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Variability of 4-Monomethylsterols and 4,4′-Dimethylsterols in Olive Oil and Their Use as Indicators of Olive Variety, Ripening Degree, and Oil Storage Temperature

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Supporting Information

ABSTRACT: To investigate the variability of 4-monomethylsterols and 4,4′-dimethylsterols in olive oil as a result of variety, ripening, and storage temperature, 36 samples were subjected to gas chromatography with flame ionization detection (GC-FID) and with mass spectrometric detection (GC-MS), and results were processed by univariate and multivariate statistics. Relative amounts (percent) of β-amyrin, cycloartenol, and 24-methylenecycloartanol accounted for the most variation due to variety, while citrostadienol (percent) and 24-methylenecycloartanol (milligrams per 100 g) were strongly affected by ripening. Multivariate statistics differentiated olive oils regardless of storage conditions, which implied the possibility to use 4-monomethyl- and 4,4′-dimethylsterols as indicators of variety and ripening degree for fresh and stored oils. Absolute changes in 4-monomethyl- and 4,4′-dimethylsterols after storage were of a much smaller magnitude, meaning the investigated olive oils essentially retained health-beneficial features that derive from these compounds. Relative changes caused by storage were specific for each storage temperature and were useful in discriminating oils by linear discriminant analysis.

KEYWORDS: *Olea europaea L., olive oil, 4-monomethylsterols, 4,4′-dimethylsterols, variety, ripening degree, storage, multivariate statistical analysis*

INTRODUCTION

Phytosterols in olive (*Olea europaea* L.) oil and in other vegetable oils can be divided into three main classes according to the number of methyl groups at the C-4 position: 4-desmethylsterols (sterols), 4-monomethylsterols, and 4,4′-dimethylsterols (triterpene alcohols).[1,2] The latter two are generally considered to be intermediates in the biosynthesis of 4-desmethylsterols;[2] they share common biosynthetic precursors up to (3*S*)-2,3-oxidosqualene (OS), which is subsequently cyclized into a large number of steroidal and nonsteroidal triterpenic compounds by the action of different OS cyclases also known as triterpene synthases. Cycloartenol synthase cyclizes the pre-chair-boat-chair OS conformer into cycloartenol, which is the first cyclic precursor of the sterol pathway. Non-sterol triterpenoids are formed from OS folded in the all-pre-chair conformation by other enzymes.[3] The most predominant 4-monomethylsterols in olive oil are obtusifoliol, cycloeucalenol, gramisterol, and citrostadienol, while the main 4,4′-dimethylsterols are amyrins, butyrospermol, cycloartenol, and 24-methylenecycloartanol.[4] 4-Monomethylsterols, 4,4′-dimethylsterols, and sterols in general are part of a larger chemical group of triterpenoids, which contribute to plant defense by producing triterpenic phytoalexins or saponins as a response to biotic and abiotic stress and have an important role in affecting plant water permeability and in plant−insect interaction.[5]

The interest of scientists to study 4-monomethyl- and 4,4′-dimethylsterols derives from the fact that they exhibit certain health benefits through their antimicrobial, antitumoral, antiviral, anti-inflammatory, analgesic, antimycotic, cytotoxic, hepatoprotective, virostatic, immunomodulatory, tonic, antifeedant, and insecticidal activities.[5−7] They were found to be important constituents in various plant materials and products such as tomato,[8,9] wild peanut,[6] maize,[10] avocado oil,[11] sea buckthorn seed oil,[12] etc., as well as, more recently, in products of animal origin such as bee pollen[13] and ham subcutaneous fat.[14] As for olive oil, it was reported that citrostadienol behaved as an antioxidant in oils kept at 180 °C,[15] while another study has proven that 4,4′-dimethylsterols can delay oil degradation when subjected to prolonged heating.[16] 4-Monomethylsterols and 4,4′-dimethylsterols were proposed as indicators of the admixture of olive oil with hazelnut oil,[1,17] as well as with other vegetable oils.[2]

Variety and ripening degree are among the most important factor influencing the levels of all important constituents in olive and olive oil, such as triacylglycerols,[18] phenols,[19] fatty acids,[20] waxes,[21] aliphatic alcohols,[22] aromas,[23] etc. The class of 4-desmethylsterols (commonly simply referred to as sterols) has already been investigated in this context in many works, and the effects of variety and ripening on their content were shown to be substantial.[20,24−29]

On the contrary, and despite their importance, the classes of 4-monomethyl- and 4,4′-dimethylsterols, which are the object of this study, have been investigated very poorly. The probable partial reason is that only recently were they introduced to the

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list of compounds for which methods of analysis are standardized and authorized by the International Olive Council (IOC).[30] Their response to even some of the most influential sources of variability is practically unknown. Only a few reports studying their intervarietal variability have been published, focusing only on particular major compounds.[27,28,31] No experimental evidence on the effect of ripening on 4-monomethyl- and 4,4′-dimethylsterols in olive oil can be found in available literature; to date, only their behavior in olive fruit has been investigated,[2,3] which can differ significantly. There are no published data on the interdependence and the interactive effect of variety and ripening on 4-monomethyl- and 4,4′-dimethylsterol concentrations. Among the olive oil research and expert community, investigations on indicators of olive oil aging are the focus of interest, mainly because olive oil quality deteriorates with storage, which is directly related to its market categorization and price. Such research is currently in full swing[32] but not a single study to date has addressed the effect of storage temperature and storage in general on the contents of 4-monomethyl- and 4,4′-dimethylsterols in any vegetable oils.

The aim of this study was to investigate the variability of the contents of major 4-monomethyl- and 4,4′-dimethylsterols in olive oil as a result of three key factors, variety, ripening degree, and storage, including the effect of storage temperature. Besides providing new knowledge about the chemistry of olive oil and its important constituents in general, the objective was to assess whether 4-monomethyl- and 4,4′-dimethylsterols have the potential for satisfactory multivariate discrimination and could be used as indicators for each of the mentioned factors of variability. Reliable and stable indicators among these constituents could contribute to the ever-growing scientific area of research on olive oil quality and authenticity and could be used for more reliable characterization, differentiation, authentication, and certification of olive oils of specific origin and attributes. It is expected that the results obtained in this work could have practical application and be useful for producers to optimize individual steps in production to obtain olive oils with targeted 4-monomethyl- and 4,4′-dimethylsterol composition.

MATERIALS AND METHODS

Sampling and Harvesting. Three important autochthonous varieties in the Istria region of Croatia were chosen for the investigation: Buža, Črna, and Rosinjola. In the crop year 2006/2007, nonirrigated trees were identified and carefully marked on different plots located in the corresponding characteristic production zone of each variety. The plots were located in the region of Istria (Croatia) within a radius of approximately 18 km and were characterized by similar pedoclimatic conditions.

