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Discrimination of Olive Oils and Fruits into Cultivars and Maturity Stages Based on Phenolic and Volatile Compounds

CURTIS M. KALUA,† MALCOLM S. ALLEN,‡ DANNY R. BEDGOOD, JR.,† ANDREA G. BISHOP,† AND PAUL D. PRENZLER*,†

E. H. Graham Centre for Agricultural Innovation, School of Science and Technology, and School of Wine and Food Science, Locked Bag 588, Charles Stuart University, Wagga Wagga 2678, Australia

Olive oil and fruit samples from six cultivars sampled at four different maturity stages were discriminated into cultivars and maturity stages. The variables—volatile and phenolic compounds—that significantly ($p < 0.01$) discriminated cultivars and maturity stage groups were identified. Separation by stepwise linear discriminant analysis revealed that Manzanilla olive cultivar was separated from cultivars Leccino, Barnea, Mission, Corregiola, and Paragon, whereas cultivars Corregiola and Paragon formed a cluster. The volatile compounds hexanol, hexanal, and 1-penten-3-ol were responsible for the discrimination of cultivars. All maturity stages were discriminated, with the separation of early stages attributed to oil phenolic compounds, tyrosol and oleuropein derivatives, whereas the volatile compounds (*E*)-2-hexenal, hexanol, 1-penten-3-ol, and (*Z*)-2-penten-3-ol characterized the separation of all maturity stages and in particular the late stages. Hexanol and 1-penten-3-ol characterized the separation of both cultivars and maturity stages.

KEYWORDS: Olive oil and fruit; stepwise linear discriminant analysis (SLDA); pattern recognition; cultivar; maturity stage; volatile profile; phenolic profile

INTRODUCTION

Olive oil is unique among the high-volume oils in that it is valued for its unique aroma and taste. As the consumption of olive oil increases in nontraditional markets (i.e., those outside the Mediterranean region), consumer preference for oil with particular sensory properties will dictate sales, pricing, and market differentiation. To this end, quantitative measures of compounds responsible for aroma and taste will be necessary to deliver a consistent product.

Although the precise relationship between chemical composition and sensory properties is yet to be elucidated for olive oil, it is now well established that phenolic compounds (*1*−*3*) and volatile compounds (*4*−*6*) have a direct influence on the taste and aroma of olive oil. Phenolic and volatile profiles of olive oil originate in the fruit and, consequently, variations in the chemical and biochemical makeup of olive fruit can have a huge influence on the resultant oil. Many factors may affect the chemical makeup of olive fruit. For example, it has been suggested that cultivar, maturity stage (degree of ripeness), geographic location, and agronomic practices (*7*−*10*) may all affect oil properties through effects on fruit. In addition, climate and environmental factors probably have an indirect effect on cultivar characteristics by modifying the degree of ripeness (*11*). This leaves olive fruit cultivar and maturity stage as the main factors that explain the variation in the characteristics of olive oil.

The application of multivariate analysis to olive oil has enabled the identification of the variables—geographic location, cultivar, etc.—that explain the variations in samples—phenols/volatiles (*10*, *12*). It has been shown that multivariate analysis with canonical discriminant analysis, using sensory attributes and chemical compounds as predictors, can efficiently authenticate some olive cultivars (*13*). Discrimination of olive oils into varietal and maturity stage groups with stepwise linear discriminant analysis (SLDA) establishes the variables that are the best predictors in separating the groups (*12*). Vichi et al. (*10*) reported the use of linear discriminant analysis (LDA) in distinguishing virgin olive oils by geographic origin and variety according to their volatile composition, with a greater success in the classification of geographic region than cultivar differences.

Identifying volatile and/or phenolic compounds that explain the variations in olive oil characteristics is a major challenge because the parameters may not be independent. Phenolic and volatile compounds are a characteristic of certain maturity stages (*14*, *15*), and discrimination of cultivars at the same maturity stage introduces bias, further necessitating multivariate analysis. Moreover, not all compounds present in olive oils and fruits at high concentrations characterize cultivar or maturity stage. For instance, lignans are among the main phenols in olive oil (*14*), but it was reported (*14*, *16*) that the amount of the lignans (+)-

* Author to whom correspondence should be addressed (telephone +61-2-6933 2978; fax +61-2-6933 2737; e-mail pprenzler@csu.edu.au).
† School of Science and Technology.
‡ School of Wine and Food Science.

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Table 1. Olive Fruit Sample Description

maturity stage	sampling date	weeks after flowering	MI[a] (without Leccino)	MI[a] (Leccino)
green	April 13, 2004	22	2.28 ± 0.68a	3.98 ± 0.01c
spotted	May 5, 2004	25	3.06 ± 0.68b	4.00 ± 0.01c
red	May 31, 2004	29	4.27 ± 0.41c	4.10 ± 0.17c
black	July 12, 2004	35	4.46 ± 0.68cd	5.13 ± 0.32d

[a] Maturity index. Different letters indicate significantly different ($p < 0.05$) mean ± standard deviation of at least three replicates.

pinoresinol and (+)-acetoxypinoresinol did not significantly ($p < 0.05$) change with ripening. It is therefore imperative to consider a wide spectrum of predictors and not necessarily the major compounds alone in the discrimination of cultivars and maturity stages.

The objective of this study was to identify the phenolic/volatile markers of maturity stages and cultivars in olive fruit and oil. In this work, 20 phenolic compounds from olive fruit and oil and 18 volatile compounds from olive oil were investigated for their ability to predict the discrimination of olive maturity stage and cultivar independent of each other. Both cultivar and maturity stage were discriminated through SLDA, and the volatile and phenolic compounds most likely to contribute to discrimination were identified. To the best of our knowledge, this is the first study to examine simultaneously the two major classes of compounds responsible for sensory quality of olive oil in order to identify cultivar and maturity stage markers.

MATERIALS AND METHODS

Materials. Reagents and phenolic and volatile standards from the indicated sources were used without further purification. The following reagents were used: acetic acid (Biolab, Sydney, Australia); hexane and methanol (Mallinckrodt Chemicals, Paris, France); acetonitrile (J. T. Baker, Phillipsburg, NJ); formic acid (Sigma, St. Louis, MO). The phenolic standards used were as follows: caffeic acid, *p*-coumaric acid, and gallic acid (Sigma); tyrosol (Aldrich, Milwaukee, WI); hydroxytyrosol (Sapphire Bioscience, Sydney, Australia); oleuropein (Extrasynthese, Genay, France). Verbascoside was kindly donated by Prof. Okuyama of Chiba University, Japan. Standards were prepared in methanol + water (50+50 v/v) and filtered through 0.45 μm plastic nonsterile filters prior to chromatographic analysis. Grade 1 water (ISO3696) purified through a Milli-Q water system was used for chromatographic preparations.

