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Discrimination and Classification of Olive Tree Varieties and Cultivation Zones by Biophenol Contents

R. JAPÓN-LUJÁN,† J. RUIZ-JIMÉNEZ,† AND M. D. LUQUE DE CASTRO*

Department of Analytical Chemistry, University of Córdoba, Campus of Rabanales, Annex C-3, 14071 Córdoba, Spain

The peak areas from a high-performance liquid chromatography−diode array (HPLC-DAD) analysis of biophenols extracted from olive leaves have been used as chemotaxonomic markers to construct chemometric models in order to discriminate and classify (1) 13 varieties of *Olea europaea* olive trees, namely, Alameño, Arbequina, Azulillo, Chorna, Hojiblanca, Lechín, Manzanillo, Negrillo, Nevadillo, Ocal, Pierra, Sevillano, and Tempranillo, from the same cultivation zone and (2) Arbequina samples from six different geoghaphical origins, namely, Córdoba, Mallorca (north and south), Ciudad Real, Lleida, and Navarra. Models based on principal component analysis (PCA) and hierarchical cluster analysis (HCA) were used for discrimination between samples as a function of the tree varieties and cultivation zone, whereas *K* nearest neighbors (KNN) and soft independent modeling of class analogy (SIMCA) models were generated to classify the samples used to validate the models into one of the groups previously established by PCA and HCA. KNN classified correctly 93 and 92% of the samples into the variety and cultivation zone, respectively; meanwhile, the SIMCA models predicted 85 and 92%, respectively.

KEYWORDS: Discrimination; classification; olive tree varieties; oleuropein; cultivation zone; chemometrics; olive biophenols

INTRODUCTION

Hundreds of olive tree varieties have been selected over centuries for their adaptation to different microclimates and soil types. Among them, some cultivars are characteristic of a given zone, whereas others can be found in several countries. With regard to the names of the different varieties, the same name is sometimes given to similar but clearly different varieties and different names are used for identical varieties (*1*, *2*).

The correct classification of the variety and cultivation zone of the olive oil, which also includes the tree and its different parts, is shown as a new problem in order to control the quality and the appellation of origin of the olive oils due to the fact that each combination of variety and cultivation zone has a different chemical composition (*3*−*5*). This fact has also been demonstrated in the case of olive leaves (*6*, *7*), thus calling for an easy way to determine both tree variety and cultivation zone.

Olive leaves are endowed with the most potent radical scavenging power of the different parts of olive trees and provide valuable concentrations of high added-value compounds, among which the main one is oleuropein, which prevents cardiac diseases by protecting membrane lipid oxidation (*8*), acting on coronary dilation and by antiarythmic action (*9*); improves lipid metabolism to protect against obesity problems (*10*); protects enzymes and hypertensive cell death in cancer patients (*8*); and presents antiviral properties (*11*, *12*). Moreover, oleuropein is not the only compound of interest in olive leaves; other biophenols present in olive leaves such as apigenin-7-glucoside—used to fight against Alzheimer's (*13*) and liver diseases (*14*)—possess strong bioactive properties; metabolites of oleuropein such as hydroxytyrosol also protect against cardiac and tumoral diseases with effects similar to those of oleuropein and show other protective abilities against atherosclerosis and diabetic neuropathies (*15*−*17*).

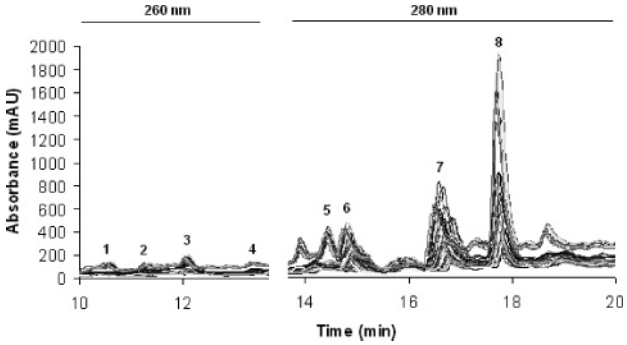

Figure 1. Chromatograms of the extracts from olive leaves. Peaks used as chemotaxonomic markers: 1, 10.6 min peak; 2, 11.3 min peak; 3, 12.1 min peak; 4, 13.5 min; 5, verbacoside; 6, luteolin-7-glucoside; 7, apigenin-7-glucoside; 8, oleuropein.

