOO proteins were extracted by mixing oil aliquots (200 mL each) with the same volume of extraction buffer (125 mM Tris-HCl, pH 7.5, 1% SDS, 1 M urea and 0.1% DTT) and vigorous shaking for 1 h at  $4\text{ }^{\circ}\text{C}$ . Samples were then spun at 3,500 rpm for 30 min at  $4\text{ }^{\circ}\text{C}$ , and proteins were precipitated overnight at  $-20\text{ }^{\circ}\text{C}$  with 20% trichloroacetic acid (TCA) in chilled acetone and 25 mM DTT. After acetone washing, proteins were pelleted by centrifugation at 7,500 rpm for 30 min. Protein pellets were further air-dried and resuspended in 0.5 mL of a solution containing 125 mM Tris-HCl (pH 7.5) and 1% SDS. Finally, all protein extracts were pooled and concentrated to a single aliquot of  $\sim 380\text{ }\mu\text{L}$  using an Amicon® Ultra-15 Ultracel®-3K centrifugal filter device (Merck Millipore Ltd., Carrigtwohill, Ireland) following the manufacturer's instructions. Two independent extractions were made, each of them from five liters of EVOO. The resulting protein samples were stored at  $-20\text{ }^{\circ}\text{C}$  until use.

Protein concentration was determined using the 2-D Quant kit (GE Healthcare Bio-Sciences, Uppsala, Sweden) following the manufacturer's standard procedure. Protein concentrations were calculated as the mean  $\pm$  SD of six ( $n = 6$ ) assays from two independent extractions (three technical replicas each).

Seed and EVOO proteins ( $\sim 50\text{ }\mu\text{g}$  per sample) were mixed with an equal volume of  $2 \times$  sample buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, 7.7% DTT, 0.0025% bromophenol blue) and electrophoresed on 4–20% polyacrylamide gel slabs using a Mini-PROTEAN® Tetra Cell (Bio-Rad, Hercules, CA, USA) following standard protocols. After electrophoresis, proteins were labelled with Flamingo fluorescent stain (Bio-Rad), following the manufacturer's instructions. Images were obtained in a Pharos FX Plus device (Bio-Rad), and the intensity of the fluorescent bands and their experimental  $M_w$  were determined with the Quantity One software (Bio-Rad) as the average  $\pm$  SD of four ( $n = 4$ ) gels from two independent extractions (two technical replicas each).

### 2.3. Immunodetection of EVOO proteins by Western blotting

EVOO proteins were electroblotted onto a PVDF membrane using a Trans-Blot® Turbo™ transfer apparatus (Bio-Rad) following standard procedures. Non-specific binding sites were blocked for 1 h at RT in Tris-buffered saline (TBS) solution (pH 7.4) containing 5% skimmed milk powder. Immunodetection of EVOO proteins was carried out by incubating each membrane overnight at  $4\text{ }^{\circ}\text{C}$  with the corresponding primary antibody diluted in blocking solution (anti-LOX Ab = 1:500; anti-profilin,  $-7\text{S}$  and  $-11\text{S}$  Abs = 1:1000). After three washes in TBST solution (TBS buffer plus 0.05% Tween-20) for 15 min each, PVDF membranes were treated for 1 h at RT with a goat anti-rabbit IgG (HRP) secondary antibody (Agrisera), diluted 1:2500 in TBS buffer. Finally, blots were rinsed as above, developed for 3 min with Clarity Western ECL substrate (Bio-Rad), and scanned in a Gel Doc imaging device (Bio-Rad). A negative control was carried out by omitting the primary Ab.

### 2.4. Lipoxygenase activity in-gel assays

*In-gel* lipoxygenase activity in EVOO was measured as described by (Heinisch, Kowalski, Ludwig & Tauscher, 1996), with minor modifications. Briefly, EVOO proteins were electrophoresed under non-denaturing conditions and gel slabs were incubated for 30 min in a solution containing  $\alpha$ -linolenic acid (18:3 *cis,cis,cis*- $\Delta^9,\Delta^{12},\Delta^{15}$ ) and 10 mM sodium cyanide. Gels were further stained for 20 min with a solution containing *N,N*-dimethyl-*p*-phenylenediamine, methanol and acetic acid. Experiments were carried out in duplicate, and a negative control was performed by excluding the enzyme substrate from the reaction mixture.

