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ARTICLE

First Ultraperformance Liquid Chromatography Based Strategy for Profiling Intact Proteins in Complex Matrices: Application to the Evaluation of the Performance of Olive (*Olea europaea* L.) Stone Proteins for Cultivar Fingerprinting

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There is a clear need for accelerating protein separations by HPLC. Different proposals have been developed including the use of perfusion and monolithic stationary phases. Nevertheless, these stationary phases, in some occasions, do not provide enough efficiency to resolve these large molecules when they are present in complex matrices. Although ultraperformance liquid chromatography (UPLC) columns have been successfully used for the efficient and rapid separation of small molecules, this is the first time these columns were proposed for the separation of intact proteins in a real complex matrix: the olive stone. Two different strategies were employed for the extraction of olive proteins: enzymatic assisted extraction and buffered extraction. Five different columns traditionally employed for the separation of proteins were used, and results were compared with those obtained when using different sub-2 μm particle columns. Separations obtained with sub-2 μm particle columns significantly improved the separations obtained with the other columns. This paper also demonstrates the applicability of protein profiles obtained from the olive stone for the discrimination among olive varieties.

KEYWORDS: Olive; stone; proteins; UPLC; differentiation

INTRODUCTION

Protein analysis by high-performance liquid chromatography (HPLC) is challenging due to the low intrapore diffusivity of these large molecules. Indeed, very lengthy analysis times jointly with broad or misshapen peaks and protein carryover are common in the separation of proteins (*1*). Two strategies focused on simplifying the intraparticle mass transfer and overcoming these problems have been developed: (i) Particle permeability could be increased by using wide pores, enhancing mass transfer, connected to smaller diffusive pores. Perfusion particles and monolithic rods are stationary phases showing this biporous structure that have been widely employed for protein separations (*2*). (ii) Diffusion paths could be reduced, thereby reducing the time required for molecules to diffuse in and out of the particle. Two different possibilities have been proposed: (a) Pellicular packings consist of a solid core covered by a thin porous shell. The main drawback associated with this kind of support is its limited loading capacity, which is important for preparative purposes and for the detection of low-abundance molecules. (b) The use of smaller diameter, sub-2 μm, porous particles has resulted in fast and efficient separations. Indeed, the increased efficiency enables the reduction of column length and, therefore, the acceleration of separations and an improved throughput of analysis (*3*). The main drawback of these phases is the high back-pressure generated.

Early works using sub-2 μm particles involved the use of nonporous particles because they are mechanically strong and relatively easy to manufacture (*4*). Nevertheless, the use of these stationary phases resulted in very high back-pressures (2500−5000 bar), limiting their application. On the other hand, sub-2 μm porous stationary phases have been extensively used for the separation of small molecules such as drugs and peptides (*5*−*8*). However, the application of these columns to the separation of intact proteins has yet to be explored, there being only two publications devoted to the separation of standard model proteins (*4*, *9*). Different reasons could be involved in the limited implementation of this technology in this area: the need for special equipment supporting the high back-pressures that are usually generated, the lack of suitable ultraperformance liquid chromatography (UPLC) columns for protein separations (e.g., most UPLC commercial columns present very narrow pore sizes (< 300 Å)), and the intrinsic difficulty in the analysis of proteins in real samples and complex matrices.

This is the first time that the evaluation of the performance of different stationary phases (perfusion, monolithic, pellicular, and conventional silica packings) in comparison with sub-2 μm porous phases for the rapid separation of intact proteins (olive stone proteins) in a real complex matrix (olive stone) is proposed.

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Our target proteins were those present in the olive stone because they seem to have an important role in the processing and quality of olives and olive oil (*10*−*12*). Olive proteins are mainly present in the stone, consisting of endocarp and seed (*13*, *14*). Despite the fact that olive proteins have been little studied, the existence of storage proteins and proteins associated with fatty bodies called *oleosins* have been documented (*15*). Storage proteins are formed from two precursor proteins of 41 kDa (Solea I) and 47.5 kDa (Solea II) that are linked by hydrogen bridges. After its reduction, the precursor of 41 kDa generates three polypeptides of 20, 22.4, and 23.5 kDa, whereas the other precursor generates two polypeptides of 27 and 30 kDa (*16*−*18*). On the other hand, two different oleosins of 22 and 50 kDa have been found in olive stone (*19*). Recently, the use of oleosins has been described for the production of recombinant proteins (*20*).