Olives were harvested at three ripening degrees denoted as green (RD1), spotted (RD2), and ripe (RD3). For Buža variety, ripening indexes were 1.4, 2.8, and 3.8; for Črna, 1.9, 2.8, and 4.1; and for Rosinjola, 1.5, 3.2, and 3.8, respectively. Each batch of olive fruits giving a single olive sample comprised olives handpicked from three single trees of a given variety at the determined stage of ripeness. Ripening index (RI) was determined from 100 olive fruits, randomly taken from each olive fruit batch. This parameter is a function of fruit color in both skin and pulp and was determined according to Beltrán et al.[33]

Oil Extraction. Each oil sample was extracted from approximately 100 kg of olive fruits transported to the mill immediately after harvest and processed within 24 h. Oils were obtained by the continuous cold automatic olive oil extraction system Oliomio Cultivar 500 (Toscana Enologica Mori Snc., Italy). Olives were washed and crushed with a stainless steel hammer crusher. For each sample, the olive paste was transported to the vertical unit where it was malaxed for 35 ± 2 min at a temperature below 27 °C. Oils were obtained by separation in a horizontal two-phase centrifuge. Oils were filtered through cotton wool to eliminate larger impurities. Samples were stored in amber glass 125 mL bottles. Nine oil samples, representing combinations of three varieties and three ripening degrees, were analyzed within a month of oil extraction (fresh oil samples).

Storage Conditions. In total, 27 oil samples representing each variety/ripening degree combination were stored in the dark for a period of 12 months under three different temperature conditions. Besides variable room temperature (VR, 10−28 °C), which was supposed to simulate common stock and domestic conditions, refrigeration (RE, 4 °C) and freezing (FR, −20 °C) temperatures, which are usually applied for conservation of various other foodstuffs and were recently found to preserve olive oil quality,[34] were also included. Stored samples were analyzed within a month after the 12 month storage period.

Extraction of 4-Monomethylsterols and 4,4′-Dimethylsterols. 4-Monomethylsterol and 4,4′-dimethylsterol analysis was conducted according to the method proposed by IOC[30] and the modified method for the determination of aliphatic alcohols adopted by EEC regulations.[35] Five grams of olive oil was added to 1-eicosanol (5 mg/100 g of olive oil) (Supelco, Bellefonte, PA), used as internal standard, and were saponified with 50 mL of potassium hydroxide ethanolic solution. After the sample was boiled, 50 mL of distilled water was added, the flask was cooled to approximately 30 °C, and the content was quantitatively transferred to a 500 mL separating funnel by use of distilled water portions of a total volume of 50 mL. The extraction of unsaponifiable fraction was carried out with three portions of diethyl ether (80 mL + 70 mL + 70 mL) by vigorous shaking. The ethyl ether extracts were combined in a separating funnel and washed with distilled water (50 mL at a time) until the washing water gave a neutral reaction. Following purification with water and drying over sodium sulfate, diethyl ether was evaporated under vacuum and nitrogen and then completing drying in an oven at 100 °C for 15 min. Unsaponifiable fraction was dissolved in chloroform, and approximately 20 mg was loaded on a basic silica thin-layer chromatography (TLC) plate (Fluka, Buchs, Switzerland). The 4-monomethylsterol and 4,4′-dimethylsterol fraction (the sum of free and esterified forms without glycosides) was separated by elution with a mixture of hexane and diethyl ether (65:35 v/v). The corresponding bands were visualized under UV light after being sprayed with 2′,7′-dichlorofluorescein 0.2% ethanolic solution, then scraped off with a spatula and extracted with chloroform and diethyl ether. After the extract was evaporated to dryness, 4-monomethylsterols and 4,4′-dimethylsterols were converted to trimethylsilyl ethers by addition of pyridine/hexamethyldisilizane/trimethylchlorosilane (9:3:1 v/v/v) (Supelco, Bellefonte, PA), left for 15 min, and then centrifuged at 3500 rpm for 5 min.

Identification of 4-Monomethyl- and 4,4′-Dimethylsterols. Identification of 4-monomethyl- and 4,4′-dimethylsterols as trimethylsilyl ethers was carried out by gas chromatography/mass spectrometry (GC-MS) on a Varian 3900 gas chromatograph coupled with a Varian Saturn 2100T ion trap mass spectrometer (Varian Inc., Harbour City, CA). The fused silica column used was a 30 m × 0.25 mm i.d. × 0.25 μm film thickness Varian VF-5 ms capillary column (5% phenylpolysiloxane/95% dimethylpolysiloxane). Injector temperature was 280 °C. The GC oven parameters were as follows: initial temperature was 180 °C, increased at 2 °C/min to 280 °C, and then kept at 280 °C (total run time approximately 65 min). Transfer line, manifold, and ion trap temperatures were 285, 80, and 220 °C, respectively. Helium was used as a carrier gas with a constant flow of 1.2 mL/min. One microliter of the analytical sample was injected in split mode (1:50). Mass spectra were acquired in the electron impact mode (70 eV) at 1 scan/s, using full scan with a mass acquisition range of 30−650 m/z. Identification of compounds was performed by careful interpretation of retention data and mass spectra and by comparisons with retention and mass spectral data from the literature[1,5,6,8−13,36−39] and NIST05 library when available (trimethylsilyl ethers or acetates).

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Table 1. Retention Time and Mass Spectrometric Data[a]

compd	t_R, min	major and characteristic ions, m/z (relative intensity)
		4-Monomethylsterols
obtusifoliol	49.83	41 (32), 67 (25), 69 (30), 73 (38), 75 (30), 81 (30), 95 (38), 109 (32), 189 (43), 227 (36), 393 (100), 394 (29), 483 (33), 498 (14)
gramisterol + cycloeucalenol	51.45	55 (28), 69 (22), 73 (50), 75 (40), 105 (31), 121 (18), 159 (31), 227 (30), 241 (43), 267 (40), 269 (20), 357 (81),, 379 (25), 393 (23), 394 (20), 407 (100), 408 (34), 469 (16), 483 (6), 484 (7), 498 (12), 512 (8)
citrostadienol	55.52	55 (19), 73 (15), 75 (15), 145 (11), 227 (10), 241 (7), 267 (40), 295 (12), 310 (8), 357 (100), 358 (29), 393 (10), 400 (17), 408 (3), 483 (5), 498 (2)
		4,4′-Dimethylsterols
δ-amyrin	49.48	69 (30), 73 (65), 75 (56), 95 (62), 109 (54), 121 (65), 135 (42), 147 (48), 189 (100), 190 (39), 203 (49), 204 (81), 218 (35), 269 (93), 284 (38), 359 (32), 393 (32), 483 (8), 498 (5)
β-amyrin	50.20	73 (23), 75 (17), 95 (15), 119 (14), 135 (10), 147 (14), 189 (41), 190 (17), 203 (100), 204 (17), 218 (40), 393 (4), 483 (2), 498 (1)
butyrospermol	50.88	41 (15), 69 (19), 73 (20), 75 (14), 81 (14), 95 (13), 109 (14), 241 (18), 393 (100), 394 (30), 483 (10), 498 (4)
cycloartenol	52.12	41 (36), 69 (45), 73 (43), 95 (38), 109 (31), 189 (19), 271 (9), 339 (35), 365 (49), 393 (100), 394 (30), 408 (17), 483 (8), 498 (3)
24-methylenecycloartanol	53.94	55 (31), 69 (63), 73 (65), 81 (50), 95 (70), 107 (58), 109 (50), 135 (50), 147 (52), 269 (24), 297 (22), 353 (44), 379 (83), 407 (100), 408 (30), 422 (28), 497 (8), 512 (2)

[a]For trimethylsilyl ethers of 4-monomethylsterols and 4,4′-dimethylsterols identified in olive oils by GC-MS analysis.