The volatile standards used were as follows: pentanal, (*E*)-2-hexenal, and nonanol (Merck, Hohenbrunn, Germany); hexanal, heptanal, (*E*)-2-octenal, (*E*)-2-nonenal, 1-penten-3-ol, 2-penten-1-ol, heptanol, octanol, hexyl acetate, methyl isobutyl ketone (MIBK), and 2-nonanone (Aldrich); octanal, octane, nonane, decane, undecane, and dodecane (Sigma); benzaldehyde (Ajax Chemicals, Auburn, Australia); ethanol and acetic acid (Biolab); ethyl acetate (Mallinckrodt Chemicals); and hexanol (Riedel de Haen, Seelze, Germany).

Fruit Harvest and Oil Extraction. Olive fruit samples (3 kg) were hand picked in duplicate from Cookathama farm, near Darlington Point in southwestern New South Wales, Australia, during the 2004 harvest season. Forty-eight fruit samples were collected at four maturity stages (**Table 1**) from six cultivars (Leccino, Barnea, Manzanilla, Mission, Corregiola, and Paragon). The maturity index (MI) was assessed using the method of the Instituto Nacional de Investigaciones Agronomicas, Estacion de Jaen (Spain), and described by IOOC (*17*). The color of the olive skin was not very useful in the description of maturity stage because different cultivars showed different rates of change in the skin pigmentation. For instance, the color of Leccino fruit remained black and was not significantly different ($p > 0.05$) throughout the maturity stages except for fruit at black maturity stage (**Table 1**). MI values for Leccino, at the same maturity stage, were significantly ($p < 0.05$) different at early maturity stages (green and spotted) but were not significantly ($p > 0.05$) different at late maturity (**Table 1**). Leccino was excluded in the calculation of the maturity index (MI) to avoid skewing the maturity description. The maturity stage description was predominantly based on the sampling date in relation to the weeks after flowering (**Table 1**), whereas MI indicated the overall range of skin pigmentation.

Oil was extracted from the olive fruit (700 g) using a cold press Abencor extraction unit (Abencor, Spain) according to the manufacturer's specifications. The oil was stored (<1 week) in the dark at room temperature prior to volatile and phenolic compound analysis.

Samples for Phenolic Compound Characterization. Ten olive samples (three oil, three fruit, and four paste) covering a wide range of phenolic compounds from different cultivars at different maturity stages were used in the characterization of phenolic compounds. The paste sample was an intermediate between the fruit and oil that was obtained after crushing of the fruit and malaxing of the paste. The paste represented phenolic compounds found in both the fruit and oil.

Samples for Volatile Compound Characterization. Characterization of volatile compounds with gas chromatography−mass spectrometry (GC-MS) was performed using fusty, rancid, and musty IOOC standard oils, Leccino oil sample, Mission oil sample, and two olive oil samples spiked with volatile standards [ethanol, 2-penten-1-ol, hexanol, heptanol, octanol, nonanol, hexyl acetate, octane, nonane, decane, undecane, acetic acid, ethyl acetate, pentanal, hexanal, (*E*)-2-hexenal, heptanal, benzaldehyde, octanal, (*E*)-2-octenal, (*E*)-2-nonenal, 1-penten-3-ol, methyl isobutyl ketone (MIBK), 2-nonanone, and dodecane].

Methods. *Extraction of Phenolic Compounds.* The method for extraction of phenolic compounds was adapted from that of Ryan et al. (*18*). Olive fruit (1 g) was crushed in liquid nitrogen and immediately blended with methanol + water (5 mL, 50+50 v/v) + gallic acid (0.5 mL, 100 mg/L) as an internal standard using an Ultra Turrax blender. The blended sample was left to stand for 30 min at ambient temperature and filtered using a Büchner funnel. The solid mass was recovered and re-extracted as before, but now the blended sample was left to stand for 15 min prior to filtering. The filtrates were combined and washed with hexane (3 × 5 mL). Hexane was discarded and the aqueous phase filtered through 0.45 μm plastic nonsterile filters.

Olive oil (15 g) was dissolved in hexane (15 mL), then gallic acid (0.5 mL, 100 mg/L) was added to the oil as an internal standard, and the mixture was extracted with 50+50 (v/v) methanol + water solutions (3 × 1 mL). The methanolic extract was washed with hexane (3 × 3 mL) and filtered through 0.45 μm plastic nonsterile filters prior to liquid chromatography−electrospray ionization−mass spectrometry (LC-ESI-MS) and high-performance liquid chromatography−diode array detector (HPLC-DAD) analysis.

Qualitative (LC-ESI-MS) Analysis of Phenolic Compounds. Phenolic compounds were identified with a Waters 2695 LC chromatograph with a Waters 2695 LC pump (Waters, Rydalmere, Australia) and a Waters Quattro micro, tandem quadrupole mass spectrometer (Waters) by electrospray ionization (ESI). Phenolic compounds were separated on an SGE Wakosil C18 column (150 mm × 2.0 mm; 5 μm) with the gradient program described for HPLC-DAD analysis below except that formic acid (0.1%) replaced acetic acid (1%) in both solvents (A and B). The flow rate of the mobile phase was 0.25 mL/min, and the sample injection volume was 5 μL. The UV detector (Waters 2487 dual-wavelength UV detector) output was monitored at 280 and 320 nm by the MassLynx 4.0 data system for alignment with the mass spectral data. The mass spectral data were acquired at four alternating scans from *m*/*z* 80 to 1000 with a scan time of 2 s using both positive (ES^+) and negative (ES^-) ion modes at cone voltages of 30 and 70 V.

Characterization of the phenolic compounds with LC-ESI-MS was reached after results from several samples were compared. Positive characterization was achieved when a phenolic compound showed the same fragmentation pattern in at least three samples and showed a similar pattern with data from literature (*19*−*22*).

Quantitative (HPLC-DAD) Analysis of Phenolic Compounds. HPLC-DAD analysis was performed using a Varian 9012 instrument (Varian, Melbourne, Australia) equipped with a 20 μL sample loop injector.