Thus, the main aim of this work has been to explore, for the first time, the potential of UPLC for the rapid profiling of intact proteins in a complex matrix such as the olive stone. Another novelty of the proposed work is the evaluation of the performance of the obtained protein profiles for olive cultivar fingerprinting. The identification of olive genotypes has been performed on the basis of plant morphological (leaves, influorescence, fruit, endorparp, etc.) and/or agronomical characters, isoenzymatic markers, or using DNA markers (*21*−*25*). Nevertheless, despite these efforts, there is still no totally satisfactory methodology due to the huge genetic diversity of olives. Therefore, the exploration of new approaches such as that based on olive proteins is needed.

MATERIALS AND METHODS

Chemicals and Samples. HPLC grade acetonitrile (AcN), supergradient HPLC grade methanol (MeOH), extrapure 2-propanol (Scharlau, Barcelona, Spain), trifluoroacetic acid (TFA) (Sigma, St. Louis, MO), and HPLC grade water (Milli-Q system; Millipore, Bedford, MA) were used in the preparation of mobile phases. Tris(hydroxymethylaminomethane), 2-mercaptoethanol, sodium dodecyl sulfate (SDS) (Merck, Darmstadt, Germany), dithiothreitol (DTT) (Sigma), disodium hydrogen phosphate (Panreac, Barcelona, Spain), and reagent grade acetone (Scharlau) were used for the extraction and precipitation of stone proteins. Bradford reagent and bovine serum albumin (BSA), used in the Bradford protein assay, were from Sigma. Six different hydrolytic enzymes were employed: Alcalase 2.4 L FG, Viscozyme L, Ultraflo L, Novozym 33102 and 33095, Lecitase Ultra (all kindly donated by Novozymes Spain S. A. (Madrid, Spain)), and lipase from *Candida rugosa* (Sigma). Fruits of 29 different olive (*Olea europaea* L.) varieties supplied by the Olive World Germplasm Bank of IFAPA (Junta de Andalucia, Córdoba, Spain) (*26*) were employed: Picual, Corbella, Changlot Real, Carrasqueño de Alburquerque, Manzanilla Cacereña, Verdalon, and Arbequina from Spain; Moraiolo T. Corsini, Frantoio, Leccino, Pendolino, and Dolce Agogia-1363 from Italy; Beyaz Yaglik, Dokkar, Erbek Yaglik, and Kiraz from Turkey; Majhol-1013, Bent al Kadi, Shami-141, and Sayfi from Syria; Cobrançosa, Cordovil de Serpa-130, and Verde Verdelho from Portugal; Mavreya and Kalokerida from Greece; Salonenque, Bouteillan, and Lucques from France; and Polinizador from Mexico. All of them were grown under the same agronomic conditions. Violet-ripened olive fruits were manually depulped. Resulting stones were dried, and remaining dried pulp was easily removed before storing at −18 °C until use.

Protein Extraction. Olive stones were ground with a domestic mill, and 0.03 g of ground 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 extraction was carried out in 5 min using an ultrasonic microprobe (Hartford, CT) at 30% amplitude. After centrifugation at 4000*g* for 10 min, proteins in the supernatant were precipitated with 10 mL of cold acetone at 4 °C for 1 h and centrifuged again during 10 min. The pellet was resuspended in 125 mM Tris-HCl (pH 7.5) and 1% (m/v) SDS, submitted to sonication for 5 min, and filtered with a 0.45 μm filter (Titan 2, Eatontown, NJ) before injection. The protein content was estimated by the Bradford protein assay (*27*).

SDS−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Proteins were separated by SDS-PAGE using a Bio-Rad Mini-Protean system (Hercules, CA). Proteins were separated in 10% (m/m) acrylamide (T) and 2.6% (m/m) bisacrylamide (C) gels using the Laemmli buffer (*28*) and stained with Coomassie Brilliant Blue.