The obtained retention time and mass spectrometric data for the corresponding trimethylsilyl ethers are reported in Table 1. Four major 4-monomethylsterols (obtusifoliol, gramisterol + cycloeucalenol, and citrostadienol) and five major 4,4′-dimethylsterols (δ-amyrin, β-amyrin, butyrospermol, cycloartenol, and 24-methylenecycloartanol) were identified. The identity of β-amyrin and cycloartenol was confirmed by comparison of their retention times and mass spectra to those of pure standards [β-amyrin analytical standard, ≥98.5%, Fluka (Buchs, Switzerland); cycloartenol, ≥90.0%, Sigma−Aldrich (St. Louis, MO)], which were the only standards of 4-monomethyl- and 4,4′-dimethylsterols analyzed in this work commercially available.

Quantitative Determination of 4-Monomethyl- and 4,4′-Dimethylsterols. Quantitative determination of 4-monomethyl- and 4,4′-dimethylsterols as trimethylsilyl ethers was carried out by capillary gas chromatography with flame ionization detection (GC-FID) on a Varian 3350 GC (Varian Inc., Harbour City, CA) equipped with the same column described above. Injector and oven were the same as described above, while detector temperature was 290 °C, for approximately 65 min. Helium was used as a carrier gas with a flow rate of 1.27 mL/min. One microliter was injected in split mode (1:50). A typical GC-FID chromatogram of 4-monomethyl- and 4,4′-dimethylsterols as trimethylsilyl ethers is presented in Figure 1. 4-Monomethyl- and 4,4′-dimethylsterol concentrations were expressed as milligrams per 100 g of internal standard, with an assumed response factor equal to 1. Relative amounts of individual compounds were expressed as percentages of total 4-monomethyl- + 4,4′-dimethylsterol peak area (all nine compounds).

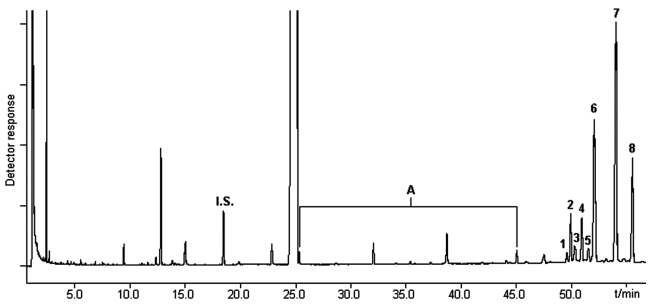

Figure 1. GC-FID chromatogram of trimethylsilyl ethers of 4-monomethyl- and 4,4′-dimethylsterols in Buža olive oil (green olives, RD1). Peak identification: I.S., internal standard (1-eicosanol); A, aliphatic alcohols; 1, δ-amyrin; 2, obtusifoliol; 3, β-amyrin; 4, butyrospermol; 5, gramisterol + cycloeucalenol; 6, cycloartenol; 7, 24-methylenecycloartanol; 8, citrostadienol.

Analyses were performed in duplicate, and average values were used in further data elaboration. The precision of the method was high, with relative standard deviations (n = 2) generally not surpassing 7% and in the case of major compounds, such as citrostadienol, butyrospermol, cycloartenol, and 24-methylenecycloartanol, not surpassing 4%.

To determine linearity, three replicate analyses of a sample spiked with three different concentrations of chemical standards of β-amyrin (2, 5, and 10 mg/100 g of oil) and cycloartenol (10, 30, and 50 mg/100 g of oil) were performed. Correlation coefficients were satisfactory: 0.997 for β-amyrin and 0.993 for cycloartenol. The same data were used for determination of recovery, which ranged from 92% to 103% with an average of 96% for β-amyrin (n = 9) and from 94% to 100% with an average of 98% for cycloartenol (n = 9).

Limits of detection (LOD) were estimated as 3 times the baseline noise and ranged from 0.056 mg/100 g for obtusifoliol to 0.092 mg/100 g for β-amyrin. Limits of quantification (LOQ) were estimated as 10 times the baseline noise and ranged from 0.185 mg/100 g for obtusifoliol to 0.306 mg/100 g for β-amyrin. Since LOD and LOQ for individual compounds change depending on FID sensitivity, average detection limits registered during method validation and analyses were reported.

Analysis of 4-Desmethylsterols. Analysis of 4-desmethylsterols was described in a previous study.[29] Only total concentrations of 4-desmethylsterols (milligrams per 100 g), obtained as the sums of concentrations of individual 4-desmethylsterols, are reported in this work.

Statistical Analysis. Duplicate analysis results were used to calculate average values, which were used in further data elaboration. Variables used for statistical analysis were average values of concentration (milligrams per 100 g) and relative amount (percent) of all analyzed compounds. These data are presented in Tables 3 and 4 without the corresponding deviations. The main-effect three-way analysis of variance (ANOVA) without replication (σ-restricted parametrization, effective hypothesis decomposition)[40] was selected to evaluate the effect of factors variety, ripening degree, and storage temperature on the content of individual and total major 4-monomethyl- and 4,4′-dimethylsterols in olive oil. The results for total 4-desmethylsterols are also presented. Because it was a nonreplicated experiment, the effects of all first- and second-order interactions were treated as random and pooled into error term. The majority of variables exhibited low variability with no statistically significant differences between means for the storage temperature factor (Table 2), so their values were not reported. Mean values for the factors variety and ripening degree are expressed together with the corresponding standard errors. Statistically significant differences were determined by Tukey's honest significant difference (HSD) posthoc analysis. Statistical significance was set at $p < 0.05$ (Tables 3 and 4).

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Table 2. Results of Analysis of Variance for Olive Oils[a]

	variable factor					
	variety		ripening degree		storage condition	
compd	*F*-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value
4-Monomethylsterols, Concentration (mg/100 g)						
obtusifoliol	13.368	0.000	4.881	0.015	2.998	0.047
gramisterol + cycloeucalenol	8.738	0.001	12.197	0.000	1.366	0.274
citrostadienol	34.851	0.000	3.801	0.035	2.826	0.057
4-Monomethylsterols, Relative Amount (%)						
obtusifoliol	2.270	0.122	39.087	0.000	5.152	0.060
gramisterol + cycloeucalenol	5.751	0.008	7.516	0.002	1.143	0.349
citrostadienol	30.543	0.000	163.903	0.000	1.112	0.361
4,4′-Dimethylsterols, Concentration (mg/100 g)						
δ-amyrin	2.731	0.083	0.684	0.513	0.571	0.638
β-amyrin	41.995	0.000	11.846	0.000	0.482	0.698
butyrospermol	38.423	0.000	0.960	0.395	0.953	0.429
cycloartenol	31.831	0.000	34.209	0.000	1.049	0.386
24-methylenecycloartanol	6.175	0.006	91.930	0.000	0.622	0.607
4,4′-Dimethylsterols, Relative Amount (%)						
δ-amyrin	0.769	0.473	45.623	0.000	0.303	0.823
β-amyrin	109.962	0.000	18.156	0.000	0.795	0.507
butyrospermol	17.361	0.000	26.116	0.000	0.018	0.997
cycloartenol	53.257	0.000	16.817	0.000	0.095	0.962
24-methylenecycloartanol	52.011	0.000	77.235	0.000	0.019	0.996
Total Concentration (mg/100 kg)						
4-monomethylsterols	31.652	0.000	4.067	0.028	1.735	0.183
4,4′-dimethylsterols	0.796	0.461	70.782	0.000	1.012	0.402

[a]Main-effect three-way analysis of variance (ANOVA) without replication for concentrations (milligrams per 100 grams) and relative amounts (percent) of 4-monomethylsterols and 4,4′-dimethylsterols in olive oils, with variety, ripening degree, and storage condition as variable factors. $p < 0.050$ is statistically significant.

