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Analytical Approaches for the Characterization and Identification of Olive (*Olea europaea*) Oil Proteins

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ABSTRACT: Proteins in olive oil have been scarcely investigated probably due to the difficulty of working with such a lipidic matrix and the dramatically low abundance of proteins in this biological material. Additionally, this scarce information has generated contradictory results, thus requiring further investigations. This work treats this subject from a comprehensive point of view and proposes the use of different analytical approaches to delve into the characterization and identification of proteins in olive oil. Different extraction methodologies, including capture via combinational hexapeptide ligand libraries (CPLLs), were tried. A sequence of methodologies, starting with off-gel isoelectric focusing (IEF) followed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) or high-performance liquid chromatography (HPLC) using an ultraperformance liquid chromatography (UPLC) column, was applied to profile proteins from olive seed, pulp, and oil. Besides this, and for the first time, a tentative identification of oil proteins by mass spectrometry has been attempted.

KEYWORDS: *olive oil, LC−MS/MS, CPLLs, protein, proteomics*

INTRODUCTION

The presence of peptides and proteins in vegetable oils has been reported in several kinds of crude and refined oils.[1,2] Proteins seem to play an important role in oil stability[3,4] and are related to the allergic reactions of sensitive individuals.[5] Nevertheless, the information available about olive oil proteins is very scarce.[6] In 2001, proteins were established, for the first time, as minor components in olive oils.[7] Hidalgo et al.[7] found that proteins in olive oil, both virgin and refined, were almost solely constituted by a polypeptide of 4.6 kDa.[8] In this study, the same polypeptide was also observed in olive pulp and seed extracts and in other seed extracts, proposing it as a new class of polypeptides in plants with oleosin-like characteristics. Nevertheless, in a more recent work, a different protein profile was obtained when separating olive oil extracts, observing the presence of proteins with molecular masses up to 30 kDa,[1] but not showing any band for the 4.6 kDa polypeptide. Additionally, the same authors also observed two protein bands with apparent molecular masses of 58 and 64 kDa in the olive oil and in other investigated oils. Furthermore, the determination of the activity of different enzymes, such as 13-lipoxygenase[3,9] or polyphenol oxidase,[3] has also demonstrated the presence of proteins in olive oil. In order to clarify this scarce and, at the same time, contradictory information, further investigation is needed.

The main problem for protein determination in olive oil has traditionally been the lack of extraction methodologies available for lipidic matrices since most of them have been developed for the extraction of proteins in aqueous solutions. The second inconvenience is the dramatically low abundance of proteins that pass from the fruit to the oil, established in the range 0.05−2.4 mg/kg,[1,7] and the presence of high amounts of interfering compounds as polyphenols. Therefore, further studies using improved extraction methods, more efficient separation techniques, and more sensitive detection techniques are needed. An additional problem limiting the determination of proteins in olive oil is the fact that the olive genome has not been sequenced yet. Consequently, at best, peptides could only be assigned to proteins by homology with other plant species whose genome had already been sequenced. Otherwise, proteins would remain unidentified.

The aim of this work was to show the results obtained using different analytical strategies to approach the characterization and identification of proteins in olive oil.

MATERIALS AND METHODS

Chemical and Samples. Supergradient HPLC grade acetonitrile (ACN) (Scharlau, Barcelona, Spain), trifluoroacetic acid (TFA) (Sigma-Aldrich, St. Louis, MO), and HPLC grade water (Milli-Q system; Millipore, Bedford, MA) were used in the preparation of mobile phases for HPLC separations. ACN and water for LC−MS/MS, and formic acid (FA) (Sigma-Aldrich) were used in the preparation of mobile phases for LC−MS/MS separations. Tris-(hydroxymethylaminomethane) (Tris), hydrochloric acid, sodium dodecyl sulfate (SDS) (all from Merck, Darmstadt, Germany), β-mercaptoethanol (β-ME), urea, dithiothreitol (DTT), 1,4-dioxane, octyl β-D-glucopyranoside (OG) (all from Sigma-Aldrich), ammonium acetate (NH_4Ac), trichloroacetic acid (TCA), acetic acid (all from Panreac, Barcelona, Spain), hexane, tetrahydrofuran (THF), methanol (MeOH), and reagent grade acetone (all from Scharlau), and all other

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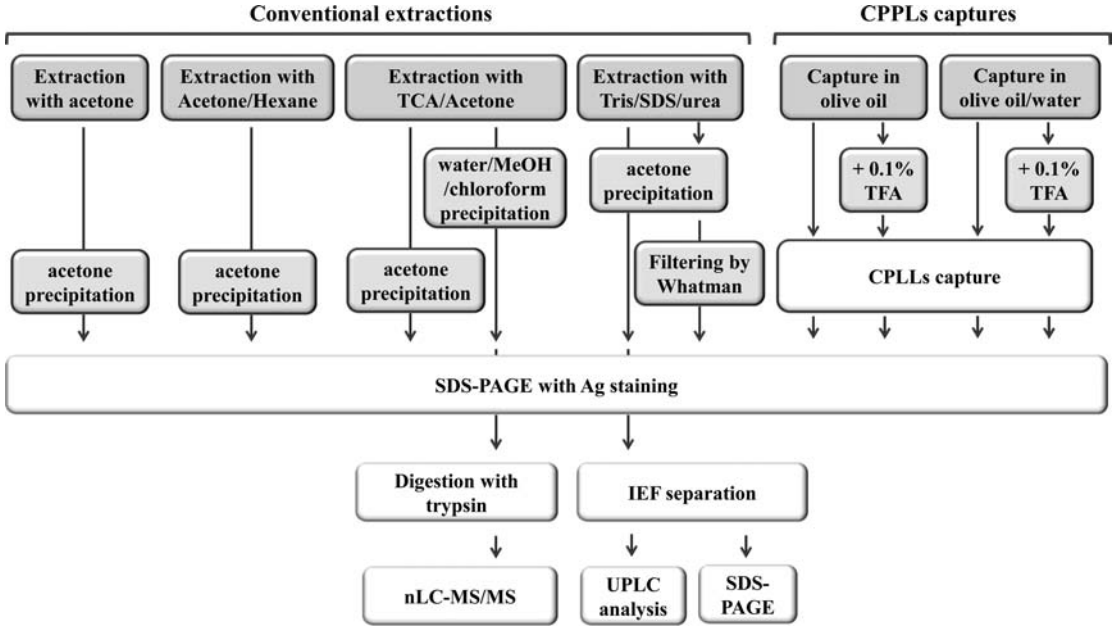

