DOI:10.1021/jf1026313

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Separation of Olive Proteins Combining a Simple Extraction Method and a Selective Capillary Electrophoresis (CE) Approach: Application to Raw and Table Olive Samples

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A simple extraction method was developed to extract proteins from olive samples based on chloroform/methanol extraction followed by a protein precipitation with cold acetone. Then, a capillary electrophoresis (CE) method was carried out using an acid buffer (1 M formic acid at pH 2) to ensure a positive net charge for proteins and a neutral charge for potential interferents as polyphenols. The method developed was applied to raw and table olive samples. Interestingly, raw olive samples showed differences in protein profiles depending upon the botanical variety of olives and their geographical region. Protein profiles obtained for table olives also showed differences according to the sample treatment. Thus, a signal reduction in the electropherograms obtained for black olives was observed in comparison to those achieved for treated green olives. In this work, the use of protein profiles was demonstrated to be a powerful tool for studying variations among olive samples.

KEYWORDS: CE; protein profile; table olive; raw olive

INTRODUCTION

Olive (*Olea europaea*) trees are among the oldest known cultivated trees in the world and are an economically important crop species mostly for the high quality of their oil that is accumulated in both mesocarp and seed tissues and for having an extreme longevity adapted to Mediterranean climates (*1*, *2*). Apart from the oil content (~22%), olive fruits are composed of water (50%), carbohydrates (19.1%), cellulose (5.8%), proteins (1.6%), and minerals (ash, 1.5%) (*3*). Olive stone is a lignocellulosic material, with hemicellulose, cellulose, and lignin being the main components of olive stone, although protein, fat, phenols, and free sugars are also presented in considerable quantities (*4*). Seeds accumulate substantial storage compounds as food reserves, principally proteins, lipids (often triacylglycerols), and carbohydrates, with these seed storage proteins being low-molecular-mass amphiphatic proteins (15−26 kDa) (*5*). The proteins in the olive mesocarp are not very well-known, although some proteins that are present in the oil bodies of the mesocarp are also passed along to the oil during olive oil extraction, contributing to some of the special characteristics of olive oils (*3*).

Spanish table olives are highly appreciated, having three types of olives commercially available according to the degree of ripeness: green olives, olives turning color, and black olives (*6*). Their industrial processing has the objective to remove the natural bitterness of this fruit caused by the glucoside oleuropein (*7*). The International Olive Oil Council (IOOC) (*6*) collects the most common preparations for table olives: natural olives placed directly in brine ("natural black olives" or "Greek style"), treated olives ("Spanish style" or "Sevillian style") that have undergone alkaline treatment and then packed in brine, and olives darkened by oxidation ("ripe olives" or "black olives"). Black olives are oxidized during processing, and a sterilization treatment is necessary for their preservation (*8*). Therefore, a possible effect in the olive characteristics and composition, specifically, in protein composition, is expected. This effect of processing on contents of selected nutrients of treated green table olives was studied by Montaño et al. (*9*), showing no significant differences in tocopherol and individual amino acid contents, except from lysine amino acid. However, Ongen et al. (*10*) found that the protein contents, calculated taking into account the total nitrogen content determined by Kjeldahl method, varied with the temperature on the drying process used in green olives. According to this fact, Casado et al. (*8*) concluded that technological factors, such as sterilization time and olive pH, had a significant influence on the total amount of amino acids in ripe olives, determined by high-performance liquid chromatography (HPLC). In addition, Lopez et al. (*11*) found that the protein content was significantly lower in black olives than in treated green olives or natural olives. This difference in protein content of different table olives was also found by Ünal et al. (*12*) using Kjeldahl analysis. They proposed that the protein content of two types of table olives was different because of the losses during treatment with sodium hydroxide and washing with water. They also indicated that some of the olive pulp proteins could be diffused into the brine.

In comparison to other samples, olive samples and plants in general are more problematic for protein extraction because plant tissues are rich in proteases and interfering compounds. Pigments, such as chlorophyll, phytochemicals, and lipid-based components, can also cause severe disturbances in protein extraction (*13*).

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 Published on Web 11/01/2010

Table 1. Table Olive Samples Studied in This Work

genetic variety	code	industrial processing	commercial presentation	size grade[a]	labeled protein content (g/100 g of olives)
Manzanilla	MAZ1	treated olives; pasteurization	green olives in brine		
	MAZ2	treated olives	green olives in brine	240/260	1.1
	MAZ3	treated olives	green olives in brine	70/80	
	MAZ4	treated olives; pasteurization	green olives in brine		
	MAZ5	treated olives	green olives in brine	240/260	1.4
Gordal	GOR1	treated olives; pasteurization	green olives in brine		
	GOR2	treated olives	green olives in brine	70/80	
	GOR3	treated olives	green olives in brine	100/110	1.3
	GOR4	treated olives	green olives in brine	100/110	1.12
	GOR5	treated olives	green olives in brine	90/160	1.3
Cacereña	CAC1	darkened by oxidation; sterilization (ferrous gluconate)	black olives	240/300	0.5
	CAC2	darkened by oxidation	black olives		
	CAC3	darkened by oxidation; sterilization	black olives		1.43
	CAC4	darkened by oxidation	black olives		1.2
	CAC5	darkened by oxidation; sterilization; stabilization	black olives		

[a] Size grade according to the number of olives per kilogram.