The column eluent was monitored through a Varian 9065 polychrome diode array detector (Varian), and data were collected at 259, 280, and 320 nm. Separation was achieved on a Phenomenex C18 column (150 mm × 4.6 mm; 5 μm) with gradient elution. The mobile phase was filtered under vacuum using Alltech Nylon 66 membranes. The flow rate of the mobile phase was 1 mL/min, and the solvents for gradient elution were solvent A (water + acetic acid; 100+1 v/v) and solvent B (methanol + acetonitrile + acetic acid; 95+5+1 v/v/v). A stepwise linear gradient commencing with 10% solvent B was employed. This was increased to 30% at 10 min, isocratic to 15 min, and then increased to 40% at 25 min, followed by further increases to 50% at 40 min, to 75% at 50 min, and to 95% at 55 min, respectively, with a 5 min isocratic run. There was a 5 min equilibration time at the end of the 60 min run.

Quantification was performed using phenolic standard calibration curves. Direct quantification of some phenolic compounds was not possible because standards were not commercially available. Therefore, the quantification of such compounds was based on oleuropein (for glycosidic phenolic compounds) and hydroxytyrosol (for simple phenols).

Qualitative (SPME-GC-MS) Analysis of Volatile Compounds. Solid-phase microextraction−gas chromatography−mass spectrometry (SPME-GC-MS) was used to qualitatively analyze volatile compounds using a Varian Star 3400CX gas chromatograph (Varian) coupled with a Saturn 2000 ion trap mass spectrometer (Varian). Qualitative analysis used the same chromatographic conditions as described for solid-phase microextraction−gas chromatography−flame ionization detection (SPME-GC-FID) in quantitative analysis below.

Electron impact ionization (EI) mode with automatic gain control (AGC) was used for MS. The electron multiplier voltage for MS was 1850 V, the AGC target was 25 000 counts, and the filament emission current was 15 μA with the axial modulation amplitude at 4.0 V. The ion trap temperature was maintained at 250 °C and the manifold temperature was maintained at 60 °C. The temperature of the transfer line, interfacing the GC and MS, was set at 250 °C. Mass spectral scan time from *m*/*z* 35 to 450 was 0.8 s (using two microscans). Background mass was set at *m*/*z* 45.

Volatile compounds were identified by comparison of the retention times with that of authentic standards on GC-FID and confirmed by GC-MS, comparing the mass spectra with the NIST 98 Library. The identity of the compounds was further confirmed by comparing the retention indices obtained with literature values (*23*−*25*). Positive characterization was achieved when a volatile compound was identified by both GC-MS and retention time of external standards. Compounds were also characterized when a compound was identified in at least three samples by GC-MS.

Quantitative (SPME-GC-FID) Analysis of Volatile Compounds. SPME-GC-FID was used to quantify volatile compounds in olive oil (*26*). Oil (1 g) in reactivials (Supelco, 10 mL) sealed with a Teflon-lined septum was placed in a thermostated oven at 40 °C. After thermal equilibration for 15 min, the SPME needle (DVB-CAR-PDMS, 50/30 μm fiber, Supelco) was inserted through the septum and left exposed in the headspace for 30 min. The sample was agitated using a magnetic stirrer throughout the equilibration and extraction process. The fiber was withdrawn after 30 min of extraction, and the volatile compounds were thermally desorbed at the GC injection port at 250 °C. The thermal desorption was done in splitless mode for 3 min, and thereafter the fiber was cleaned in split mode for 10 min at the injection port prior to reuse.

Volatile compounds were analyzed using a Varian Star 3400CX gas chromatograph (Varian). The column temperature program was as follows: 40 °C for 8 min, increasing at 5 °C/min to 200 °C with a final isothermal period of 10 min. Separation was achieved on an SGE BPX5 column (length = 30 m, 0.25 mm id, film thickness = 0.25 μm) using nitrogen carrier gas at a flow rate of 2 mL/min (pressure = 23 psi). The injection temperature was 250 °C, and the FID detector was maintained at 300 °C. Dodecane (5 μg/g) was used as an internal standard in the quantification (*26*).

Statistical Data Analysis. Data were analyzed using SPSS 11.5 (SPSS Inc., Chicago, IL). Unlike the other multivariate exploratory procedures, standardizing the variables in linear discriminant analysis has no effect on the outcome but merely rescales the axes (*27*). However, in this study, all predictors had an almost normal distribution, so that no transformation was done to the data set.

SLDA was used to find patterns that best separated groups of maturity stages and cultivars with concentrations of volatile and phenolic compounds as grouping variables (predictors). SLDA involves variable selection, evaluation of variable contribution to discrimination, and pattern recognition.

1. Variable Selection. Variables are sequentially entered into the model in stepwise variable selection. The variable considered for entry into the discriminant function is the one with the largest positive or negative correlation that significantly improves the prediction of the outcome. The variable is entered into the discriminant function only if it satisfies the criterion for entry. The variable entry procedure stops when there are no variables that meet the entry criterion (*28*). A stringent criterion ($p = 0.01$) for entry was chosen to select the most likely predictors of cultivar and maturity stage patterns. Phenolic and volatile compounds (**Tables 2** and **3**) in the olive fruit and oil were the variables used in the discrimination of cultivars and maturity stages (**Tables 4** and **5**).

2. Variable Contribution. The relative contribution of the variables toward discrimination can be explained with the standardized discriminant function coefficients, which is equivalent to the standardized beta in regression and indicates the contribution of each variable to the variates (*28*). The variates are the linear combinations of dependent variables that predict which group a sample belongs to. These variates can be described in terms of linear regression equations called linear discriminant functions that are used in calculating scores for discriminating different objects. The magnitude of the canonical discriminant function coefficient is equivalent to the relative contribution of the predictor in the variate, whereas the positive or negative sign of the coefficient indicates either a positive or negative contribution respectively (*28*).

3. Pattern Recognition. The first two discriminant functions were used to show the cultivar and maturity stage patterns, which were represented as combined-group scatter plots in two dimensions, *x*-axis (function 1) and *y*-axis (function 2). The significance of the discriminant functions in the scatter plots was tested with the Wilks' lambda statistic, where values close to 0 indicate that the group means are different and values close to 1 indicate that the group means are not different. Small significance values ($p < 0.05$) indicate that the group means differ, and large significance values ($p > 0.05$) indicate that the group means are the same. The group differences explained by the canonical discriminant functions should be significant ($p < 0.05$) to necessitate discrimination in the underlying dimension.

RESULTS AND DISCUSSION

Discrimination of olive oils into cultivars and maturity stages was studied by initially identifying the volatile and phenolic compounds present in the olive oil and fruit (**Tables 2** and **3**) and then using SLDA with the identified compounds as predictors. Those compounds that significantly ($p < 0.01$) separated cultivars and maturity stages into recognizable and mutually exclusive clusters were classified as discriminating compounds (**Tables 4** and **5**), and whether fruit or oil compounds contributed more to the discrimination of olive cultivars and maturity stages was examined. The relative contributions of the predictors were reached after examination of the canonical discriminant coefficients. Similarities between groups that were not separated by volatile and phenolic compounds were also recognized.