Figure 1. Analytical procedures designed for the extraction of proteins from olive oil.

chemicals used along the experimental work were pure analytical grade products. ProteoMiner (combinatorial hexapeptide ligand library beads), Laemmli buffer, 40% acrylamide/Bis solution, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), Mini-PROTEAN precast gels, Tris/glycine/SDS running buffer, and molecular mass standards were from Bio-Rad Laboratories (Hercules, CA). Silver staining kits were purchased from Sigma-Aldrich and InvitroGen (Carlsbad, CA). Sequencing grade trypsin was from Roche Diagnostics (Basel, Switzerland). In-house-made capture peptide ligand libraries (CPLLs) were produced in the laboratory of Prof. Righetti. Amicon cutoff filters (3 kDa) (Millipore) and OMIX C_4 pipet tips (Agilent Technologies Inc., Waldbron, Germany) were also used. Raw olives of the 'Picual' variety with violet maturity index were kindly donated by the Olive World Germplasm Bank of IFAPA (Junta de Andalucía, Cordova, Spain). Olive fruits were manually depulped and stone cut to extract the olive seed. The seed and pulp were stored at −20 °C until use. Virgin olive oil of 'Picual' variety was acquired in a local supermarket (Alcalá de Henares, Spain), and also olive oil of 'Gentile di Chieti' variety was kindly donated by the Masseria Don Vincenzo (Vasto, Italy).

Protein Extraction Protocols. *Olive Seed Protein Extraction.* The method employed was similar to the method previously developed by our research group for the olive stone.[10] Briefly, olive seeds were ground with a domestic mill and passed through a sieve with a light path of 0.355 mm. 0.03 g of sample was mixed with 5 mL of an extracting solution containing 125 mM Tris-HCl (pH 7.5), 1% (m/v) SDS, and 0.5% (m/v) DTT. The mixture was vigorously shaken during 1 min and centrifuged twice at 4000*g* for 10 min. Proteins in the supernatant were precipitated with 25 mL of cold acetone at −20 °C for 1 h, collected by centrifugation during 10 min at 4 °C, and dried at room temperature.

Olive Pulp Protein Extraction. Protein extraction was based on a method previously developed by our research group.[11] Olive pulp was frozen and ground in a domestic miller. For 0.5 g of pulp, 10 mL of 10% (m/v) TCA in acetone was added. After vortexing thoroughly for 1 min, the sample was kept at −20 °C for 30 min to allow complete protein precipitation. The sample was centrifuged for 5 min, and the resulting pellet was washed twice with 10% (m/v) TCA in acetone, then with aqueous 10% (m/v) TCA twice, and finally with 80% (v/v) acetone in water (all solutions employed were at 4 °C). The resulting pellet was mixed with 10 mL of an extracting solution containing 125 mM Tris-HCl (pH 7.5), 1% (m/v) SDS, and 0.5% (m/v) DTT. After centrifugation for 10 min, 50 mL of acetone was added to the supernatant and kept at −20 °C during 1 h. The proteins were recovered by centrifugation for 10 min and dried at room temperature.

Olive Oil Protein Extraction. Different extraction methods, including capturing via CPLLs, were designed (see Figure 1).

a. Extraction with Acetone. Extraction of olive oil proteins was performed using the method described by Martín-Hernández et al.[1] with some modifications. To 100 g of olive oil of the 'Picual' variety (Spain) was added 250 mL of acetone. The mixture was shaken vigorously, kept for 1 h at 4 °C, and shaken every 15 min. The mixture was then centrifuged, and the supernatant was discarded. The precipitate was collected with approximately 2 mL of 125 mM Tris-HCl (pH 7.5) and 1% (m/v) SDS, mixed with 25 mL of cold acetone, and kept at −20 °C during 1 h. Proteins were collected by centrifugation at 4000*g* during 10 min at 4 °C.

b. Extraction with Acetone/Hexane. The method was identical to that previously described but using a mixture of acetone/hexane (1:1) instead of acetone.

