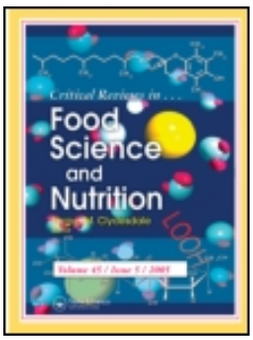

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Proteins in Olive Fruit and Oil

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This paper is a comprehensive review grouping the information on the extraction, characterization, and quantitation of olive and olive oil proteins and providing a practical guide about these proteins. Most characterized olive proteins are located in the fruit, mainly in the seed, where different oleosins and storage proteins have been found. Unlike the seed, the olive pulp contains a lower protein content having been described a polypeptide of 4.6 kDa and a thaumain-like protein. Other important proteins studied in olive fruits have been enzymes which could play important roles in olives characteristics. Part of these proteins is transferred from the fruit to the oil during the manufacturing process of olive oil. In fact, the same polypeptide of 4.6 kDa found in the pulp has been described in the olive oil and, additionally, the presence of other proteins and enzymes have also been described. Protein profiles have recently been proposed as an interesting strategy for the varietal classification of olive fruits and oils. Nevertheless, there is still a lot of knowledge without being explored requiring new studies focused on the determination and characterization of these proteins.

Keywords Olive, olive oil, protein, oleosin, seed storage protein

INTRODUCTION

Olive (*Olea europaea L.*) is a long-life tree from which humans have obtained oil, fruit, and wood for more than 5000 years (Rallo et al., 2005). The olive is the only tree of *Oleaceae* family with edible fruit. Olive fruits have been used for several years to produce olive oil and table olives. In addition to human and animal consumption, olive oil has also been used for other purposes such as in the cosmetic and the pharmaceutical industries. According to the International Olive Oil Council, world olive oil production has been calculated in 2.67 million tons in the season 2008–2009 (last year season with final data) and the table olive world production has reached values of two million tons in the last seasons. Although the production of olive is extended worldwide, the main olive oil producer is still the European Union (EU), especially the Mediterranean area, accounting for 73% of the world total. Main consumers are located in EU (66%) and USA (9%), although other countries such as China, Russia, and India have increased the olive oil consumption during last years.

Olive oil consumption has been associated with the decrease of the incidence of important illness like cardiovascular diseases, cancer, and Alzheimer's disease in the Mediterranean area (López-Miranda et al., 2010). Due to this fact, olive fruits and, especially, olive oils have been investigated in deep to determine their nutritional and sensory values, their composition, their uses, and the great benefits above-mentioned for human health. So far, some compounds such as fatty acids, polyphenols, or sterols have greatly been investigated. However, despite their high informative value and role in food stability and allergenicity, proteins have scarcely been investigated in comparison with the other olive components.

The aim of this review is to provide comprehensive information on proteins present in olive fruits and oils. To achieve this goal, protein extraction procedures will be critically described together with the methods employed for the characterization of olive proteins in the fruit and oil. Finally, information on total protein contents in fruits and oils as well as on the varietal discrimination potential of proteins will also be provided.

EXTRACTION OF PROTEINS FROM OLIVE FRUIT AND OIL

Sample preparation is the first critical step that affects the outcome of the entire protein analysis. The first problem for protein determination in olive fruits and, especially, in the case of the olive oil is the limited number of extraction methodologies applicable to these lipidic matrices. Table 1 gathers the extraction methods developed for that purpose.

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Table 1 Proteins in olive fruits and olive oils. Extraction and characterization methodologies

Type	Name	M	Sample	Extraction stages	Analytical technique	Reference
Proteins in olive fruit						
			Seed (endosperm and cotyledon)	1. Extraction buffer: 125 mM Tris-HCl (pH 6.8), 125 mM Tris-HCl (pH 6.8) with 0.2% (m/v) SDS, or 125 mM Tris-HCl (pH 6.8) with 0.2% (m/v) SDS and 1% (m/v) 2-mercaptoethanol. 2. Centrifugation, boiling, and centrifugation. 3. Protein precipitation with acetone.	SDS-PAGE and 2D-SDS-PAGE (silver staining)	Alché et al., 2006
SSPs	Solea I/ Pro 2 Solea II/Pro 1	41 kDa 47.5 kDa	Seed	1. Extraction buffer: 125 mM Tris-HCl (pH 6.8) with 0.2% (m/v) SDS and 1% (m/v) 2-mecaptoethanol. 2. Centrifugation. 3. Protein precipitation with cold acetone.	Native PAGE and SDS-PAGE (Coomassie blue or silver staining)	Wang et al., 2001; Jiménez et al., 2007
			Stone	1. Extraction buffer: 125 mM Tris-HCl (pH 7.5) with 1% (m/v) SDS and 0.5% (m/v) DTT. 2. Ultrasonic microprobe. 3. Centrifugation. 4. Protein precipitation with cold acetone.	SDS-PAGE (Coomassie blue staining)	Esteve et al., 2010
Seed oleosins	–	20 kDa	Seed (Oil bodies and microsomes)	1. Homogenization medium: 0.4 M sucrose, 100 mM Hepes/NaOH (pH 7.5) with 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 1% (m/v) ascorbic acid. 2. Filtration through four layers of cheesecloth and centrifugation. 3. Oil bodies purification (chloroform-methanol (2:1, v/v)). 4. Protein isolation from defatted oil bodies: buffer extraction (25 mM sodium borate buffer (pH 8.35) with 25 mM SDS) and heating (100°C for 1 h). 5. Centrifugation 6. Drying.	SDS-PAGE (silver staining)	Hidalgo et al. 2001
			Seed (oil bodies)	1. Homogenization medium: 0.4 M sucrose, 100 mM Hepes/NaOH (pH 7.5) with 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 1% (m/v) ascorbic acid. 2. Filtration and centrifugation. 3. Oil bodies purification (diethylether extraction and centrifugation).	SDS-PAGE (Coomassie blue staining), Western blotting, and electron microscopy	Ross et al., 1993
			Stone	1. Extraction buffer: 125 mM Tris-HCl (pH 7.5) with 1% (m/v) SDS and 0.5% (m/v) DTT. 2. Ultrasonic microprobe. 3. Centrifugation 4. Protein precipitation with cold acetone.	SDS-PAGE (Coomassie blue staining)	Esteve et al., 2010
	–	50 kDa	Seed (oil bodies)	1. Homogenization medium: 0.4 M sucrose, 100 mM Hepes/NaOH (pH 7.5) with 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 1% (m/v) ascorbic acid. 2. Filtration and centrifugation. 3. Oil bodies purification (diethylether extraction and centrifugation).	SDS-PAGE (Coomassie blue staining), Western blotting, and electron microscopy	Ross et al., 1993