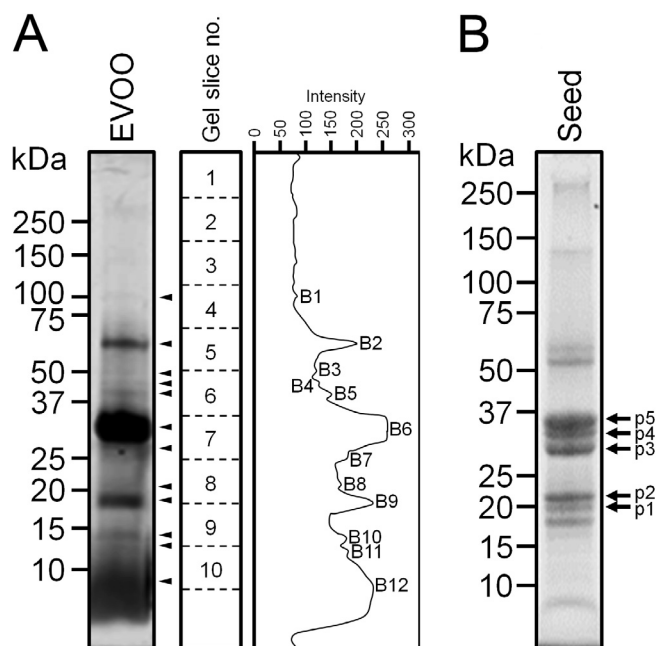
### 2.5. Protein identification by nLC-MS

nLC-MS analysis was performed using a nanoACQUITY UPLC® system (Waters, Milford, MA, USA) and a Synapt G2Si ESI Q-Mobility-TOF spectrometer (Waters) equipped with an ion mobility chamber (T-Wave-IMS), as described by Castro, Lima-Cabello & Alché (2020). Accurate LC-MS data were collected in high definition direct data analysis (HD-DDA) mode using the ion mobility separation step (Helm et al., 2014). Mascot searches against an *ad hoc* olive protein database were carried out at a final peptide FDR of 1%. An error of  $> 15\text{ ppm}$  and 0.2 Da of peptide and fragment mass tolerance, respectively, was tolerated. Peptide matches were also manually validated when their score was close to the Mascot homology threshold for a given Mascot *p* value (Figs. S1 to S24 from Castro et al., 2020).

## 3. Results and discussion

### 3.1. SDS-PAGE profiling of EVOO proteins

The protein concentration in EVOO samples was determined using the 2-D Quant kit. This method was designed for the accurate determination of protein concentration in samples having chemical reagents (SDS, DTT, etc.) and/or secondary metabolites that can interfere with common protein assays (Bradford, Lowry, etc.). Our data showed that EVOO contains about  $36.18 \pm 1.47\text{ }\mu\text{g}$  of proteins per 100 g of oil. These values are similar to those obtained by amino acid analysis (Hidalgo et al., 2001; Martín-Hernández et al., 2008), which is likely the most accurate procedure for determining protein concentration (Ozols, 1990). However, this approach is quite laborious, and requires expensive equipment and a considerable expertise to operate. In contrast, the 2-D Quant kit is simple to use, fast and inexpensive. To our knowledge, this method has never been used before to measure the protein concentration in vegetable oils. However, our data should be taken with precaution since this and other protein quantifying methods should be compared from the same (origin, quality, etc.) oil sample.



**Fig. 1.** (A) Flamingo-stained polyacrylamide gel showing protein 1-D profile of extra virgin olive oil (EVOO). The densitometric profile of EVOO protein extracts is also plotted. This gel was systematically cut into slices of ~1 cm width each from the top (slice S1) to the bottom (slice S10) for further mass spectrometry analysis. (B) Flamingo-stained polyacrylamide gel showing protein 1-D profile of olive seeds. Protein markers are displayed on the left of each figure.

