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ARTICLE

Exploratory Characterization of the Unsaponifiable Fraction of Tunisian Virgin Olive Oils by a Global Approach with HPLC-APCI-IT MS/MS Analysis

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The unsaponifiable fraction of six Tunisian monovarietal virgin olive oils from the region of Medenine was evaluated within a single chromatographic run by using HPLC-APCI-tandem MS. Separation of the compounds under study was achieved by the RP-LC method, giving a reasonable analysis time and good resolution. Detection was done by an ion trap (working alternatively in MS and MS/MS modes), the fact which made our method suitable to unequivocally identify a high number of compounds belonging to different families of the unsaponifiable fraction of oil and to carry out their reliable and sensitive quantification. A great amount of qualitative information was generated in every analysis, although we focused on the quantification of sterols, tocopherols, and triterpenic dialcohols since their standards were commercially available. The limits of detections achieved were within the range of 1.21 and 10.31 μg/kg for sitostanol and β-sitosterol, respectively. Significant differences were observed in the composition of the studied olive cultivars. Jemri Ben Guerdane oil was the richest one in terms of all of the sterols under study. α-Tocopherol was the main vitamin E isomer in all samples, ranging from 70.14 to 130.72 mg/kg. Principal component analysis (PCA) and cluster analysis were applied to the whole data set in order to explore the distribution of the olive cultivars according to their oil composition.

KEYWORDS: Monovarietal olive oils; sterols; tocopherols; triterpenic dialcohols; LC-APCI-tandem MS; multiple reaction monitoring

INTRODUCTION

The beneficial effect derived from the consumption of virgin olive oil (VOO) on human health is well-known and is mainly attributed to its characteristic fatty acid composition and the presence of minor components, such as squalene, phytosterols, and antioxidants (phenolic compounds and tocopherols) (*1*). Chemical, sensory, and nutritional characteristics of olive oil depend on the olive variety used, and thus, in the past few years there has been increasing interest of consumers in monovarietal olive oils (*2*). Indeed, the main olive oil-producing countries are being particularly active studying the chemical composition of olive oils produced by using just one variety of olive fruit or oils produced in a specific area and how the quality of the oils is influenced by that (*2*−*7*). In Tunisia, olive cultivation is dominated only by two main cultivars: Chemlali and Chétoui; the others are local varieties with very few areas of distributions. Moreover, many types of Tunisian extra-VOO are produced by mixing oils from different cultivars, that is, from different varieties of tree species coming from different geographical origins of cultivation. In order to improve the quality of the olive oil produced in Tunisia and to satisfy consumers' requirements, oils from local olive varieties must be easily distinguishable and identified by presenting the same compositional quality. In fact, the identification of local cultivars and their genetic and sanitary certification processes is a crucial point in the improvement of olive oil production (*8*). Nowadays, introduction into the market of different monovarietal virgin olive oils, with different sensory and chemical characteristics, is opening great expectations. Moreover, bearing in mind the different types of olives, it is very important to explore, evaluate, and conserve their existing genetic diversity, still preserved in spite of the influence of the environments where they are cultivated.

In the current work, we used a global approach by HPLC-APCI-MS/MS to get as much information as possible about the unsaponificable fraction of different Tunisian VOOs. Then, we quantified sterols (4-desmethyl sterols and 4,4′-dimethyl sterols), tocopherols, and triterpenic dialcohols. The selection of the families of analytes was motivated by the importance that they have; phytosterols and tocopherols are bioactive components occurring in all vegetable foods, and a wide number of publications have demonstrated their beneficial health effects (*9*, *10*). Likewise, both erythrodiol and uvaol, belonging to the triterpenic dialcohols family, have been shown to possess vasodilatory (*11*),

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antioxidant (*12*), and anti-inflammatory (*13*) properties. Analytical evaluation of erythrodiol and uvaol, which are usually studied together with sterols, has been performed both with GC and LC methods.