			Stone	1. Extraction buffer: 125 mM Tris-HCl (pH 7.5) with 1% (m/v) SDS and 0.5% (m/v) DTT. 2. Ultrasonic microprobe. 3. Centrifugation 4. Protein precipitation with cold acetone.	SDS-PAGE (Coomassie blue staining)	Esteve et al., 2010
Seed polypeptide	–	4.6 kDa	Seed (oil bodies)	1. Homogenization medium: 0.4 M sucrose, 100 mM Hepes/NaOH (pH 7.5) with 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 1% (m/v) ascorbic acid. 2. Filtration through four layers of cheesecloth and centrifugation. 3. Oil bodies purification (chloroform-methanol (2:1, v/v)). 4. Protein isolation from defatted oil bodies: buffer extraction (25 mM sodium borate buffer (pH 8.35) with 25 mM SDS) and heating (100°C for 1 hour). 5. Centrifugation. 6. Drying.	SDS-PAGE (silver staining)	Hidalgo et al. 2001; Hidalgo et al. 2002
Olive mesocarp proteins	–	4.6 kDa	Mesocarp (oil bodies)	1. Homogenization medium: 0.4 M sucrose, 100 mM Hepes/NaOH (pH 7.5) with 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 1% (m/v) ascorbic acid. 2. Filtration through four layers of cheesecloth and centrifugation. 3. Oil bodies purification (chloroform-methanol (2:1, v/v)). 4. Protein isolation from defatted oil bodies: buffer extraction (25 mM sodium borate buffer (pH 8.35) with 25 mM SDS) and heating (100°C for 1 hour).	SDS-PAGE (silver staining)	Hidalgo et al. 2001; Hidalgo et al. 2002
			Mesocarp (oil bodies)	5. Centrifugation. 6. Drying.	SDS-PAGE (silver staining) and HPLC	Zamora et al. 2001
	Thaumatin-like (allergen)	23 kDa	Pulp (mesocarp and epicarp)	1. Washing with ether-ethanol (3:1, v/v). 2. Extraction buffer: phosphate (pH 7.0). 3. Centrifugation. 4. Dialyzation.	SEC, SDS-PAGE (Coomassie blue staining)	Palomares et al., 2008
	–	70 kDa	Mesocarp (oil bodies)	1. Homogenization medium: 0.4 M sucrose, 100 mM Hepes/NaOH (pH 7.5) with 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 1% (m/v) ascorbic acid. 2. Filtration and centrifugation. 3. Oil bodies purification (diethylether extraction and centrifugation).	SDS-PAGE (Coomassie blue staining), Western blotting, and electron microscopy	Ross et al. 1993
	–	20–25 kDa	Pulp (mesocarp and epicarp)	1. Pre-cleaning steps: 10% (m/v) TCA/acetone, acetone, 10% (m/v) TCA/water, and 80% acetone (v/v). 2. Extraction buffer: 125 mMTris-HCl (pH 7.5) with 1% SDS (m/v) and 0.5% (m/v) DTT 3. Centrifugation. 4. Protein precipitation with cold acetone.	SDS-PAGE (Coomassie blue staining)	Esteve et al., 2011
Olive pomace proteins	–	30 kDa and some around 20 kDa	Pomace	1. Olive incubation with Alcalase for 1 hour at 50°C and pH 8. 2. Enzyme inactivation: heating at 80°C for 15 minutes. 3. Centrifugation and lyophilization. 4. Precipitation by adjusting the pH to the isoelectric point. 5. Centrifugation. 6. Drying.	SDS-PAGE (Coomassie blue staining)	Vioque et al., 2000

(Continued on next page)

Table 1 Proteins in olive fruits and olive oils. Extraction and characterization methodologies *(Continued)*

Type	Name	M	Sample	Extraction stages	Analytical technique	Reference
Enzymes	LOX	>100 kDa	Whole fruit	1. Extraction buffer: 50 mM sodium phosphate (pH 6.8) with 0.2 mM EDTA, 0.2% (m/v) TritonX-100, 0.3 mM DTT, 10 mM sodium metabisulfite, and 20% (m/v) hydrated PVPP. 2. Filtration in vacuum to remove PVPP and centrifugation. 3. Filtration to exclude lipid material.	Spectrophotometric method and HPLC	Olías et al., 1993
			Seed (oil bodies)	1. Extraction buffer: 50 mM Tris-HCl (pH7.5) with 15% (m/m) sucrose. 2. Centrifugation and filtration. 3. Washing and centrifugation. 4. Oil bodies purification by SEC.	Spectrophotometric method, HPLC, and H^1NMR	Georgalaki et al. 1998a
			Pulp (mesocarp and epicarp)	1. Extraction buffer: 50 mM Hepes (pH 7.5) with 330 mM sorbitol, 20 mM KCl, 2 mM $MgCl_2$, 5 mM EDTA, 7 mM 2-mercaptoethanol, 3 mM DTT, 0.1% (m/v) ascorbate, 10% (v/v) glycerol, and acid-washed PVP. 2. Filtration through commercial tissue and centrifugation.	Spectrophotometric and colorimetric methods, and HPLC	Salas et al., 1999
	Alcohol dehydrogenase	–	Pulp (mesocarp and epicarp)	1. Preparation of an acetone powder extract. 2. Extraction buffer: 50 mM potassium phosphate (pH 7.2) with 14 mM 2-mercaptoethanol, 2 mM DTT, and 10% (v/v) glycerol. 3. Centrifugation. 4. Ammonium sulfate precipitation. 5. Desalting with Sephadex G-25.	IEC and affinity chromatography	Salas and Sánchez, 1998
			Tissues	1. Extraction buffer: 50 mM sodium phosphate (pH 6.8) with 0.2 mM EDTA, 0.2% (m/v) TritonX-100, 0.3 mM DTT, 10 mM sodium metabisulfite, and 20% (m/v) hydrated PVPP. 2. Filtration in vacuum to remove PVPP and centrifugation. 3. Filtration to exclude lipidic material.	CG-FID	Olías et al., 1993
	Acylhydrolase	–	Whole fruit	1. Extraction buffer: 50 mM sodium phosphate (pH 6.8) with 0.2 mM EDTA, 0.2% (m/v) TritonX-100, 0.3 mM DTT, 10 mM sodium metabisulfite, and 20% (m/v) hydrated PVPP. 2. Filtration in vacuum to remove PVPP and centrifugation. 3. Filtration to exclude lipid material.	Colorimetric method	Olías et al., 1993
Enzymes	Fatty acid hydroperoxide lyase (FAHL)	–	Whole fruit	1. Extraction buffer: 50 mM sodium phosphate (pH 6.8) with 0.2 mM EDTA, 0.2% (m/v) TritonX-100, 0.3 mM DTT, 10 mM sodium metabisulfite, and 20% (m/v) hydrated PVPP. 2. Filtration in vacuum to remove PVPP and centrifugation. 3. Filtration to exclude lipidic material.	GC-FID	Olías et al., 1993
	Alcohol acetyl-transferase	–	Tissues	1. Extraction buffer: 50 mM sodium phosphate (pH 6.8) with 0.2 mM EDTA, 0.2% (m/v) TritonX-100, 0.3 mM DTT, 10 mM sodium metabisulfite, and 20% (m/v) hydrated PVPP. 2. Filtration in vacuum to remove PVPP and centrifugation. 3. Filtration to exclude lipidic material.	GC-FID	Olías et al., 1993