Phenolic Compound Characterization. Olive maturity stages and cultivars have been characterized by either the presence or absence of compounds and by a significant increase or reduction of compounds in a sample (*14*, *29*−*32*). A study of eight olive cultivars (*31*) based on hydroxytyrosol, elenolic acid glucoside, demethyloleuropein, quercetin-3-rutinoside, luteolin-7-glucoside, and oleuropein proposed demethyloleuropein

Table 2. Characterization of the Phenolic Compounds Used To Discriminate Olive Oils and Fruits into Cultivars and Maturity Stages

compound	UV[a]	MS[b]	RT[c] (min)	oil	fruit	major ES$^-$ peaks	major ES$^+$ peaks
hydroxytyrosol glucoside		X	6.2 (0.1)		Y[d]	315, 153	339, 317, 155, 137
hydroxytyrosol	X	X	6.76 (0.09)	Y	Y	153, 151, 123	155, 137
tyrosol glucoside		X	8.50 (0.08)		Y	399, 299	323, 301, 225
tyrosol	X		9.72 (0.07)	Y	Y	no trace	no trace
luteolin -7-rutinoside		X	11.18 (0.02)		Y	593, 285	595, 287
caffeic acid	X	X	13.0 (0.1)		Y	179, 139, 135	165, 151
p-coumaric acid	X	X	17.9 (0.3)	Y		195, 165, 163	no clear trace
3,4-DHPEA-DEDA[e]		X	19.1 (0.2)	Y	Y	319, 195, 165	343, 321, 303, 137
verbascoside	X	X	23.1 (0.3)		Y	623, 461, 161	647, 471, 325
luteolin-7-glucoside		X	25.3 (0.5)		Y	447, 381	449, 297, 225, 165, 137
dialdehyde form of ligstroside		X	26.4 (0.5)	Y		303, 285, 179, 165	327, 297, 225, 165
hesperidin		X	27.1 (0.3)		Y	609, 463, 377, 361	633, 611, 465, 433, 303, 137
hemiacetal of ligstroside		X	27.9 (0.2)	Y		335, 275, 377	359, 361, 137, 433
oleuropein	X	X	29.8 (0.5)		Y	539, 415, 377	563, 379, 361 137
(+)-pinoresinol		X	32.57 (0.02)	Y		459, 377, 361, 303, 285, 179	359, 319, 121, 417
(+)-acetoxypinoresinol		X	33.2 (0.2)	Y		459, 377, 361, 333	811, 439, 417, 357, 233
ligstroside		X	35.8 (0.3)		Y	523, 495	547, 417, 363, 345
oleuropein aglycon		X	41.0 (0.7)	Y		755, 377, 307, 275	843, 433, 361, 137
luteolin		X	48.9 (0.3)	Y	Y	285, 223	287, 225, 173
hemiacetal of oleuropein		X	49.7 (0.5)	Y		409, 377, 361	433, 411, 245, 173, 137

[a] Detection by HPLC-DAD denoted X. [b] Characterization by LC-ESI-MS denoted X. [c] Retention time. [d] Presence of compound denoted Y. [e] 3,4-dihydroxyphenylethyl alcohol–decarboxymethyl elenolic acid dialdehyde.

as a varietal marker. The same study proposed hydroxytyrosol as a maturity marker, although the work did not include the black maturation stage. A decrease in secoiridoid concentrations with an increase of olive maturity has been reported (*14*), demonstrating that phenolic compounds may be used to identify maturity stages.

The present study used 20 phenolic compounds (**Table 2**) as predictors in the discrimination of olive oils and fruits into cultivars and maturity stages. As the phenolic profiles of olive fruit and oil are different, separate fruit and oil phenolic groups were used for discriminant analysis. Glycosylated phenolic compounds found only in olive fruit included hydroxytyrosol glucoside, luteolin-7-rutinoside, verbascoside, and oleuropein (**Table 2**). These molecules showed fragmentation in both ES$^-$ and ES$^+$ modes, formed sodium adducts in the ES$^+$ mode (with the exception of luteolin-7-rutinoside), and gave weaker peaks but more fragmentation in the ES$^+$ mode.

Phenolic compounds detected in olive oil, but absent in the fruit, were derivatives of oleuropein and ligstroside (dialdehydes and hemiacetals), lignans (pinoresinol and acetoxypinoresinol), aglycons such as oleuropein aglycon, and *p*-coumaric acid (**Table 2**). Fragmentation of these compounds showed fewer, but more intense, peaks in the ES$^-$ mode and, in some cases, no trace in the ES$^+$ mode, as with *p*-coumaric acid. Sodium adducts were not observed in the ES$^+$ mode for tyrosol, hydroxytyrosol, luteolin, luteolin-7-glucoside, and luteolin-7-rutinoside. Apart from luteolin-7-glucoside and luteolin-7-rutinoside, all compounds that did not form sodium adducts were components of the oil, suggesting that they may be less polar and preferentially partition into the oil.

For discriminant analysis, the concentrations of tyrosol and hydroxytyrosol were combined with those of their respective glycosides and, for oleuropein and ligstroside, the hemiacetals and dialdehydes were combined and classified as oleuropein and ligstroside derivatives, respectively.

Volatile Compound Characterization. Olive oil volatile compounds have been used previously to characterize maturity stages and cultivars using multivariate analysis, unlike olive phenolic compounds. Differences in four cultivars were characterized in six European varieties of virgin olive oil using 55 volatile compounds (*6*), and 10 C6 volatile compounds have been used to characterize three maturity stages (*15*).

Table 3. Characterization of Volatile Compounds Used To Discriminate Olive Oils and Fruits into Cultivars and Maturity Stages

volatile compound	FID[a]	MS[b]	RI (exptl)[c]	RI
acetic acid	X	X	718	710 (*25*)
1-penten-3-one		X	733	682 (*24*)
1-penten-3-ol	X	X	733	686 (*23*)
pentanal	X	X	738	732 (*23*)
(*Z*)-2-penten-1-ol	X		771	767 (*23*)
octane	X	X	800	800 (*23*)
hexanal	X	X	794	800 (*24*)
(*E*)-2-hexenal	X	X	855	854 (*23*)
(*E*)-2-hexen-1-ol		X	869	870 (*25*)
hexanol	X	X	874	858 (*25*)
6-methyl-5-hepten-2-one		X	1011	965 (*25*)
5-methyl-5-hepten-2-one		X	1012	
2-pentylfuran		X	1012	993 (*23*)
octanal	X	X	1029	1006 (*23*)
hexyl acetate	X	X	1036	1014 (*23*)
octanol	X	X	1089	1072 (*23*)
(*E*)-2-nonen-1-ol[d]		X	1120	
1-dodecene[d]		X	1187	

[a] Detection by GC-FID denoted X. [b] Characterization by GC-MS denoted X. [c] Experimental retention index based on BPX5 column. [d] Tentative assignment based on MS.