The protein pattern of EVOO is displayed in Fig. 1A. A very prominent band (B6) with a calculated Mw of 30.5 kDa accounted for  $19.0 \pm 4.2\%$  of the total protein content. Three other secondary protein bands of ~ 60.5 (B2), ~18.5 (B9) and ~ 14 kDa (B10), respectively, represents altogether about  $13.5 \pm 3.8\%$  of the total protein content. In addition, a large and diffuse band just below the 10-kDa marker (B12) was also visible after staining with Flamingo. Finally, six bands of much less intensity of about 100.5 (B1), 50.5 (B3), 44.5 (B4), 40 (B5), 27 (B7), 21 (B8) and 12 kDa (B11), respectively, completed the visible EVOO proteome. This electrophoretic pattern is rather similar to that reported by Martín-Hernández et al. (2008) with the exception of the 30.5-kDa band. Moreover, we did not detect the 4.6-kDa band previously reported in olive oil and other non-related vegetable oils (Hidalgo et al., 2001). Nevertheless, some authors suggested that this electrophoretic band is actually an artifact coming from the Whatman filter used in the extraction method (Esteve et al., 2013).

### 3.2. Seed storage proteins (SSP) are the major protein constituents of EVOO

In order to identify the EVOO proteins, the gel lane containing the Flamingo-stained proteins was systematically cut into slices of ~ 1 cm width each from the top (slice S1) to the bottom (slice S10) (Fig. 1A). Protein identification searches were carried out using recently published olive transcriptomic and genomic records (Carmona et al., 2015; Cruz et al., 2016). The lack of such information has been often a bottleneck for protein determination in olive oil matrices (Esteve et al., 2013). Here, by using the ion mobility cell and wide-band enhancement TOF synchronization, both the sensitivity and quantity/quality of MS spectra obtained per run was improved over standard DDA acquisition. All gel slices, except S2, delivered positive matches against the databases surveyed (Table 1; Table S1 from Castro et al., 2020). Overall, the determined EVOO proteome comprises nearly 40 unique proteins.

Among the identified proteins in the EVOO, it was noticeable the presence of several seed storage proteins (SSP), including a salt-soluble globulin of vicilin-type (7S) and several 11S globulins, also known as

legumins (Table 1; Table S1 from Castro et al., 2020). To validate identifications, we carried out Western blotting experiments using two specific antibodies against olive seed 11S and 7S proteins, respectively (Alché et al., 2006; Zafra et al., 2018). The immunoblot probed with the anti-7S Ab revealed the presence of a single protein band of around 50 kDa in the EVOO proteome (Fig. 2A). This reactive band might correspond to one of the two 7S protein precursors recently reported in olive seeds (Zafra et al., 2018), and can be tentatively assigned to band B3 on the basis of its Mw. On the other hand, the anti-11S Ab was able to react with a protein that clearly corresponds to band B6 on the basis of its size and intensity (Fig. 2A). It is plausible that this massive band contains the  $\beta$ (basic) subunits of *Solea* I (p3 and p4) and *Solea* II (p5) precursors of 11S globulins, by far the most abundant protein of olive seeds (Alché et al., 2006). These proteins likely overlap due to their abundance and vicinity, making them indistinguishable on 1-D mini-gels.

Beta-conglutin proteins of lupine seeds show anti-inflammatory properties (Lima-Cabello et al., 2017; Lima-Cabello et al., 2018). Interestingly, the olive seed flour promotes a significant attenuation of key pro-inflammatory mediators of  $\beta$ -cell damage in type 2 diabetes (T2D) (Lima-Cabello et al. 2015). Recently, Lima-Cabello and coworkers observed that olive seed  $\beta$ -conglutins also show anti-inflammatory activity in the PANC-1 induced inflammation cell model (personal communication). These preliminary data suggest that SSP have potential as functional food components, providing alternative therapies for the prevention and treatment of inflammatory-related diseases. However, it is not clear whether SSP might exert any therapeutic effect at their present concentration levels in EVOO, so this question is currently being investigated in our laboratory.