Because of these problems, there are many extraction procedures reported for protein extraction in plants, which included several washing and precipitation steps. In this context, chloroform/methanol is a solvent widely used for lipid solubilization and allows for membrane protein extraction (*14*). Chloroform is known as a protein denaturant, and according to Wang et al. (*14*), in the mixture of protein and chloroform/methanol (2:1), the forces between subunits of lipid−protein complexes are weakened. For this reason, the separation of proteins from lipids during the washing and precipitation steps is favored (*14*). However, the main problem is that the chloroform/methanol extracts can contain protein, triglycerides, sphingolipids, glycolipids, and phospholipids (*15*). To increase the separation selectivity to separate proteins from other interfering compounds, capillary electrophoresis (CE) is a high-performance separation technique very valuable for proteins. In fact, in comparison to conventional gel electrophoresis, which allows for protein separation according to molecular mass, CE is much more versatile, because very diverse separation modes based on different separation principles may be investigated, and shows higher resolution and automatization than gel electrophoresis. As a consequence, the aim of the present work was to develop an efficient extraction method combined with a selective CE approach for the separation of proteins contained in olive samples. Then, the usefulness of the protein profiles as a tool for the differentiation of olives (raw and table olive samples) was also studied because discrimination among cultivar or geographical origin may be useful to control the quality of these samples, also showing the possible influence of the different treatments of table olives in the protein content.

MATERIALS AND METHODS

Reagents and Materials. HPLC-grade acetonitrile (ACN), 2-mercaptoethanol, and boric acid were obtained from Scharlau Chemie (Barcelona, Spain). Hydrochloric acid, sodium hydroxide pellets, tris-(hydroxymethyl)aminomethane, sodium dodecyl sulfate (SDS), formic acid, methanol, chloroform, and acetone were obtained from Merck (Darmstadt, Germany). 2-(*N*-Cyclohexylamino)ethanesulfonic acid (CHES) was obtained from Sigma (St. Louis, MO). All solutions were prepared with ultrapure water from a Milli-Q system. UltraTrol LN dynamic precoating was purchased from Target Discovery (Palo Alto, CA). Regenerated cellulose (RC) filters were obtained from Millipore (Millipore, Bedford, MA). The extract was evaporated using a centrifugal concentrator CentriVap (Labconco, Barcelona, Spain). A pH meter (Metrohm, Herisau, Switzerland) was used to adjust the pH. ProteomeLab SDS-MW analysis kit was purchased from Beckman (Beckman Coulter, Inc., Fullerton, CA). The preliminary study was carried out on olives of three different botanical origins (Arbequina, Hojiblanca, and Picual), growing in different geographical locations (Toledo and Jaén) and harvested on December 2009. Table olive samples were purchased in local markets (Madrid, Spain). In **Table 1**, the genetic variety together with the codes used in this work, the industrial processing, the commercial presentation, the size grade, and the labeled protein content of the table olives studied in this work are shown.

Apparatus. After protein extraction, protein separation was achieved using a $HP^{3D}CE$ instrument (Agilent Technologies, Palo Alto, CA) equipped with an on-column diode array detector (DAD) for ultraviolet (UV) detection and spectra collection. The experiments were performed in fused-silica capillaries (Polymicro Technologies, Phoenix, AZ), with an internal diameter of 75 μm and effective length of 50 cm. Formic acid (1 M, pH 2) was used as separation buffer. The capillary conditioning between sample injections was performed with Milli-Q water (1 bar) for 2 min, 0.1 M HCl (1 bar) for 2 min, Milli-Q water (1 bar) for 2 min, and the separation buffer (1 bar) for 4 min. The selected CE conditions were as follows: capillary temperature, 15 °C (from the 15, 25, and 35 °C tested); applied voltage, −20 kV; and UV detection, 254 nm, with a bandwidth of 5 nm in all cases. Protein peaks were assigned according to the typical protein UV spectra containing three different absorption maxima at 210 nm for peptide bonds, at 254 nm for phenylalanine residues, and at 280 nm for tyrosine and tryptophan residues. The sample injection was performed by pressure, 50 mbar for 15 s (~4% of the total capillary length). For capillary gel electrophoresis (CGE), the conditions used were as follows: a sieving matrix provided by Beckman Coulter (Beckman Coulter, Inc., Fullerton, CA); effective length, 23 cm (total length, 31.5 cm); internal diameter, 75 μm; capillary temperature, 25 °C; electrokinetic injection, −20 kV for 5 s; voltage, −15 kV; and UV detection, 210 nm, with a bandwidth of 5 nm.