	PPO	–	Tissues	1. Preparation of an acetone powder extract with cold acetone and polyethyleneglycol. 2. Filtration and reextraction. 3. Extraction buffer: 50 mM phosphate (pH 6.2) with 1 M KC1. 4. Stirring and centrifugation.	Spectrophotometric method	Hornero-Méndez et al., 2002; Sciancalepore and Longone, 1984
		50–55 kDa (two subunits of 27.7 kDa)	Whole fruit	1. Preparation an acetone powder extract with cold acetone and polyethyleneglycol. 2. Filtration and reextraction. 3. Buffer: 0.1 M sodium phosphate (pH 6.2) with 0.3 mg ml(1 of type-II trypsin inhibitor. 4. Filtration through glass wool and centrifugation.	Spectrophotometric method, SDS-PAGE, Western blotting, and HPLC	Ortega-García et al., 2008
		55, 36, and 21 kDa	Tissues	1. Extraction buffer: 0.1 M sodium phosphate (pH 8.0) with 50 g L^{-1} PVP, 3 g L^{-1} soybean trypsin inhibitor type II, and 2 mM 2-mercaptoethanol. 2. Filtration and centrifugation. 3. Precipitation by slow addition of ammonium sulfate to 80% saturation. 4. Centrifugation. 5. Dialyzation.	Spectrophotometric method, SDS-PAGE, Western blotting, and HPLC	Ortega-García and Peragón, 2009
		–	Tissues	1. Extraction buffer: 100 mM Tris-Glycine (pH 6.8) with 2.5% (m/v) PVPP. 2. Centrifugation.	Spectrophotometric method and SDS-PAGE	Ebrahimzadeh et al., 2003
Enzymes	β-glycosidase	–	Pulp (mesocarp and epicarp)	1. Extraction buffer: 0.1 M borate buffer (pH 9.0) with 5.0 mM EDTA, 1.0 mM PMSF, 1% (m/v) 2-mercaptoethanol, and 10% (m/v) PVP 2. Shaking and centrifugation.	Spectrophotometric method	Briante et al., 2002
		66 kDa	Seed	1. Extraction buffer: water. 2. Stirring and centrifugation. 3. Filtration and precipitation with acetone. 4. Extraction buffer: 125 mM Tris–HCl (pH 6.8) with 0.2% (m/v) SDS and 1% (m/v) 2-mercaptoethanol. 5. Centrifugation, boiling, and centrifugation. 6. Protein precipitation with acetone.	SDS-PAGE (Coomassie blue staining)	de Nino et al., 2008
	Esterase	–	Pulp (mesocarp and epicarp)	1. Extraction buffer: 0.1 M borate buffer (pH 9.0) with 5.0 mM EDTA, 1.0 mM PMSF, 1% (m/v) 2-mercaptoethanol, and 10% (m/v) PVP. 2. Shaking and centrifugation.	Spectrophotometric method	Briante et al., 2002
	Phenylalanine ammonia-lyase	75 kDa	Tissues	1. Extraction buffer: 0.1 M sodium phosphate (pH 8.0) with 50 g L^{-1} PVP, 3 g L^{-1} soybean trypsin inhibitor type II, and 2 mM 2-mercaptoethanol. 2. Filtration and centrifugation. 3. Precipitation by slow addition of ammonium sulfate to 80% saturation. 4. Centrifugation. 5. Dialyzation.	Spectrophotometric method and Western blotting	Ortega-García and Peragón, 2009; Ortega-García et al., 2009
	Superoxide dismutase (SOD)	–	Tissues	1. Extraction buffer: 50 mM Tris–HCl (pH 7.5) with 0.1 mM EDTA, 5 mM cysteine, and 0.2% (v/v) Triton X-100. 2. Filtration through four layers of gauze and centrifugation. 3. Dialyzation.	Spectrophotometric method	Hornero-Méndez et al., 2002

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Table 1 Proteins in olive fruits and olive oils. Extraction and characterization methodologies *(Continued)*

Type	Name	M	Sample	Extraction stages	Analytical technique	Reference
	Peroxidase	68.6–18.4 kDa	Pulp (mesocarp and epicarp)	1. Preparation an acetone powder extract. 2. Filtration and washing with acetone. 4. Extraction buffer: 50 mM sodium phosphate (pH 7.0). 5. Stirring and centrifugation. 6. Precipitation by slow addition of ammonium sulfate to 30% saturation. 7. Centrifugation. 8. Desalting and lyophilization.	Spectrophotometric method, SDS-PAGE (silver staining), FT-IR, GC-FID	Saraiva et al., 2007
		–	Tissues	1. Extraction buffer: 100 mM Tris-Glycine (pH 6.8) with 2.5% (m/v) PVPP. 2. Centrifugation.	Spectrophotometric method and SDS-PAGE	Ebrahimzadeh et al., 2003
Proteins in olive oil						
Polypeptide	–	4.6 kDa	–	1. Protein precipitation with cold acetone. 2. Filtration. 3. Drying.	SDS-PAGE (silver staining) and HPLC	Hidalgo et al. 2001; Hidalgo et al. 2002
Protein	–	10-s-40 kDa	–	1. Extraction buffer: 50 mM Tris-HCl (pH7.5) with 0.1 M NaCl and 10% (v/v) glycerol. 2. Centrifugation. 3. Filtration. 4. Protein precipitation with 15% TCA or 70% ammonium sulfate. 5. Centrifugation. 6. Washing with diethyl ether for TCA precipitation and with 50% (v/v) ethanol for ammonium sulfate. 7. Dialysis step for the ammonium sulfate precipitation.	SDS-PAGE (silver staining) and FPLC	Georgalaki et al., 1998b
Protein	–	<14, 18, 27, 29, 58, 64 kDa	–	1. Solvent extraction with acetone:hexane (1:1, v/v). 2. Storage at 4°C for 1 hour. 3. Centrifugation. 4. Washing with acetone:hexane (1:1, v/v).	SDS-PAGE (silver staining)	Martín-Hernández et al., 2008
Enzymes	PPO	10 kDa	–	1. Extraction buffer: 50 mM Tris-HCl (pH7.5) with 0.1 M NaCl and 10% (v/v) glycerol. 2. Centrifugation. 3. Filtration.	Spectrophotometric method and FPLC	Georgalaki et al., 1998b
	LOX	–	–	1. Extraction buffer: 50 mM Tris-HCl (pH7.5) with 0.1 M NaCl and 10% (v/v) glycerol. 2. Centrifugation. 3. Filtration.	Spectrophotometric method, HPLC, and H^1NMR Spectrophotometric method and FPLC	Georgalaki et al., 1998a Georgalaki et al., 1998b

Protein Extraction from Olive Fruit

Proteins are distributed in the different parts of the fruit. The stone is constituted by the endocarp and the seed. The seed contains the higher amount of proteins, mainly oleosins and seed storage proteins (SSPs). The olive pulp consists of the mesocarp and the epicarp and contains lower protein content.

Extraction of SSPs has been mostly performed using a Tris-HCl buffer at pH 6.8–7.5 and different additives to make easier their extraction, like detergents as sodium dodecyl sulfate (SDS) to solubilize the proteins, reducing agents as 2-mercaptoethanol or dithiothreitol (DTT) to reduce disulfide bonds or urea for protein denaturation (Wang et al., 2001; Alché et al., 2006; Jiménez et al., 2007; Esteve et al., 2010). Proteins are later isolated by precipitation with acetone and separation by centrifugation. Extraction of seed oleosins is frequently performed by homogenization in buffered sucrose at pH 7.5 containing salts, coordination complexes, and ascorbic acid (Ross et al., 1993; Hidalgo et al., 2001). This medium allows extracting the fat layer from olive fruits that contains the oil bodies. Oil bodies are then isolated and purified, removing fatty acids and triacylglycerols by adding different solvents such as a chloroform:methanol (2:1, v/v) mixture (Hidalgo et al., 2001) or diethylether (Ross et al., 1993).

