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Separation of olive proteins by capillary gel electrophoresis

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ABSTRACT

Olive proteins are not well known and there are still a lot of unknown information requiring further studies focused on the determination and characterization of these proteins. Despite the widely use of gel electrophoresis, this is the first time that capillary gel electrophoresis (CGE) is applied to separate proteins extracted from olive fruits. Seven common peaks were identified in the twenty olive varieties studied in this work. According to their migration times, these seven peaks could correspond to molecules with molecular masses of 11.0 ± 0.4, 13.9 ± 0.5, 16.3 ± 0.8, 22.1 ± 0.6, 30 ± 1, 48 ± 1, and 53 ± 2 kDa. All of the determined molecular masses could be attributed to proteins and four of them have been previously observed by SDS-PAGE. The electrophoretic profiles were also evaluated for their capability to differentiate olive varieties according to their presumed geographical origin. Results demonstrated that this method could successfully classify the studied olive varieties by its combination with multivariate chemometrics tools.

1. Introduction

Olives are composed of water (50%), oil (22%), carbohydrates (19.1%), cellulose (5.8%), proteins (1.6%), and minerals (ash) (1.5%) [1]. As it is known so far, olive proteins are distributed between the olive mesocarp and the olive stone (constituted by the seed and the endocarp), being mainly present in the olive stone [2,3]. Olive proteins present in the olive stone, most of them contained in the olive seed, belong to two main families: seed storage proteins (SSPs) and oleosins. Storage proteins are formed during seed development [4]. According to Alché [4], the most abundant storage proteins in the mature olive seed belong to the 11 S protein family, accounting for approximately 70% of the total seed proteins. A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that storage proteins in olive seed mainly consisted of two hydrogen-bonded subunits of 41 and 47.5 kDa [4–6]. Moreover, the precursor of 41 kDa generated three polypeptides of 21.5, 25.5, and 27.5 kDa when reduced, while the other precursor yielded two polypeptides of 20.5 and 30.0 kDa [4]. In addition to these storage proteins, proteins associated with fatty bodies called oleosins are hydrophobic proteins from oleaginous seeds stabilizing oil bodies. The oil bodies are composed of a core of triacylglycerols surrounded by a monolayer of phospholipids in which different proteins are inserted [7]. Oleosins are insoluble in water, but could be easily dissolved using a surfactant such as SDS, according to Huang et al. [8]. Ross et al. [9] found two different oleosins of 22 and 50 kDa in olive seeds. The 22 kDa protein was assigned to an oleosin located on the surface of the oil bodies. These authors [9] also analyzed the oil bodies from the olive mesocarp tissue not detecting any oleosin. However, the mesocarp contains proteins (1.3–1.8% of the dry weight of the olive fruit) that are still not well-known [1]. Indeed, Hidalgo et al. [10] reported that some proteins present in the oil bodies of the olive fruit mesocarp could pass to the oil during their extraction. Moreover, Zamora [1] found a polypeptide of 4.6 kDa by SDS-PAGE analysis regardless of the variety of the olive fruit or the maturity stage.

Up to date, SDS-PAGE is the most used technique to separate olive proteins and to determine their molecular masses [1,4,5,9–13]. Further refinement of the technique has been recently observed by the off-line analysis of the SDS-PAGE bands using MALDI-TOF MS and/or nanoLC-MS/MS [14,15]. Capillary electrophoresis (CE) presents a high potential for the separation, detection, and determination of biomolecules such as proteins. In this context, capillary gel electrophoresis (CGE) is an electrophoretic mode which uses a sieving matrix as buffer for the separation of high molecular weights compounds [16]. CGE presents the advantages of automation, on-column detection, and higher resolution than the traditional SDS-PAGE. Thus, the aim of this work was to use CGE as an alternative to SDS-PAGE for

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the separation of olive proteins and to study the potential of obtained protein profiles to differentiate olive varieties according to their presumed geographical origin.