Sample Preparation. Frozen olives were defreezed and destoned, with the stone and pulp being homogenized separately in a domestic miller (Kenwood Ibérica, Barcelona, Spain). A mixture of the homogenized stone and pulp of 10 olives was made. A total of 20 mL of chloroform/methanol (2:1, v/v) were added to 2 g of the mixture and vortexed vigorously for 1 min. Then, a centrifugation (Heraeus Instrument, Hanau, Germany) at 1500*g* for 15 min was performed twice. After centrifugation, two strategies were performed to isolate the proteins: filtering through a membrane filter or protein precipitation with acetone. Filtering through a membrane filter was made using Whatman filters (grade 1) to retain the proteins, washing the filter paper with 10 mL of chloroform/methanol (2:1, v/v), and shaking in an ultrasonic bath (3 min) for protein recuperation. On the contrary, the protein precipitation strategy was performed precipitating proteins in the supernatant with 2 volumes of cold acetone at −20 °C for 1 h. To separate precipitated proteins, the mixture was centrifuged (Multifuge 3 LR Heraeus, Buckinghamshire, U.K.) at 10000*g* for 5 min. Finally, the proteins were redissolved in 0.5 mL of formic acid at pH 2 with 20% (v/v) ACN and filtered through 0.45 μm Titan filters (Rockwood, TN) prior to injection on the CE system.

Statistical Analysis. The electropherograms were prepared using the computer program Origin version 7.0 software.

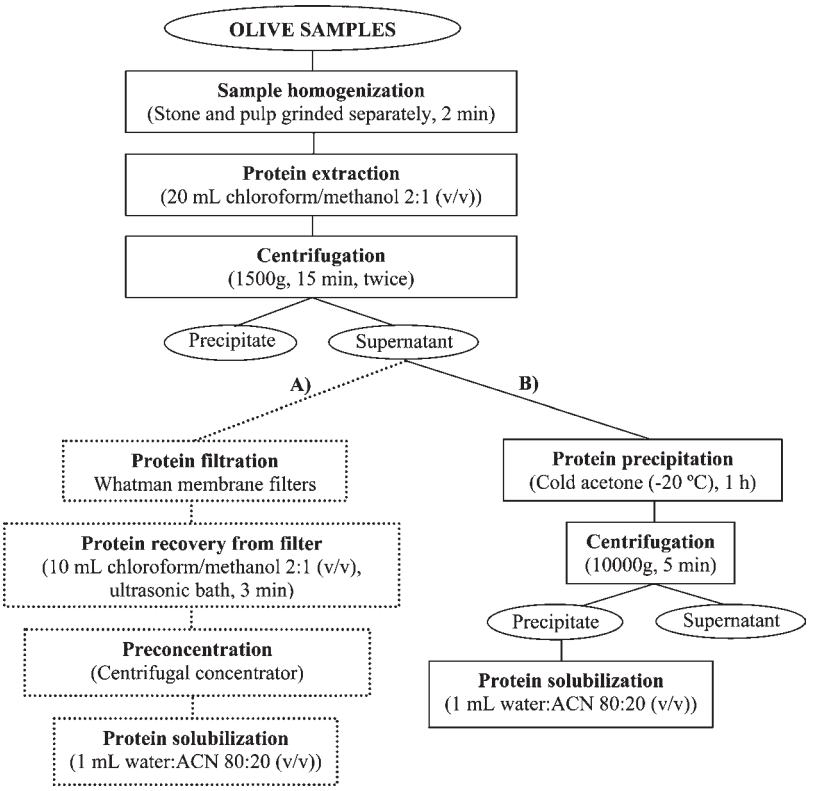

Figure 1. Schematic procedure developed for protein extraction from olive samples following the two strategies studied in this work for protein isolation: (**A**) protein filtration through regenerated cellulose filters or (**B**) protein precipitation with cold acetone.