Olive mesocarp proteins are extracted from mesocarp and olive pulp (mesocarp and epicarp) using very varied strategies (see Table 1). Two main difficulties have been found in this extraction: the low amount of proteins in comparison with olive seeds and the high amount of interfering compounds. Indeed, polyphenols and other components are also extracted together with proteins when aqueous buffers are used for extraction and when organic solvents are employed for protein precipitation (Wang et al., 2003). The presence of these nonproteinaceus components can be critical in next steps aimed to the characterization and analysis of proteins. An extraction method to remove these interfering compounds was developed by Wang et al. (2003) to characterize proteins by one-dimensional (1D) and two-dimensional (2D)-polyacrylamide gel electrophoresis PAGE based on the use of a mixture of phenol and SDS Tris-HCl buffers at pH 8.0. Afterward, the same authors developed a general method for protein extraction in recalcitrant plant tissues. The method combined trichloroacetic acid (TCA)/acetone and methanol washes to remove the interferences followed by protein extraction with phenol (Wang et al. 2006; Wang et al., 2008). Esteve et al. (2011) have recently observed the same interfering problems when olive pulp was analyzed by SDS-PAGE. Phenol, chloroform/methanol, and Tris/SDS/PAGE extractions with different precleaning steps were tested in the extraction of pulp proteins for removing interfering compounds. The optimum extraction procedure consisted of a cleaning step to remove interfering compounds using different TCA solutions to remove phenols and other compounds, extraction of proteins with a Tris/SDS/DTT buffer, and subsequent protein precipitation with acetone.

To our knowledge, only one procedure has been employed in the extraction of proteins from the whole olive fruit. The method was based on a solvent extraction with a chloroform/methanol (2:1, v/v) mixture followed by protein precipitation with cold acetone (Montealegre et al., 2010c). This simple extraction method was combined with a capillary electrophoresis (CE) strategy to allow the selective separation of proteins from raw and table olive samples, avoiding potential interferents such as polyphenols.

The olive pomace is the solid residue remaining after olive oil extraction. Vioque et al. (2000) studied the protein composition of this residue previous extraction of proteins by an enzymatic-assisted method and the proteolytic enzyme Alcalase.

Moreover, different enzymes have also been described in the oil bodies of the olive fruit. Extraction of these enzymes usually involves the use of a phosphate buffer at pH from 6.2 to 8.0. It is also common the use of polyvinylpyrrolidone (PVP) to eliminate phenolic compounds, metabisulfite to prevent the rapid oxidation of phenolic compounds, and EDTA/DTT to stabilize the enzymes (Olías et al., 1993). In some cases, the fat layer containing these oil bodies is later purified by SEC (Georgalaki, 1998a) or IEC (Salas and Sánchez, 1998).

Protein Extraction from Olive Oil

The significant low concentration of proteins together with the high amount of lipids and interfering compounds make very difficult the extraction of proteins from the olive oil. Like in the case of the olive fruit, the number of methods developed for this purpose is rather limited. Extraction of proteins from those matrices has usually been carried out using a first extraction with aqueous/organic solvents, followed by a protein isolation step. More recently, extraction of proteins from olive oils have been based on their precipitation with solvents as acetone (Hidalgo et al., 2001; Hidalgo et al., 2002; Lerma-García et al., 2007) or acetone:hexane (1:1, v/v) (Matín-Hernandez et al., 2008). Precipitation of proteins with cold acetone at 4°C for 30 minutes followed by filtration through a Whatman filter, elution with dioxane and tetrahydrofurane, and concentration by evaporating under nitrogen has been the most employed procedure. This method has later been applied with some improvements (Zamora et al., 2001; Koidis et al., 2006; Montealegre et al., 2010b).

With the aim of establishing a universal protein extraction procedure from refined and non-refined oils, Martín-Hernández et al. (2008) evaluated five different extractions methods. They were based on the direct addition of different organic/aqueous solvents such as acetone:hexane (1:1, v/v), acetone:methanol (1:1, v/v), acetone, and a mixture of hexane:isopropanol:water (3:2:1, v/v/v). These extracting methods were evaluated by the amino acidic quantification and SDS-PAGE analysis of extracts. The results showed the highest protein content and the lowest interference problems when the method based on acetone:hexane extraction was used. On the other hand, Georgalaki et al. (1998b) employed an aqueous buffer to extract enzymes present in olive oil in order to study their activity and content. This methodology consisted of the addition of a Tris-HCl buffer at pH 7.5 with

some additives to 0.75 mL of olive oil. In this case, the protein precipitation was performed on ice during 30 minutes employing two different reagents: 15% (m/v) TCA or 70% (m/v) ammonium sulfate. A dialysis step was necessary in the case of the ammonium sulfate precipitation. The very low volume of olive oil used in this work could explain the poor results obtained.

CHARACTERIZED PROTEINS IN OLIVE FRUIT AND OIL

Characterization of proteins has widely been performed using 1D- and 2D-PAGE. Nevertheless, other techniques also employed for protein detection or characterization were gas chromatography-flame ionization detector (GC-FID), size-exclusion chromatography (SEC), ion exchange chromatography (IEC), fast protein liquid chromatography (FPLC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and spectroscopic techniques as spectrophotometry, H^1NMR, or FT-IR.

Proteins in Olive Fruit

Table 1 shows the classification of the proteins present in olive fruit depending on their strategic location: olive seed proteins, olive mesocarp proteins, and olive pomace proteins. Enzymes have been grouped together due to their distinct character in comparison with the rest of the olive fruit proteins.

Olive Seed Proteins

Olive stone, comprising the endocarp and the seed, does not exceed the 3% of fruit fresh weight and contains less than 5% of olive oil (Ross et al., 1993). However, the protein content in seeds is higher than in the rest of the olive fruit (Rodríguez and Lama, 2008) and, as above mentioned, great amounts of SSPs similar to the 11S globulins present in other plants can be found. These proteins represent 70% of the total proteins in the olive seed (Rodríguez and Lama, 2008). They are formed during seed development and deposited predominantly in specialized storage tissues (Alché et al., 2006). The SSPs are formed from two precursor proteins of 41 kDa (Solea I) and 47.5 kDa (Solea II) that are linked by hydrogen bridges (Wang et al., 2001; Alché et al., 2006). After its reduction, the precursor of 41 kDa generates three polypeptides of 22.4, 23.5, and 27.0 kDa, whereas the precursor of 47.5 kDa generates two polypeptides of 20 and 30 kDa (Wang et al., 2001; Jiménez et al., 2007). These polypeptides have been observed by SDS-PAGE analysis in extracts obtained from the whole olive stone (Esteve et al., 2010). Alché et al. (2006) analyzed the composition of these SSPs by SDS-PAGE finding that precursors were formed by individual polypeptides, named as p1-p5 (20.5, 21.5, 25.5, 27.5, and 30 kDa, respectively), which differed slightly from the above mentioned polypeptides. Figure 1A shows the SDS-PAGE protein profiles obtained in reducing and non-reducing conditions. In the first line, the two intact SSP precursors were clearly observed, while in line two, where reducing conditions were applied, different polypeptides resulting of breaking disulfide bonds appeared. In order to carry out the purification of these polypeptides, gel slices were excised and proteins were extracted with Tris/SDS and separated again by SDS-PAGE, as it is shown in Figures 1B and Figure 1C. In the same work, 2D-PAGE was used for the separation of SSPs, determining basic character for p1 and p2, and acidic character for p3, p4, and p5 polypeptides.