c. Extraction with TCA/Acetone. Extraction with TCA/acetone was first performed by mixing 100 g of olive oil of the 'Picual' variety (Spain) with 250 mL of 10% (m/v) TCA in acetone in a similar way to previous methods. An improved extraction was later performed by using 400 g of olive oil of the variety 'Gentile di Chieti' (Italy), and adding 400 mL of cold acetone containing 25% (m/v) TCA. The mixture was kept at −20 °C during 1 h and shaken every 10 min. Proteins were collected by centrifuging at 9000*g* during 10 min at 4 °C. The supernatant was discarded, and the precipitate was dissolved by boiling during 10 min in 1.5 mL of 4% (m/v) SDS and 25 mM DTT. A water/methanol/chloroform precipitation[12] was next applied, obtaining a very thin white precipitate that was dissolved in 40 μL of Laemmli buffer containing 5% (v/v) of β-ME by boiling during 10 min. The insoluble part of the precipitate was removed by centrifugation at 13000 rpm during 5 min.

d. Extraction with Tris/SDS/DTT/Urea. This extraction method was based on the previous one employed for the extraction of olive stone proteins by our research group,[10] with some modifications. To 100 g of olive oil of the variety 'Picual' (Spain) was added 100 mL of a mixture consisting of 125 mM Tris-HCl (pH 7.5), 1% (m/v) SDS, 0.1% (m/v) DTT, and 1 M urea. The mixture was shaken vigorously during 30 min and then centrifuged. The supernatant corresponding to the organic phase was discarded. To the aqueous phase was added 400 mL of cold acetone, and the mixture was kept at −20 °C for 1 h. The mixture was distributed in 25 mL vials, and proteins were collected by centrifuging at 4000*g* during 10 min at 4 °C. The

supernatant was discarded and the precipitate was collected from the different vials with 0.5 mL of 125 mM Tris-HCl (pH 7.5) and 1% (m/v) SDS. 25 mL of cold acetone was added, and the mixture was kept at −20 °C during 1 h. Proteins were collected by centrifugation at 4000*g* for 10 min at 4 °C.

e. Extraction with Tris/SDS/DTT/Urea and Filtration. This method is a combination of the previous one and the method described by Hidalgo et al.[7,8] 100 g of oil was extracted with a Tris-HCl/SDS/DTT/urea buffer, vigorously shaken, and centrifuged. The aqueous phase was mixed with 400 mL of cold acetone and kept at −20 °C during 1 h. Then the mixture was filtered through a Whatman filter (grade 1), and the filter was cleaned twice with 20 mL of cold acetone. The proteins were eluted from the filter first with 5 mL of dioxane and then with 5 mL of THF by shaking in an ultrasonic bath during 10 min. Finally, the solvents were evaporated in a centrifugal evaporator at 30 °C. A blank of the extraction method was performed by passing through a Whatman filter (grade 1) 40 mL of cold acetone, shaking with 5 mL of dioxane and 5 mL of THF in the ultrasonic bath, and evaporating solvents in a centrifugal evaporator.

f. Olive Oil Protein Capturing by CPLLs. The isolation of olive oil proteins was also tried by capturing with CPLLs. Commercially available combinatorial libraries (ProteoMiner) and diverse homemade CPLLs presenting different physicochemical properties were used. As no previous examples were reported for the capture in such a lipidic matrix, new methods were designed. Starting from 400 mL of olive oil of the variety 'Gentile di Chieti', two strategies were performed: capturing the proteins directly in the olive oil and capturing the proteins in a mixture of olive oil/water (1:4) forming micelles by continuous agitation. In each case, two different captures were performed, in the absence and in the presence of 0.1% (v/v) TFA, mimicking reverse-phase conditions.[13] The first capture was performed by adding 50 μL of a mixture of three homemade CPLLs and ProteoMiner to the olive oil and keeping the mixture overnight at room temperature under gentle agitation. On the other hand, the capturing mimicking reverse-phase conditions, in the presence of 0.1% (v/v) TFA as ion-paring reagent, was implemented via a special hydrophobic library.[13−17] To the olive oil was added 50 μL of hydrophobic beads, and the capture was performed overnight under gentle shaking. The libraries were recovered by filtering with Bio-Spin chromatographic columns (Bio-Rad) under vacuum. The captured proteins from each sample were desorbed (twice, with 150 μL each time) with a solution containing 4% SDS (m/v) and 25 mM DTT, for 20 min, under boiling conditions.[18] In order to remove the remaining oil, a water/methanol/chloroform precipitation was next applied.[12] The resulting pellet was dried at room temperature and resuspended in 40 μL of Laemmli buffer containing 5% (v/v) of β-ME by boiling during 10 min and centrifuging at 13000 rpm.

SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Electrophoresis was performed in a Bio-Rad Mini-Protean system (Hercules, CA) according to standard protocols[10,17] and using commercial Mini-PROETEAN Precast Gels or homemade gels. Homemade gels consisted of a 4% polyacrylamide stacking gel cast over a 15% resolving polyacrylamide stacking gel.

Off-Gel Isoelectric Focusing (IEF). For the p*I*-based protein separation, a 3100 OFFGEL Kit pH 3−10 (Agilent Technologies Inc.) with a 12-well setup was employed according to the supplier protocol. Isolated proteins were resuspended in a solution consisting of 0.36 mL of water and 1.44 mL of 8 M urea, 2.5 M thiourea, 0.08 M DTT, 12% (v/v) glycerol, and ampholytes. IPG gel strips with a linear pH gradient ranging from 3 to 10 were rehydrated in the assembled device with 40 μL of focusing buffer (either 8 M urea, 2.5 M thiourea, 0.08 M DTT, 12% (m/v) glycerol, and ampholytes) per well. The pellet from each matrix was diluted with focusing buffer to a final volume of 1.8 mL, and 150 μL of sample was loaded in each well. The sample was then focused until 50 kV h was reached using a maximum current of 50 μA and voltages ranging from 500 to 4000 V. The recovered fractions (volumes ranging from 100 to 150 μL) were analyzed both by SDS−PAGE and HPLC.