RESULTS AND DISCUSSION

Protein Extraction in Olive Samples. All of the experiments for the optimization of the extraction method were performed using the CE conditions previously applied to obtain protein profiles for olive oil samples (*16*). First of all, different extraction mixtures were tested using 20 mL of solvent per 2 g of a homogenized olive sample from the Picual variety. To a great extent, the yield of total proteins extracted from a plant tissue depends upon the fineness of the tissue powder (*17*). For this reason, the homogenization was made in different phases to ensure a fine powder: fruits were manually depulped and the stones were ground first to obtain a fine powder. Subsequently, pulp was added and also ground. To this mixture, a 125 mM Tris-HCl (pH 6.8) containing 0.2% (m/v) SDS and 1% (v/v) 2-mecaptoethanol buffer (*18*) was added to extract the proteins of olive samples. In this case, the fat layer and the oil bodies remained at the top, making protein isolation difficult. These results were attributed to the fact that, although detergents are used to promote membrane protein solubility (*13*), they are not suitable for systems, such as seeds or oil bodies, that contain a high lipid/protein ratio (*19*). Then, another extraction mixture was tested on the basis of a chloroform/methanol mixture (*14*, *20*) for the extraction of proteins contained in the olive samples. This mixture is typically used to dissolve lipids and extract the membrane proteins in oil bodies (*14*). However, the solvent mixture was not only selective for proteins (*15*), and two other strategies were investigated for protein isolation: the use of membrane filters or a step of protein precipitation (see **Figure 1**). On one hand, membrane filters were tested to separate precipitated proteins (*21*) according to their size. The method consisted of adding 20 mL of chloroform/methanol (2:1, v/v) to 2 g of the homogenized olive samples. This mixture was vigorously vortexed for 2 min. Then, centrifugation at 1500*g* for 15 min twice was performed to remove the pellets. After centrifugation, denaturalized proteins were filtered through the filter. To desorb

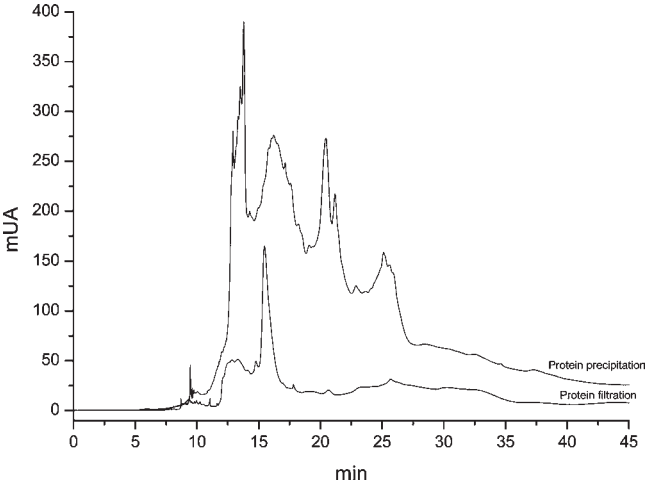

Figure 2. Comparison of the electropherograms obtained for a Picual olive sample under the two different strategies for protein isolation studied in this work: protein filtration or protein precipitation. CE conditions: buffer solution, 80 mM borate buffer with 20% (v/v) ACN (apparent pH 8.5); effective length, 50 cm; internal diameter, 75 μm; capillary temperature, 15 °C; pressure injection, 50 mbar for 100 s; voltage, −20 kV; UV detection, 254 nm with a bandwidth of 5 nm, dynamic precoating with UltraTrol LN.

retained proteins from the filter, 10 mL of the same extraction buffer was added to the filter paper and shaken in an ultrasonic bath for 3 min. Finally, the extract obtained was evaporated and redissolved in 1 mL of water/ACN (80:20, v/v) (see **Figure 1A**). On the other hand, an extract obtained in the same way was precipitated with 2 volumes of cold acetone at −20 °C for 1 h. To separate precipitated proteins, the mixture was ultracentrifuged at 10000*g* for 5 min and redissolved in 1 mL of water/ACN (80:20, v/v) (see **Figure 1B**). A comparison of the electropherograms

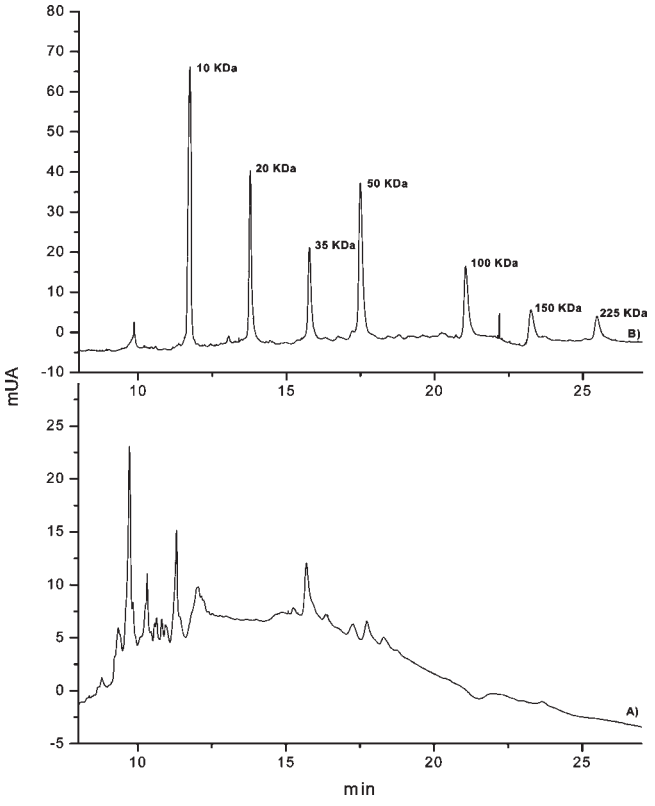

Figure 3. Protein profiles obtained by SDS CGE analysis for (**A**) SDS-MW size standards of different molecular weight between 10 and 225 kDa and (**B**) Cacereña table olive sample (CAC2). CE conditions were the same as provided by the manufacturer (Beckman Coulter, Inc., Fullerton, CA), with the exception of the effective length (23 cm) and the UV detection of 210 with a bandwidth of 2.5 nm.