2. Experimental section

2.1. Reagents and Materials

HPLC grade acetonitrile (ACN) and 2-mercaptoethanol were obtained from Scharlau Chemie (Barcelona, Spain). Hydrochloric acid, sodium hydroxide pellets, tris(hydroxymethyl)aminomethane (Tris), SDS, methanol, chloroform, and acetone were obtained from Merck (Darmstadt, Germany). All solutions were prepared with ultrapure water from a Milli-Q system (Millipore, Bredford, MA). ProteomeLab^{TM} SDS-MW Analysis Kit was purchased from Beckman (Beckman Coulter, Inc., Fullerton, CA). Olive samples from twenty different varieties were supplied by the Olive World Germplasm Bank of IFAPA (Junta de Andalucía, Córdoba, Spain). To avoid possible pedoclimatic influences, all varieties were ground in the same geographical zone, under the same climatic factors. Moreover, fruit samples were harvested at the same time and at the same stage of maturity, being the own variety the only difference among them. Table 1 shows the varieties, their origin and geographical extension and different denominations found in Spain, according to Rallo et al. [17].

2.2. Apparatus

Electropherograms were obtained using a HP^{3D}CE instrument (Agilent Technologies, Palo Alto, CA) equipped with an on-column DAD for UV detection and spectra collection. The experiments were performed in fused-silica capillaries (Polymicro Technologies Phoenix, Arizona, USA) of 50 μm id and an effective length of 23 cm (31.5 cm of total length). A replaceable gel from ProteomeLab^{TM} SDS-MW Analysis Kit from Beckman (Beckman Coulter, Inc., Fullerton, CA) designed for the separation of protein-SDS complexes was used to fill the capillary. A capillary conditioning was performed every six injections consisting of a 0.1 M sodium hydroxide rinse for 15 min, an acidic rinse (4 min) with 0.1 M HCl followed by Milli-Q water for 4 min, and a gel rinse for 15 min. The CGE selected conditions were: capillary temperature 25 °C, applied voltage −20 kV, and UV detection at 210, 254, and 280 nm with a bandwidth of 5 nm in all cases. Sample injection was performed electrokinetically at −5 kV for 20 s.

2.3. Sample preparation

Olive samples were prepared according to a previous procedure developed in our research group [18]. Briefly, the stone and pulp were homogenized separately in a domestic miller (Kenwood Ibérica, Barcelona, Spain). 2 g of the stone and pulp mixture was added to 20 mL of chloroform/methanol (2:1, v/v) and it was vortexed vigorously. A centrifugation (Heraeus Instrument, Hanau, Germany) at 1500 *g* for 15 min was carried out twice. After centrifugation, the pellets were removed and the proteins in the liquid phase were precipitated with 40 mL of cold acetone at −20 °C for 1 h. Precipitated proteins were separated by centrifugation (Multifuge 3 LR Heraeus, Buckinghamshire, England) at 10,000 *g* for 5 min. Proteins were solubilized in 0.5 mL of 100 mM Tris–HCl buffer (pH 9.0) containing 1% (m/v) SDS and filtered through 0.45 μm Titan filters (Rockwood, Tennessee) prior to their injection in the CE system.

2.4. Molecular masses determination

In order to determine the molecular masses corresponding to the electrophoretic peaks, a calibration curve was obtained using seven molecular size standards from Beckman (Beckman Coulter, Inc., Fullerton, CA): 10, 20, 35, 50, 100, 150, and 225 kDa. Within this size range, the logarithm of the protein molecular mass is

Table 1
Olive varieties studied indicating their country of origin, the most common zones for their cultivation in Spain and different Spanish denominations found (Rallo et al., 2005; http://www.oliveoilsource.com/).