Before being subjected to unsupervised (principal component analysis, PCA) and supervised (stepwise linear discriminant analysis, SLDA) multivariate statistical analysis, GC data were standardized (centered) via the autoscale procedure, in which a variable is subtracted from its mean value and the obtained difference is divided by the standard deviation of this variable throughout the data set. For the factors variety and ripening degree, unrotated PCA was carried out by use of variables selected according to their high *F*-statistic values. Kaiser's rule was followed for selection of the number of principal components (PCs), and only factors with eigenvalues higher than 1.00 were retained. PCA models with the best separation capacity are presented. For a valid statistical classification of olive oil samples into categories according to variety or ripening degree, forward SLDA was applied, with Wilk's λ as a selection criterion and an *F*-statistic factor to establish the significance of the changes when a new variable is tested. The value of *F* for a variable to enter the differentiation model was fixed at 1.00, and minimum tolerance was set at 0.01. For presentation of changes as a result of storage by SLDA, variables were previously standardized through all levels of each single subject separately (the same sample fresh and stored at three temperatures) to eliminate the effect of variety and ripening degree. In this way relative changes, expressed as an increase or a decrease, are presented.

Statistical data elaboration was carried out with Statistica v 8.0 software (Stat-Soft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Results of three-way analysis of variance (ANOVA) for individual (milligrams per 100 g and percent) and total 4-monomethyl- and 4,4′-dimethylsterols in different samples of olive oil, with variety, ripening degree, and storage condition as factors, are presented in Table 2. A significant effect of variety ($p < 0.05$) was determined for the majority of compounds. The highest *F*-statistic values were found for concentrations of *β*-amyrin, butyrospermol, citrostadienol, and cycloartenol, as well as for relative amounts of *β*-amyrin, cycloartenol, and 24-methylenecycloartanol. A significant effect of ripening degree ($p < 0.05$) with the highest *F* values was found for concentrations of 24-methylenecycloartanol and for relative amounts of citrostadienol, 24-methylenecycloartanol, and *δ*-amyrin. Total 4-monomethylsterols were found to be more affected by variety, while total 4,4′-dimethylsterols varied more significantly due to ripening degree. Evidently, variability arising from the effect of storage was much lower than that caused by the other two factors. For this reason, mean values for the storage condition factor are not reported in Tables 3 and 4 together with those for variety and ripening degree.

Variability as a Result of Olive Variety. Buža variety was found to contain the lowest concentrations and relative amounts of 4-monomethylsterols, while Rosinjola had the highest citrostadienol level (Table 3). Among 4,4′-dimethylsterols, Buža was characterized by the highest 24-methylenecycloartanol and the lowest butyrospermol levels. Rosinjola was the most abundant in *β*-amyrin and cycloartenol, and it exhibited the lowest 24-methylenecycloartanol content. The lowest content of *β*-amyrin was found in Črna oils (Table 4). The differences in total 4,4′-dimethylsterols between mean values for the investigated varieties were not significant (Table 2).

When PCA was applied on the data set comprising 36 cases (olive oil samples) and variables with the highest *F* values for the variety factor (Table 2), a model with butyrospermol (milligrams per 100 g), citrostadienol (milligrams per 100 g), *β*-amyrin (percent), and cycloartenol (percent) as variables was found to have the best separation capacity (Figure 2). Two

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Table 3. Concentrations and Relative Amounts of 4-Monomethylsterols in Olive Oils[a]

	olive variety												
	Buža				Črna				Rosinjola				
ripening degree	CO	VR	RE	FR	CO	VR	RE	FR	CO	VR	RE	FR	mean (ripening degree)
Obtusifoliol Concentration (mg/100 g)													
green	6.06	6.33	6.30	7.37	8.94	8.74	8.62	10.62	7.33	8.35	9.56	8.31	8.04 ± 0.40 b
spotted	7.02	8.63	8.75	10.13	8.42	8.71	9.40	9.11	9.67	11.41	10.14	8.85	9.19 ± 0.32 a
ripe	7.51	6.88	7.64	7.91	8.33	9.31	9.88	10.97	7.76	9.89	9.48	8.45	8.67 ±0.35 ab
mean (variety)		7.54 ± 0.34 b				9.25 ± 0.24 a				9.10 ± 0.33 a			
Gramisterol + Cycloeucalenol Concentration (mg/100 g)													
green	2.34	2.56	2.37	2.22	3.70	3.84	3.64	3.61	2.08	2.27	2.30	1.89	2.74 ± 0.21 b
spotted	2.71	3.05	2.90	2.80	3.90	3.95	4.08	3.86	5.50	5.85	5.25	4.43	4.02 ± 0.31 a
ripe	3.46	3.35	3.53	2.91	3.82	4.03	3.89	3.62	4.15	5.44	4.38	3.22	3.82 ± 0.19 a
mean (variety)		2.85 ± 0.13 b				3.83 ± 0.05 a				3.90 ± 0.43 a			
Citrostadienol Concentration (mg/100 g)													
green	16.60	14.84	17.40	15.80	22.89	22.24	23.42	22.60	20.64	22.71	23.08	19.74	20.16 ±0.92 ab
spotted	15.91	17.88	17.25	16.87	21.54	21.41	22.75	21.28	25.29	27.58	24.15	20.42	21.03 ± 1.04 a
ripe	15.49	13.67	16.19	12.39	16.89	17.30	17.87	16.22	25.13	27.75	26.03	17.81	18.56 ± 1.43 b
mean (variety)		15.86 ± 0.46 c				20.53 ± 0.77 b				23.36 ± 0.92 a			
Total 4-Monomethylsterol Concentration (mg/100 g)													
green	25.01	23.73	26.08	25.40	35.53	34.82	35.68	36.82	30.04	33.34	34.93	29.94	30.94 ± 1.40 b
spotted	25.63	29.56	28.90	29.80	33.86	34.07	36.24	34.25	40.45	44.84	39.54	33.71	34.24 ± 1.58 a
ripe	26.46	23.90	27.36	23.20	29.04	30.63	31.64	30.81	37.04	43.08	39.88	29.48	31.04 ±1.77 ab
mean (variety)		26.25 ± 0.65 b				33.62 ± 0.72 a				36.36 ± 1.52 a			
Obtusifoliol Relative Amount (%)													
green	5.88	6.78	5.87	7.30	6.38	6.33	6.01	7.42	6.51	6.76	7.55	7.76	6.71 ± 0.19 a
spotted	5.42	5.99	6.20	7.19	5.60	5.78	5.83	6.11	5.38	5.91	5.79	5.82	5.92 ± 0.14 b
ripe	3.65	3.75	3.60	4.28	4.97	5.38	5.44	6.61	3.99	4.86	4.62	4.69	4.65 ± 0.26 c
mean (variety)		5.49 ± 0.39				5.99 ± 0.19				5.80 ± 0.34			
Gramisterol + Cycloeucalenol Relative Amount (%)													
green	2.26	2.75	2.21	2.20	2.64	2.78	2.54	2.52	1.85	1.84	1.81	1.77	2.26 ± 0.11 ab
spotted	2.09	2.12	2.06	1.99	2.59	2.62	2.53	2.59	3.06	3.03	3.00	2.91	2.55 ± 0.12 a
ripe	1.69	1.83	1.66	1.57	2.28	2.33	2.14	2.17	2.13	2.67	2.13	1.79	2.03 ± 0.10 b
mean (variety)		2.04 ± 0.09 b				2.48 ± 0.06 a				2.33 ± 0.16 ab			
Citrostadienol Relative Amount (%)													
green	16.03	15.92	16.20	15.64	16.33	16.10	16.33	15.80	18.34	18.38	18.23	18.45	16.81 ± 0.33 a
spotted	12.29	12.41	12.23	11.97	14.32	14.20	14.11	14.27	14.07	14.28	13.77	13.43	13.45 ± 0.27 b
ripe	7.54	7.45	7.63	6.70	10.08	10.00	9.83	9.77	12.92	13.64	12.68	9.90	9.85 ± 0.66 c
mean (variety)		11.84 ± 1.07 c				13.43 ± 0.79 b				14.84 ± 0.81 a			