GC-FID is the most frequently applied technique for the analysis of sterols; however, normal-phase and reversed-phase LC techniques have been used for the analysis of the title compounds (*14*). A sterol detection system coupled to the LC has been carried out by photodiode array detection (DAD), refractive index (RI) detection, evaporative light scattering detection (ELSD), nuclear magnetic resonance (NMR) detection, and mass spectrometry (MS) (*15*). For tocopherols analysis, normal and reversed-LC have been applied, using DAD, mass spectrometry, or even fluorescence as detection systems (*16*).

As far as the interfaces in mass spectrometry are concerned, there are not many publications in which atmospheric pressure chemical ionization (APCI) has been used (*17*, *18*), although in principle that interface will suit quite properly for the analyses of the mentioned kinds of compounds. For the analyzer, we can confirm that quadrupole, ion trap, or triple quadrupole have been the most widely used (*17*, *18*).

The objective of this study was to demonstrate the usefulness of a very sensitive HPLC-APCI-IT MS^n method to produce reliable quantitative data about the sterols, tocopherols, and triterpenic dialcohols present in six Tunisian VOOs from the southeast of Tunisia (arid region of Medenine). Using MS/MS detection, we assured very low detection limits and unequivocal identification of the compounds under study. The LC-MS/MS method can give to the analyst information about some other compounds present in the unsaponifiable fraction of the studied oils which could be very interesting.

MATERIALS AND METHODS

Oil Samples. Six different monovarietal extra virgin olive oils (EVOOs) from southeastern Tunisia were analyzed. The EVOO samples chosen for this study were from various olive cultivars: Chemlali Zarzis, Zalmati, Zarrazi Zarzis, Jemri Ben Guerdane, Dhokar Ben Guerdane, and Indouri Jerba. These cultivars were growing in the arid region of Medenine (rainfall average of 150 mm per year) under the same pedoclimatic conditions. Olive samples studied were harvested in mid December, 2007. For each cultivar, three representative 5 kg samples from three olive trees were collected. Oil extraction was performed using an Abencor laboratory oil mill (MC2 Ingenierias y sitemas, Sevilla, Spain), kneading the olive paste at 28 °C for 30 min, and oil samples were stored at 4 °C in darkness using amber glass bottles without headspace until analysis.

Reagents and Chemicals. Cholesterol was acquired from Riedel-de Haën (Germany), and β-sitosterol and erythrodiol were from Fluka (Buchs, Switzerland). Sitostanol, uvaol, Δ5-avenasterol, stigmasterol, campesterol, and brassicasterol were from Sigma-Aldrich. α-, β-, γ-, and δ-tocopherols were purchased as an isomer kit from Merck (Darmstadt, Germany).

Stock solution (0.1 mg/mL) was prepared by dissolving all standards in acetonitrile and stored in the dark at −20 °C over a maximum period of 2 months. Working standard solutions were prepared daily by dilution of this concentrated solution in acetonitrile. All reagents used were of analytical grade. HPLC grade acetonitrile, methanol, diethyl ether, and ethyl acetate were purchased from Panreac (Barcelona, Spain). Potassium hydroxide and anhydrous sodium sulfate were purchased from Sigma. Milli-Q water of 18.2 MΩ cm^{-1} resistivity was used throughout (Millipore, Billerica, MA, USA).

Sample Preparation. Olive oil sample (5 g oil) was saponified at 80 °C by refluxing with a 50 mL ethanolic solution of 2 M KOH for 30 min. After cooling at room temperature, 100 mL of distilled water was added. After phase separation in a separation funnel, the aqueous phase was washed three times with diethyl ether. The diethyl ether fractions were collected and successively washed three times with distilled water and with an ethanolic solution of 0.5 N KOH. Finally, the ether extracts were washed with distilled water until the wash water gave a neutral reaction and were dried with anhydrous sodium sulfate. They were then filtered and evaporated to dryness using a rotary evaporator at reduced pressure, and the residue was dissolved in acetonitrile. Sample extracts were filtered through a membrane filter (0.45 μm) before being analyzed by HPLC-APCI-MS.