Chromatographic System. Separations were carried out in an Agilent Technologies 1100 series liquid chromatograph (Agilent Technologies, Pittsburgh, PA) equipped with diode array and fluorescence detectors, an automatic injector, a degasser system, a quaternary pump, and a thermostated column compartment. HP Chemstation software was used for instrument control and data acquisition. A reversed-phase UPLC column, Hypersil Gold (100 × 3 mm i.d., 1.9 μm particle size, 175 Å pore size) from Thermo Scientific (Cheshire, U.K.) was employed for the separation of proteins. Moreover, an in-line filter with a 0.2 μm pore size from Agilent Technologies was also used as precolumn. Mobile phases consisted of 0.1% (v/v) TFA in Milli-Q water (mobile phase A) and 0.1% (v/v) TFA in ACN (mobile phase B) were employed. Separations were performed with a linear gradient: 36−45% B in 16 min, 45−95% B in 2 min, 95−5% B in 2 min, and 5−36% B in 3 min to return to starting conditions. The flow rate was 0.4 mL/min, and the column temperature was kept at 55 °C. Fluorescence detection at a λ_{ex} of 280 nm and a λ_{em} of 360 nm was used. The injection volume was 1 μL for the seed samples, 3 μL for the pulp samples, and 10 μL for the oil samples.

In Gel Tryptic Digestion. SDS−PAGE bands were cut into thin slices along the migration path. Proteins were reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, and digested with 1 ng/μL trypsin in 25 mM ammonium bicarbonate at 37 °C overnight. Taking into account the hydrophobic nature of the matrix, it is expected that the proteins passing to and remaining in the olive oil were those with more hydrophobic behavior. The hydrophobic peptides are more difficult to extract from the polyacrylamide gel than the hydrophilic ones, and because of this we used an additional step for the specific extraction of those peptides.[19] The gel was separated from the hydrolysis solution and treated with 30 μL of isopropanol containing 10% (v/v) of FA, during 10 min at room temperature. The solution was then collected, and the operation was repeated by increasing the incubation time to 30 min. Thereafter, 20 μL of pure FA was added to the sample, which was allowed to stand for 15 min, and the supernatant was collected and saved. Finally, the gel spots were washed with 20 μL of isopropanol and further incubated for 15 min. All the washes containing peptides were combined and dried in a vacuum concentrator. The residues were resuspended in 15 μL of water containing 0.1% (v/v) of TFA. Peptides were desalted using reverse-phase ZipTip C_{18} minicolumns (Millipore, Bedford, MA) according to the manufacturer's recommendations and eluted with 10−15 μL of 70% (v/v) ACN containing 0.1% (v/v) TFA, dried in a vacuum concentrator, and resuspended in water containing 0.1% (v/v) of FA.

NanoLC−MS/MS. 8 μL of peptide extract obtained from tryptic digestion of every band was injected in a nano chromatographic system, UltiMate 3000 RSLC nanosystem (Dionex). The peptide mixtures were loaded on a reversed-phase trap column (Acclaim PepMap100, C18, 100 Å, 100 μm i.d. × 2 cm, Dionex) for its cleanup and preconcentration. After cleanup, the valve was switched to place the trap column in series with a fused silica reversed-phase column (picoFrit column, C18, 2.7 μm, New Objective). Peptides were eluted in a 30 min gradient from 4% phase A (2% (v/v) ACN and 0.1% (v/v) FA in water) to 60% phase B (2% (v/v) water and 0.1% (v/v) FA in ACN) at a constant flow rate of 300 nL/min. The chromatographic system was connected to an LTQ-XL or an LTQ-Orbitrap-XL mass spectrometer (Thermo Scientific), both equipped with a nano spray ion source. Full scan mass spectra were acquired in the mass range from m/z 350 to m/z 1800, and the four most intense ions were automatically selected and fragmented in the ion trap. The raw data were analyzed by Mascot search engine (version 2.3.01) using Proteome Discover software (v. 1.2.0 Thermo) and consulting Uniprot_viridiplantae (30264 sequences, 184678199 residues) and without taxonomy restriction (All entries). Oxidation of methionine residues was set as variable modifications; two missed cleavages were allowed to trypsin; peptide mass tolerance was set to 1 Da for low resolution data, fragment mass tolerance to 0.8 Da, and an ion source cutoff of 20 was chosen. The false discovery rate obtained by Proteome Discoverer, consulting the Mascot decoy database, was less than 0.05.