Principal component analysis (PCA) is a powerful visualization tool. It provides a way to reduce the dimensionality of the

* Corresponding author (e-mail qa1lucam@uco.es; telephone/fax +34 957 21 86 15).
† These authors have contributed equally to this investigation.

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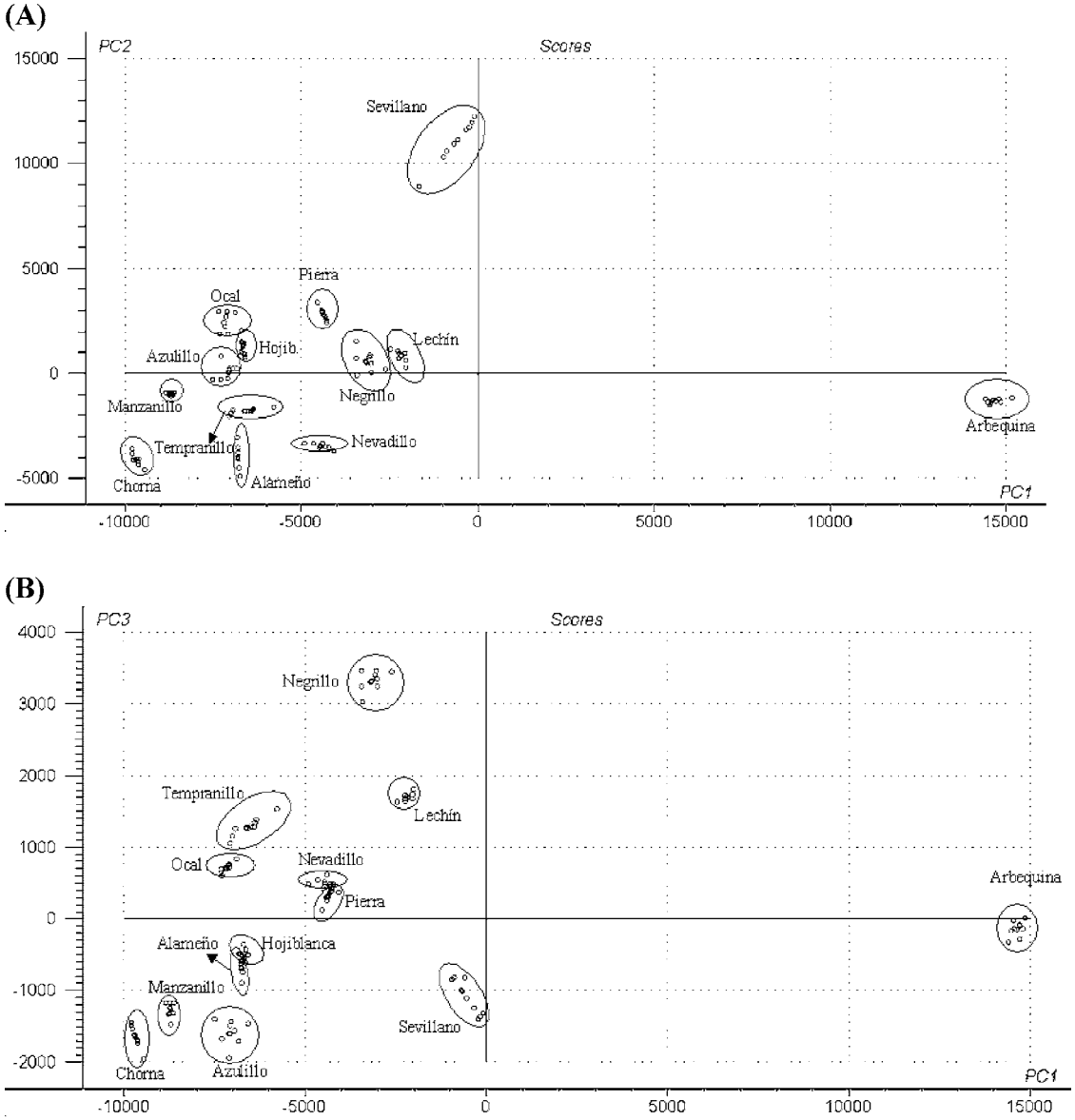

Figure 2. PCA plots for the 13 varieties of olive trees: PC1–PC2 (**A**) and PC1–PC3 (**B**).

data and finds linear combinations of the original independent variables which are used to explain the maximal variance of the data. This concept is the basis for chemometric methods such as soft modeling and multivariate calibration methods, among others (*18*).