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. Eight different reversed-phase HPLC (RP-HPLC) columns were employed: a monolithic silica column Chromolith Performance RP-18e (100 × 4.6 mm i.d.) from Merck, two different POROS R2/H perfusion columns (50 × 4.6 mm and 100 × 2.1 mm i.d., 10 μm particle size) from Perseptive Biosystems (Framingham, MA), a pellicular Zorbax Poroshell 300SB-C18 column (75 × 1 mm i.d., 5 μm particle size, and 300 Å pore size), a conventional C8 Zorbax column (150 × 4.6 mm i.d., 5 μm particle size, and 300 Å pore size), and a UPLC C18 Zorbax SB (50 × 4.6 mm i.d., 1.8 μm particle size, and 80 Å pore size), all from Agilent Technologies, and two UPLC Hypersil Gold columns (50 × 4.6 mm and 100 × 3 mm i.d., 1.9 μm particle size, 175 Å pore size) from Thermo Scientific (Cheshire, U.K.). Moreover, an in-line filter with a 0.2 μm pore size from Agilent Technologies was employed with columns of sub-2 μm particle size. Mobile phases used in all cases consisted of 0.1% (v/v) TFA in Milli-Q water (mobile phase A) and 0.1% (v/v) TFA in an organic modifier (MeOH or AcN) (mobile phase B). The volume injected in the non-UPLC columns was 20 μL and in the UPLC columns was 5−3 μL.

Data Treatment. Peak areas from stone proteins were integrated by setting the baseline from valley to valley. The area percentage for every peak was calculated as the average of two replicates (injected by duplicate). The application of multivariate analysis was performed using the computer program Statgraphics Plus for Windows 5.1 (Statistical Graphics Corp.).

RESULTS AND DISCUSSION

The development of a methodology enabling the characterization of olive genotypes from olive stone proteins involved the optimization of a protocol for extracting olive stone proteins and a separation method to obtain protein profiles from these extracts. All of these experiments were performed using olive stones from the Picual variety.

With regard to protein extraction, there is a great difficulty in the extraction of proteins from a lipid matrix. To our knowledge, no method enabling the extraction of proteins from the whole stone has been described yet, and only one bibliographic method was found for the extraction of proteins from the olive seed (*16*−*18*). Briefly, ground seeds (0.1 g/mL) were mixed with a Tris-HCl buffer (pH 6.8) containing 0.2 or 1% (m/v) SDS and/or a reducing agent such as 2-mercaptoethanol (1% (v/v)) or DTT (0.1 M). After centrifugation for 5 min, the supernatant was boiled and centrifuged again, and proteins in the supernatant were precipitated with cold acetone during a whole night at 4 °C. Finally, the precipitated proteins were resuspended in the extracting buffer. The conditions used by Wang et al. (*16*−*18*) for the extraction of proteins from the olive seed were applied for the extraction of proteins from the olive stone. For that purpose, an ultrasonic microprobe at 30% amplitude and for 5 min was employed to favor the extraction of proteins, and the obtained extracts were analyzed by RP-HPLC using a monolithic column (100 mm × 4.6 mm i.d.). The chromatographic conditions employed were established on the basis of our group experience and consisted of the following: temperature, 25 °C; flow rate, 3 mL/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); binary gradient, 30−55% B in 10 min; detection, 210 nm. Nevertheless, it was not possible to obtain any separation using these conditions. Therefore, to reduce the complexity of the extraction procedure

and increase its throughput and to obtain a suitable protein profile from the whole olive stone, the sample extraction procedure and the protein separation conditions were optimized.

Sample Extraction Optimization. Two different strategies for extracting olive stone proteins were proposed: enzymatic assisted extraction and buffered extraction. Olive stone proteins were extracted using different hydrolytic enzymes: proteases (Alcalase), carbohydrases (Viscozyme L, Ultraflo L, Novozym 33102 and 33095), and lipases (Lecitase Ultra and Lipase). The experimental conditions used were obtained from the information included in the product data sheet or in the bibliography (*29*−*32*): Alcalase (pH 8.0 and 50 °C), Viscozyme L (pH 4.1 and 45 °C), Ultraflo L (pH 6.0 and 40 °C), Lipase (pH 6.0 and 30 °C), Lecitase (pH 7.0 and 25 °C), and Novozym 33102 and 33095 (pH 6.2 and 55 °C). The extraction time ranged from 1 to 2 h. After digestion, extracts were centrifuged, proteins in the slurry were precipitated with acetone at 2 °C, and the precipitated proteins were dissolved in 0.125 M Tris-HCl buffer containing 0.1% (m/v) SDS. The obtained extracts were injected into the chromatographic system (using the same chromatographic conditions previously employed and a binary gradient from 5 to 95% B in 15 min); identical chromatograms with and without enzyme were observed, and it was concluded that enzymes did not improve extraction of olive stone proteins.