### 3.3. EVOO contains an active 13S-lipoxygenase enzyme

During VOO storage, oxidation of unsaturated fatty acids (UFA) leads to its oxidative rancidity, adversely affecting its flavor and nutritional properties (Morales & Przybylski, 2013). Beside autoxidation and photooxygenation processes, it has been suggested that lipoxygenases (LOX) might be also responsible for the oxidative deterioration of UFA and pigments in vegetable edible oils (Mínguez-Mosquera, Gandul-Rojas, Garrido-Fernandez & Gallardo-Guerrero, 1990). The presence of lipoxygenase (LOX) activity in VOO was first reported by Georgalaki et al. (1998a), but our proteomic analyses failed to identify this enzyme in EVOO. In order to shed light to this controversy, we carried out Western blot experiments using a commercial anti-soybean 13S-LOX polyclonal Ab that shows high cross-reactivity with an olive seed 13S-LOX (Zienkiewicz, Zienkiewicz, Rejón, Alché, Castro & Rodríguez-García, 2014). This anti-LOX Ab was able to detect a major protein band of ~ 100 kDa in EVOO (Fig. 2A). The presence of an active full-size 13S-LOX in EVOO was further confirmed by *in-gel* enzyme assays (Fig. 2B). An additional smaller protein fragment with enhanced LOX activity was also visible (Fig. 2B). This partial LOX protein was also recognized by the anti-LOX Ab as a very weak band of ~ 27 kDa (Fig. 2A). Interestingly, limited proteolysis of soybean LOX1 also generated a fragment of the enzyme with enhanced activity and membrane binding ability (Maccarrone et al., 2001). 13S-LOX activity is mainly located in protein bodies (PB) and on the surface of lipid bodies of olive seed endosperm and cotyledon cells (Zienkiewicz et al., 2014). It has been suggested that this seed 13S-LOX might be the enzyme detected originally in VOO (Georgalaki et al., 1998b). However, another 13S-LOX gene, called *Oep2LOX2*, which is involved in the biosynthesis of volatile compounds of VOO, is also highly expressed in the olive ripe mesocarp (Padilla, Hernández, Sanz & Martínez-Rivas, 2009). Therefore, more investigations are needed to clearly define the tissue origin of olive oil 13S-LOX.

**Table 1**

List of proteins identified in EVOO. Seed storage proteins are highlighted in bold.