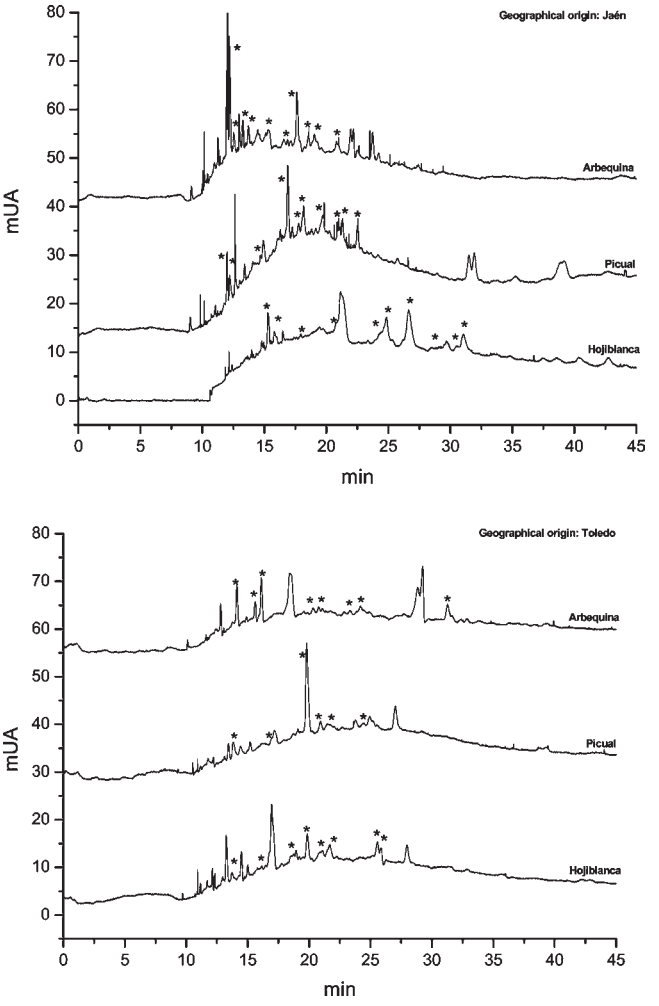

Figure 4. Protein profile obtained for Arbequina, Picual, and Hojiblanca olive samples from two different geographical origins (Toledo and Jaén) extracted following a sequential chloroform/methanol extraction and protein precipitation. The peaks assigned to protein are labeled with an asterisk. CE conditions: buffer solution, 1 M formic acid buffer (pH 2); effective length, 50 cm; internal diameter, 75 μm; capillary temperature, 15 °C; pressure injection, 50 mbar for 15 s; voltage, 20 kV; UV detection, 254 nm with a bandwidth of 5 nm.

obtained for a Picual olive sample by these two strategies (**Figure 2**) showed the highest number and size of peaks for the method including a protein precipitation. In addition, this precipitation step is normally used for protein isolation (*22*, *23*). Shen et al. (*24*) affirmed that acetone, as an organic solvent, could destroy the combination of enzymes and plant polyphenols, leaving polyphenols dissolved in the solution. However, the elimination of polyphenols could not be complete. For this reason, to confirm the presence of proteins in these olive samples obtained by a sequential chloroform/methanol extraction and acetone precipitation, a CGE separation was performed. It was achieved using the gel from the ProteomeLab SDS-MW analysis kit made for the separation of protein−SDS complexes. This kit provides an effective sieving range from 10 to 225 kDa. In this range, compounds with low molar masses, such as polyphenols, are not separated. **Figure 3** shows the protein profile obtained applying this ProteomeLab SDS-MW analysis kit to a table olive sample of Cacereña variety, CAC2 (see **Table 1**). As can be seen in this **3**figure, several peaks were obtained in the sieving range between 10 and 30 min, as compared to the SDS-MW size standards. The results obtained with this kit allowed for the demonstration of the existence of peaks with molecular weight above 10 kDa, which could be assigned to proteins.