The following parameters were next studied to optimize buffered extraction: buffer composition, buffer pH, and ultrasonic microprobe conditions. The need to add a reducing agent (2-mercaptoethanol (from 1 to 5% (v/v)) or DTT (from 0.5 to 1% (m/v)) to the extracting medium (0.125 M Tris-HCl buffer (pH 6.8) containing 1% (m/v) SDS) was investigated. The best separation was observed when 0.5% (m/v) DTT was added. The effect of the pH was evaluated using buffers at pH values ranging from 6.5 to 9.0. The use of a Tris-HCl buffer at pH 7.5 enabled the best chromatographic separation. The effect of adding urea to the extracting buffer was also studied, showing an increase in the signal but a significant reduction in the resolution between peaks. Despite the modifications performed in the separation gradient (reducing the gradient slope) and the reduction in the concentration of proteins injected, it was not possible to achieve a suitable separation of proteins. Ultrasonic microprobe conditions (time and amplitude) were also optimized, observing that extractions longer than 5 min and amplitudes higher than 30% did not result in an improvement in the extraction. Finally, a precipitation step of proteins with cold acetone was considered. The study of the effect of using different precipitation times (15 min, 30 min, 1 h, 2 h, and whole night) led to the conclusion that precipitation times longer than 1 h did not result in an increase in protein recovery or peak sizes.

The obtained extract was analyzed by using the Bradford protein assay. Moreover, the obtained extract was also separated by SDS-PAGE. The estimated protein content given by the Bradford method was 1070 ± 80 μg/mL. Analysis of extracts by SDS-PAGE (applied volume, 50 μL) resulted in different bands that enabled confirmation of the protein nature of the extracts. As examples, **Figure 1** shows the SDS-PAGE separation corresponding to Picual variety. It was possible to observe a region (a) that could match an oleosin of 50 kDa (*19*); a second region (b) could match storage proteins from the Solea II precursor (27 and 30 kDa) (*16*−*18*), and a third one (c) could match an oleosin of 22 kDa and with the storage proteins from the Solea I precursor of 20, 22.4, and 23.5 kDa (*16*−*19*).

Optimization of the Chromatographic Separation. The initial chromatographic conditions employed did not enable the profiling of olive stone proteins. To improve the separation, these conditions were optimized by the evaluation of different parameters: column temperature, gradient, and detection. Flow rate was not optimized because monolithic supports show efficiencies independent of the flow rate (*2*). UV detection was performed at typical wavelengths for protein absorption (210, 254, and 280 nm), and fluorescence detection was performed at the wavelength of maximum fluorescence emission of tryptophane (λ_{exc} = 280 nm and λ_{em} = 360 nm). The optimized conditions were as follows: elution gradient, 30−40% B in 30 min and 40−47% B in 6 min; flow rate, 3 mL/min; separation temperature, 40 °C. Nevertheless, the performance of the initially chosen monolithic column could not resolve properly this separation even when optimized chromatographic conditions were used (see **Figure 2**). Consequently, other chromatographic columns were next tried: two perfusion columns, a conventional C8 column, a pellicular C18 column, and three sub-2 μm particle columns. All columns used were commercially available. In every case, a slight optimization of experimental conditions consisting mainly on the modification of the elution gradient, the flow rate (only for the conventional and the pellicular columns because perfusion and UPLC columns show efficiencies independent of the flow rate), and the temperature was carried out. **Figure 2** compares the profile obtained using the initial monolithic column with those observed when using the other columns. There was no significant improvement when using the perfusion, C8, and pellicular columns. Better separations resulted with the sub-2 μm particles stationary phases. Nevertheless, the Zorbax SB (50 × 4.6 mm i.d., 1.8 μm particle size) presented a reduced pore size (80 Å), which resulted in the collapse of the column after the injection of a few protein extracts. Both stationary phases from Thermo with sub-2 μm particles presented a higher pore size (175 Å) than the Zorbax, which avoided the column collapse. As a consequence, the Thermo 100 × 3 mm i.d. column (particle size of 1.9 μm and pore size of 175 Å) was selected for further optimization. The back-pressure observed with this column at 60% of mobile phase B, 0.4 mL/min of flow rate, and 55 °C temperature was 240 bar.