[a]Olive oils are Buža, Črna, and Rosinjola varieties obtained from green, spotted, and ripe olives (control, CO) and stored for 12 months at variable room temperature (VR, 10−28 °C), refrigeration (RE, 4 °C), and freezing (FR, −20 °C) temperatures. Different letters within a group represent statistically significant different means at $p < 0.05$ for the variety and ripening degree factors obtained by main-effect three-way ANOVA and Tukey HSD posthoc test. Mean values are reported together with the corresponding standard errors. Mean values for the storage temperature factor are not reported because of low variability with no statistically significant differences for the majority of compounds. Average values of two GC determinations for each variety/degree of ripening/storage temperature combination are represented without standard deviation.

principal components (PCs) were extracted, explaining 86.9% of total variance of the set. Figure 2a shows the projection of olive oil samples along the directions of PC1 and PC2, while the corresponding factor loading plots that establish relative importance of the variables are reported in Figure 2b. Rosinjola oils were differentiated from Buža and Črna along the direction of PC1 by higher percentages of β-amyrin and cycloartenol. Črna oils were associated with higher concentrations of butyrospermol and were differentiated from Buža and Rosinjola oils by PC2.

SLDA was applied on the whole data set (36 cases, 16 variables) and succeeded in classifying oils according to varietal origin correctly. Seven variables were selected according to Wilks' λ criterion. β-Amyrin (percent) entered the model as the first variable and classified 100% of Rosinjola oils correctly. Inclusion of butyrospermol (percent) and δ-amyrin (percent) resulted in 100% correct classification of all 36 oils. Gramisterol + cycloeucalenol (percent), cycloartenol (percent), obtusifoliol (milligrams per 100 g), and citrostadienol (milligrams per 100 g) additionally improved the differentiation capacity of the model.

Single variables (milligrams per 100 g and percent) or their combination, which were found to account for the most variability arising from varietal origin, could be considered as relatively stable indicators of variety, since the intravarietal variability due to ripening degree and storage condition did not compromise their differentiating power. 4-Monomethyl- and 4,4′-dimethylsterols, mostly the major components 24-methylenecycloartanol, cycloartenol, and citrostadienol, were previously found to be useful for characterization and differentiation of monovarietal olive oils, but only in combination

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Table 4. Concentrations and Relative Amounts of 4,4′-Dimethylsterols in Olive Oils[a]

ripening degree	olive variety: Buža CO	Buža VR	Buža RE	Buža FR	Črna CO	Črna VR	Črna RE	Črna FR	Rosinjola CO	Rosinjola VR	Rosinjola RE	Rosinjola FR	mean (ripening degree)
δ-Amyrin Concentration (mg/100 g)													
green	1.47	1.61	1.40	1.38	2.07	2.05	1.96	2.03	2.02	2.01	2.16	1.85	1.83 ± 0.08
spotted	1.60	1.71	1.67	1.71	1.96	1.80	2.02	1.92	1.77	1.80	1.73	1.52	1.77 ± 0.04
ripe	2.18	1.95	2.18	1.96	1.80	1.87	1.88	1.81	1.69	1.81	1.73	1.52	1.86 ± 0.05
mean (variety)		1.73 ± 0.08				1.93 ± 0.03				1.80 ± 0.06			
β-Amyrin Concentration (mg/100 g)													
green	3.14	2.29	3.25	3.19	3.93	3.90	3.96	4.27	5.12	5.52	5.76	4.89	4.10 ± 0.31 b
spotted	3.68	4.22	4.15	4.43	3.52	3.58	3.91	3.70	6.41	6.96	6.54	5.53	4.72 ± 0.37 b
ripe	5.89	5.42	6.01	5.58	3.45	3.72	3.86	3.82	6.81	7.20	7.36	6.27	5.45 ± 0.41 a
mean (variety)		4.27 ± 0.35 b				3.80 ± 0.07 b				6.20 ± 0.24 a			
Butyrospermol Concentration (mg/100 g)													
green	5.86	5.13	6.08	5.67	12.02	11.60	12.50	12.29	11.92	12.82	13.01	10.95	9.99 ± 0.93
spotted	6.68	7.45	7.17	7.08	11.51	11.52	12.13	10.89	9.44	9.97	9.37	8.10	9.28 ± 0.56
ripe	9.06	7.87	9.34	8.24	10.77	10.74	11.69	10.33	9.35	10.37	9.82	8.60	9.68 ± 0.33
mean (variety)		7.14 ± 0.39 b				11.50 ± 0.20 a				10.31 ± 0.46 a			
Cycloartenol Concentration (mg/100 g)													
green	27.66	24.83	28.74	26.61	37.18	36.80	38.46	37.78	42.84	46.96	47.77	40.32	36.33 ± 2.25 b
spotted	31.75	34.93	34.38	33.99	37.15	37.37	40.22	36.74	49.66	53.11	48.84	42.77	40.08 ± 2.02 b
ripe	53.97	49.47	57.20	50.13	39.85	41.00	43.18	38.82	64.44	60.71	67.58	62.05	52.37 ± 2.93 a
mean (variety)		37.81 ± 3.34 b				38.71 ± 0.58 b				52.25 ± 2.67 a			
24-Methylenecycloartanol Concentration (mg/100 g)													
green	40.37	35.56	41.88	38.77	49.44	48.95	50.86	49.85	20.59	22.93	22.95	19.07	36.37 ± 3.57 c
spotted	60.06	66.27	64.80	63.89	62.39	62.40	66.78	61.59	71.87	76.42	69.28	60.42	65.52 ± 1.43 b
ripe	107.76	94.91	110.00	95.69	82.63	84.97	89.46	80.45	75.22	80.27	78.88	71.96	87.68 ± 3.53 a
mean (variety)		68.33 ± 7.92 a				65.81 ± 4.35 a				55.82 ± 7.49 b			
Total 4,4′-Dimethylsterol Concentration (mg/100 g)													
green	78.51	69.43	81.36	75.61	104.63	103.30	107.74	106.22	82.48	90.23	91.65	77.09	89.02 ± 3.91 c
spotted	103.76	114.58	112.17	111.09	116.52	116.67	125.07	114.85	139.15	148.26	135.76	118.35	121.35 ± 3.80 b
ripe	178.86	159.62	184.73	161.60	138.50	142.30	150.07	135.22	157.52	160.36	165.37	150.39	157.05 ± 4.34 a
mean (variety)		119.28 ± 12.04				121.76 ± 4.66				126.39 ± 9.49			
δ-Amyrin Relative Amount (%)													
green	1.42	1.73	1.30	1.36	1.48	1.49	1.36	1.42	1.79	1.63	1.71	1.73	1.54 ± 0.05 a
spotted	1.23	1.19	1.19	1.21	1.30	1.19	1.25	1.29	0.99	0.93	0.99	1.00	1.15 ± 0.04 b
ripe	1.06	1.06	1.03	1.06	1.07	1.08	1.03	1.09	0.87	0.89	0.84	0.85	0.99 ± 0.03 c
mean (variety)		1.24 ± 0.06				1.26 ± 0.05				1.19 ± 0.11			
β-Amyrin Relative Amount (%)													
green	3.05	2.47	3.03	3.16	2.80	2.83	2.76	2.98	4.55	4.47	4.55	4.57	3.44 ± 0.24 a
spotted	2.85	2.93	2.94	3.14	2.34	2.38	2.42	2.48	3.57	3.60	3.73	3.64	3.00 ± 0.15 b
ripe	2.87	2.95	2.83	3.02	2.06	2.15	2.12	2.30	3.50	3.54	3.59	3.48	2.87 ± 0.17 b
mean (variety)		2.94 ± 0.05 b				2.47 ± 0.09 c				3.90 ± 0.14 a			
Butyrospermol Relative Amount (%)													
green	5.66	5.51	5.66	5.61	8.58	8.40	8.72	8.59	10.59	10.37	10.28	10.24	8.18 ± 0.59 a
spotted	5.16	5.17	5.08	5.03	7.65	7.65	7.52	7.31	5.25	5.16	5.35	5.33	5.97 ± 0.34 b
ripe	4.41	4.29	4.41	4.46	6.43	6.21	6.43	6.23	4.81	5.10	4.78	4.78	5.20 ± 0.25 b
mean (variety)		5.04 ± 0.15 b				7.48 ± 0.28 a				6.84 ± 0.76 a			
Cycloartenol Relative Amount (%)													
green	26.72	26.66	26.75	26.35	26.53	26.64	26.82	26.42	38.07	38.00	37.74	37.67	30.36 ± 1.60 a
spotted	24.53	24.23	24.37	24.12	24.70	24.79	24.93	24.65	27.65	27.51	27.87	28.13	25.62 ± 0.47 c
ripe	26.31	26.95	26.97	27.13	23.79	23.71	23.76	23.39	33.12	29.84	32.92	34.50	27.70 ± 1.16 b
mean (variety)		25.93 ± 0.35 b				25.01 ± 0.37 b				32.75 ± 1.28 a			