Category[a]	Variety origin	Most common zones for olive variety cultivation in Spain	Other Spanish denominations
Mean Varieties	*'Arbequina' ESP*	Cataluña, Aragón, and less common in Andalucía	*Blanca*
	'Empeltre' ESP	Aragón and Baleares principally, Castellón, Tarragona, and Navarra	*Aragonesa, Común, de Aceite, Fina, Injerto, Macho, Mallorquina, Navarro, Negral, Payesa, Salseña, Terra Alta,* and *Zaragona*
	'Lechín Sevilla' ESP	Sevilla, Córdoba, Cádiz, and Málaga	*Lechín, Ecijano, Zorzaleño, Alameño, Cordobés, Lechino,* and *Manzanilla Serrana*
	'Morrut' ESP	Castellón and Tarragona	*Morruda, Montserratina, Regués, Rocha,* and *Roig*
	'Villalonga' ESP	Province of Valencia and north zone of Alicante	*Forna, Manzanet, Manzanilla, Sevillano*, and *Valenciana*
Secondary Varieties	*'Lucio' ESP*	Granada	*Lucio Gordo* and *Plateado*
Spread Varieties	*'Alameño de Cabra' ESP*	Córdoba, Granada, and Sevilla	*Alameño (H)*[b]
	'Azul' ESP	Alhama de Granada (Granada) and Huelma (Jaén)	–
	'Negrillo de Estepa' ESP	Estepa (Sevilla) and Campiña-Penibética (Córdoba)	*Negrillo*
Local Varieties	*'Bolvino' ESP*	Belchite (Zaragoza)	–
	'Caballo' ESP	Huelma (Jaén)	–
	'Carrasquillo' ESP	Montefrío (Granada)	*Negrete*
	'Negro de el Carpio' ESP	El Carpio (Córdoba)	*Negro*
	'Nevado Basto' ESP	Baena and Cabra (Córdoba)	–
	'Nevado Rizado' ESP	Cabra (Córdoba)	–
	'Torcío de Cabra' ESP	Cabra (Córdoba)	*Torcío*
Other Varieties	*'Belluti' TUR*	Turkey	–
	'Samsun Tuzlamalik' TUR	Turkey	–
	'Rosciola' ITA	Italy	–
	'Barnea' ISR	Israel	–

[a] Category established according to the cultivar expansion.
[b] H (homonymia): different varieties with the same name.

linear with its reciprocal electrophoretic mobility. Size standards were injected under the same CGE conditions as olive samples. This meant that they should be denatured before CE injection such as olive proteins were after their extraction from the olive. For that purpose, 2 μL of standard solutions were treated with 5 μL of 2-mercaptoethanol and 85 μL of sample buffer, heated in a water bath at 100 °C for 3 min, and cooled to stop the reaction. Each size standard was injected by triplicate. Electrophoretic mobility for every peak was calculated from the migration times obtained for the standard proteins, using the equation:

$$\mu_e = \frac{l_{ef} \cdot l}{t \cdot V}$$

where μ_e is the electrophoretic mobility, l_{ef} the effective length, l the total length, t the migration time, and V the applied voltage. The logarithm of the standard protein molecular masses was plotted against the inverse of the electrophoretic mobility to obtain the calibration curve. The calibration curve obtained was as follow: log MW= −0,0791-7,397E-05 ($1/\mu_e$) (r=0.993), where *MW* is the molecular mass. The molecular masses of proteins from olive samples were determined by interpolation in the calibration plot. Data of migration times of olive protein peaks were obtained from two individual samples of the same olive variety subjected to the same procedure and injected by triplicate. Errors were calculated taking into account the standard deviation (n=6).

2.5. Statistical analysis

Peak areas were integrated by setting the baseline from valley to valley to calculate the migration time and the electrophoretic mobility. The electropherograms were treated using the computer program Origin® version 7.0 software. To apply multivariate methods, the Statgraphics Centurion XV program was used.

3. Results and discussion

3.1. Olive proteins separation by CGE

An extraction method previously developed by our research group [18] and consisting of a simple chloroform/methanol extraction followed by an acetone precipitation was used for the extraction of olive proteins. However, this extraction method was not very selective since acetone, as organic solvent, may destroy the combination of enzymes and plant polyphenols, leaving polyphenols dissolved in the solution [19]. For a selective separation of olive proteins, the use of CGE with a sieving gel discriminating among low and high-molecular mass compounds allowed the separation of low-molecular mass polyphenols from high-molecular mass proteins. Thus, a gel formulated to separate compounds in an effective sieving range of approximately 10 to 225 kDa was proposed in this work.