Finally, the effect of using other solvents besides AcN in mobile phase B and the effect of reducing the extract concentration were

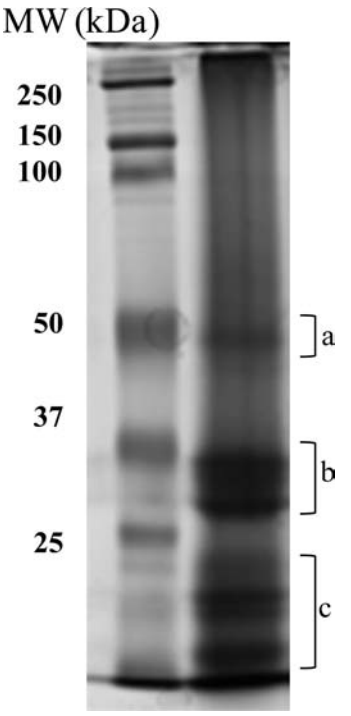

Figure 1. Coomassie-stained 10% T gel showing separation of olive stone proteins from *O. europaea* cv. Picual.

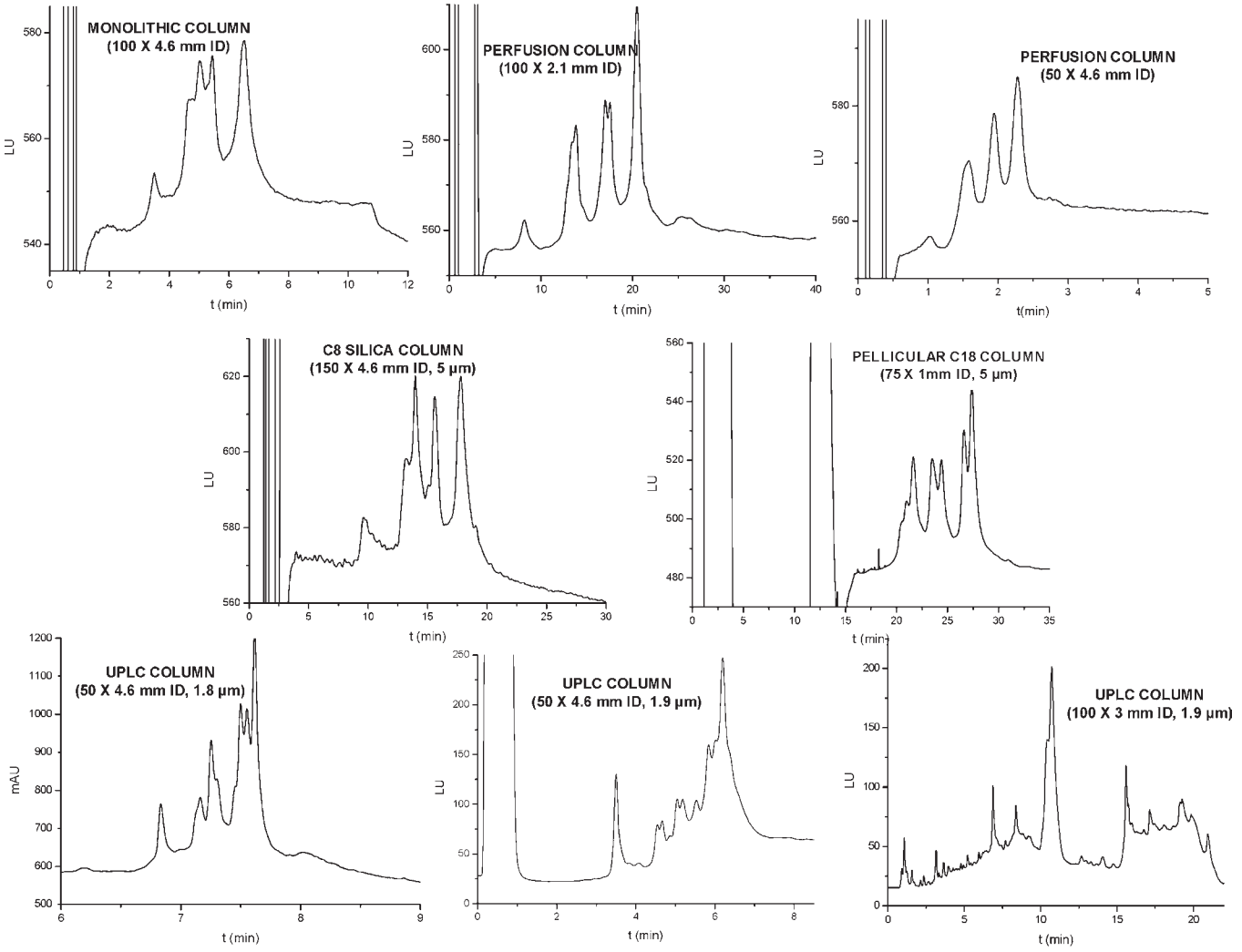

Figure 2. Separation of olive stone proteins from the Picual variety using different stationary phases: monolithic column (100 × 4.6 mm i.d., Merck) (30−40% B in 30 min and 40−47% B in 6 min, 3 mL/min, 40 °C); perfusion column (100 × 2.1 mm i.d., 10 μm, Perseptive Biosystems) (30−40% B in 24 min, 0.5 mL/min, 40 °C); perfusion column (50 × 4.6 mm i.d., 10 μm, Perseptive Biosystems) (30−40% B in 2.4 min, 5 mL/min, 40 °C); C8 silica column (150 × 4.6 mm i.d., 5 μm, Agilent Technologies) (30−47% B in 15 min, 1 mL/min, 40 °C); pellicular C18 column (75 × 1 mm i.d., 5 μm, Agilent Technologies) (30−47% B in 30 min, 0.1 mL/min, 40 °C); UPLC column (50 × 4.6 mm i.d., 1.8 μm, Agilent Technologies) (5% B for 2 min, 5−60% B in 10 min, 1.4 mL/min, 25 °C); UPLC column (50 × 4.6 mm i.d., 1.9 μm, Thermo Scientific) (30−60% B in 12 min, 2.4 mL/min, 55 °C); UPLC column (100 × 3 mm i.d., 1.9 μm, Thermo Scientific) (5−45% B in 5 min; 45% B for 8 min; 45−95% B in 5 min, 0.7 mL/min, 55 °C). Other chromatographic conditions: mobile phases, 0.1% (v/v) TFA in water (phase A) and in AcN (phase B); fluorescence detection (λ_{exc} = 280 nm and λ_{em} = 360 nm); injected volume in non-UPLC columns was 20 μL (total protein mass, 20 μg) and in UPLC columns was 5 μL (total protein mass, 5 μg).

studied. Due to the world shortage in AcN and its subsequent high cost, other organic solvents (MeOH and 2-propanol) were employed as alternatives in mobile phase B, observing that methanol yielded a separation similar to that obtained with AcN but at a lower cost. Moreover, better resolution was obtained when the injected volume was reduced from 5 μL (total protein mass, 5 μg) to 3 μL (total protein mass, 3 μg).