On the other hand, seed oleosins are alkaline proteins with molecular masses ranging between 15–26 kDa, depending on the bibliographic source (Huang, 1992; Ross et al., 1993). They are structural proteins with a strategic localization on the surface of oil bodies, as it has been demonstrated by immunogold electron microscopy (Ross et al., 1993). SDS-PAGE analysis allowed the determination of two different oleosins of 22 and

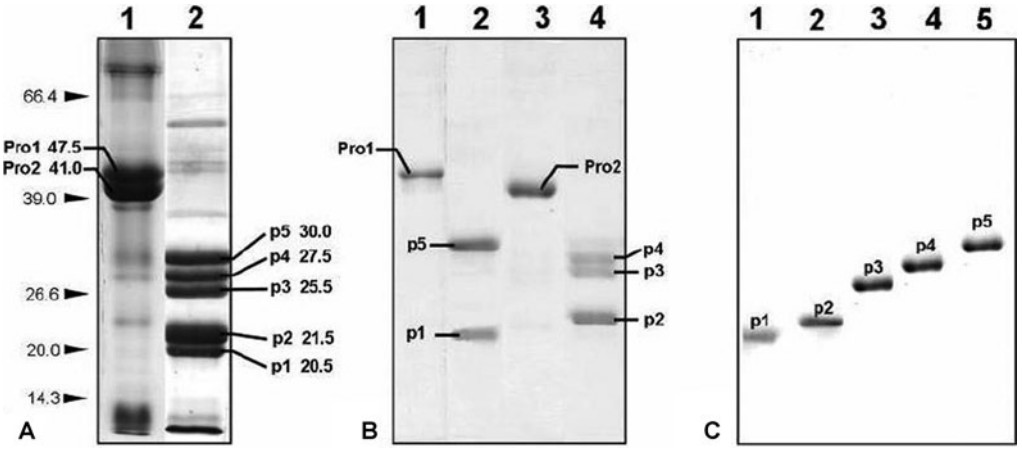

Figure 1 Coomassie-stained gels showing purification of individual components of 11S proteins from mature seeds of *O. europaea* cv. Picual. (A) Protein profiles obtained after SDS-PAGE of nonreducing (lane 1) and reducing (lane 2) crude protein extracts prepared from olive seeds. (B) Purification and SDS-PAGE separation of precursor forms (41.0 and 47.5 kDa) under nonreducing conditions (lanes 1 and 3, respectively). After reduction, individual components resulting from each precursor were separately resolved by SDS-PAGE (lanes 2 and 4, respectively). Molecular masses of protein standards are displayed on the left. (C) Reduced mature forms of 11S proteins after purification of individual components (Alché et al., 2006).

50 kDa in the olive seed (Ross et al., 1993). Oleosins were also identified by SDS-PAGE analysis in the oil bodies isolated from olive seeds as two bands of about 20 kDa (Hidalgo et al., 2001). Their presence was also confirmed when the whole stone was analyzed (Esteve et al., 2010).

Finally, a polypeptide of 4.6 kDa has also been described in the olive seed (Hidalgo et al., 2001). As it will be described below, this polypeptide has also been found in the olive pulp and in the olive oil.

Olive Mesocarp Proteins

Olive mesocarp tissue accounts for 65–85% of fresh weight and not less than 95% of the total oil of the fruit (Ross et al., 1993). A polypeptide of 4.6 kDa has been found in olive mesocarp. This peptide was firstly isolated and characterized from the oil bodies of the mesocarp and seeds using SDS-PAGE analysis (Hidalgo et al., 2001; Zamora et al., 2001). The only detection of this 4.6 kDa polypeptide band by SDS-PAGE analysis made the authors to establish it as the unique protein form in the olive mesocarp. Moreover, although oleosins have not been described in the olive mesocarp, a protein of an estimated molecular mass of 70 kDa in the olive body fraction of this olive part was described by Ross et al. (1993). Recently, Esteve et al. (2010) observed the presence of a protein in the range 20–25 kDa when the whole olive pulp (mesocarp and epicarp) from different olive varieties was analyzed by SDS-PAGE. This protein could correspond to a 23 kDa protein observed by other authors in the olive pulp which was demonstrated to be allergenic in an allergy clinic case of an olive oil mill worker (Palomares et al., 2008). The protein was purified by SEC and subjected to Edman degradation to determine part of its amino acidic sequence, showing homology to allergenic thaumatin-like proteins from plant foods and pollen.

Olive Pomace Proteins

The olive pomace protein composition was determined using SDS-PAGE by Vioque et al. (2000). A 30 kDa protein band and a smear of proteins at around 20 kDa that probably corresponded to oleosins, were found. These proteins could be partially hydrolyzed because they were subjected to an enzymatic-assisted extraction with Alcalase. In addition, the SDS-PAGE analysis showed bad protein resolution due to interferences caused by the high fiber content of olive pomace.

Enzymes in Olive Fruits

Several enzymes present in olive fruits have been studied. In these studies, a general procedure has been used. Firstly, the extracted proteins are fractionated using analytical techniques such as SEC, IEC, or affinity chromatography. Afterward, the enzymatic activity is determined in the isolated fraction by using established spectrophotometric methods. In other occasions, the by-products generated in the enzymatic reactions are also measured, allowing the identification of the enzyme. For this purpose techniques such as HPLC or GC-FID are usually employed. As observed in Table 1, lipoxygenase (LOX) has been one of the most studied olive enzymes due to its implication in the biogenesis of six carbon volatile aldehydes, the major constituents of virgin olive oil aroma (Salas et al., 1999). This enzyme has been widely characterized, in the whole fruit, pulp, and seed of olive fruits (Georgalaki et al., 1998b; Olías et al., 1993; Salas et al., 1999). Olive lypoxigenase is a linolate 13-LOX active on free linoleic acid present in the olive endosperm with a molecular mass higher than 100 kDa (Olías et al., 1993; Georgalaki et al., 1998a). Besides this, some other enzymes have been detected in olive fruits: alcohol dehydrogenase (Olías et al., 1993; Salas and Longone, 1994), acylhydrolase (Olías et al., 1993), fatty acid hydroperoxide lyase (Olías et al., 1993), alcohol acetyltransferase (Olías et al., 1993), polyphenol oxidase (Sciancalepore and Longone, 1984; Hornero-Méndez et al., 2002; Ebrahimzadeh et al., 2003; Ortega-García et al., 2008; Ortega-García and Peragón, 2009), β-glycosidase (Briante et al., 2002; De Nino et al., 2008), esterase (Briante et al., 2002), phenylalanine ammonia-lyase (Ortega-García and Peragón, 2009; Ortega-García et al., 2009), superoxide dismutase (Hornero-Méndez et al., 2002), and peroxidase (Ebrahimzadeh et al., 2003; Saraiva et al., 2007). These enzymes are responsible for several organoleptic and nutritional properties of the olive oil and olive fruits (Ortega-García et al., 2008). Thus, Olías et al. (1993) characterized several enzymes in olive fruit, proposing a scheme for the biogenesis of the compounds that contribute significantly to the green odor notes of virgin olive oil aroma. Moreover, some of these enzymes such as polyphenols oxidase and peroxidase have been considered as markers of maturity state of the olive fruit due to the modification of their activities during fruit development (Ebrahimzadeh et al., 2003).