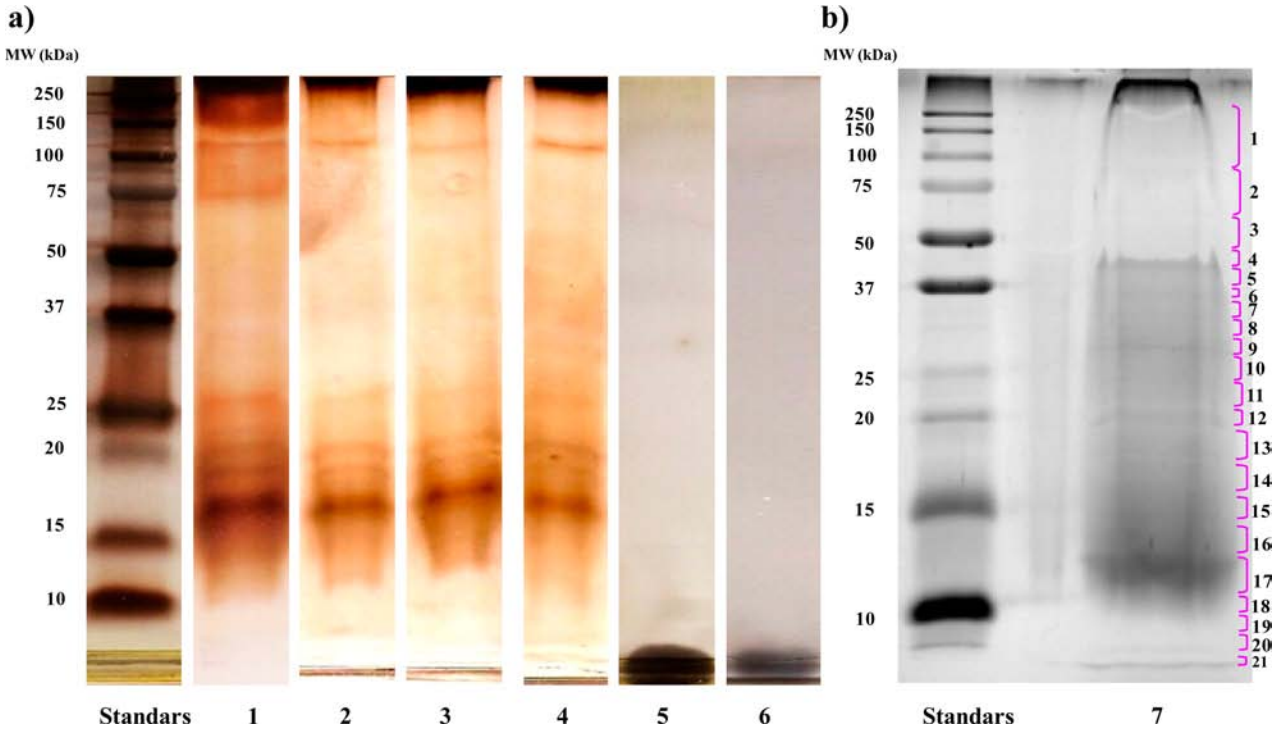

Figure 2. Silver-stained SDS−PAGE gels corresponding to protein extracts from olive oil obtained with different extraction methods: lane 1, extraction with acetone; lane 2, extraction with acetone/hexane; lane 3, extraction with TCA/acetone and precipitation with acetone; lane 4, extraction with Tris/SDS/DTT/urea; lane 5, extraction with Tris/SDS/DTT/urea and filtration; lane 6, blank of the extraction method with Tris/SDS/DTT/urea and filtration; lane 7, extraction with TCA/ecetone and precipitation with water/methanol/chloroform. Experimental conditions in (a): precast gel; olive oil variety, 'Picual' (Spain); initial olive oil mass, 100 g; silver-staining kit from Bio-Rad. Experimental conditions in (b): homemade gel, 15% T; olive oil variety, 'Gentile di Chieti' (Italy); initial olive oil mass, 400 g; silver-staining kit from InvitroGen.

RESULTS AND DISCUSSION

Development of a Method for the Extraction of Proteins from Olive Oil. Different extraction methods were designed (see Figure 1). The first one, extraction with acetone, was based on the method employed by Martín-Hernández et al.[10] These authors described the presence of two proteins with 58 and 64 kDa in the olive oil and other proteins up to 30 kDa but not the presence of the polypeptide of 4.6 kDa, previously described by Hidalgo et al.[7,8] The second method was very similar but used a mixture of acetone/hexane instead of acetone for the precipitation of proteins. This mixture had been employed previously to remove proteins from soybean oil.[20] The third and fourth methods employed a first extraction with TCA/acetone, a commonly used procedure for the extraction of plant proteins. The fifth method was based on an extraction with a Tris-HCl buffer containing SDS and urea that had been previously employed by our research team for the extraction of olive seed proteins.[10] The last method consisted of a combination of the previous method and that of Hidalgo et al.,[7,8] who described the presence of a 4.6 kDa peptide in the olive oil.

All extracts and the blank of the Whatman filter employed in the last method were separated by SDS−PAGE and silver stained. Figure 2 shows the electrophoretic profiles obtained for each olive oil protein extract (lanes 1−5 and 7) and the blank obtained from the Whatman filter (lane 6). Similar protein profiles, with molecular masses up to 30 kDa, were observed by using the extraction methods with acetone (lane 1), acetone/hexane (lane 2), TCA/acetone (lanes 3 and 7), and Tris/SDS/DTT/urea (lane 4). It is important to remark that slight differences observed in lane 7 could be attributed to the different sample employed, the larger amount of oil, and the different gel and silver-staining kit used. These bands were also observed by Martín-Hernández et al.[1] However, the same authors also observed two additional bands with molecular masses of 55 and 65 kDa that are not observed in the profiles of Figure 2. In the mentioned article, these bands appeared for a lot of different vegetable and animal oils, suggesting that they could correspond to a contamination during manipulation with the most abundant human skin keratins, with molecular masses of 58 and 64 kDa. On the other hand, when the Whatman filter was used (lane 5), a very intense band was obtained at a molecular mass below 10 kDa, corresponding to the smallest standard. This band probably corresponded to the 4.6 kDa band previously described by Hidalgo et al.[7,8] for the olive oil and by Zamora et al.[21] for the olive pulp and seed. However, the same band appeared for the blank of the extraction method (lane 6). The fact that the proteins observed in all other extracts disappeared when using a Whatman filter and that a new band appeared also in the filter blank could suggest that this band is coming from the filter. This hypothesis becomes more significant taking into account that this was the unique band observed by Hidalgo et al.[7] in very different nonrelated vegetable oils and plants. Despite the fact that separations obtained using these different extraction methods look very similar (except when using the Whatman filter), the method using Tris/SDS/DTT/urea was the one yielding the cleanest sample, free of oil. Moreover, Tris/SDS/DTT/urea was the extraction method more similar to those used for the extraction of seed and pulp proteins, being the method selected for further analysis of oil proteins.