Instrumentation. A model MicropH 2002 m from Crison (Barcelona, Spain) was used to adjust the pH of the mobile phase with a precision of 0.01 pH units. A Heidolph VV rotary evaporator (Schwabach, Germany) coupled to Büchi B-169 Vacuum System (Flawill, Switzerland) was used to concentrate the sample extracts.

Analyses were carried out operating at 25 °C on System Gold HPLC (Beckman Couter, Fullerton, CA, USA), including a 126 solvent module, a 168 diode array detector module, and a manual sample valve injector with a 20 μL loop (Rheodyne, Cotati, CA, USA). The HPLC column used was Phenomenex C18 (5 μm, 3.0 × 250 mm) fitted with a Phenomenex C-18 guard column (Phenomenex, Phenomenex, Torrance, CA). Solvents were filtered using Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA) prior to degassification by ultrasonication.

Chromatographic Conditions. Compounds belonging to the nonsaponificable fractions of the oil samples were analyzed by LC-APCI-IT MS^n. The compounds were separated by using a gradient consisting of acetonitrile/water (0.01% acetic acid) at a flow rate of 1.5 mL min^{-1}. Phase A was acetonitrile, and phase B was acidified water, and the composition of the mobile phase was changed according to the following gradient: from 0 to 60 min, 90 to 92% A; from 60 to 63 min, 92 to 100% A; and from 63 to 65 min, 100 to 90% A. The column temperature was set at 25 °C during the whole run, and the injection volume was 20 μL. The examined compounds were monitoring as well by a DAD detector, which was set at 280/240 nm.

Once the compounds under study were separated by LC, they were ionized in an APCI interface and then detected by an ion trap MS analyzer.

LC-MS and LC-MS/MS Analyses. The mass spectrometer coupled to our LC system was a Bruker Esquire 2000 ion trap purchased from Bruker Daltonics (Bremen, Germany) equipped with an APCI interface. The mobile phase flow was split into the source at 500 μL min^{-1}. The ion trap full scan sensitivity is necessary for all data dependent experiments; it enables intelligent acquisition of MS/MS data, especially on low abundance precursor ions. The ionization was positive-ion APCI, and mass spectra were acquired in real time, up to mass m/z 800. In addition, tandem experiments using MS/MS studies to identify fragments ions and quantify them were carried out in positive mode as well.

APCI interface was operating in positive ion mode using the following parameters: optimized temperature and flow rate of the dry gas (nitrogen) were 350 °C and 5 L/min, respectively, and the APCI interface temperature was 450 °C. The pressure of the nebulizer gas (nitrogen) was set to 50 psi, and the voltage of the corona discharge needle was +3500 V. Capillary voltage was set at −3000 V and the end-plate offset at −500 V. Source and transfer parameters were optimized by direct infusion experiments with individual standard solutions and with solutions containing all of the compounds under study, which we had as commercially available standards looking for the best conditions regarding peak intensity and resolution. We confirmed that the MS conditions found were the most appropriate for every compound used in direct infusion experiments with individual solutions.

The use of tandem mass spectrometry improves the selectivity of the analysis and often produces good evidence about the identity of the compounds under study. Both auto-MS^n and multiple reaction monitoring (MRM) modes were used in the study. Nitrogen was used as the nebulizing and drying gas, and helium gas was used to induce dissociation for MS/MS acquisition data. In MS/MS experiments, the maximum accumulation time was set at 50 ms with two averages per experiment in order to obtain 10 spectra per peak. In the auto-MS^n mode, the MS^n product-ion spectra were produced by CID of the main detected ion for all of the analytes at their respective HPLC retention times.