In hierarchical cluster analysis (HCA), distances between pairs of samples are calculated and compared. Relatively short distances between samples indicate similarity; dissimilar samples will be separated by relatively large distances. The primary purpose of HCA is to present data in a manner that emphasizes natural grouping. Plotting HCA results as a dendrogram facilitates visual recognition of such categories (*19*).

K nearest neighbors (KNN), a supervised statistical pattern recognition method, attempts to categorize an unknown sample by means of its proximity to others already classified into categories (*20*). A new sample is classified by calculating the distance to the nearest training case: the KNN classifier takes the *k* nearest points—which can correspond to different classes—and assigns a new sample to the class from which more points have been taken. KNN is quite simple, but very computationally intensive. Each of the *K* closest training set sample votes once for its class; the unknown is then assigned to the class with more votes. The determination of the optimal value for *K* is the most important part of this process because it should be the maximum number of neighbors with the minimum possible error and gives an idea of the robustness of the model.

Soft independent modeling of class analogy (SIMCA) was introduced by Wold et al. in 1974 (*21*). Since then, the acronym has changed, but the functionality of the method has been demonstrated and enhancements have been offered by a number of researchers (*22*). In contrast to KNN, SIMCA develops principal component models for each training set category and is sensitive to the quality of the data used to generate the models. As a result, there are diagnostics to assess the quality of the data, such as the modeling power and the discriminatory power: the former describes how well a variable helps the principal components to model variation and the latter describes how well the variable helps the principal components to classify the samples in the data set. Variables with low modeling power and low discriminatory power are usually deleted from the data because they contribute only to the noise the principal component models. In the prediction step of SIMCA a new sample is projected into the principal component space of each class and then assigned to the class it best fits (*23*). An attractive feature