The current study is based on 18 volatile compounds (**Table 3**) from six cultivars at four different maturity stages over a period of 3 months (**Table 1**). The first 10 early-eluting volatile compounds in **Table 3** [acetic acid, 1-penten-3-one, 1-penten-3-ol, pentanal, (*Z*)-2-penten-1-ol, octane, hexanal, (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, and hexanol] are predominantly C5 and C6 compounds and were common in all olive oils except oil from Manzanilla, which had C8 compounds (octane, octanal, and octanol) as the predominant volatiles. Two volatile compounds, (*E*)-2-nonen-1-ol and 1-dodecene, were identified by GC-MS only, without either reference retention index or comparison with external standards by GC-FID (**Table 3**). Volatile compounds that were positively identified showed a high probability (>70%) when compared with the reference compounds in the NIST 98 library.

Multivariate Approach toward Cultivar and Maturity Stage Discrimination. SLDA was used to identify the compounds that predict cultivar and maturity stage patterns. It

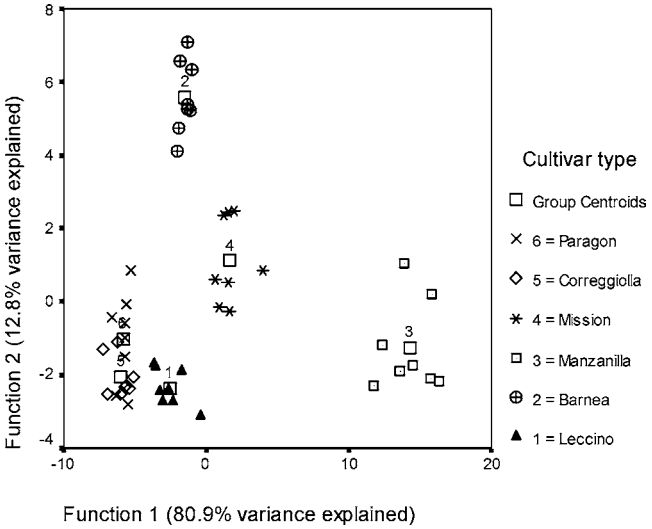

Figure 1. Scatter plot for scores of olive oil volatile compounds based on the first two canonical discriminant functions separating cultivars.

involves variable selection, evaluation of variable contribution to discrimination, and pattern recognition as outlined under Materials and Methods. Important to the successful implementation of SLDA are a stringent criterion ($p = 0.01$) for entry of variables, and evaluation of the Wilks' lambda statistic to indicate the significance of the discriminant functions. The outcome of a discriminant analysis can be visualized in two dimensions by a combined-group scatter plot (e.g., **Figure 1**), where the *x*-axis plots the values of discriminant function 1 and the *y*-axis plots the values of discriminant function 2.

The "percent variance explained" indicates the extent to which the discriminant functions explain the patterns (**Tables 4** and **5**), with higher values indicating a better discrimination. The cumulative percent variance explained for the first two functions in the discrimination of olive cultivars in this study ranged from 84.3 to 93.7% (**Table 4**), values higher that those gained through other multivariate statistical analysis methods, such as principal component analysis (PCA) on monovarietal olive oils, which gave a cumulative percent variance explained on the first two components of 37.2−56.8% (*33*).

Cultivar Discrimination. Discriminant analysis of cultivars was investigated with the olive fruit phenolic compounds, the oil phenolic compounds, the oil volatile compounds, and combined oil phenolic/volatile compounds (**Table 4**). The highest cumulative percent variance explained (93.7%) was observed with oil volatile compounds. Discrimination based on phenolic compounds produced a lower percent variance explained than volatile compounds (~84% for both fruit and oil phenols, **Table 4**). It was observed that scatter plots with higher cumulative percent variance explained on the first two functions had a better discrimination of the cultivars (cf. maturity stage discrimination, below).

In addition to a better discrimination with volatile compounds, the Wilks' lambda statistic for the first two canonical discriminant functions was close to 0 and significantly different ($p < 0.05$), indicating the suitability of the functions to discriminate the cultivar groups. These functions separated the six cultivars into five distinct clusters that were mutually exclusive (**Figure 1**).

In the current study, Manzanilla (3) was separated from Barnea (2) and Mission (4); a cluster was formed for Corregiola (5) and Paragon (6); and Leccino (1) was close to this cluster (**Figure 1**).

Table 4. Cultivar Discrimination by Volatile and Phenolic Compounds from the Olive Oil and Fruit

sample (compounds)	cultivar discriminating compound	% variance explained function 1	function 2	cumulative
fruit (phenols)	hesperidin verbascoside tyrosol luteolin-7-rutinoside hydroxytyrosol	68.0	16.3	84.3
oil (phenols)	tyrosol DHPEA-DEDA ligstroside dialdehyde acetoxy-pinoresinol oleuropein aglycon luteolin	55.7	29.0	84.7
oil (volatiles)	hexanal 1-penten-3-ol hexanol (*E*)-2-nonen-1-ol hexyl acetate 1-dodecene	80.9	12.8	93.7
oil/oil (volatiles/phenols)	hexanal 1-penten-3-ol hexanol (*E*)-2-nonen-1-ol hexyl acetate 1-dodecene tyrosol ligstroside dialdehyde	77.7	12.4	90.1

The best *x*-axis separation (function 1, 80.9% variance explained) was observed for Manzanilla (**Figure 1**), indicating a big difference from the other cultivars. This is consistent with our observation (above) that the C8 compounds, octane, octanal, and octanol, were the predominant volatile compounds for Manzanilla only. The smallest separation on the *x*-axis was between Paragon and Corregiola (**Figure 1**), supporting a report (*34*) that the two cultivars might be from the same Frantoio family. The closeness of Leccino to the Paragon/Corregiola cluster (**Figure 1**) indicates similarities in the volatile profiles of the three cultivars. Function 2 (*y*-axis, 12.8% variance explained) was successful at discriminating Barnea from the rest of the cultivars as shown by the wide separation between the centroids (**Figure 1**). This good separation of the cultivars provided by the olive oil volatile compounds is consistent with earlier studies (*35*) in which three olive cultivars, Leccino, Frantoio, and Cipressino, were distinguished on the basis of their volatile composition.