In MRM mode, the appropriate precursor ion for every compound was isolated, and the fragment ions were monitored. As the collision energy required was slightly different for every compound, we created some segments in our MS method, and each one had its own isolation and fragmentation conditions. MRM transitions were monitored in six different elution time windows. The selection of fragmentation products for each compound was based on the most specific transitions that produced the highest signal. Fragmentation was carried out by means of collision-induced

Table 1. Ions Observed in the APCI MS and MS/MS Spectra of Sterols, Tocopherols, and Triterpenic Dialcohols in Positive Mode, Their Retention Time, and the Selected MS/MS Conditions for All of the Compounds under Study[a]

analytes	MW	$[M+H-H_2O]^+$	$[M+H-2H_2O]^+$	$[M]\cdot^+$	precursor ion	fragment ions	width (*m/z*)	cutoff (*m/z*)	amplitude (V)	identification points	retent. time	segment period
erythrodiol	442	425.2	407.2		425.2	407.2* 396.2 297.1/190.9	4.0	110	1.00	4	3.7	I (0−8 min)
					407.2	295.0* 389.2 214.9	4.0	120	0.90	3		
uvaol	442	425.2	407.2		425.2	407.2* 397.2 244.9/190.0	4.0	110	1.00	4	3.7	I (0−8 min)
					407.2	295.0* 389.2 311.0/283.1	4.0	120	0.90	4		
δ-tocopherol	402			402.2	402.2	136.8* 176.8 150.8	4.0	108	0.95	3	15.9	II (8−18 min)
lupeol	426	409.2			409.2	271.0* 257.0 285.0/202.9	4.0	110	0.80	4	19.8	III (18−20.7 min)
brassicasterol	398	381.2			381.2	255.1* 297.1 311.1	4.0	102	0.85	3	20.2	III (18−20.7 min)
β-tocopherol	416			416.2	416.2	150.8* 190.9 369.2/381.2	4.0	115	0.65	4	20.6	III (18−20.7 min)
γ -tocopherol	416			416.2	416.2	150.8* 190.9 276.0 373.2/122.9	4.0	115	0.65	5	20.6	III (18−20.7 min)
cholesterol	386	369.2			369.2	243.0* 287.1 160.9 257.0/146.9	4.0	99	0.85	5	20.8	IV (20.7−25.2 min)
Δ5-avenasterol	412	395.2			395.2	255.0* 327.1 297.1/285.2	4.0	106	0.90	4	21.0	IV (20.7−25.2 min)
campesterol	400	383.2			383.2	243.0* 315.1 227.9/239.9	4.0	103	0.90	4	25.0	IV (20.7−25.2 min)
β-amyrin	426	409.2			409.2	313.1* 270.9 299.0 340.2/285.0	4.0	110	0.80	5	25.5	V (25.2−29.0 min)
stigmasterol	412	395.2			395.2	297.1* 311.1 255.0/283.0	4.0	106	0.90	4	26.6	V (25.2−29.0 min)
α-tocopherol	430			430.2	430.2	164.8* 204.9 136.8	4.0	116	1.0	3	27.2	V (25.2−29.0 min)
β-sitosterol	414	397.2			397.2	243.0* 315.1 257.0/146.9	4.0	107	0.85	4	31.1	VI (after 29.0 min)
sitostanol	416	399.2			399.2	245.0* 317.1 347.0/226.9	4.0	107	0.9	4	38.9	VI (after 29.0 min)

[a] MW: molecular weight. Fragments with * are the most intense ones in the MS spectra. In general, the first transition was used for quantification in all of the cases; the other transitions were used for confirmation.

dissociation with the helium present in the trap for 40 ms in MRM mode. Ions observed in the APCI MS and MS/MS spectra of sterols, tocopherols, and triterpenic dialcohols in positive mode, their retention time, and the selected MS/MS for all of the compounds under study can be observed in **Table 1**.

Working with this MS method and alternating the capability of our IT to make MS and MS^n, we could achieve within the same run the MS base peak chromatogram (BPC), unequivocal identification, and sensitive quantitative determination of the compounds under study.