An alternative and novel approach based on protein capture in CPLLs was also proposed (see Figure 1). Two different methodologies consisting of the direct extraction of proteins from the olive oil or from an oil−water micelle were employed by using CPLLs of different nature. Nevertheless, the eluates of the CPLL captures resulted in clean lanes with no band appearing in the gel. This could be explained taking into account that the interaction between proteins and CPLLs are prominently ionic[22,23] and they may be extremely hampered in such an apolar matrix.

Comparison of SDS−PAGE Profiles in the Olive Seed, Pulp, and Oil. The electrophoretic profile obtained with olive oil was compared with those observed for the olive pulp and seed in order to characterize the protein bands observed in olive oil. Figure 3 shows these SDS−PAGE profiles. Olive seed

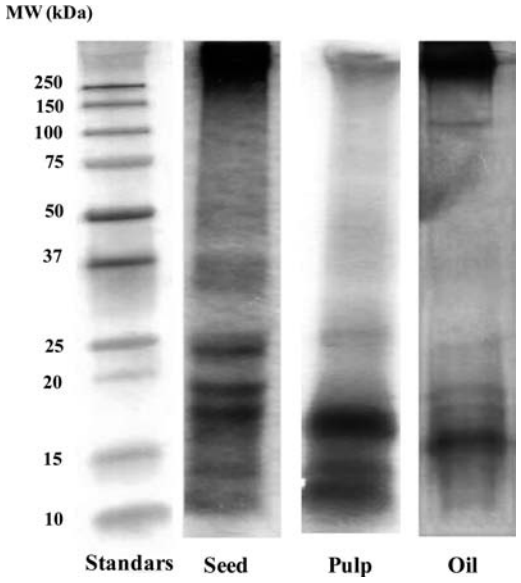

Figure 3. SDS−PAGE gel showing the separation of olive seed, pulp, and oil proteins using the Tris/SDS/PAGE/urea extraction method. Experimental conditions as in Figure 2a.

showed the most crowded profile consisting of different bands distributed in three regions. From 10 to 25 kDa, bands could be attributed to seed storage proteins (SSPs) from the Solea I precursor (20, 22.4, and 23.5 kDa) and an oleosin of 22 kDa.[10] Two close bands in the range from 25 to 37 kDa could match with SSPs from the Solea II precursor (27 and 30 kDa). Finally, bands with higher molecular masses could be attributed to oleosins. This profile was similar to that observed by our research team when using Coomassie staining.[10]

Olive pulp yielded a main band at 17 kDa and three less intense bands at approximately 11, 13, and 25 kDa. This profile is different from that obtained by our research group in a previous work when using Coomassie staining.[11] In this case, just the band at 25 kDa, that was mainly attributed to a thaumatin-like protein, was observed.[11,24] Regarding the olive oil, it was possible to observe a main band at 15 kDa and two additional less intense bands (bands at 17 and 19 kDa). All these bands could be attributed to proteins coming from the pulp or the seed. No band with molecular mass higher than 19 kDa was observed in the oil, suggesting that only smaller proteins from the olive fruit can pass to the oil while bigger proteins such as the oleosin of 50 kDa and the SSPs of 25 and 37 kDa of the seed or the thaumatin-like protein of the pulp would remain in the solid residue obtained after oil extraction.

2-D Separation of Olive Seed, Pulp, and Oil Proteins. In order to make a better comparison of protein profiles from the olive seed, pulp, and oil, a two-dimensional separation strategy was next tried. Protein extracts were first separated by off-gel IEF and the collected fractions separated by SDS−PAGE and by RP-HPLC.

Ampholytes required for IEF significantly interfered with the SDS−PAGE separation producing an unpleasantly high background. Different strategies were tried to remove ampholytes such as the reduction of ampholyte concentration, protein precipitation prior to SDS−PAGE separation using cold acetone, TCA/acetone, or H_4Ac/MeOH, filtering through molecular mass cutoff (Mwco) filters of 3 kDa, or cleaning with C_4 OMIX tips. Nevertheless, the removal of ampholytes was generally accompanied with a loss of proteins, and this procedure was abandoned (see Table 1). Even so, for the