Gel slide	Band no.	Band Mw (kDa)	Proteins identified (species)	Database matched
S1	None	–	Actin 7 ( <i>Arabidopsis thaliana</i> )	G, Se, Pi, Po
S3	None	–	Protein TOPLESS ( <i>Arabidopsis thaliana</i> )	Pi
S4	B1	~100.4	Chaperone protein ClpB3, mitochondrial ( <i>Oryza sativa</i> subsp. Japonica)	Pi
			Serine hydroxymethyltransferase 1, mitochondrial ( <i>Arabidopsis thaliana</i> )	Pi
			Protein translocase subunit SECA1, chloroplastic ( <i>Arabidopsis thaliana</i> )	Pi
			Heat shock cognate 70 kDa protein 2 ( <i>Solanum lycopersicum</i> )	G, Se, Pi
			Ribonucleotide-diphosphate reductase subunit alpha ( <i>Nevskia soli</i> )	G
S5	B2	~61.0	Ribonucleotide-diphosphate reductase subunit alpha ( <i>Nevskia soli</i> )SUMO-activating enzyme subunit 1B-1-like isoform X1 ( <i>Nicotiana tomentosiformis</i> )	G
			Fasciclin domain protein ( <i>Medicago truncatula</i> )	G
			Acetyl-coenzyme A synthetase, chloroplastic/glyoxysomal isoform X1 ( <i>Sesamum indicum</i> )	G
			Calcium-dependent protein kinase 7 ( <i>Arabidopsis thaliana</i> )	Po
			ATP-dependent CLP protease ATP-binding subunit CLPa homolog CD4A, chloroplastic ( <i>Solanum lycopersicum</i> )	Po
			Putative L-cysteine desulfhydrase 1 ( <i>Oryza sativa</i> subsp. Japonica)	Pi
			Ras-related protein RABF2a ( <i>Arabidopsis thaliana</i> )	Pi
			Putative uncharacterized protein ( <i>Vitis vinifera</i> )	Pi
			ATP synthase subunit alpha ( <i>Olea europaea</i> subsp. <i>europaea</i> )	UniProt
			<b>Vicilin-like antimicrobial peptides 2-2</b> ( <i>Erythranthe guttata</i> )	G
			Probable U6 snRNA-associated Sm-like protein LSm4 ( <i>Nicotiana tabacum</i> )	G, Pi
			ATP synthase subunit alpha, mitochondrial ( <i>Marchantia polymorpha</i> )	Po
			Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex 2 ( <i>Arabidopsis thaliana</i> )	SeSe
S6	B3 B4 B5	~50.3 ~44.4 ~39.9	Vacuolar protein sorting-associated protein 52A ( <i>Arabidopsis thaliana</i> )	Po
			Uncharacterized protein ( <i>Erythranthe guttata</i> )	Po
			Elongation factor Tu, mitochondrial ( <i>Arabidopsis thaliana</i> )	Pi
			<b>11S globulin subunit beta-like</b> ( <i>Erythranthe guttata</i> )	G
			Exocyst subunit exo70 family protein E2 ( <i>Arabidopsis thaliana</i> )	G
			Pollen-specific protein C13 ( <i>Zea mays</i> )	Po
			Probable UDP-N-acetylglucosamine-peptide N-acetylglucosaminyltransferase <i>SEC</i> ( <i>Arabidopsis thaliana</i> ) <b>Glycinin G3</b> ( <i>Glycine max</i> )	Po Se
S7	B6B7	~30.7~27.1	Elongation factor Tu, mitochondrial ( <i>Arabidopsis thaliana</i> )	Pi
			<b>11S globulin subunit beta-like</b> ( <i>Erythranthe guttata</i> )	G
			Exocyst subunit exo70 family protein E2 ( <i>Arabidopsis thaliana</i> )	G
			Pollen-specific protein C13 ( <i>Zea mays</i> )	Po
			Probable UDP-N-acetylglucosamine-peptide N-acetylglucosaminyltransferase <i>SEC</i> ( <i>Arabidopsis thaliana</i> ) <b>Glycinin G3</b> ( <i>Glycine max</i> )	Po Se
S8	B8 B9	~20.7 ~18.4	Elongation factor Tu, mitochondrial ( <i>Arabidopsis thaliana</i> )	Pi
			Pollen-specific protein C13 ( <i>Zea mays</i> )	Po
			Serine/threonine protein phosphatase 2A, 59 kDa regulatory subunit B' gamma isoform ( <i>Arabidopsis thaliana</i> )	Po
			Uncharacterized protein ( <i>Morus notabilis</i> )	Po
			<b>11S globulin subunit beta</b> ( <i>Cucurbita maxima</i> )	Se
			<b>11S globulin seed storage protein 2</b> ( <i>Sesamum indicum</i> ) <b>12S seed storage protein CRA1</b> ( <i>Arabidopsis thaliana</i> ) DNA-directed RNA polymerases II, IV and V subunit 6A ( <i>Arabidopsis thaliana</i> )Probable succinyl-coa ligase [ADP-forming] subunit alpha, mitochondrial ( <i>Oryza sativa</i> )	Se Se Se Se Pi
S9	B9 B10 B11	~18.4 ~13.8 ~12.0	<b>11S globulin subunit beta-like</b> ( <i>Nicotiana attenuata</i> )	G
			Pollen-specific protein-like At4g18596 ( <i>Arabidopsis thaliana</i> )	Po
			<b>11S globulin seed storage protein 2</b> ( <i>Sesamum indicum</i> )	Se
S10	B11 B12	~12.0 ~8.0	Profilin-1 ( <i>Ricinus communis</i> )	G, Se, Pi, Po
			Vesicle-associated protein 4-1 ( <i>Arabidopsis thaliana</i> )	Pi

G, olive (cv. Farga) genome (Cruz et al., 2016); Se, olive (cv. Picual) seed transcriptome (unpublished); Pi and Po, olive (cv. Picual) pistil (Pi) and pollen (Po) transcriptomes (Carmona et al., 2015).