Proteins in Olive Oil

The number of research articles describing the presence of proteins in olive oils is quite limited. Table 1 groups the few proteins characterized in olive oils. In 2001, Hidalgo et al. (2001) established, for the first time, proteins as minor components in olive oils. In this work, the analysis by SDS-PAGE of the extracts obtained from 40 different olive oils only allowed to determine the presence of a 4.6 kDa polypeptide. This polypeptide, previously reported in olive fruits, is the most widely described protein in olive oils and it is mainly derived from oil bodies in olive mesocarps (Hidalgo et al., 2001; Hidalgo et al., 2002).

Georgalaki et al. (1998a) suggested the presence of a group of proteins with molecular masses ranging from 10 to 40 kDa in the olive oil from the results obtained by SDS-PAGE and FPLC analysis. Nevertheless, the identification was not complete and the authors themselves came up with the possible correspondence of these signals with nonprotein olive oil components. Figure 2 shows the protein profiles obtained from different vegetable oils. Lane 9 shows the separation obtained in the case of olive oil proteins. Firstly, it is possible to observe the presence of four bands with molecular masses under 30 kDa.

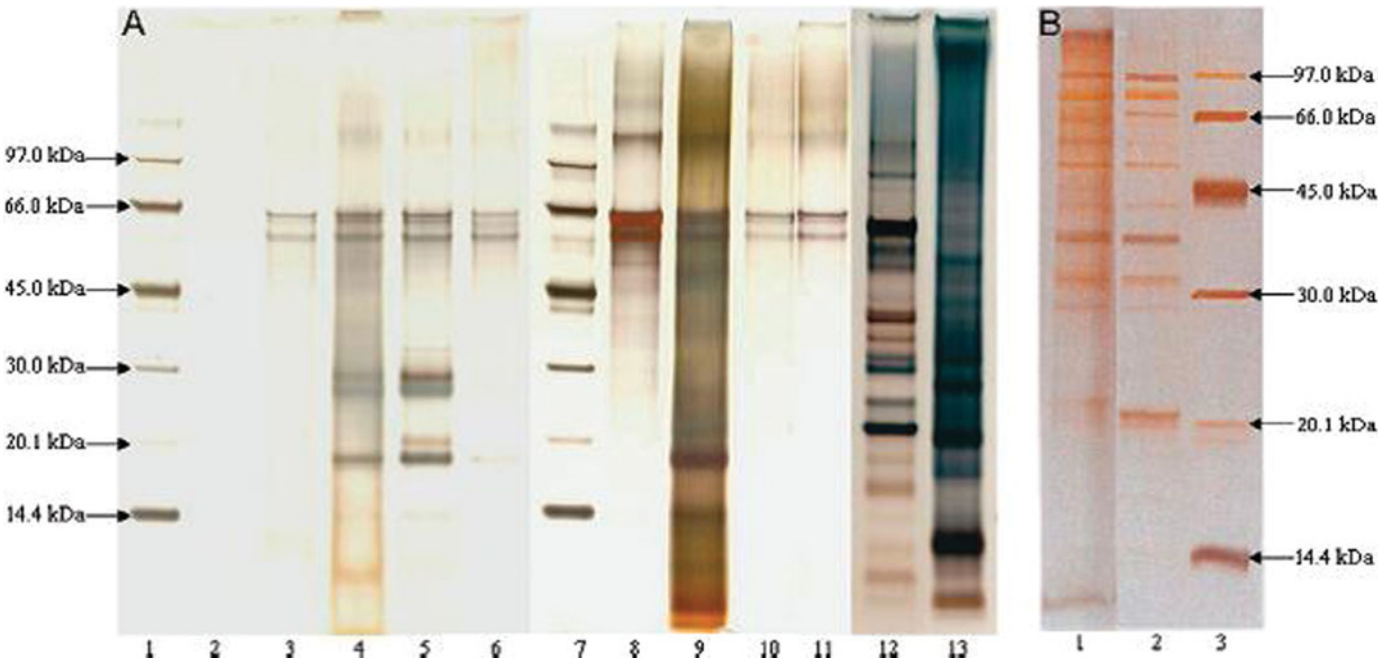

Figure 2 Electrophoretic pattern obtained by SDS-PAGE analysis for different oils. The extra virgin olive oil is showed in gel A lane 9 in which protein bands of 64, 58, 29, 27, 18, and 14 kDa can be observed. The rest of the lanes correspond to different refined and nonrefined oils (Martín-Hernandez et al., 2008). (Color figure available online.)

Moreover, a band around 100 kDa is also observed in the region of higher molecular masses. Besides this, two bands with molecular masses between 55 and 65 kDa can be observed in all vegetable oils. Although it was not indicated by the authors, the presence of those bands in all the samples could correspond to human keratins that could appear due to lack of care during the analysis. In this case, the higher sensitivity of silver staining comparing with Coomassie blue staining for the detection of proteins after electrophoretic separations allowed the determination of minor olive oil proteins not observed previously.

In addition to these proteins, enzymes, such as polyphenol oxidase (PPO) or LOX, have also been described by Georgalaki et al. (1998a, 1998b) in olive oils. The presence of both enzymes, LOX and PPO, in olive oils was demonstrated by applying activity tests based on spectrophotometric measurements (Gerogalaki et al., 1998a, 1998b) and, in the case of LOX enzyme, determining the content of LOX oxygenated fatty acid derivatives in the olive oil by HPLC and ^{1}H NMR. Moreover, authors concluded these enzymes were in very low proportion by the comparison of enzyme activity of olive oil with other foods such as soybean (Georgalaki et al., 1998a). It is important to remark that these authors determined a molecular mass of around 10 kDa for the PPO enzyme in olive oil. This molecular mass disagrees with that observed for the same enzyme in the olive fruit (42 kDa). These differences were explained assuming that PPO activity in olive oil resulted from the proteolytic fragmentation of the native enzyme present in the olive fruit. Moreover, Georgalaki et al. (1998a) also demonstrated that LOX, initially located in the olive endosperm, did not suffer any damage during olive processing to obtain oil and remained active in the olive oil.

QUANTITATION OF PROTEINS IN OLIVE FRUIT AND OIL

Total protein content in the olive fruit and oil has been evaluated using different methods generally employed for protein determination: Kjeldhal method, turbidimetric assays, different spectrofotometric-based methods as Bradford, Lowry, and Folin-Lowry assays, as well as BioRad protein assay, and amino acid analysis. Table 2 summarizes the protein contents obtained for the different olive tissues and olive oil and the methodologies employed in every case. In general, it is possible to observe a great disagreement in these contents which were greatly influenced by the methodology employed. Indeed, problems like overestimation of the protein content when using the turbidimetric assay or interference with chemical reagents or secondary metabolites in the spectrofotometric methods could explain these differences.