Compounds That Discriminate Cultivars. To investigate which volatile compounds contribute to the cultivar discrimination in **Figure 1**, it is necessary to examine the "standardized discriminant function coefficients" for the first and second discriminate functions (function 1, V_1, and function 2, V_2, respectively). The relative contribution of the volatile compounds toward the discrimination of cultivars along the *x*-axis of **Figure 1** is given in the linear discriminant equation (V_1, **1**) below.

$$V_1 = 0.84[\text{hexanal}] - 0.72[\text{1-penten-3-ol}] + 0.60[\text{hexanol}] + 0.76[(E)\text{-2-nonen-1-ol}] - 0.10[\text{hexyl acetate}] + 1.18[\text{1-dodecene}] \quad (1)$$

The contributions of the variables were similar in magnitude but different in sign. Group centroids for Mission and Manzanilla lie on the positive side of the *x*-axis (**Figure 1**), indicating

that volatile compounds with positive coefficients [hexanal, hexanol, (*E*)-2-nonen-1-ol, and 1-dodecene] have a greater contribution than the volatile compounds with negative coefficients (1-penten-3-ol and hexyl acetate). Similarly, it can be deduced that 1-penten-3-ol and hexyl acetate discriminate the cultivars on the negative side of the *x*-axis in **Figure 1**—Leccino, Corregiola, and Paragon. This discrimination on V_1 explained more variance (80.9%) than V_2 (12.8%). Of the six cultivars under study, the discrimination of all but one (Barnea) was explained by V_1.

Barnea was discriminated on the *y*-axis of the scatter plot (**Figure 1**) by the second discriminant function (V_2, **2**):

$$V_2 = 1.44[\text{hexanal}] - 0.55[\text{1-penten-3-ol}] + 1.33[\text{hexanol}] - 0.55[(E)\text{-2-nonen-1-ol}] + 0.58[\text{hexyl acetate}] - 0.37[\text{1-dodecene}] \quad (2)$$

In fact, group centroids for both Barnea and Mission, lie on the positive side of the *y*-axis in **Figure 1**, indicating that volatile compounds with positive coefficients (hexanal, hexanol, and hexyl acetate) were important in discriminating these cultivars. The volatile compounds with negative coefficients [1-penten-3-ol, (*E*)-2-nonen-1-ol, and 1-dodecene] were important in discriminating the cultivars on the negative side of the *y*-axis in **Figure 1**—Leccino, Corregiola, Paragon, and Manzanilla.

By combining the effect of both linear discriminant functions (V_1 and V_2), 93.7% of the variance is explained. Thus, it can be concluded that, in this study, pattern recognition in olive cultivars is strongly dependent on volatile compounds. Not all volatile compounds present in the oil are responsible for cultivar discrimination. Of the six compounds listed in **Table 4**, the greatest effects were observed with hexanal and hexanol in the discrimination of Mission, Barnea, and Manzanilla and with 1-penten-3-ol in the discrimination of Leccino, Corregiola, and Paragon.

Various volatile compounds have previously been identified as cultivar markers. Morales et al. (*6*) reported that (*E*)-2-hexenal, (*E*)-3-hexenal, hexanal, butyl acetate, and 2-butanone were responsible for olive cultivar differences among Koroneiki, Koratina, Arbequina, and Picual. The variation in the compounds identified by that study and this may be due to the different cultivars studied, although both studies observed that the occurrence of hexanal is cultivar dependent.

Esti et al. suggested the use of demethyloleuropein as a varietal marker (*31*). It was reported in only two cultivars (Coratina and Leccino) of eight olive cultivars examined [Gentile (Larino), Gentile (Colletorto), Gentile (Santacroce), Coratina, Peranzana, Rosciola, Saligna, and Leccino]. In the current study, however, demethyloleuropein did not significantly ($p < 0.01$) discriminate cultivars. Our results (**Table 4**) indicate that both fruit and oil phenolic compounds explained a lower variance in cultivar groups than oil volatile compounds did. This suggests that oil volatile compounds are better varietal markers than phenolic compounds.

Maturity Stage Discrimination. Pattern recognition of maturity stages was done with olive fruit phenolic compounds, oil phenolic compounds, oil volatile compounds, and oil phenolic/volatile compounds (**Table 5**). All of these provided a cumulative percent variance explained close to 100%, indicating a strong discriminating potential with both volatile and phenolic compounds.

The maximum cumulative percent variance explained (99.9%) was observed with oil volatile compounds. However, the strong influence of the first discriminant function, explaining 93.8% of the variance (**Table 5**), limited the ability of the *y*-axis to discriminate different maturity stages (**Figure 2**). Although group centroids were separated on the *x*-axis, different maturity groups were not mutually separated, except for green and black olives. Points for oil from spotted olives (2) were scattered all over the plot along the *y*-axis (**Figure 2**), indicating that the linear discriminant function 2 was not good at discriminating spotted olives. The Wilks' lambda statistic for function 2 was close to 1, and the means of the scores of the maturity stage groups were not significantly different ($p > 0.05$), confirming the unsuitability of using olive oil volatile compounds to discriminate maturity stages. The lack of good separation of the centroids for a large cumulative percent variance explained (99.9%) illustrates the importance of considering the loading of the scores on the respective discriminant functions to achieve a recognized pattern in samples.

The largest percent variance explained for function 2 was gained when both the volatile and phenolic compounds, from olive oil samples, were included in the analysis (**Figure 3**). Moreover, this was achieved without significant loss in the cumulative percent variance explained (98.2%). The Wilks'

Table 5. Maturity Discrimination by Volatile and Phenolic Compounds from the Olive Oil and Fruit

sample (compounds)	maturity discriminating compounds	% variance explained function 1	function 2	cumulative
fruit (phenols)	hydroxytyrosol luteolin-7-rutinoside ligstroside derivatives	80.6	18.0	98.6
oil (phenols)	oleuropein derivatives oleuropein aglycon luteolin oleuropein hemiacetal	92.8	6.2	99.3
oil (volatiles)	(*E*)-2-hexenal 1-penten-3-ol (*Z*)-2-penten-1-ol hexanol	93.8	6.1	99.9
oil (volatiles/phenols)	(*E*)-2-hexenal 1-penten-3-ol (*Z*)-2-penten-1-ol hexanol tyrosol oleuropein derivative	63.9	34.3	98.2