Profiling of Olive Stone Proteins. The method was applied to the profiling of 29 different olive varieties from different parts of the world but all grown under the same agronomic conditions in the Olive World Germplasm Bank of IFAPA (Junta de Andalucía, Córdoba, Spain). Two different kinds of profiles were observed. Most genotypes (23 of 29) yielded profiles like those grouped in **Figure 3** in which three different groups of peaks could be observed: a first group consisting mainly of peaks A and B, a second group comprising peaks C−J, and a third group constituted by peaks K, L, and M. The differences in area percentages observed in these peaks enabled the discrimination among these olive cultivars. On the other hand, there were seven genotypes showing profiles different from those observed in **Figure 3**. These profiles were also very different from each other and could be easily differentiated by their direct observation. **Figure 4** shows the profiles obtained for five of them, as examples.

Repeatability (in peak area) for five injections of the same sample performed in the same day was better than 4%, except for peak A. Reproducibility (in peak area), evaluated as the RSD value corresponding to the injection of five individual samples, was less than 10%, except for peak A. Consequently, only peaks B−M in **Figure 3** were employed to evaluate the performance of the UPLC profiles for olive genetic fingerprinting. Moreover, the robustness of the method was demonstrated by comparing the profiles obtained for the same variety at different maturation states (yellowish green, green with reddish spots, and violet), obtained from different trees or corresponding to different years, and observing that there was no significant difference among profiles.