Olive Protein Separation by CE. The extraction method was optimized using a CE separation method previously used to obtain protein profiles of olive oils (*16*). It consisted of capillary precoating with UltraTrol LN to avoid the absorption of proteins to the wall of the capillary and 80 mM borate (pH 8.5) with 20% (v/v) ACN as a separation buffer. The other CE conditions were as follows: capillary temperature, 15 °C; applied voltage, −20 kV; and UV detection, 254 nm with a bandwidth of 5 nm in all cases. However, the high price of this dynamic precoating and the low reproducibility of migration times at the basic conditions used lead us to study another condition. A 100 mM CHES−Tris buffer (pH 8.6) with 0.1% (m/v) SDS was tested. The resulting electropherogram showed a great reduction in signal intensity. For this reason, a new CE strategy using an acid buffer was used. It consisted of 1 M formic acid at pH 2 to achieve a neutral inner capillary wall and, therefore, avoid adsorption of proteins. These separation conditions were selected to ensure a positive net charge for proteins and a neutral charge for potential interferents, such as the above-mentioned polyphenols. The other CE conditions remained with the exception of the applied voltage, which was applied at normal polarity (20 kV). Because of the acidic nature of the separation buffer, different solubilization media for the extracted proteins were studied: 1 M formic acid (pH 2) and 20% (v/v) ACN, water and 20% (v/v) ACN, and 100 mM Tris-HCl (pH 9) containing 1% (m/v) SDS. The most acidic mixture [1 M formic acid at pH 2 with 20% (v/v) ACN] was selected because it allowed us to ensure the net positive charge of all olive proteins before their introduction in the separation capillary. To

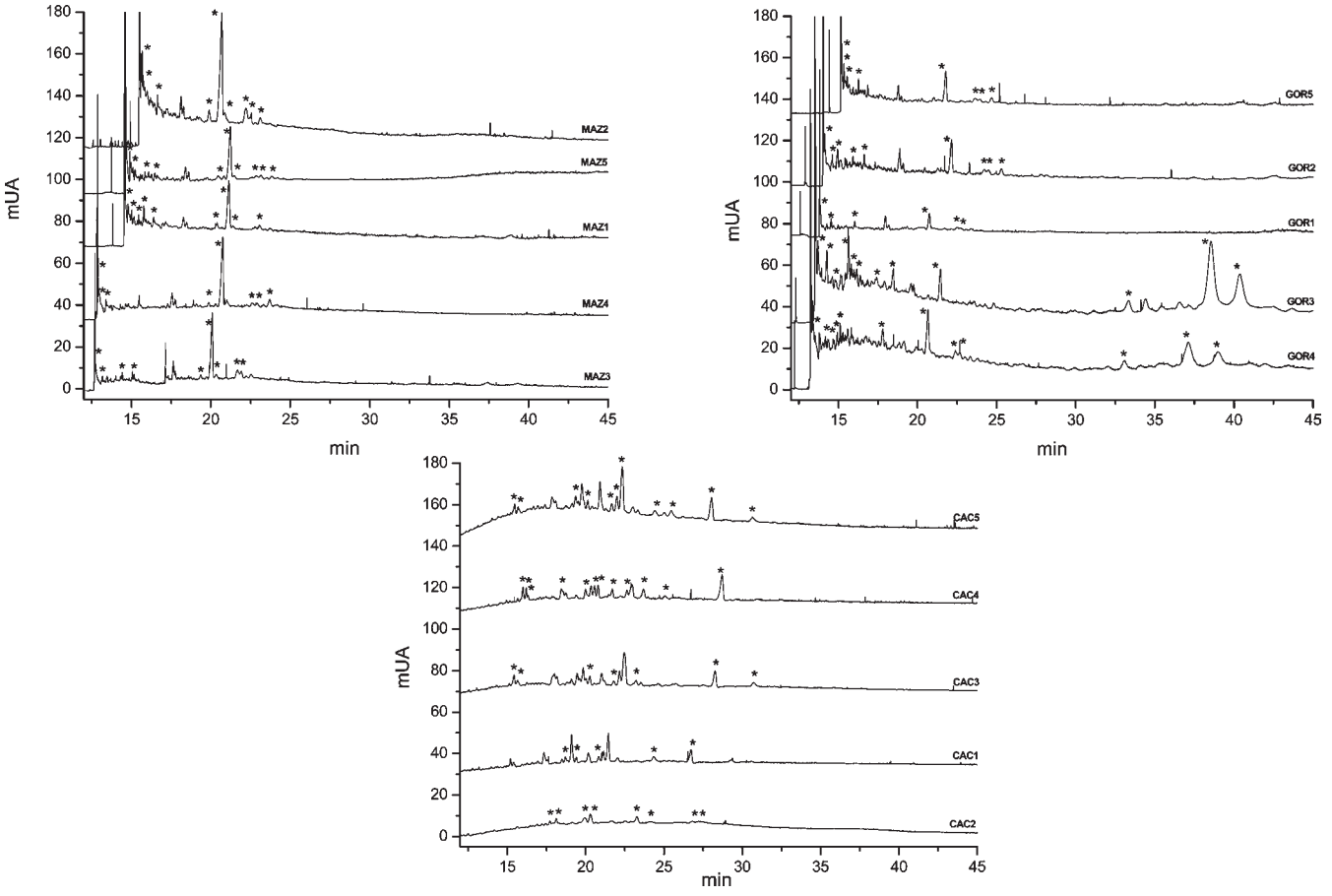

Figure 5. Protein profiles obtained for the different table olive samples analyzed (see further information in **Table 1**) from 10 g of olive samples extracted following a sequential chloroform/methanol extraction and protein precipitation. The peaks assigned to protein are labeled with an asterisk. CE separation conditions are the same as in **Figure 4**.

preconcentrate proteins, the sample was finally redissolved in 0.5 mL instead of 1 mL of this separation medium.