Table 1. Protein Recovery Results Obtained by RP-HPLC for Each Method Employed in the Removal of Ampholytes

methodologies	protein recovery
precipitation with acetone (4 °C)	90
precipitation with acetone (−20 °C)	80
precipitation with 10% TCA/acetone (4 °C)	95
precipitation with 10% TCA/acetone (−20 °C)	110
precipitation with NH_4Ac/MeOH (4 °C)	98
precipitation with NH_4Ac/MeOH (−20 °C)	96
cut-off filter in presence of 1% OG	50
cut-off filter in presence of 1% SDS	90
C_4 OMIX tips	5

olive seed it was possible to observe clear bands at molecular masses between 20 and 25 kDa in the most basic wells that probably corresponded with the SSPs from the Solea I precursor. These results matched with those observed by Alché et al.[25] by 2D-PAGE. At basic p*I*s it was also possible to observe a diffuse band at molecular masses ranging from 45 to 50 kDa that could match with the oleosin of 50 kDa.[26] Regarding the chromatographic separation of the IEF fractions (see Figure 4), no interferences were observed from ampholytes. In fact, in this case the separation of IEF wells enabled observation of not only the presence of basic proteins in the seed but also proteins with acidic nature. The more basic wells, corresponding to p*I*s 8.4, 8.9, and 9.5, showed one peak at retention time of 3 min, two peaks between 9 and 10 min, and a fourth peak at 11.5 min that mainly appeared in the well corresponding to p*I* 8.4. According to the results observed when SDS−PAGE separation was achieved, those peaks probably corresponded to subunits of the SSP Solea I. On the other hand, the result highlights the profile observed in the chromatogram corresponding to the fraction at p*I* 5.3. While the separation by SDS−PAGE did not allow to observation of the presence of proteins in this well, the use of HPLC using a UPLC column as a second separation dimension permitted detection of three signals that could correspond to subunits of the other SSP, Solea II.[25]

In the case of the olive pulp, SDS−PAGE separation also resulted in an important background for almost all the p*I*s with the exception of the most basic ones, where a band of approximately 17 kDa was observed. This band matched with the main band appearing for the pulp in Figure 3 that was

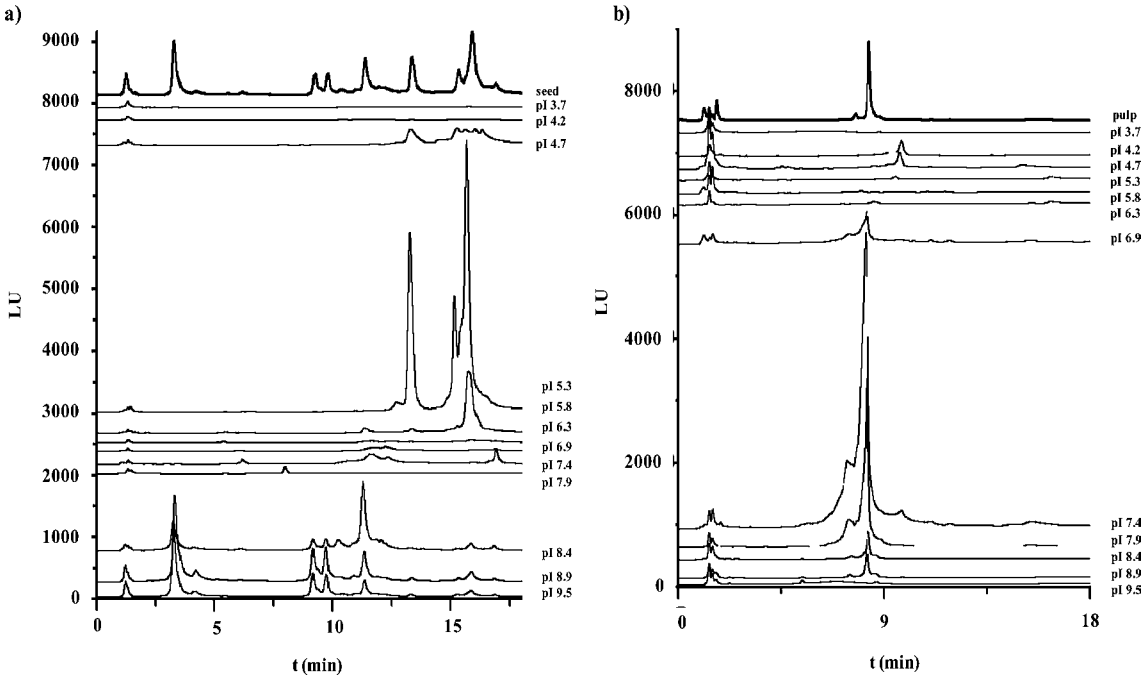

Figure 4. Protein chromatographic profiles corresponding to the samples collected from each off-gel IEF well and to the whole sample: (a) seed and (b) pulp. Chromatograms are ordered by their average isoelectric point. Chromatographic conditions: gradient, 36−45% B in 16 min, 45−95% B in 2 min, 95−5% B in 2 min, and 5−36% B in 3 min; mobile phases, 0.1% (v/v) TFA in Milli-Q water (mobile phase A) and 0.1% (v/v) TFA in ACN (mobile phase B); flow rate, 0.4 mL/min; column temperature, 55 °C; fluorescence detection, λ_{ex} 280 nm and λ_{em} 360 nm.