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Table 4. continued

ripening degree	olive variety: Buža CO	Buža VR	Buža RE	Buža FR	Črna CO	Črna VR	Črna RE	Črna FR	Rosinjola CO	Rosinjola VR	Rosinjola RE	Rosinjola FR	mean (ripening degree)
	24-Methylenecycloartanol Relative Amount (%)												
green	38.98	38.18	38.98	38.37	35.27	35.44	35.46	34.85	18.30	18.55	18.13	17.82	30.69 ± 2.70 c
spotted	46.42	45.98	45.94	45.34	41.49	41.39	41.40	41.30	40.02	39.58	39.51	39.74	42.34 ± 0.79 b
ripe	52.47	51.72	51.87	51.77	49.33	49.14	49.24	48.45	38.66	39.46	38.43	40.01	46.71 ± 1.66 a
mean (variety)	45.50 ± 1.65 a				41.90 ± 1.70 b				32.35 ± 3.02 c				

[a]Olive oils are Buža, Črna, and Rosinjola varieties obtained from green, spotted, and ripe olives (control, CO) and stored for 12 months at variable room temperature (VR, 10−28 °C), refrigeration (RE, 4 °C), and freezing (FR, −20 °C) temperatures. Different letters within a group represent statistically significant different means at $p < 0.05$ for the variety and ripening degree factors obtained by main-effect three-way ANOVA and Tukey HSD posthoc test. Mean values are reported together with the corresponding standard errors. Mean values for the storage temperature factor are not reported because of low variability with no statistically significant differences for the majority of compounds. Average values of two GC determinations for each variety/degree of ripening/storage temperature combination are represented without standard deviation.

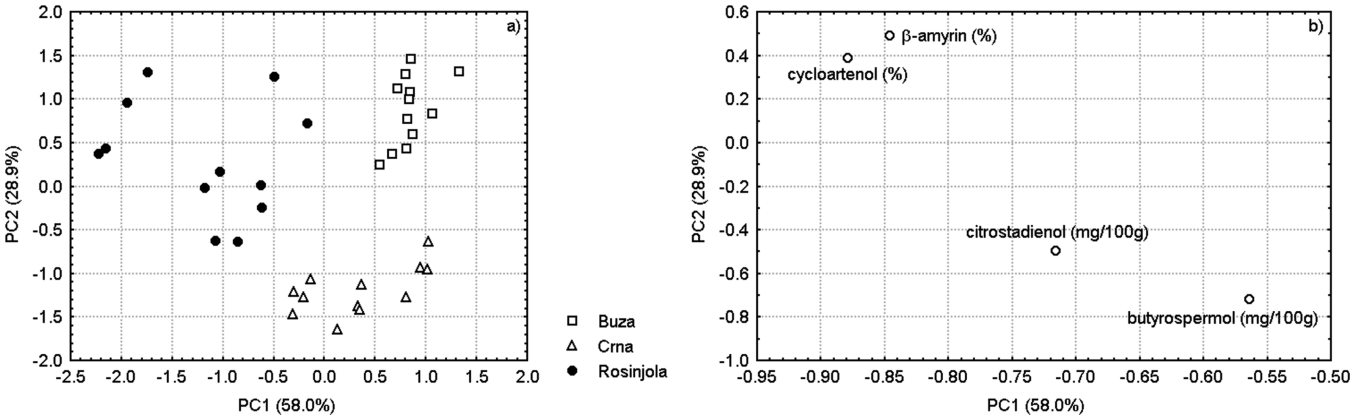

Figure 2. (a) Separation according to variety of olive oils from Buža, Črna, and Rosinjola varieties, harvested at three different ripening degrees and stored for 12 months, along the directions of principal components PC1 and PC2. (b) Factor loadings of selected variables (concentrations in milligrams per 100 g and relative amounts in percent of 4-monomethyl- and 4,4′-dimethylsterols) on PC1 and PC2.

with other constituents and without considering intravarietal variability due to ripening degree and storage.[27,28,31]

Throughout the investigated period of ripening, the major 4-monomethylsterol was citrostadienol, while the most abundant 4,4′-dimethylsterols were 24-methylenecycloartanol and cycloartenol (Tables 3 and 4). The same compounds were also the most abundant in oils from Picholine and Sayali varieties cultivated in Tunisia,[2,41] in Italian varieties Bosana, Coratina, Dritta, Frantoio, Moraiolo, Provenzale, and Leccino,[27,31] and in Spanish olive oils,[4] but with different relative proportions. In two of the most comprehensive and detailed studies on triterpenoids in olive fruit, Stiti and Hartmann[5] and Stiti et al.[3] identified a very large number of major and minor sterol and non-sterol compounds including 4-monomethyl- and 4,4′-dimethylsterols, with triterpenic acids as the most abundant. They used a pattern for the classification of triterpenoids into chemical groups according to their molecular structure which was different from that applied in this work and by other authors. In addition, Stiti et al.[3] did not report quantitative data for all compounds and reported the amounts of free and esterified forms separately. All the aforementioned reasons made these data hard to interpret and compare in terms of olive oil relative composition.