Application of the Developed Method to the Discrimination of Olive Samples. The separation method developed was applied to raw and table olive samples.

Raw olives were harvested at the same time but in different Spanish geographical locations. Arbequina, Picual, and Hojiblanca olives were picked on December 2009 from Toledo and Jaén. **Figure 4** shows the protein profiles obtained for these raw olive samples. As this figure shows, the protein profiles vary according to the olive botanical origin. However, this figure also showed that the geographical origin could influence the protein profile (*25*). Although this preliminary result should be studied deeply, supporting more than two samples of different geographical origin, it is interesting to observe the potential of proteins to differentiate based on the geographical origin of olive samples because it is already known that the genetic material is less influenced by environmental conditions, such as the geographical origin, than other macromolecules and metabolites of olive fruits (*25*).

Three varieties of Spanish table olives belonging to two different types of olives (green and tuning color olives) were also analyzed. As shown in **Table 1**, the olives were treated with the two industrial processing treatments most widely used in Spain. Manzanilla and Gordal olives were alkaline-treated olives, packed in brine and presented as green olives. Cacereña olives are darkened by oxidation, producing a low acid product, preserved in a sterilized container, and presented as black olives. Therefore, as other authors have shown determining the amino acid content (*8*, *9*, *11*) or the total nitrogen content (*10*, *12*), an effect on protein exists as a function of the olive processing. In our study, protein profiles were used to investigate differences according to these two olive treatments. It is important to consider that, with drastic pH values for olive treatments, the total amount of amino acid increases, which could be due to an increase of the hydrolysis of protein (*8*). As a consequence, increasing amounts of sample (2, 4, 8, 10, and 20 g) were studied because of the poor signal obtained with 2 g of olive sample. The selection of 10 g of sample provided the highest number of peaks without compromising peak resolution. When the different olive samples analyzed were compared (**Figure 5**), differences among the protein profiles of table olives were observed. For the 15 table olive samples studied, 2 independent samples (injected by duplicate) were analyzed. The protein profiles of green treated olives are more similar in comparison to black olives. However, there is a peak at about minute 20 that is the highest in all olive samples from Manzanilla compared to Gordal variety but that is not so notable in Cacereña olive samples. A clear signal reduction in black olives was also observed. This observation is in agreement with López et al. (*11*), who affirmed that the numerous treatments and water washes in the processing of black olives could result in a greater loss of nitrogenous compounds when compared to Spanish-style (treated) olives, with the calculated protein content in treated olives being 1.2% (m/m) and the calculated protein content in black olives being 0.9% (m/m).

In this work, a simple and efficient method to extract protein from olive samples consisting of a sequential chloroform/methanol extraction and protein precipitation with cold acetone was developed. A CE strategy using an acid buffer (1 M formic acid at pH 2)

to ensure a positive net charge for proteins and a neutral charge for polyphenols enabled the selective separation of proteins from raw and table olive samples, avoiding potential interferents as polyphenols. Results obtained showed the interesting use of proteins for discrimination among olive samples according to the protein profile obtained by CE. This is the first time that proteins from olives are separated by CE and that protein profiles are investigated for the differentiation of raw and table olive samples.

LITERATURE CITED

(1) Giannoulia, K.; Banilas, G.; Hatzopoulos, P. Oleosin gene expression in olive. *J. Plant Physiol.* **2007**, *164*, 104−107.

(2) Muzzalupo, I.; Stefanizzi, F.; Salimonti, A.; Falabella, R.; Perri, E. Microsatellite markers for identification of olive accessions. *Sci. Agric.* **2009**, *66*, 685−690.

(3) Zamora, R.; Alaiz, M.; Hidalgo, F. J. Influence of cultivar and fruit ripening on olive (*Olea europaea*) fruit protein content, composition, and antioxidant activity. *J. Agric. Food Chem.* **2001**, *49*, 4267−4270.

(4) Rodríguez, G.; Lama, A.; Rodríguez, R.; Jiménez, A.; Guillén, R.; Fernández-Bolaños, J. Olive stone an attractive source of bioactive and valuable compounds. *Bioresour. Technol.* **2008**, *99*, 5261−5269.

(5) Huang, A. H. C. Oil bodies and oleosins in seeds. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1992**, *43*, 177−200.

(6) International Olive Oil Council (IOOC). *Trade Standard Applying to Table Olives*; IOOC: Madrid, Spain, 2004; COI/OT/NC 1.