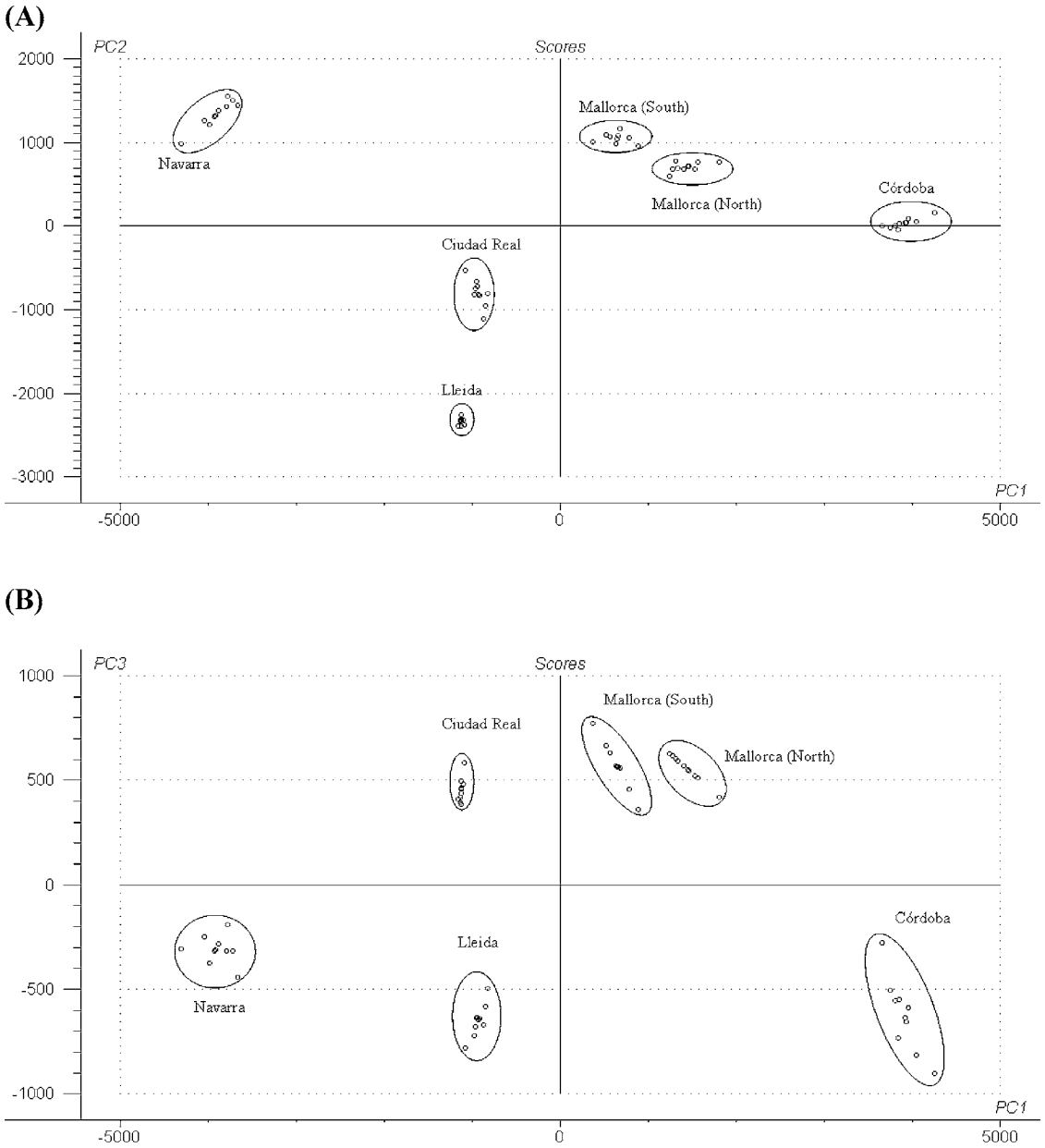

Figure 3. PCA plots for the six different cultivation zones: PC1–PC2 (**A**) and PC1–PC3 (**B**).

of SIMCA is its realistic prediction options as compared to KNN. Note that KNN always assigns every sample to exactly one training class, the so-called nearest neighboring class, but this may be near or not. In contrast, SIMCA provides three possible prediction outcomes: (1) the sample fits only one predefined category, (2) the sample does not fit any predefined category, and (3) the sample fits more than one predefined category.

These chemometric methods have been used to achieve the main aims of this research: (1) differentiate between varieties of olive trees cultivated in the same geographical zone; (2) differentiate between samples of the same variety of olive tree, Arbequina, cultivated in zones with different climates; and (3) classify validation sample sets (VS) into the correct cultivation zone or tree variety. The peak areas—obtained from a high-performance liquid chromatography—diode array (HPLC-DAD) analysis—of the main biophenols in olive leaves—extracted with microwave assistance—have been used as chemotaxonomic markers to generate the chemometric models.

MATERIALS AND METHODS

Apparatus. A Microdigest 301 digestor of 200 W maximum power (Prolabo, Paris, France) furnished with a microprocessor programmer (Prolabo) to control the microwave unit was used for favoring extraction.

A Selecta Angular 6 centrifuge was used to remove particles in the extract. An Agilent 1100 liquid chromatograph consisting of a G1322A vacuum degasser, a G1315A diode array detector, and a Rheodyne 7725 high-pressure manual injection valve (20 μL injection loop) was used for the analysis of the target analytes by HPLC. The analytical column was a Lichrospher 100 RP-18 (250 $\times$ 4 mm i.d., 5 μm) from Análisis Vínicos (Ciudad Real, Spain). A Kromasil 5 C-18 column (15 $\times$ 4.6 mm i.d., 5 μm) protected with a steel holder, both from Scharlab (Barcelona, Spain), was also used.

Data analysis was performed using Pirouette 2.6, Infometrix Inc. (Woodinville, WA), and The Unscrambler 7.6., Camo Inc. (Oslo, Norway).