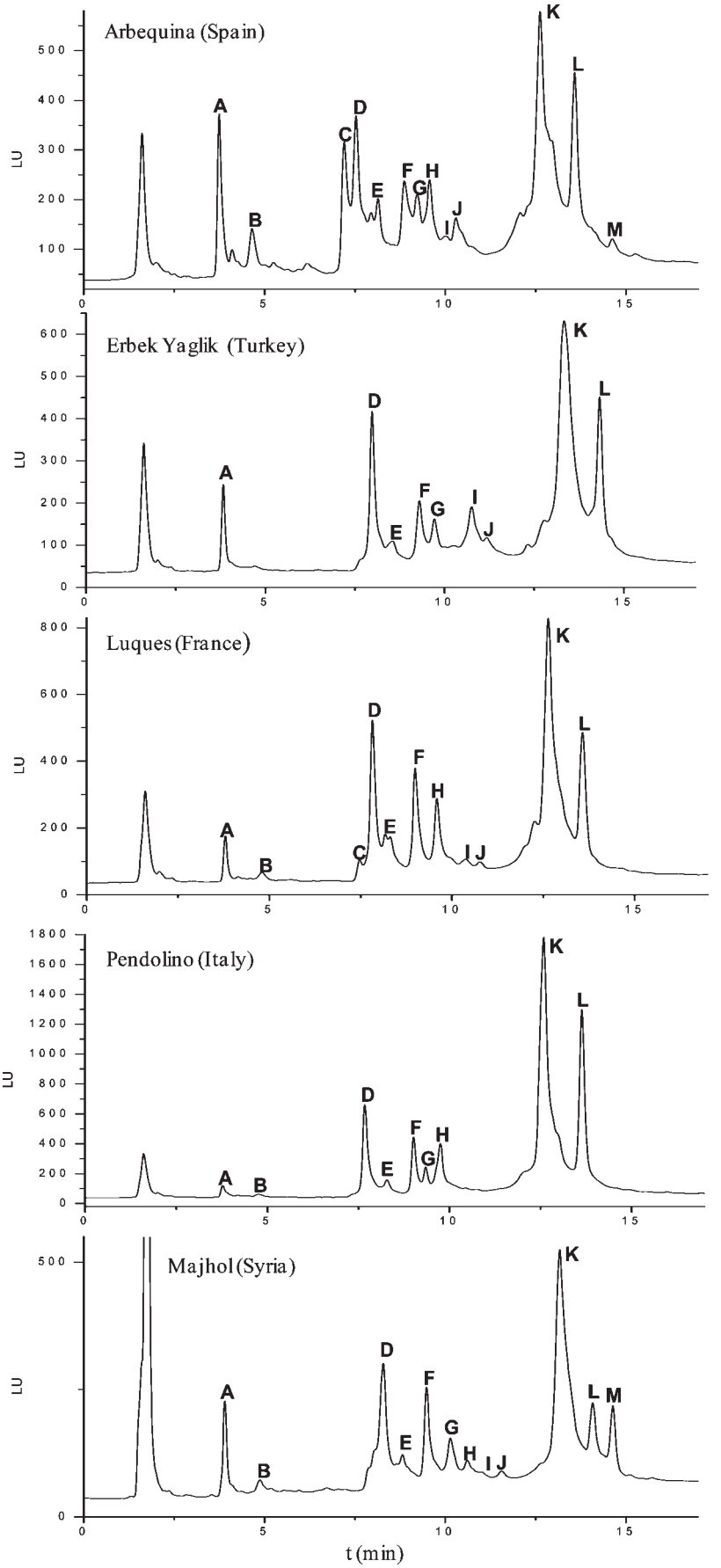

Figure 3. Protein profiles corresponding to different olive stone genotypes. Chromatographic conditions: column, 100 × 3 mm i.d., 1.9 μm of Thermo Scientific; gradient, 60−80% B in 16 min; 80−95% B in 2 min, and 95−5% B in 2 min; flow rate, 0.4 mL/min; temperature, 55 °C; mobile phases, 0.1% (v/v) TFA in water (phase A) and in MeOH (phase B); fluorescence detection (λ_{exc} = 280 nm and λ_{em} = 360 nm); injected volume, 3 μL (total protein mass, 3 μg).