The total amount of proteins in the olive fruit has been established between 0.3 and 46 mg/g (Ajana et al., 1999; Ortega-Garcia et al., 2008). In addition to the influence of the method employed for protein determination (Ajana et al., 1999; Ortega-García et al., 2008), there are different factors that can affect the protein content in the olive fruit. In fact, proteins are the result of genetic expression, and are affected by the environmental conditions in which the plant grew and the maturity state of the fruit (Ajana et al., 1999; Briante et al., 2002).

Table 2 Total protein content and analytical assay or procedure used for protein determination in olive fruits and olive oils

	Location	Protein content	Analytical assay or procedure used for protein determination	Reference
Olive fruit	Pulp	13–18 mg/g	Amino acid analysis	Zamora et al., 2001
		1.5 mg/g	BioRad protein assay	Wang et al., 2006
		3–12 mg/g	Lowry assay	Briante et al., 2002
		4.7 mg/g	Folin-Lowry method modified	Salas et al., 1999
	Stone	11 mg/g	Bradford	Esteve et al., 2010
		32 mg/g	–	Rodriguez et al., 2008
	Whole olive	0.3–1.2 mg/g	Bradford	Sciancalepore et al., 1984
		23–46 mg/g	Kjeldhal method	Ajana et al., 1999
Olive oil	–	307–570 mg/kg	Lowry assay	Georgalaki et al., 1998b
		11–44 mg/kg	Bradford assay	Georgalaki et al., 1998b
		440 mg/kg	Turbidimetric assay	Georgalaki et al., 1998b
		0.07–0.51 mg/kg	Amino acid analysis	Hidalgo et al., 2001; Hidalgo et al., 2002
		0.05–2.40 mg/kg	Bradford assay	Koidis et al., 2006
		0.2–0.6 mg/kg	Amino acid analysis	Martín-Hernández et al., 2008

Moreover, different authors have also demonstrated how olive fruit processing to obtain table olives can affect the protein content (Ünal and Nergiz, 2003; Montaño et al., 2005; Öngen et al., 2005; Casado et al., 2007; López et al., 2007; Lanza et al., 2010). Furthermore, results demonstrate that proteins are distributed in different proportions in the olive fruit. In fact, the seed is the region with the highest protein content (11–32 mg/g) because proteins are accumulated there acting as food reserve compounds, like fat, phenols, and free sugars. Unlike the olive seed, very low amounts of protein exist in the olive mesocarp (Ross et al., 1993). In fact, it has been determined a protein concentration in the range of 1.5–18 mg/g in this part.

The information available about the amount of proteins passing from the fruit to the oil during its extraction is very scarce. The analysis of the solid waste (olive pomace) generated during olive oil extraction yields a fiber content of around 70% and a protein content of about 6%. Vioque et al (2000) observed these proteins consisting mainly in oleosins probably coming from the seed. Moreover, SSPs have also been determined in olive oil byproducts (alpeorujos and orujos), which suggested that the major proportion of the olive fruit proteins did not pass to the olive oil (Jiménez et al., 2007). Nevertheless, Zamora et al. (2001) demonstrated that some proteins present in the oil bodies of the olive fruit mesocarp passed to the oil during olive oil extraction, constituting the main protein component in these oils. As Table 2 shows, values between 0.07–0.51 mg/kg were established by analyzing the amino acid content after acid hydrolysis of olive oil proteins (Hidalgo et al., 2002; Hidalgo and Zamora, 2006) and 0.05–2.40 mg/kg by using the Bradford method (Koidis and Boskou, 2006). Moreover, Georgalaki et al. (1998b) determined, using a turbidimetric procedure, values clearly overestimated in comparison with those above reported. Different authors have studied the influence of the oil extraction procedure on the protein content of the resulting oil. Indeed, the manufacture of virgin olive oils sometimes involves a final filtration step to remove suspended solid and humidity (Lozano-Sánchez et al, 2010). The influence of this filtration step on the protein content was studied by using a modified Bradford method, determining similar contents in unfiltered and filtered olive oils (Koidis and Boskou, 2006). However, differences in the protein content were found among refined and nonrefined oils. Amino acid analysis of both refined and nonrefined oils showed a decrease of protein amount from 0.2–0.6 mg/kg to less than 0.2 mg/kg in refined oils (Martín-Hernández et al., 2008).

USE OF PROTEINS FOR VARIETAL DIFFERENTIATION

Nowadays, there are more than 1500 olive varieties known all over the world (Bartolini et al., 1998). This huge genetic diversity of the olive crop has hindered its identification and classification. Olive fruit and oil authenticity involves not only to detect possible adulterations but also to determine whether an olive oil is genuine with regard to authenticity issues, such as geographical origin and botanical variety (García-Gonzalez and Aparicio, 2010). While, traditionally, differentiation among olive cultivars has been supported by morphological and pomological traits, nowadays investigations have been focused on some compounds present in olive fruits and oils with differentiation potential (Montealegre et al., 2010a). In this sense, various DNA markers have been applied to solve traceability and provenance issues (Consolandi et al., 2008; Pollastri, 2008; Montealegre et al., 2010a). Olive fruit and olive oil proteins have also been studied as possible markers for olive variety classification. The analysis of amino acids resulting from the hydrolysis of oil proteins with hydrochloric acid by HPLC using UV (Hidalgo et al., 2002) or MS detection (Lerma-García et al., 2007) was proposed for the classification of vegetable oils according to their botanical origin. Unfortunately, the differentiation among genetic varieties of olive oils based on these amino acidic profiles was not possible.

The use of intact proteins as traceability markers of the olive variety showed higher differentiation potential than amino acids