Analytical Characteristics of the Developed Method. We carried out a study to check the repeatability and reproducibility of the proposed method as well as to establish the calibration curves to quantify the compounds under study and calculate the detection, quantification limit, and precision (as relative standard deviation (RSD) of the intermediate concentration value of the linear range) of the method.

Calibration curves were obtained for each standard with good linearity ($r^2 > 0.97$) by plotting the standard concentration as a function of the peak area obtained from HPLC-APCI-IT MS/MS analysis. For this purpose, the stock solutions (1000 mg/L) of the standards were diluted with ACN to have different concentrations. Each concentration was analyzed by triplicate injections. Method detection limits (DL) and method quantification limits (QL) were assumed as the minimum detectable amount of compound with a signal-to-noise (S/N) ratio of 3 for DL and 10, with a RSD ≤10% ($n = 3$), for QL.

The accuracy of the method was determined by means of the recovery study. The apparent recovery R is the ratio of the concentration found for each analyte by the considered method (c) versus the reference value ($cref$) and is calculated as follows: $R = (c/cref)\cdot 100$.

Identity Confirmation and Transition Used for the Quantification of Sterols, Tocopherols, and Triterpenic Dialcohols. Identification of the analytes was based on the comparison of their retention times, MS, and MS/MS spectra with those of authentic reference compounds. Peak identification was also done with spiked samples at different concentration levels.

In any ideal situation, when using LC-tandem MS as an instrumental technique, a minimum of three identification points (IPs) is required. In this study, several MRM transitions were monitored for all of the compounds, and the MRM ratio was calculated as the relationship between the abundances of the different transitions. The first transition was generally used for quantification and the rest for confirmatory purposes. Quantification was performed by plotting the peak area against the concentration of each compound. Calibration standards were analyzed at the beginning and at the end of a sample sequence. The variations in signal intensity were monitored by the analyses of three quality control samples after approximately 10 injections. All samples that were quantified above the highest concentration of the calibration curves were diluted with ACN and reanalyzed. In order to guarantee the accuracy and precision of the procedure, the dilutions were validated with the use of a standard solution.

Statistical Analysis. Significant differences among cultivars were determined by analysis of variance which applied a Duncan's test. Differences were considered statistically significant when probability was greater than 95% ($p < 0.05$). All collected data were submitted to principal component analysis (PCA). The statistical analysis was performed using the XLSTAT software, version 2008.1.02 (Addinsoft) and SPSS 13.0 for Windows (SPSS Inc., 2004).

RESULTS AND DISCUSSION

Identification of Compounds at Optimal Conditions and Potential Use of the Developed Method. LC separation was achieved by gradient elution using acetonitrile and aqueous solution with acetic acid as mobile phases. The selection of the mobile phase solvents was based on previous chromatographic separations published by our research group (*18*). In APCI-IT MS, the source, transfer, and detection parameters were optimized by direct infusion experiments with solutions containing all of the compounds under study, looking for the best conditions of resolution and intensity. The triterpenic dialcohols (erythrodiol and uvaol) produced in MS their maximum signal at m/z 407.2 ($[M+H-2H_2O]^+$), although 425.2 ($[M+H-H_2O]^+$) could be also observed. In the case of tocopherols, we consistently saw $[M]\bullet^+$, and for the sterols (both 4-desmethylsterols and 4,4′-dimethylsterols), the major m/z signal in the MS spectra was $[M+H-H_2O]^+$. Thus, in MRM mode, we used as precursor ions $[M+H-2H_2O]^+$ for triterpenic dialcohols, $[M]\bullet^+$ for tocopherols, and $[M+H-H_2O]^+$ for sterols. As the collision energy required was slightly different for every compound, we created some segments in our MS method, and each one had its own isolation and fragmentation conditions (see **Table 1**). MRM transitions were monitored in six different elution time windows. The selection of fragmentation products for each compound was based on the most specific transitions that produced the highest signal. In this study, several MRM transitions were monitored for all of the compounds; the first transition was generally used for quantification and the rest for confirmatory purposes. **Figure 1** shows the optimum chromatogram (full scan MS) of a mixture containing sterols (4-desmethylsterols and 4,4′-dimethylsterols), tocopherols, and triterpenic dialcohols and includes the time range of the 6 segments used in MRM mode.