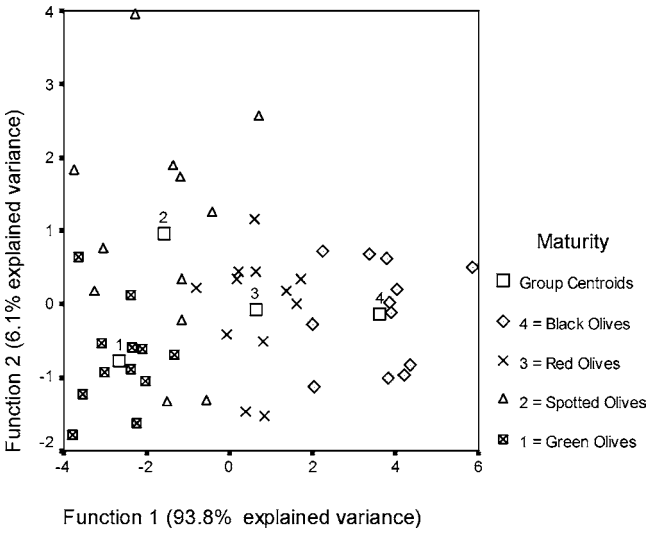

Figure 2. Scatter plot for scores of olive oil volatile compounds based on the first two canonical discriminant functions separating maturity stages.

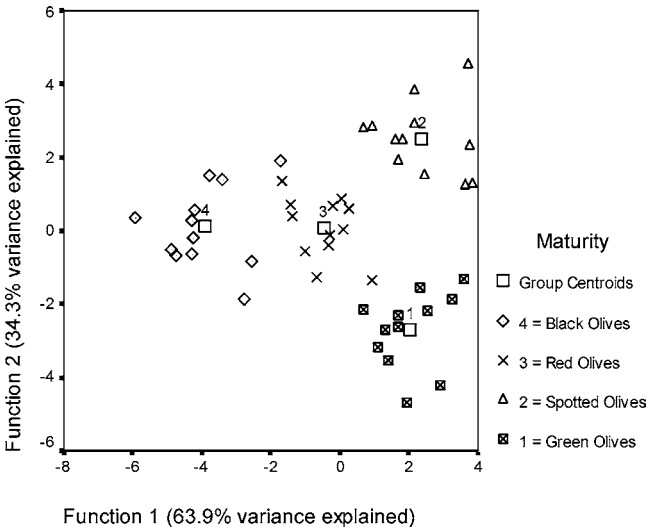

Figure 3. Scatter plot for scores of olive oil volatile and phenolic compounds based on the first two canonical discriminant functions separating maturity stages.

lambda statistic of both functions was close to 0, with the means of the maturity stages scores calculated from both functions significantly different ($p < 0.05$). The combination of olive oil volatile and phenolic compounds clearly separated the green (1) and spotted (2) fruits on the *y*-axis (function 2), and the two maturity stages were further separated from oil of red (3) and black (4) fruits on the *x*-axis (**Figure 3**). Olive oil from black olives (4) had the largest separation with respect to all maturity stage centroids on the *x*-axis. Quantitative data (**Table 6**) support the significant ($p < 0.05$) differences between late (black) and early (green) maturity stages. The two discriminant functions therefore successfully separated the different maturity stages of olives at the green and spotted maturity stages, which were not well separated on function 1, achieving a good separation on the second function. These results show that oil extracted from olives at the late maturity stage (black olives) has chemical characteristics different from those of oil extracted olives at the other maturity stages (**Table 6**) and that a combination of volatile and phenolic compounds achieves a reasonable separation of the maturity stages.

Compounds That Characterize Maturity. The relative contribution of the compounds toward the discrimination of cultivars along the *x*-axis of **Figure 3** is given by the coefficients in the linear discriminant equation below.

$$V_1 = 0.52[(E)\text{-2-hexenal}] - 2.06[\text{1-penten-3-ol}] + 2.28[(Z)\text{-2-penten-1-ol}] - 0.79[\text{hexanol}] - 0.07[\text{tyrosol}] + 0.46[\text{oleuropein derive}] \quad (3)$$

The red (3) and black (4) maturity stages, which are on the negative side of the *x*-axis on the scatter plot (**Figure 3**), are discriminated by compounds with negative coefficients, particularly 1-penten-3-ol and hexanol, which have larger coefficients than tyrosol. However, the compounds with positive coefficients, (*E*)-2-hexenal, (*Z*)-2-penten-1-ol, and oleuropein derivatives, contributed little to discriminating the green (1) and spotted (2) maturity stages on the positive side of the *x*-axis (**Figure 3**).

The green (1) and spotted (2) maturity stages were discriminated on the *y*-axis (**Figure 3**), and the relative contribution of the compounds that discriminated the maturity stages is given through function 2 (V_2, 4) below.

$$V_2 = -1.84[(E)\text{-2-hexenal}] + 2.13[\text{1-penten-3-ol}] - 0.75[(Z)\text{-2-penten-1-ol}] + 1.18[\text{hexanol}] - 1.07[\text{tyrosol}] + 1.42[\text{oleuropein derive}] \quad (4)$$

Discrimination of the green (1) maturity stage, on the negative side of the *y*-axis of the scatter plot (**Figure 3**), is influenced by those compounds with negative coefficients, (*E*)-2-hexenal, (*Z*)-2-penten-1-ol, and tyrosol. The compounds with positive coefficients, 1-penten-3-ol, hexanol, and oleuropein derivatives, had an important contribution in discriminating the spotted (2) maturity stage, which appears on the positive side of the *y*-axis in the scatter plot (**Figure 3**).

Not all compounds available in olive oil contributed to the discrimination of maturity stage groups. The results from both linear discriminant functions (V_1 and V_2), discussed above, show that the volatile compounds (*E*)-2-hexenal and (*Z*)-2-penten-1-ol characterized olive oils extracted from green fruits, whereas 1-penten-3-ol and hexanol discriminated olive oils from spotted, red, and black olives. An earlier study (*15*) concluded that the unripe stage was best characterized by C6 volatile compounds, and this was attributed to alcohols, which had levels of concentrations far apart in different maturity stages. This was not the case in our study as those volatile compounds failed to separate the green maturity stage from the other stages.

In the current study, phenolic compounds characterized the early maturity stages only, in contrast to volatile compounds, which characterized all olive fruit maturity stages. Tyrosol contributed to the discrimination of oil from green olive fruits, whereas oleuropein derivatives contributed to discrimination of oil from spotted olives. Oil from red and black olives had a slight contribution from tyrosol (coefficient of −0.07) in their separation from the early maturity stages. Our findings, showing that oleuropein derivatives (dialdehydes and hemiacetals) significantly ($p < 0.01$) discriminate early from late maturity stages, are consistent with earlier observations (*14*) in which it was reported that the amount of secoiridoids decreased with ripening.