Reagents and Working Solutions. Ethanol, acetonitrile, and acetic acid from Panreac (Barcelona, Spain) were used; 18 mΩ deionized water from a Millipore Milli-Q water purification system was used to

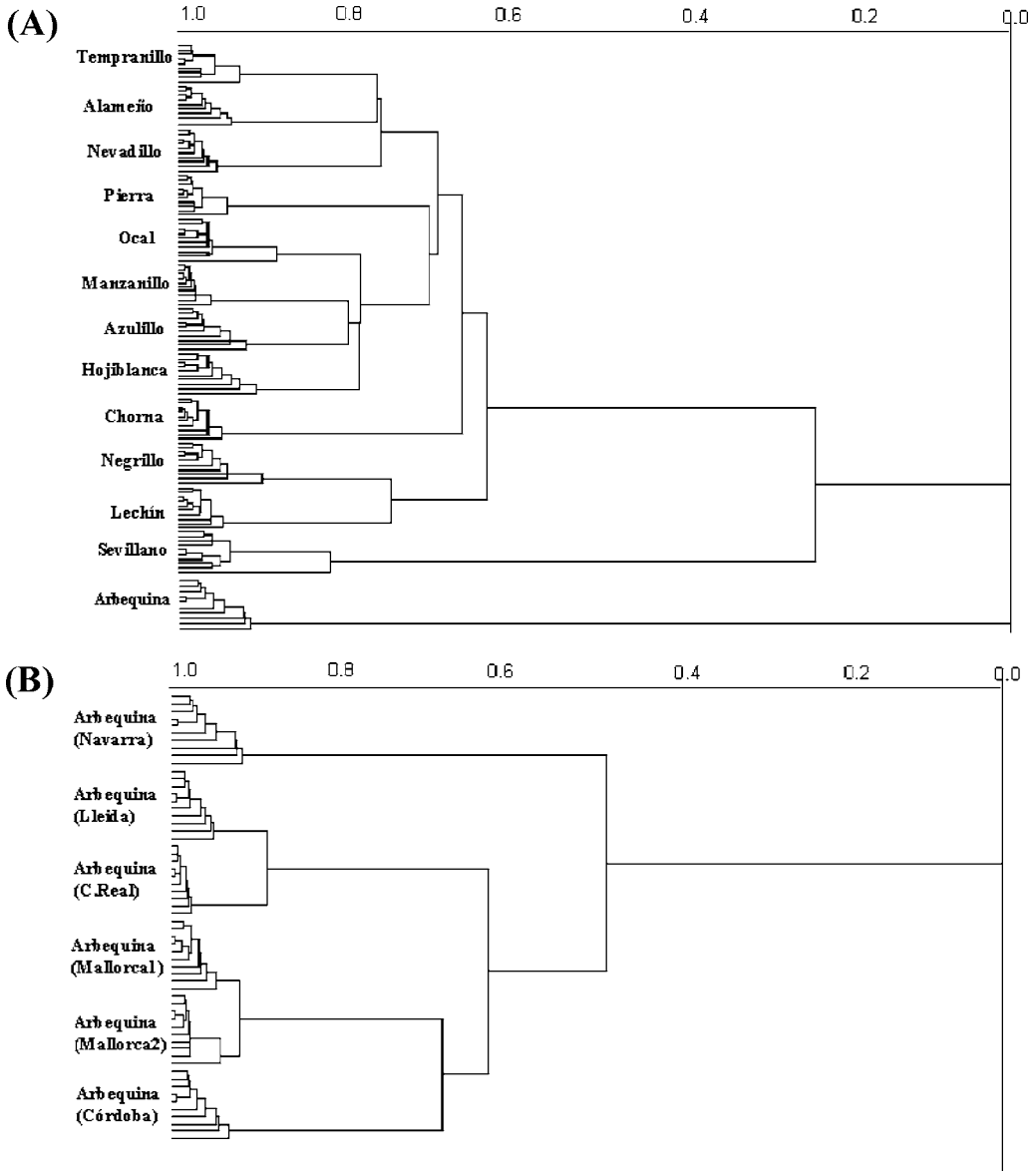

Figure 4. HCA dendrograms for the 13 varieties of olive trees (**A**) and for the six different cultivation zones (**B**).

prepare both water−ethanol extractant mixtures and mobile chromatographic phases.

Oleuropein, apigenin-7-glucoside, verbacoside, and luteolin-7-glucoside from Extrasynthèse (Genay, France) were used for the identification of these compounds, the most abundant in olive leaves.