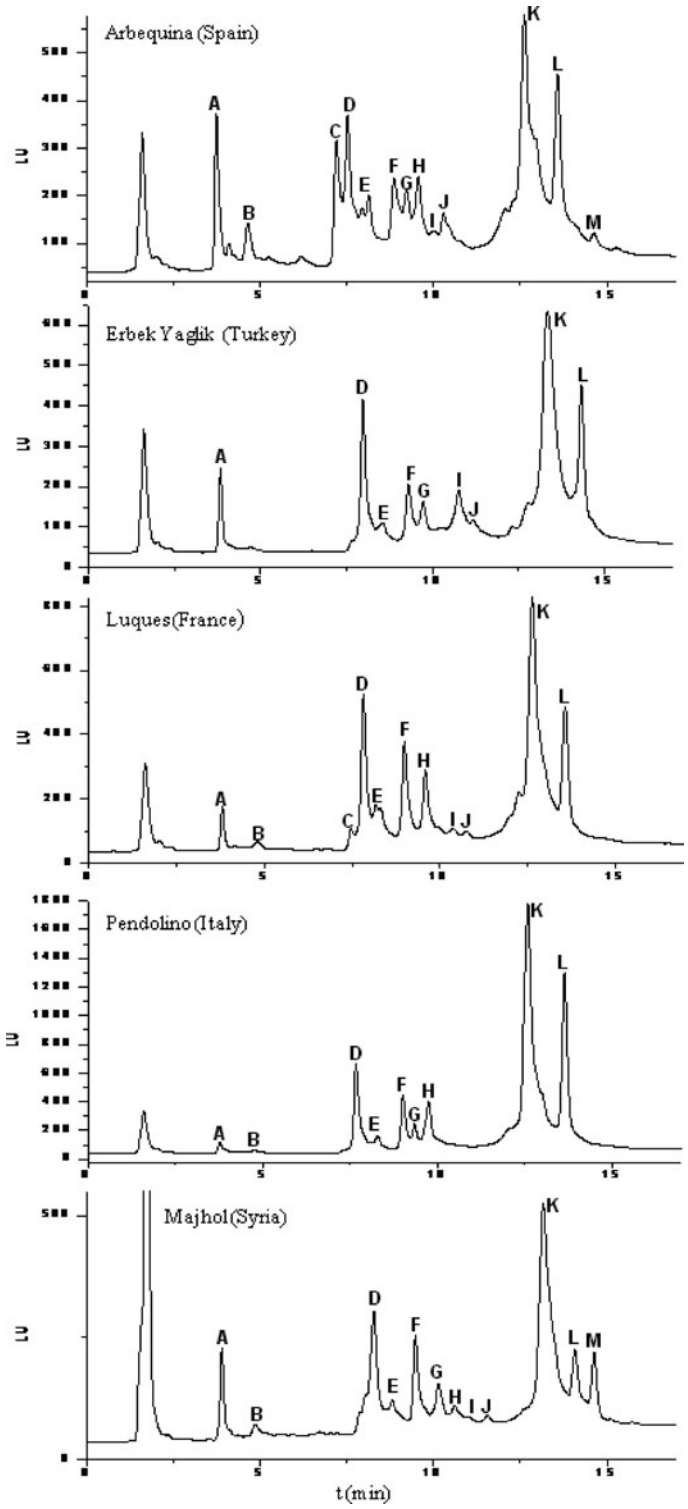

Figure 3 Protein profiles corresponding to different olive stone genotypes. Letters from A to M indicate the protein peaks. Chromatographic conditions: column, 100 × 3 mm i.d., 1.9 μm of Thermo Scientific; gradient, 60–80% B in 16 min; 80–95% B in two minutes, and 95–5% B in two minutes; flow rate, 0.4 mL/minute; temperature, 55°C; mobile phases, 0.1% (v/v) TFA in water (phase A) and in MeOH (phase B); fluorescence detection ($\lambda_{exc} = 280$ nm and $\lambda_{em} = 360$ nm); injected volume, 3 μL (total protein mass, 3 μg) (Esteve et al., 2010).

obtained from proteins. However, a first study comparing forty different olive oils by SDS-PAGE analysis did not provide significant results (Hidalgo et al., 2001). Afterward, the comparison of protein profiles obtained using analytical techniques with higher resolution capacity than gel electrophoresis allowed the differentiation among olive fruits and among olive oil varieties (Esteve et al., 2010; Montealegre et al., 2010b, Montealegre et al., 2010c; Esteve et al., 2011). Specifically, an ultrahigh performance liquid chromatography (UHPLC) method for profiling intact olive proteins was recently developed by Esteve et al. (2010, 2011). This methodology was applied to separate the proteins extracted from the stone in 29 different olive varieties (Esteve et al., 2010). Figure 3 shows the protein profiles corresponding to five different varieties. Clear differences were observed, demonstrating that analysis of the proteins present in the olive stone was a suitable tool for cultivar fingerprinting. In fact, olive stone proteins enabled the suitable classification of 98.9% of the olive samples. The same chromatographic methodology was applied to separate proteins from the olive pulp (Esteve et al., 2011). Three peaks corresponding to proteins were obtained. In this case, differences were less significant and a common protein was observed in all varieties. CE has also demonstrated to be a suitable analytical technique for protein separation from raw and table olive samples (Montealegre et al., 2010c) and olive oil samples (Montealegre et al., 2010b). Raw olive samples showed differences in protein profiles depending upon the botanical variety and their geographical region. Olive oils were also classified according to their olive variety using protein profiles obtained by CE (Montealegre et al.,

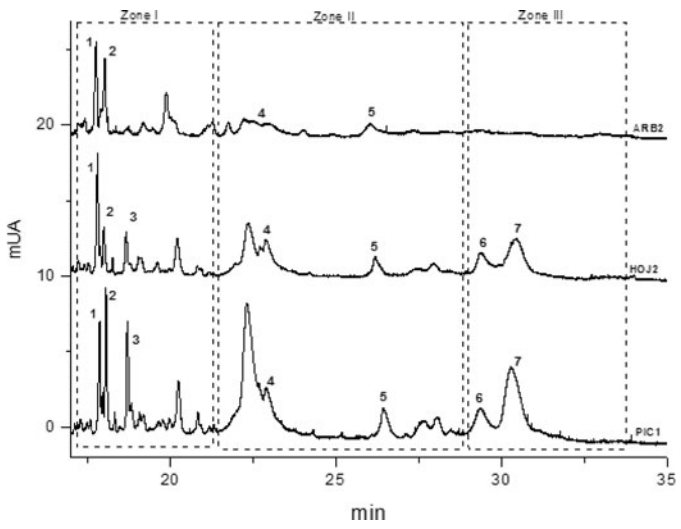

Figure 4 Protein profiles of monovarietal olive oils of three different varieties Arbequina (ARB), Picual (PIC), and Hojiblanca (HOJ) showing three characteristic migration zones: Zone I (similar zone; peaks 1,2,3), Zone II (black zone; peaks 4 and 5) and Zone III (differential zone; peaks 6 and 7). Separation conditions: 80 mM borate buffer with 20% v/v ACN (apparent pH 9.1); UltraTrol^{TM}-LN dynamic precoated capillary with 75 μm i.d. and 50 cm effective length (58.5 cm total length); injection by pressure, 50 mbar for 100 s; capillary temperature, 15°C; applied voltage, −20 kV; UV detection at 254 nm (5 nm bandwidth) (Montealegre et al., 2010b).

2010b). As observed in the electropherograms of Figure 4, seven electrophoretic peaks were assigned to proteins and the three main monovarietal Spanish olive oils showed visual differences. These studies demonstrated the enormous potential of protein profiles for the differentiation of olive crops.

CONCLUSIONS AND FUTURE TRENDS

The protein fraction of the olive fruit and oil has received little attention in comparison with other components of these samples. Proteins are distributed in the different parts of the olive fruit and it has been demonstrated that some of them can also be present in the oil. The highest protein content of the olive seed has allowed a better characterization of these proteins in comparison with olive pulp and oil proteins. Among olive proteins, enzymes have attracted more attention since they could play an essential role in olive oil characteristics and stability.

The development of analytical methods for the detection and characterization of proteins in olive fruit and, above all, in olive oil is currently necessary. Existing procedures for the extraction of proteins in these oily matrices are very scarce and use to be non selective and time consuming which makes necessary the development of more efficient procedures.

The use of high-resolution separation techniques combined with sensitive detection techniques is also an important key for the determination of proteins, especially, in oil. The recent use of UHPLC and CE in combination with UV, fluorescence, or MS detection seems to be very promising for this purpose.

Important efforts should also be focused on the identification of the still unknown olive proteins and their variability due to factors such as the botanical origin, stress conditions, geographical and environmental conditions, processing, storage, etc. The application of proteomic techniques would be essential but advances in this field are limited by the absence of olive DNA databases. At this respect, it would be necessary a parallel sequencing of the olive genome. The determination of olive protein functionality is also a subject to be explored in the future.

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