A CGE method was optimized using olive samples from the 'Lechín de Sevilla' variety. Three different sample injections were tested: −10 kV for 20 s and −5 kV for 20 or 100 s. The smaller injection (−5 kV for during 20 s) was the best in order to have a good peak resolution. The other electrophoretic conditions used were: capillary temperature 25 °C, applied voltage −15 kV, and UV detection at 210, 254, and 280 nm with a bandwidth of 5 nm in all cases. As Fig. 1 shows, the electropherogram obtained for the 'Lechín de Sevilla' variety consisted of several peaks separated in the range from 10 to 30 min. According to the selectivity of the CGE method and the typical protein UV spectra, showing absorption maxima at 210 nm, 254 nm, and 280 nm, seven peaks were assigned to potential proteins (see Fig. 1). In addition, only those peaks with a S/N ratio higher than 3 were considered for their molecular mass determination.

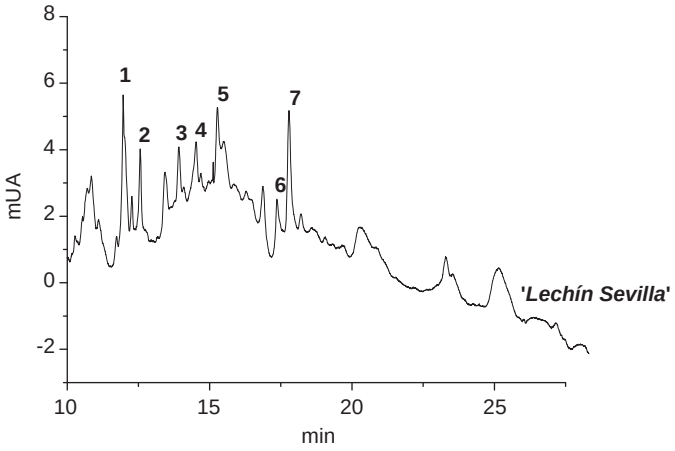


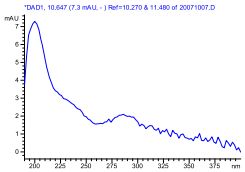
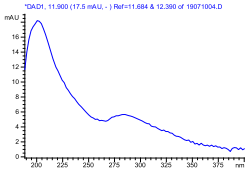
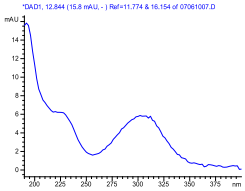
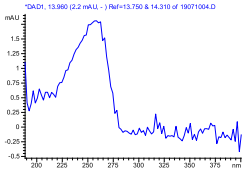
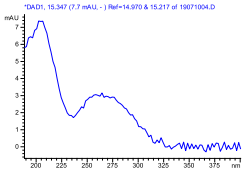
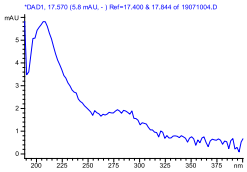
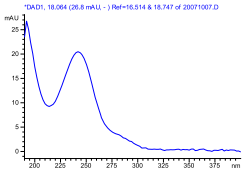
Fig. 1. Electropherogram corresponding to the olive variety *'Lechín de Sevilla'* subjected to a sequential chloroform/methanol extraction followed by a protein precipitation in cold acetone prior to its separation by CGE using the Beckman sieving gel (Beckman Coulter, Inc., Fullerton, CA). Other CGE conditions: 50 μm id capillary with an effective length of 23 cm; sample injection −5 kV for 20 s, capillary temperature 25 °C, applied voltage −20 kV, and UV detection at 254 nm with 5 nm bandwidth. The numbers correspond to the peaks assigned to proteins.

3.2. Determination of molecular masses for olive proteins by CGE

The twenty different varietal samples included in Table 1 were analyzed by the CGE method optimized to determine the molecular masses of the olive proteins. The analysis of the protein profiles obtained by CGE showed seven peaks that were observed in most of the olive varieties studied. Table 2 shows the migration times for these seven peaks, the UV spectra, the number of olive varieties that present the peak, the abundance of the peak considering the seven selected peaks, the molecular masses calculated using CGE data, and their tentative assignment to proteins already reported in literature.