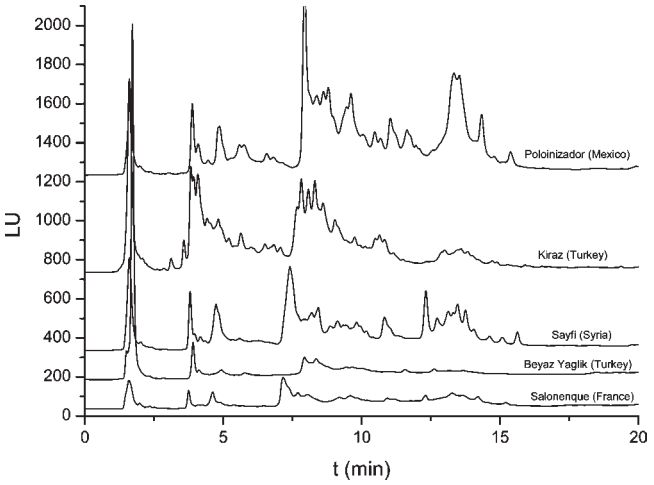

Figure 4. Protein profiles corresponding to different olive stone genotypes. Chromatographic conditions were as in **Figure 3**.

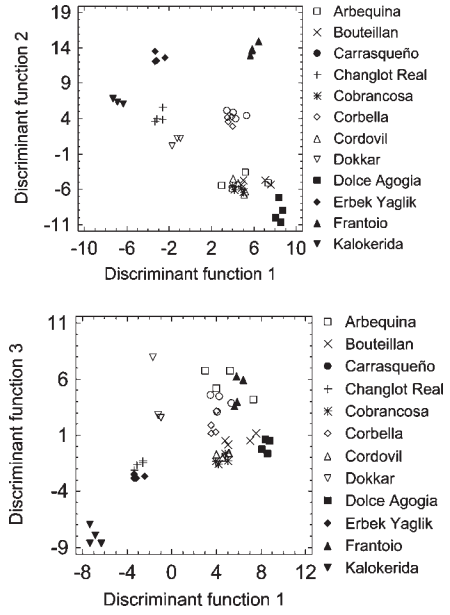

Figure 5. Classification of results of linear discriminant analysis using a representation of the first and second and the first and third discriminant functions found from relative peak areas.

Finally, discriminant analysis was applied to the area percentages of peaks B−M for the olive varieties showing profiles like those grouped in **Figure 3**. A total of 91 different samples were employed and 11 discriminating functions with P values of < 0.05 were statistically significant at the 95% confidence level. Of the samples, 98.9% were correctly classified, observing a 94.5% prediction capability (using cross-validation). Peaks D, I, and M presented the highest discrimination capability with a 75.8% correct classification with only these three peaks. **Figure 5** shows the discrimination among varieties observed using the three main discriminating functions accounting for 33.58, 32.26, and 10.91% of discrimination, respectively. Most varieties were correctly classified using only discriminating functions 1 and 2, although discriminant function 3 was essential for the differentiation in some cases (e.g., Arbequina, Bouteillan, and Cordovil). Therefore, the optimized methodology yielded differentiable profiles that could be attributed only to the different genetic map of every variety because the rest of the conditions, both agronomics and analytical, were identical. Consequently, the developed method was found to be useful for the rapid throughput and low-cost analysis of olive identity.

In conclusion, separation of proteins in complex matrices requires new chromatographic phases because the ones usually employed for this purpose are not able to resolve them or do it within a short analysis time. There is no example on the use of UPLC columns for the analysis of intact proteins in complex samples. A chromatographic methodology using a sub-2 μm particle stationary phase is proposed, for the first time, for the analysis of intact proteins in a complex sample: the olive stone. There is an inherent difficulty in the extraction of proteins from lipid matrices, which together with the absence of any methodology for the extraction of proteins from the whole olive stone increases the value of this proposal. A buffered extraction assisted with an ultrasonic microprobe followed by centrifugation and protein precipitation enabled the extraction of proteins from the olive stone in a total time of 3 h. The employment of hydrolytic enzymes (proteases, lipases, or hydrolases) did not improve the results obtained with the buffered extraction. The protein nature of the obtained extracts was demonstrated. UPLC columns were the only ones providing suitable protein profiling of the olive stone with reasonable analysis time (15 min). These protein profiles enabled differentiation among olive varieties by the direct observation of chromatograms, in some cases, or by using discriminant analysis. The proteins present in the olive stone could be considered suitable for cultivar fingerprinting of olives, yielding a very high prediction capability, close to 100%.

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