Table 2. Peptides Identified by Homology with Plant Proteins in the Digested Extract of the Isolated Olive Oil Proteins

protein					peptide	
accession number	description	score	mass	no. of peptides	score	sequence
tr|C1E6T8|C1E6T8_MICSR	histone H4, OS = *Micromonas* sp. (strain RCC299/NOUM17)	105	11388	2	24.12	VFLENVIR
					60.87	ISGLIYEETR
tr|D8RCL6|D8RCL6_SELML	putative uncharacterized protein, OS = *Selaginella moellendorffii*	82	19606	1	31.23	RCSDLNYGIK
tr|B9R7T9|B9R7T9_RICCO	cytochrome P450, OS = *Ricinus communis*	70	71448	1	37.91	LNDLSLDLIDAK

Table 3. Peptides Corresponding to Bacteria Proteins Identified in the Digested Extract of the Isolated Olive Oil Proteins

protein					peptide	
accession number	description	score	mass	no. of peptides	score	sequence
gi|227819381	AsnC family transcriptional regulator [*Sinorhizobium fredii* NGR234]	155	17537	1	77.05	MMNTLNLDLVDRK
gi|103488202	OmpA/MotB [*Sphingopyxis alaskensis* RB2256]	78	31223	1	46.50	FAEQTAATEALR
gi|46203495	hypothetical protein Magn03005587 [*Magnetospirillum magnetotacticum* MS-1]	86	29861	1	86.12	VPIGLGSLTIEAEALDAR
gi|91788123	periplasmic phosphate-binding protein [*Polaromonas* sp. JS666]	76	36516	1	66.17	INYQSVGSGAGLR
gi|13476844	unnamed protein product [*Mesorhizobium loti* MAFF303099]	75	32272	1	74.53	TWVSGVGDDANPCSR
gi|103488296	30S ribosomal protein S10 [*Sphingopyxis alaskensis* RB2256]	73	11683	1	73.15	VLDQATTDIADTAR
gi|103486299	MotA/TolQ/ExbB proton channel [*Sphingopyxis alaskensis* RB2256]	64	28703	1	64.43	APTLADGAAKLEK

identified as a thaumatin-like protein. When IEF wells were injected into the chromatographic system, a main peak at 8.5 min highly focused between p*I* 7.4 and p*I* 7.9 was observed (Figure 4b). Moreover, these signals coeluted with another peak appearing at 8 min. The coelution of two peaks and the focusing in more than one well could suggest the presence of different isoforms of the thaumatin-like protein. These would confirm the data obtained in a previous work where some modifications in the amino acid sequence of the olive thaumatin-like protein were observed when digesting with trypsin and analyzing by nanoLC−MS/MS.[24] It is also possible to observe a minute peak appearing at 10 min in the wells with p*I* 4.2 and 4.7. This peak, which could correspond to one of the less intensive bands observed by SDS−PAGE, is not visible in the whole extract.

Unfortunately, the comparison of protein profiles corresponding to the olive seed and pulp with that of the olive oil was not possible since the IEF separation was not successful for the olive oil. Indeed, the intrinsic lipidic nature of the oil prevented the separation of oil proteins. Different strategies, such as reduction of the oil volume or use of Mwco filters, were

tried, but all resulted unsuccessful. Moreover, direct injection of oil extract in the UPLC column did not yield any signal.

Protein Digestion and Analysis by NanoLC−MS/MS. The last analytical strategy for the identification of olive oil proteins was the trypsin digestion of the SDS−PAGE bands (see Figure 2, lane 7) and their analysis by nanoLC−MS/MS. For that purpose, LTQ-XL mass spectrometer was first employed, but the instrument sensitivity was not high enough for these experiments. Consequently, a very sensitive LTQ-Orbitrap-XL mass spectrometer was next tried. This constitutes the first time that olive oil proteins have been identified by MS. Three proteins corresponding to plant proteins (see Table 2) and seven proteins corresponding to bacterial proteins (see Table 3) were identified. Regarding those identified as plant proteins (Table 2), a histone H4, an uncharacterized protein, and a cythochrome P450 were identified. It should be noted that histone H4 was homologous to a histone H4 previously observed in the olive fruit seed.[17] In fact, the histone H4 observed in the olive oil presented a peptide (ISGLIYEETR) that was also observed in the olive seed. These data suggested that this protein could be transferred from the seed to the oil. On the other hand, three of the bacterial proteins corresponded to the same bacterium, named *Sphingopyxis alaskensis*. Proteins corresponding to four other different bacteria were also identified. These results could be explained taking into account that it is very common to find bacteria in food obtained by conventional procedures like those employed in wine or olive oil production.[27] Such an unwieldy matrix and the low abundance of proteins in the olive oil render our results rather preliminary. Further developments in separation and mass spectrometry technology will hopefully enable in the future to increase the knowledge on the olive oil proteome.

In conclusion, despite the evidence of the presence of proteins in the olive oil, the information available about them is very scarce and contradictory. This is the first time that a comprehensive study of olive oil proteins has been performed, involving the use of different extraction methods, including the use of CPLLs, different electrophoretic separations, HPLC and MS analysis. It has been possible to demonstrate that some of the proteins previously observed in the olive oil came from a nonsuitable manipulation of extracts (bands at 55 and 65 kDa probably from human keratins) or an incorrect extraction procedure (band at 4.6 kDa). It has also been possible to optimize a procedure for the suitable extraction of olive oil proteins yielding bands by SDS−PAGE up to 20 kDa. The comparison of electrophoretic profiles obtained with the olive oil and those corresponding to the olive seed and pulp enabled observation that only the smaller proteins in the seed and the pulp seem to pass to the oil while the bigger proteins probably remain in the pellet resulting from the oil extraction. Olive oil, an intrinsically difficult matrix, did not permit the separation of oil proteins by isoelectric focusing. A tentative identification of olive oil proteins by the trypsin digestion of the SDS−PAGE bands and their subsequent analysis by an Orbitrap mass analyzer has been possible. It is clear, though, that proteins in olive oil are only present in ultratraces and that finding additional species not yet identified in oils might be an impossible task.

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Notes

The authors declare no competing financial interest.

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