Samples. Sixty samples of Arbequina olive leaves from Córdoba, Mallorca (northern and southern areas of the island), Ciudad Real, Lleida, and Navarra (Spain) and 10 samples of each variety (namely, Alameño, Arbequina, Azulillo, Chorna, Hojiblanca, Lechín, Manzanillo, Negrillo, Nevadillo, Ocal, Pierra, Sevillano, and Tempranillo), all from Córdoba, were collected in December 2005. In all cases, the samples were obtained from at least five different and healthy trees. All samples were dried, milled, and kept at 4 °C until use.

Extraction and Separation−Detection of Biophenols from Olive Leaves. *Microwave-Assisted Extraction of Biophenols from Olive Leaves.* One gram of milled leaves and 8 mL of 80:20 ethanol/water were placed into the quartz extraction vessel located in the microwave-irradiation zone. After extraction (8 min of microwave irradiation at 200 W), the suspension was centrifuged at 3000 rpm for 5 min and the extract 1:1 diluted prior to injection into the liquid chromatograph to avoid overpressure problems in the chromatographic column.

HPLC-DAD Separation−Detection. The mobile phase was a 6% acetic acid + 2 mM sodium acetate aqueous phase in mixture with acetonitrile, and the injection volume was 20 μL. An initial linear gradient from 0 to 50% acetonitrile at 0.8 mL/min in 25 min was used, followed by another linear gradient from 50 to 100% acetonitrile and an increase of the flow rate from 0.8 to 1.2 mL/min in 2 min. These conditions were maintained for 13 min and, finally, a 5 min equilibration step enabled the initial conditions to be reached and stabilization of the mobile phase. No single wavelength is appropriate for monitoring all phenolics as they display absorbance maxima at different wavelengths. Thus, oleuropein was monitored at 280 nm, verbacoside at 330 nm, apigenin-7-glucoside at 340 nm, and luteolin-7-glucoside at 350 nm. Repeatability and reproducibility of the injections were high; therefore, the use of an internal standard was not necessary, and biophenol quantification was expressed as area units. The chromatograms obtained for all samples used in the study are shown in **Figure 1**.

RESULTS AND DISCUSSION

Optimization of the Extraction and Separation−Detection of Biophenols from Olive Leaves. Microwave assistance was used to accelerate the extraction of biophenols from the samples. The three variables potentially influential on the extraction step (i.e., irradiation power, irradiation time, and extractant composition) were previously optimized in a multivariate way using the extraction efficiency as response variable to obtain the best working conditions for proper extraction without degradation of the target compounds (*24*).

Table 1. KNN Model Predictions

(**A**) Olive Tree Varieties

sample	best prediction	real variety
150	Hojiblanca	Ocal
174	Tempranillo	Tempranillo
122	Negrillo	Negrillo
7	Arbequina	Arbequina
178	Tempranillo	Tempranillo
170	Sevillano	Sevillano
71	Azulillo	Azulillo
169	Sevillano	Sevillano
115	Manzanillo	Manzanillo
84	Chorna	Chorna
157	Pierra	Pierra
101	Lechín	Lechín
88	Chorna	Chorna
4	Arbequina	Arbequina
70	Alameño	Alameño
99	Hojiblanca	Hojiblanca
143	Ocal	Ocal
80	Hojiblanca	Azulillo
136	Nevadillo	Nevadillo
106	Lechín	Lechín
154	Pierra	Pierra
139	Nevadillo	Nevadillo
117	Manzanillo	Manzanillo
63	Alameño	Alameño
127	Negrillo	Negrillo
98	Hojiblanca	Hojiblanca
	correctly classified samples: 93%	

(**B**) Cultivation Zone

sample	best prediction	real cultivation zone
35	Ciudad Real	Ciudad Real
12	Mallorca (south)	Mallorca (south)
51	Navarra	Navarra
59	Navarra	Navarra
4	Córdoba	Córdoba
30	Mallorca (south)	Mallorca (north)
46	Lleida	Lleida
7	Córdoba	Córdoba
45	Lleida	Lleida
16	Mallorca (north)	Mallorca (north)
36	Ciudad Real	Ciudad Real
28	Mallorca (south)	Mallorca (south)
	correctly classified samples: 93%	