Identification of some electrophoretic signals could be performed by comparison with the molecular masses previously reported when using SDS-PAGE. Nevertheless, it is important to highlight that the correspondence between SDS-PAGE and SDS-CGE is tentative due to the different migration behavior of proteins in both techniques. Just four signals with molecular masses of 22.1 ± 0.6, 30 ± 1, 48 ± 1, and 53 ± 2 kDa, could be assigned to proteins previously observed by SDS-PAGE. Indeed, the fourth peak observed by SDS-CGE analysis showed a molecular mass of 22.1 ± 0.6 kDa that could be related to a polypeptide found by Alché [4] and derived from the precursor of 41 kDa. This peak was present in 18 of the 20 analyzed varieties although its abundance was very low (only 4.4%). The peak with a molecular mass of 30 ± 1 kDa could be attributed to a polypeptide of 30.0 kDa [4,20]. The last two peaks had the worst resolution and could be assigned to the same protein, an oleosin of 50 kDa reported by Ross [9] and Esteve [20]. The rest of peaks were never observed using SDS-PAGE with traditional extraction methodologies. Only a recent work of the group using protein extraction with peptide ligand libraries [15] enabled the detection of more SDS-PAGE bands that could be correlated with the additional three signals observed in this work by SDS-CGE. Finally, the last peak appearing at 17.7 min presented the highest area (35.0%). Fig. 2 shows, as example, the electropherogram corresponding to the olive variety 'Alameño de Cabra', presenting all common peaks, compared with the size standards used to determine the different molecular masses.

Table 2
Information obtained for the seven common peaks found for the twenty olive varieties studied in this work.

COMMON PEAKS					
Time ($_{min}$)	**UV spectra**	**Olive varieties containing these peaks**	**% Presence ($A_{peak}/A^{*}_{total}100$)**	**Estimated MW**	**Correspondence with MW found in literature (*references*)**
10.8 ± 0.1		15	22.0[a]	11.0 ± 0.4	–
11.8 ± 0.1		17	16.9	13.9 ± 0.5	–
12.5 ± 0.2		10	4.1	16.3 ± 0.8	–
13.8 ± 0.1		18	4.4	22.1 ± 0.6	**21.5** KDa, polypeptide derived from the precursor of 41 KDa (SSP) (*Alché* et al., *2006*)
15.2 ± 0.2		17	5.8	30 ± 1	**30.0** KDa, polypeptide derived from the precursor of 47.5 KDa (SSP) (*Alché* et al., *2006; Esteve* et al., *2010*)
17.3 ± 0.1		16	5.6	48 ± 1	**50** KDa, oleosin (*Alché* et al., *2006; Ross* et al., *1993; Esteve* et al., *2010*)
17.7 ± 0.2		18	35.0	53 ± 2	**50** KDa, oleosin (*Alché* et al., *2006; Ross* et al., *1993; Esteve* et al., *2010*)

[a] Overestimated because the peak was overlapped with other peaks in several olive varieties.

3.3. Differentiation of olive varieties based on their CGE protein profiles

Clear differences among the protein profiles were observed when comparing the electropherograms obtained for the analyzed olive samples. For a better understanding of these differences, multivariate classification methodologies were applied to the area percentages obtained from protein profiles. Cluster analysis did not result in a suitable classification according to their geographical origin. Therefore, a supervised multivariate method such as discriminant analysis was chosen to construct linear discriminant functions to classify olives according to their geographical origin. For that purpose, the area percentages of the selected seven peaks in the twenty olive varieties studied was used. The classification factor used was the geographical origin of every olive sample, using the following four denominations "North east", "South east", "South west", and "Other countries" for those olive varieties with a geographical origin different from Spain (see Table 1). At this point, it is necessary to point out that olive cultivars from different geographic origins often show significant variability in their genetic and phenotypic traits. In this work, the olive varieties were grown under the same pedoclimatic conditions, avoiding the possible influence of these conditions on their classification. Fig. 3 shows the distribution of olive varieties in the plane defined by the two first discriminating functions comprising the mathematical model. A clear classification