Previously, when fruit phenolic compounds were used as predictors, hydroxytyrosol was reported (*31*) as an indicator of maturation, in agreement with our results when olive fruits were considered (**Table 5**). However, the low percent variance explained for function 2 of 18.0%, compared to a value of 34.3% for the same function when using a combination of oil volatile and phenolic compounds (**Table 5**), justifies the use of the latter for the discrimination of maturity stages. Interestingly, when using olive oil phenolic and volatile compounds as maturity predictors, hydroxytyrosol was not among the compounds that significantly ($p < 0.01$) discriminated the maturity stages (**Table 5**).

Just as maturity predictors may differ depending on whether olive fruit or oil is considered as the basis for discrimination, so, too, maturity markers may change if different discriminating variables or cultivars are used. A study (*15*) based on 10 C6 volatile compounds from Arbequina, Picual, Koroneiki, and Coratina olive cultivars showed that the major indicators of ripeness in olive oil were (*E*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, hexanal, and hexyl acetate. Our results indicate otherwise. This may be due to the different cultivars and volatile compounds studied. The SLDA method used in the present study did not presuppose which volatiles should be included in the analysis, whereas the earlier study (*15*) preselected the volatile compounds for consideration; this preselection may have

Table 6. Quantitative Data for Early (Green) and Late (Black) Maturity Stages Showing the Different Levels for Predictors of Maturity Stages and Cultivars in Olive Oil

cultivar	(*E*)-2-hexenal	hexanal	hexanol	1-penten-3-ol	(*Z*)-2-penten-1-ol	tyrosol	oleuropein derivatives
			Green Maturity				
Leccino	8.0 ± 1.0abcd	2.86 ± 0.02a	<0.03	0.26 ± 0.02a	0.17 ± 0.01def	<1.0	<3.0
Barnea	13.0 ± 0.9def	19.44 ± 0.07d	<0.03	0.30 ± 0.01a	0.155 ± 0.007cdef	<1.0	106 ± 1a
Manzanilla	4.7 ± 0.2a	18.4 ± 0.9d	<0.03	0.14 ± 0.01a	0.15 ± 0.01cde	<1.0	58 ± 5a
Mission	16.7 ± 1.2ef	14.2 ± 0.5c	<0.03	1.12 ± 0.07c	0.24 ± 0.03g	4.0 ± 0.6a	246 ± 16b
Corregiola	37.4 ± 0.9g	3.7 ± 0.4a	<0.03	1.1 ± 0.1c	0.19 ± 0.03ef	<1.0	286 ± 97b
Paragon	38.6 ± 6.3g	4.2 ± 0.9a	<0.03	1.0 ± 0.2c	0.20 ± 0.01fg	<1.0	267 ± 6b
			Black Maturity				
Leccino	6.4 ± 2.2abc	3.2 ± 1.3a	<0.03	0.26 ± 0.07a	0.07 ± 0.02a	<1.0	<3.0
Barnea	17.6 ± 0.7f	7.3 ± 0.4b	0.28 ± 0.03b	0.31 ± 0.01a	0.095 ± 0.007ab	<1.0	<3.0
Manzanilla	5.4 ± 0.2ab	6.7 ± 0.3b	0.215 ± 0.007a	0.36 ± 0.02a	0.115 ± 0.007bc	<1.0	<3.0
Mission	11.2 ± 1.8cd	6.7 ± 0.7b	0.20 ± 0.03a	0.70 ± 0.06b	0.12 ± 0.04bc	3.3 ± 0.2a	<3.0
Corregiola	11.9 ± 0.4 de	4.3 ± 0.1a	<0.03	1.1 ± 0.2c	0.125 ± 0.007bcd	<1.0	<3.0
Paragon	10.4 ± 1.7bcd	4.1 ± 1.3a	<0.03	0.7 ± 0.1b	0.065 ± 0.007a	<1.0	<3.0

[a] Different letters in a column indicate significantly different ($p < 0.05$) mean ± standard deviation in μg/g of duplicates.

influenced the outcome of the analysis. Our results show that the volatile compounds (*E*)-2-hexenal, hexanol, 1-penten-3-ol, and (*Z*)-2-penten-1-ol (**Table 5**) had a significant ($p < 0.01$) contribution toward the discrimination of maturity stages. The C5 compounds, 1-penten-3-ol and (*Z*)-2-penten-1-ol, were not included in the earlier study (*15*).

Maturity Stage and Cultivar Dependence. The contributions of compounds to the discrimination of cultivars and maturity stages are not independent of each other. For instance, in our study, the volatile compounds hexanol and 1-penten-3-ol characterized both cultivar and maturity discrimination. An earlier study (*15*), based on cultivars different from ours, concluded, however, that hexanol did not contribute to ripeness characterization. Another volatile compound that has shown cultivar and maturity dependence is (*E*)-2-hexen-1-ol. A study of Arbequina, Picual, Koroneiki, and Coratina reported (*15*) that (*E*)-2-hexen-1-ol was one of the major contributors toward ripeness characterization, but significant differences in concentration of this compound were not observed in Carolea and Gentile di Chieti olive cultivars (*36*). This dependence of compounds responsible for characterizing both cultivar and maturity calls for careful consideration in the identification of maturity and varietal markers.

The results from this study illustrate the value of multivariate analysis with SLDA in identifying compounds that are responsible for cultivar and maturity stage patterns. Olive cultivar strongly influenced the abundance of volatile compounds, in particular, hexanol, hexanal, and 1-penten-3-ol. Maturity stage was discriminated best by both volatile and phenolic compounds. This approach may be applied to selectively produce olive oil with particular attributes (sensory or stability) from chosen cultivars at certain maturity stages.

ABBREVIATIONS USED

DVB-CAR-PDMS, divinylbenzene−carboxen−polydimethylsiloxane; GC-MS, gas chromatography−mass spectrometry; HPLC-DAD, high-performance liquid chromatography−diode array detector; IOOC, International Olive Oil Council; LC-ESI-MS, liquid chromatography−electrospray ionization−mass spectrometry; LDA, linear discriminant analysis; MI, maturity index; PCA, principal component analysis; SLDA, stepwise linear discriminant analysis; SPME-GC-FID, solid-phase microextraction−gas chromatography−flame ionization detection.

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Supporting Information Available: Tables 7 and 8 showing dissimilarity matrices for the cultivar discrimination and maturity stage discrimination, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

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