Table 2. SIMCA Model Predictions

(**A**) Olive Tree Varieties

sample	best prediction	second-best prediction	real variety
150	UD[a]	UD	Ocal
174	Tempranillo	Tempranillo	Tempranillo
122	UD	UD	Negrillo
7	Arbequina	Arbequina	Arbequina
178	Tempranillo	Tempranillo	Tempranillo
170	Sevillano	Sevillano	Sevillano
71	Azulillo	Azulillo	Azulillo
169	Sevillano	Sevillano	Sevillano
115	Manzanillo	Manzanillo	Manzanillo
84	Chorna	Chorna	Chorna
157	Pierra	Pierra	Pierra
101	Lechín	Lechín	Lechín
88	Chorna	Chorna	Chorna
4	Arbequina	Arbequina	Arbequina
70	UD	UD	Alameño
99	Hojiblanca	Hojiblanca	Hojiblanca
143	Ocal	Ocal	Ocal
80	UD	UD	Azulillo
136	Nevadillo	Nevadillo	Nevadillo
106	Lechín	Lechín	Lechín
154	Pierra	Pierra	Pierra
139	Nevadillo	Nevadillo	Nevadillo
117	Manzanillo	Manzanillo	Manzanillo
63	Alameño	Alameño	Alameño
127	Negrillo	Negrillo	Negrillo
98	Hojiblanca	Hojiblanca	Hojiblanca
	correctly classified samples: 85%		

(**B**) Cultivation Zone

sample	best prediction	second-best prediction	cultivation zone real
35	Ciudad Real	Ciudad Real	Ciudad Real
12	Mallorca (south)	Mallorca (south)	Mallorca (south)
51	Navarra	Navarra	Navarra
59	UD	UD	Navarra
4	Córdoba	Córdoba	Córdoba
30	Mallorca (north)	Mallorca (north)	Mallorca (north)
46	Lleida	Lleida	Lleida
7	Córdoba	Córdoba	Córdoba
45	Lleida	Lleida	Lleida
16	Mallorca (north)	Mallorca (north)	Mallorca (north)
36	Ciudad Real	Ciudad Real	Ciudad Real
28	Mallorca (south)	Mallorca (south)	Mallorca (south)
	correctly classified samples: 92%		

[a] UD, undefined.

The experimental variables to obtain an appropriate HPLC separation−detection were also optimized. Thus, different columns, guard columns, wavelengths, and composition and flow rate of the mobile phase were checked (*25*).

Data Analysis. *Principal Component Analysis.* PCA using as chemotaxonomic markers the peak area of eight phenolic compounds—four of them are the most important biophenols in olive leaves extract (namely, oleuropein, verbacoside, apigenin-7-glucoside, and luteolin-7-glucoside) and the rest are the highest unidentified peaks at 260 nm, a typical wavelength for the monitoring of phenolics—present in the olive leaves extract was carried out for each sample set. The peak area of each compound and sample can be seen in the Supporting Information.

Figure 2 shows that differentiation between samples with the same geographical origin and different variety is possible: the 13 varieties are clearly separated. The samples corresponding to the Arbequina variety are the most different; Negrillo, Lechín, and Sevillano varieties can also be easily distinguished.

The main contributions to PC1, which explains 68% of the variance of the samples, were from the four identified peaks and the peak at 11.30 min with a small contribution from the other analytes. PC2 explains 29% of the variance, the most influential variables being apigenin-7-glucoside and luteolin-7-glucoside, oleuropein, and the peaks at 11.30 and 13.50 min, with a small contribution from the other analytes.

For Arbequina samples with different geographical origins, it is easy to differentiate the six zones under study. As can be seen in **Figure 3**, the leaves cultivated in zones with opposite climates such as Navarra—a cold and wet region—and Córdoba—an extremely hot and very dry zone—appear clearly separated in panels **A** (PC1−PC2) and **B** (PC1−PC3) of **Figure 3**. In both plots the samples from zones with mild climatic conditions (e.g., Ciudad Real, Lleida, and Mallorca) are in the center of the plot, between the samples from Navarra and Córdoba. The two groups of samples from Mallorca (north and south) are very close and separated from the rest. In this case the main contributions to PC1, which explains 74% of the variance of the samples, are from oleuropein, verbacoside, and luteolin-7-glucoside; apigenin-7-glucoside and the peak at 10.60 min have a null influence, and a small contribution is made by the other analytes. PC2 explains 23% of the variance, and the most

influential variables are apigenin-7-glucoside and the peak at 10.60 min, with a small influence from other variables.