(7) Sánchez, A. H.; García, P.; Rejano, L. Trends in table olive production. *Grasas Aceites* (*Sevilla, Spain*) **2006**, *57*, 86−94.

(8) Casado, F. J.; Sánchez, A. H.; Rejano, L.; Montaño, A. D-Amino acid formation in sterilized alkali-treated olives. *J. Agric. Food Chem.* **2007**, *55*, 3503−3507.

(9) Montaño, A.; Casado, F. J.; de Castro, A.; Sánchez, A. H.; Rejano, L. Influence of processing, storage time, and pasteurisation upon the tocopherol and amino acid contents of treated green table olives. *Eur. Food Res. Technol.* **2005**, *220*, 255−260.

(10) Ongen, G.; Sargin, S.; Tetik, D.; Kose, T. Hot air drying of green table olives. *Food Technol. Biotechnol.* **2005**, *43*, 181−187.

(11) López, A.; Garrido, A.; Montaño, A. Proteins and amino acids in table olives: Relationship to processing and commercial presentation. *Ital. J. Food Sci.* **2007**, *19*, 221−233.

(12) Ünal, K.; Nergi, C. The effect of table olive preparing methods and storage on the composition and nutritive value of olives. *Grasas Aceites* (*Sevilla, Spain*) **2003**, *54*, 71−76.

(13) Mitra, S. K.; Walters, B. T.; Clouse, S. D.; Goshe, M. B. An efficient organic solvent based extraction method for the proteomic analysis of *Arabidopsis* plasma membranes. *J. Proteome Res.* **2009**, *8*, 2752−2767.

(14) Wang, W.; Vignani, R.; Scali, M.; Sensi, E.; Tiberi, P.; Cresti, M. Removal of lipid contaminants by organic solvents from oilseed protein extract prior to electrophoresis. *Anal. Biochem.* **2004**, *329*, 139−141.

(15) Thakarea, K.; Shi, W.; Barbeau, W. E.; Bassaganya-Riera, J.; Hontecillas, R.; Scott, F. Investigation of chloroform−methanol soluble wheat proteins and sphingolipids as potential dietary triggers of diabetes in BBdp. *Food Agric. Immunol.* **2008**, *19*, 25−48.

(16) Montealegre, C.; Marina, M. L.; García-Ruiz, C. Separation of proteins from olive oil by CE: An approximation to the differentiation of monovarietal olive oils. *Electrophoresis* **2010**, *31*, 1−8.

(17) Wang, W.; Scali, M.; Vignani, R.; Spadafora, A.; Sensil, E.; Mazzuca, S.; Cresti, M. Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. *Electrophoresis* **2003**, *24*, 2369−2375.

(18) Wang, W.; de-Dios-Alché, J.; Castro, A. J.; Rodríguez-García, M. I. Characterization of seed storage proteins and their synthesis during seed development in olea europaea. *Int. J. Dev. Biol.* **2001**, *45*, 63−64.

(19) D'Andréa, S.; Jolivet, P.; Boulard, C.; Larré, C.; Froissard, M.; Chardot, T. Selective one-step extraction of *Arabidopsis thaliana* seed oleosins using organic solvents. *J. Agric. Food Chem.* **2007**, *55*, 10008−10015.

(20) Vertommen, A.; Panis, B.; Swennen, R.; Carpentier, S. C. Evaluation of chloroform/methanol extraction to facilitate the study of membrane proteins of non-model plants. *Planta* **2010**, *231*, 1113−1125.

(21) Paschke, A.; Zunker, K.; Wigotzki, M.; Steinhart, H. Determination of the IgE-binding activity of soy lecithin and refined and non-refined soybean oils. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2001**, *756*, 249−254.

(22) Simpson, D. M.; Beynon, R. J. Acetone precipitation of proteins and the modification of peptides. *J. Proteome Res.* **2010**, *9*, 444−450.

(23) Jiang, L.; He, L.; Fountoulakis, M. Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. *J. Chromatogr., A* **2004**, *1023*, 317−320.

(24) Shen, W.; Jin, Z.; Xu, X.; Zhao, J.; Deng, L.; Chen, H.; Yuan, C.; Li, D.; Li, X. New source of α-D-galactosidase: Germinating coffee beans. *Food Chem.* **2008**, *110*, 962−966.

(25) Montealegre, C.; Marina Alegre, M. L.; García-Ruiz, C. Traceability markers to the botanical origin in olive oils. *J. Agric. Food Chem.* **2010**, *58*, 28−38.

Received for review July 8, 2010. Revised manuscript received October 13, 2010. Accepted October 13, 2010. We thank the Ministry of Science and Innovation (Spain) and the Comunidad Autónoma of Madrid (Spain) for financial support (projects CTQ2009-11252 and S-2009/AGR-1464, respectively). Cristina Montealegre thanks the University of Alcalá for her predoctoral grant.