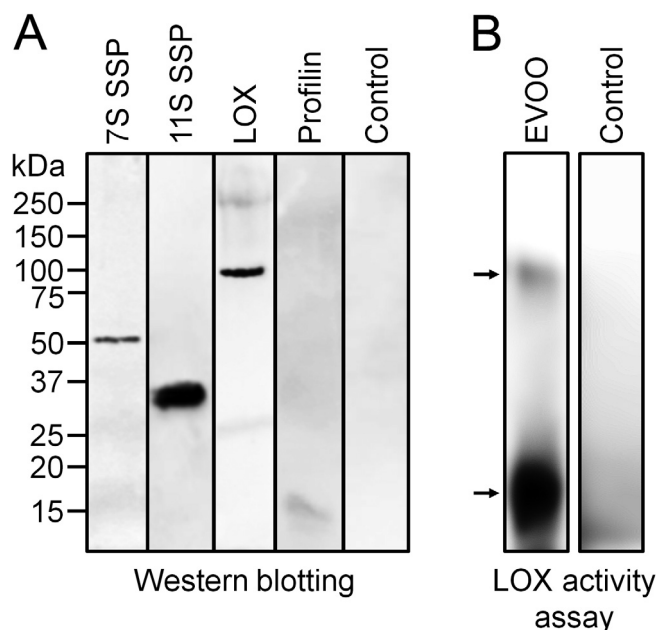
### 3.4. Identification of potential allergenic proteins in EVOO

Among the different edible plant oils, olive oil is considered non-allergenic. The low protein concentration in EVOO might explain its low allergenicity despite its high consumption. However, several cases of allergy to olives and olive oil by either contact, inhalation or consumption have been reported (Esteve, Montealegre, Marina & García, 2012). To date, a thaumatin-like protein (TLP), called Ole e 13, is the only allergen described in the olive fruit (Palomares, Alcántara, Quiralte & Villalba, 2008). Here, we have identified several potentially allergenic proteins in EVOO, including 11S and 7S globulins, and a profilin (Ole e 2). The presence of these proteins in EVOO was validated by immunoblotting (Fig. 2A). On the contrary, we could not confirm the presence of Ole e 13 allergen in EVOO. This protein might be degraded during the oil extraction process, as reported for edible olives (Torres et al., 2014). It is interesting to notice that a new pollen-food (olive-olive) syndrome was previously proposed, but the allergen responsible for this cross-reactivity have not been yet identified (Ünsel et al., 2009). Since pollen and plant food profilins show equivalent IgE reactivity

(Sirvent et al., 2011), this 'pan-allergen' might be a good candidate to be further explored.

### 3.5. Fungal proteins in EVOO

We identified up to 15 fungal proteins from the yeast-like fungus *Aureobasidium pullulans* (Table S2 from Castro et al., 2020), which is ubiquitous in the phyllosphere and carposphere of olive (Abdelfattah, Nicosia, Cacciola, Droby & Schena, 2015). Thus, draft genomes of both the wild and domesticated olive contain contaminating gene sequences of *Aureobasidium pullulans* (Cruz et al., 2016; Unver et al., 2017). A recent study showed that both acidity and peroxide levels increase in the olive oil extracted from fruits that were inoculated with this fungus (Torbati, Arzanlou, Azadmard-damirchi, Babai-ahari & Alijani, 2014). However, it is not clear whether fungal proteins are responsible for this detrimental effect on olive oil quality.



**Fig. 2.** (A) Immunodetection of 7S and 11S globulin, lipoxygenase (LOX) and profilin (Ole e 2) proteins in EVOO total protein extracts by Western blotting. A negative control was performed by omitting the primary Ab. Protein markers are displayed on the left. (B) *In-gel* assay of lipoxygenase activity (arrows) from EVOO total protein extracts. A negative control was performed by excluding the substrate from the reaction mixture.

#### 4. Conclusions

Some fruit proteins overcome the milling and extraction process and are transferred intact to EVOO. Seeds are the main source of EVOO proteins, being storage proteins of globulin-type the most abundant constituents of the EVOO proteome. The EVOO also contains other fruit proteins, including an active lipoxygenase and several potential allergenic proteins. The presence of fungal proteins in EVOO might be circumstantial and suggests that harvested olive fruits were contaminated either in the field or during their storage. More investigations are needed to evaluate the potential impact of EVOO proteins on its oxidative stability and quality, and their relevance to human health.

#### CRedit authorship contribution statement

**Antonio Jesús Castro:** Data curation, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Elena Lima-Cabello:** Investigation, Methodology, Writing - original draft. **Juan de Dios Alché:** Conceptualization, Investigation, Funding acquisition, Writing - original draft, Writing - review & editing, Supervision.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2020.100099>.

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