In this work, the level of total 4,4′-dimethylsterols was approximately 4−6 times higher than that of 4-monomethylsterols at different ripening stages, which reasonably corresponds to literature data where Sakouhi et al.[2,41] reported an average ratio of approximately 3:1.

Variability as a Result of Ripening Degree. The average concentrations (milligrams per 100 g) of 4-monomethylsterols, as well as of δ- and β-amyrin and butyrospermol, were found to be stable throughout ripening or slightly increased in oils produced from ripe olives. However, the patterns were not quite uniform for all varieties (Tables 3 and 4). 24-Methylenecycloartanol and cycloartenol increased constantly from RD1 to RD3, which was more pronounced in the case of the latter constituent. Such an increase has caused a significant drop in the relative amounts of other 4-monomethyl- and 4,4′-dimethylsterols in olives produced from spotted (RD2) and especially ripe (RD3) olives (Table 4). The increase in 24-methylenecycloartanol and cycloartenol contributed the most to the increase in total 4,4′-dimethylsterol concentration (Table 4). A constant increase in 4-monomethyl- and 4,4′-dimethylsterols (free + esterified) during olive fruit ontogeny was reported by Stiti et al.[3] The same authors observed a significant rise in 24-methylenecycloartanol and cycloartenol concentration at the moment olive epidermal color began to change from green to spotted, coinciding with the start of the synthesis of anthocyanins and loss of chlorophylls. This phenomenon was explained as a result of a redirection of carbon flux from the triterpenoid toward the sterol pathway, with cycloartenol being a precursor to 24-methylenecycloartanol and a major early

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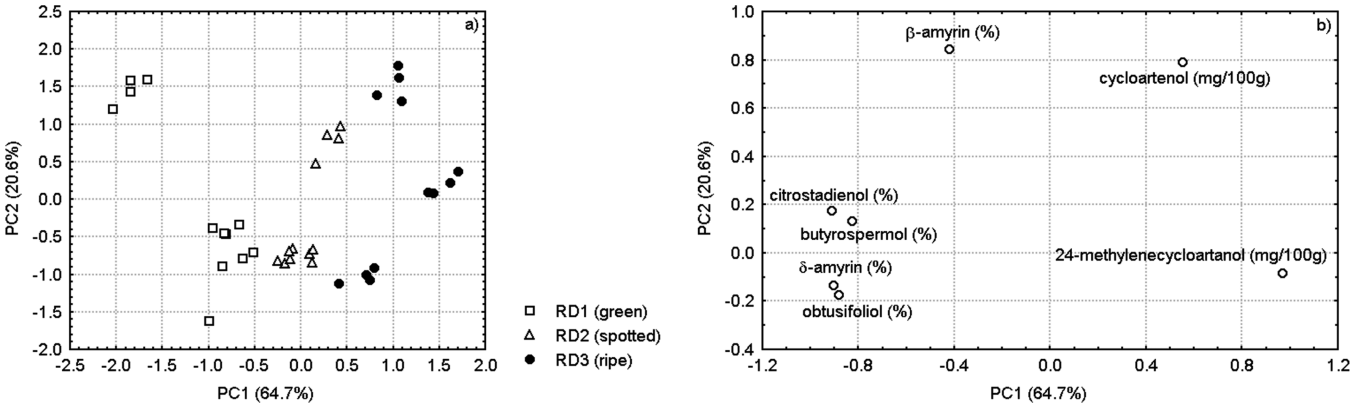

Figure 3. (a) Separation according to ripening degree of olive oils from Buža, Črna, and Rosinjola varieties, harvested at three different ripening degrees (RD1−RD3) and stored for 12 months, along the directions of principal components PC1 and PC2. (b) Factor loadings of selected variables (concentrations in milligrams per 100 g and relative amounts in percent of 4-momomethyl- and 4,4′-dimethylsterols) on PC1 and PC2.

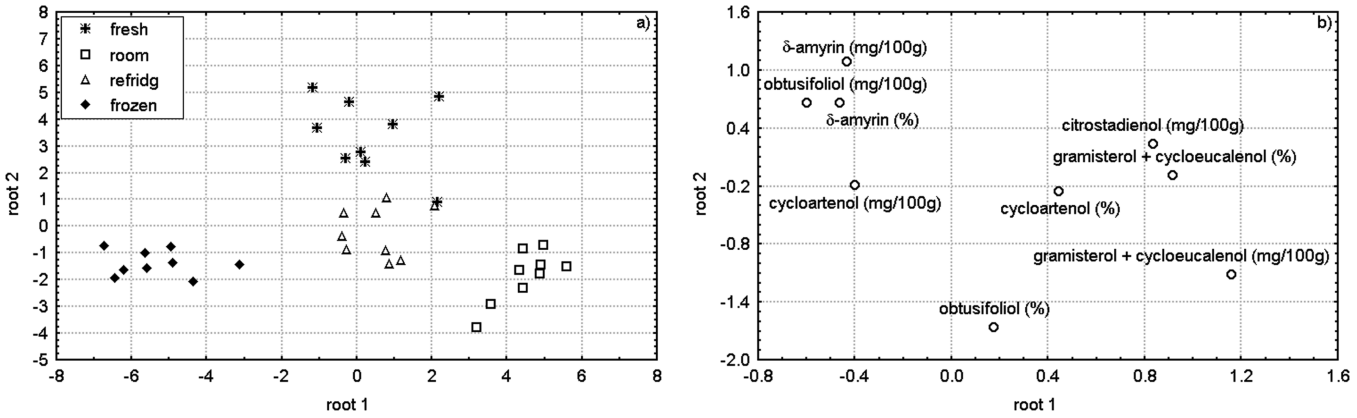

Figure 4. (a) Separation according to storage conditions of olive oils from Buža, Črna, and Rosinjola varieties, harvested at three different ripening degrees and stored for 12 months, along the directions of two discriminant functions. (b) Projection of standardized coefficients of selected variables (concentrations in milligrams per 100 g and relative amounts in percent of 4-methylsterols and 4,4′-dimethylsterols) in two-dimensional space.

biosynthetic intermediate in 4-desmethylsterol synthesis. In this work, the highest average total 4-desmethylsterol concentration was found in oils produced from spotted olives (RD2), which was possibly a result of increased carbon flux toward sterol biosynthesis. The mean values were (in milligrams per 100 g) for Buža, RD1 111.33, RD2 135.65, and RD3 122.16; for Črna, RD1 199.11, RD2 206.61, and RD3 197.16; and for Rosinjola, RD1 135.16, RD2 174.74, and RD3 138.19. Near the end of the olive ripening process, Stiti et al.[3] observed a specific accumulation of 24-methylenecycloartanol, indicating a slowing down of the metabolic flux through the sterol pathway. A sharp increase in 24-methylenecycloartanol concentration (Table 4) together with a drop in total 4-desmethylsterol concentration in RD3 oils were observed in this investigation and are in line with such findings. 4-Monomethyl- and 4,4′-dimethylsterols are components of surface waxes of olive fruit.[5] Therefore, the increase in their concentration in oils made from ripe olives (RD3) is possibly partly a result of a more fragile exocarp that fragments better during milling, as reported by Sánchez Casas et al.[42] Sakouhi et al.[2] noted quite a different behavior of 4-monomethyl- and 4,4′-dimethylsterols during olive ripening, which included much more fluctuation of their content, with the concentrations of cycloartenol and 24-methylenecycloartanol inversely proportional throughout the whole ripening period.