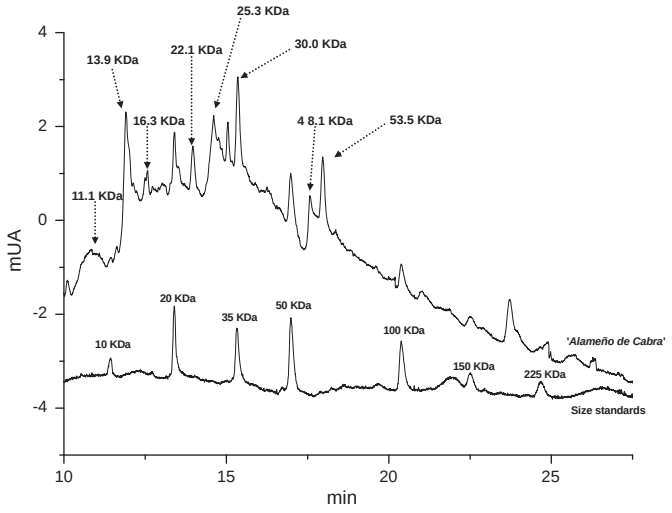


Fig. 2. Protein molecular masses for the olive variety *'Alameño de Cabra'* compared to the molecular masses of the size standards used for calibration. CGE conditions as in Fig. 1.

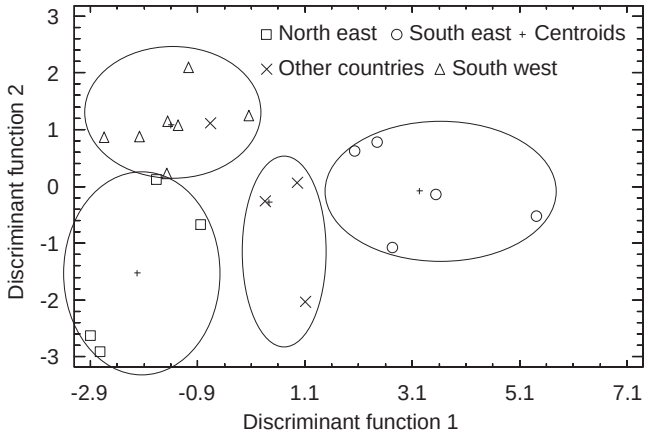


Fig. 3. Distribution of the twenty olive varieties studied in the plane defined by the two first discriminant functions.

according to the geographical origin was achieved demonstrating that the variety origin could be a suitable classification factor [21]. Indeed, four different groups were observed and two discriminating functions with P-values lower than 0.05 were statistically significant at the 95% confidence level. The model enabled the correct classification of 16 of the 20 olive samples (80% of prediction capability). For the evaluation of this model, a cross-validation procedure was performed by the treatment of n-1 out of n observations as training dataset to determine the discrimination rule and to classify the observation left out observing a 78.5% of correct classification.

4. Conclusions

Olive proteins have scarcely been studied and, in most cases, SDS-PAGE has been the preferred technique for this purpose. The separation of olive proteins by SDS-CGE presents several advantages over SDS-PAGE, such as high resolution, on-line detection, ultra-small amount of sample required, and full automation. This is the first time that olive proteins have been separated by CGE. This technique allowed the separation of seven peaks that were common to most of the twenty olive varieties studied in this work. Four of these signals could be tentatively attributed to proteins previously observed by SDS-PAGE. Nevertheless, three of these peaks presented molecular masses that could not be attributed to any protein or molecule previously reported using this SDS-PAGE with traditional extraction procedures. All the seven common peaks were evaluated for their capability to differentiate among olive cultivars. Interestingly, the application of discriminant analysis allowed the correct classification of 16 of the 20 studied olive varieties according to their presumed geographical origin.

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