These results prove that PCA can be used as a tool for the identification of olive tree varieties and differentiation of the cultivation zone.

Hierachical Cluster Analysis. The single-linkage method, with the Euclidean distance as the similarity measurement, was used in this case.

The results obtained with HCA are analogous to those from PCA: Arbequina is the most dissimilar of the varieties, and low similarity exists between Negrillo, Lechín, and Sevillano and the rest of varieties (see **Figure 4A**). HCA corroborates the differences between Arbequina samples from the six cultivation zones (see **Figure 4B**). The lowest and highest similarities are found between the samples from Córdoba and Navarra and both samples from Mallorca (north and south), respectively.

Classification Analysis. Due to the low number of samples (10 for each variety and geographical origin), the results obtained in this case should be considered as a first approach to classification for prediction of the variety and geographical origin of the olive tree. A larger number of samples would be necessary to obtain better models and higher accuracy in the application.

The samples sets—SS1 and SS2—divided into training sets (TS) including 80% of the components of each sample set (TS1 and TS2, 104 and 48 samples, respectively) are used for constructing the models; the validation sets (VS), which include 20% of the components of each sample set (VS1 and VS2, 26 and 12 samples, respectively), are used to validate the models. The training and validation sets were randomly selected from all samples.

K Nearest Neighbors. The objective of the application of this method was to classify an unknown sample in terms of variety or cultivation zone into one of the groups previously established and demonstrated by PCA and HCA.

All of the models were made for a maximum neighbor value of 10 and without any data preprocessing. The optimization of the model shows that the optimal neighbor value was the sixth with 0% of prediction error for all of the models built. The proposed models predict 93% of correct variety and 92% of the cultivation zone of the validation sets (see **Table 1**), which show the capability of the model for the prediction of the variety as well as cultivation zone.

Soft Independent Modeling of Class Analogy. The aim of SIMCA is the same as KNN analysis; the difference lies in the methodology used to optimize the models. Each training class is considered independently; the training sets TS1 and TS2 include the samples belonging to the 13 varieties with the same geographical origin and the Arbequina samples cultivated in the six geographical zones, respectively. For this reason, optimization of the number of factors in each class and elimination of outliers were necessary. In all models generated for either samples of different varieties and the same geographical origin or samples of the same variety and different geographical origin, a factor of three—PCs chosen independently for each model—was selected as optimal value for all of the classes. The variance explained was 100%, with a 5% significance level providing the best results.

The prediction capacity of the SIMCA models was determined by analyzing the validation sets of samples that had not been used at any time to generate the model. With 3 as factor, the proposed models predict correctly 85 and 92% of the varieties and cultivation zones, respectively. As can be seen in **Table 2**, the false predictions were not classified into an erroneous category and remained as undefined.

Conclusions. Discrimination and classification between varieties of olive trees, namely, Alameño, Arbequina, Azulillo, Chorna, Hojiblanca, Lechín, Manzanillo, Negrillo, Nevadillo, Ocal, Pierra, Sevillano, and Tempranillo, from the same cultivation zone and of Arbequina olive trees cultivated in different geographical zones, that is, Córdoba, Mallorca (north and south), Ciudad Real, Lleida, and Navarra, have been achieved using as variables the peak areas of eight phenolic compounds, which were obtained by HPLC-DAD analysis of extracts from olive leaves obtained by the assistance of microwaves.

The most influential variables on the development of the PCA models were the main biophenols in olive leaves (namely, oleuropein, verbacoside, apigenin-7-glucoside, and luteolin-7-glucoside). This shows that the relative concentrations of these compounds—of relevance because of their healthy properties—can also be useful to differentiate both varieties and cultivation zones. The results obtained by HCA are in agreement with those from PCA.

Concerning classification, 93 and 85% of varieties from the same cultivation zone were well classified by KNN and SIMCA, respectively, and those of the same variety from different geographical zones were classified at better than 92% in both cases.

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Supporting Information Available: Peak area of each compound and sample. This material is available free of charge via the Internet at http://pubs.acs.org.

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