In PCA, the most successful separation between groups of olive oil samples produced at different ripening degrees was achieved by the model that included 36 cases and seven variables: citrostadienol (percent), *δ*-amyrin (percent), *β*-amyrin (percent), obtusifoliol (percent), butyrospermol (percent), 24-methylenecycloartanol (milligrams per 100 g), and cycloartenol (milligrams per 100 g). Two principal components (PCs) were extracted, explaining 85.3% of total variance. The groups were separated along the direction of PC1 (Figure 3). Citrostadienol, *δ*-amyrin, *β*-amyrin, obtusifoliol, and butyrospermol were loaded on the negative side of PC1 and were characteristic for RD1 oils. RD3 oils were associated with higher concentrations of 24-methylenecycloartanol and cycloartenol, which had positive PC1 loadings. RD2 oils were scattered between other two groups in all four quadrants, gravitating toward the intersection of PC1 and PC2. Interestingly, on the scatterplot, three subgroups each comprising four cases/samples could be observed within each of the three groups of oils separated according to ripening degree (Figure 3a). These subgroups corresponded to different varieties, confirming that certain variables can exhibit high variability as a result of both varietal origin and ripening degree.

SLDA, applied on the whole data set (36 cases, 16 variables), classified 97.22% of oil samples correctly according to ripening degree. 24-Methylenecycloartanol (milligrams per 100 g) entered the model as the first variable, which resulted in the

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correct classification of 94.44% of all samples. Other variables included were cycloartenol (percent), β-amyrin (milligrams per 100 g), δ-amyrin (percent), and obtusifoliol (percent).

Variability as a Result of Storage Temperature. The results presented in Tables 3 and 4 suggest that 4-monomethyl- and 4,4′-dimethylsterols in oils have undergone certain changes during storage. Among the probable causes are chemical and microbial transformations involving oxidation,[43] cleavage of glycosides into free 4-monomethyl- and 4,4′-dimethylsterol aglycons by acid or enzymatic hydrolysis, and further transformation of reactive sterol precursors such as squalene and (3*S*)-2,3-oxidosqualene.[7] Upon comparison to the magnitude of variability of 4-monomethyl- and 4,4′-dimethylsterols caused by variety and ripening degree, it can be stated that their levels were relatively consistent after the storage period. The concentration of obtusifoliol was the only variable for which a significant effect of storage condition factor was determined by three-way ANOVA. However, certain subtle, and more important, regular patterns were observed after the application of SLDA to the normalized data set (36 cases, 16 variables). The obtained SLDA model classified 100% of all samples according to storage temperature correctly. Nine variables were selected, and 100% correct classification was obtained by the first four variables: gramisterol + cycloeucalenol (percent and milligrams per 100 g), obtusifoliol (percent), and δ-amyrin (milligrams per 100 g). The prediction capacity of the model was evaluated by leave-one-out cross-validation, where each oil sample was removed from the model and classified by the functions derived from all samples other than that sample. Average percentage of correct prediction was 94.44%, with the origin of only two samples not correctly predicted. As seen in Figure 4, very good and clear SLDA differentiation of olive oils was obtained. Fresh oils were discriminated from others along the second discriminant function by higher δ-amyrin (milligrams per 100 g and percent) and obtusifoliol (milligrams per 100 g) and lower gramisterol + cycloeucalenol (milligrams per 100 g) and obtusifoliol (percent) amounts. The first function discriminated samples stored at room temperature and at freezing temperatures from each other as well as from fresh and refrigerated samples, characterizing the former by higher amounts of δ-amyrin (both milligrams per 100 g and percent), obtusifoliol (milligrams per 100 g), and cycloartenol (milligrams per 100 g) and the latter by higher amounts of gramisterol + cycloeucalenol (milligrams per 100 g and percent) and citrostadienol (milligrams per 100 g). It can be said that the composition of oils stored at refrigeration temperature was generally the most similar to that of the fresh oils since Mahalanobis distances between these groups were the shortest (data not shown).

The present investigation demonstrated that olive variety and ripening degree are important sources of variability of 4-monomethyl- and 4,4′-dimethylsterol content in olive oil. Relative amounts of β-amyrin, cycloartenol, and 24-methylenecycloartanol accounted for the most variation due to varietal origin, while the largest influence of ripening degree was observed for relative amounts of citrostadienol and concentrations of 24-methylenecycloartanol. The total concentration of 4,4-dimethylsterols was found to increase during ripening and peak in oils obtained from ripe olives. It should be mentioned that this is the first experimental evidence on the effect of ripening on 4-monomethyl- and 4,4′-dimethylsterols in olive oil. Another novel finding is that the complex interactive effect of variety and ripening degree, which can complicate olive oil characterization, can be overcome by selecting appropriate 4-monomethyl- and 4,4′-dimethylsterols by use of chemometric tools, similarly as recently reported by our group for 4-desmethylsterols[29] and aliphatic alcohols.[22] Successful differentiation of olive oils achieved by multivariate PCA and SLDA models pointed to their potential use as indicators of varietal origin that are independent of ripening degree and vice versa, applicable for both fresh and stored oils. It practically means that there are 4-monomethyl- and 4,4′-dimethylsterols which are more dependent on variety and those which depend more on ripening degree, which is, to our knowledge, the first finding of this type. The presented approach also has practical relevance. It could be especially useful for technical and scientific studies aiming at the authentication and certification of olive oil varietal origin, which often overlook the impact of olive maturity, and vice versa. For example, the approach tested in this work could be utilized for developing more robust olive oil chemical composition specifications for various protected designations, because it considers the interrelationship between three factors of variability and extracts only the most robust and useful indicators. The results obtained in this work could be useful for producers to optimize individual steps in production to obtain olive oils with targeted 4-monomethyl- and 4,4′-dimethylsterol composition.

Variability as a result of storage temperature and storage in general was of a much smaller magnitude. It suggests that olive oil essentially retains its original characteristics and provides health benefits related to 4-monomethyl- and 4,4′-dimethylsterols throughout its shelf life. It also means that, despite the notorious loss of phenols and tocopherols during ripening and storage, oils obtained from ripe olives, even the aged ones, are not inferior with respect to all valuable phytonutrients. Regardless of their small absolute magnitude, relative changes caused by storage were specific for each investigated storage temperature and were found useful in discriminating stored from fresh oils as well, which has not been accomplished by applying the same approach for 4-desmethylsterols[29] and aliphatic alcohols.[22] A clear and regular dependency of 4-monomethyl- and 4,4′-dimethylsterol composition on storage temperature, identified for the first time in our investigation, offers new possibilities for tracking new reliable markers of olive oil age and quality.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Eight figures showing mass spectra of 4-monomethyl- and 4,4′-dimethylsterols. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b01638.

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Notes

The authors declare no competing financial interest.

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