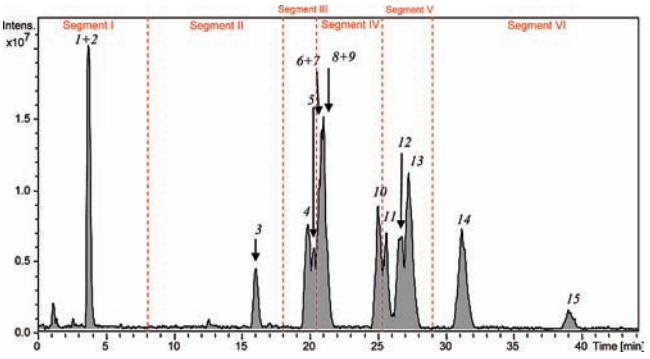

Figure 1. HPLC-APCI-IT MS chromatogram of a standard mixture (200 ppm) containing sterols (4-desmethyl sterols and 4,4′-dimethyl sterols), tocopherols, and triterpenic dialcohols. Different segments were created to improve the quality of MS/MS data; they are shown in the current figure. Identity of peaks: *1*, erythrodiol; *2*, uvaol; *3*, δ-tocopherol; *4*, lupeol; *5*, brassicasterol; *6*, β-tocopherol; *7*, γ-tocopherol; *8*, cholesterol; *9*, Δ5-avenasterol; *10*, campesterol; *11*, β-amyrin; *12*, stigmasterol; *13*, α-tocopherol; *14*, β-sitosterol; and *15*, sitostanol.

The developed method was then used to analyze several Tunisian VOO samples. In all of the cases, we first made one MS full scan acquisition; second, we carried out the analysis in MRM mode to get unequivocal identification and reliable and sensitive quantitative results about the three families under study, and finally, we carried out an auto-MS/MS analysis to get as much information (qualitative) as possible about some other compounds present in the unsaponifiable fractions of the oils. **Figure 2** shows the UV chromatograms (240 and 280 nm (signal was saved in the whole range 190−600 nm)) and the base peak chromatogram of the unsaponifiable fraction of Chemlali Zarzis VOO. Apart from the identification and quantification of compounds belonging to sterols, tocopherols, and triterpenic dialcohols, our method has great potential and could give to the analyst a great amount of information about some other compounds from the unsaponifiable fraction of oil. We tried to take advantage of the great potential of our LC-MS/MS method, and in **Table 2**, we summarize the *m*/*z* signal of the new compounds detected by our method, their fragments in MS/MS mode (auto MS/MS), and their presence/abscence in the different VOOs studied. Tocotrienols, aliphatic alcohols, carotenes, other kinds of sterols, etc. are compounds from the unsaponifiable fraction of oil which could be perfectly determined by our method. We have studied the MS and MS/MS data of the compounds present in the analyzed extracts to generate a wide amount of qualitative information for the exploratory characterization of the Tunisian VOOs under study.

Analytical Parameters of Our Method. Calibrations curves were constructed with standard solutions containing all of the commercially available analytes. They were obtained by plotting the standard concentration as a function of the peak area obtained from HPLC-APCI-IT MS/MS analyses. In order to calculate the regression equations and DLs, we considered the extracted ion chromatogram (EIC) of the most intense transition in MRM mode in the mass spectrum for each compound, although it is important to bear in mind that for carrying out the identification of these compounds in real samples, we should consider all of the detected ion fragments in the MS/MS spectra as well as their relative abundance.

Each point of the calibration plot was repeated three times in an independent solution prepared in the same way. Linear calibration curves were obtained, indicating a good correlation between peak areas and analyte concentrations since the regression coefficients

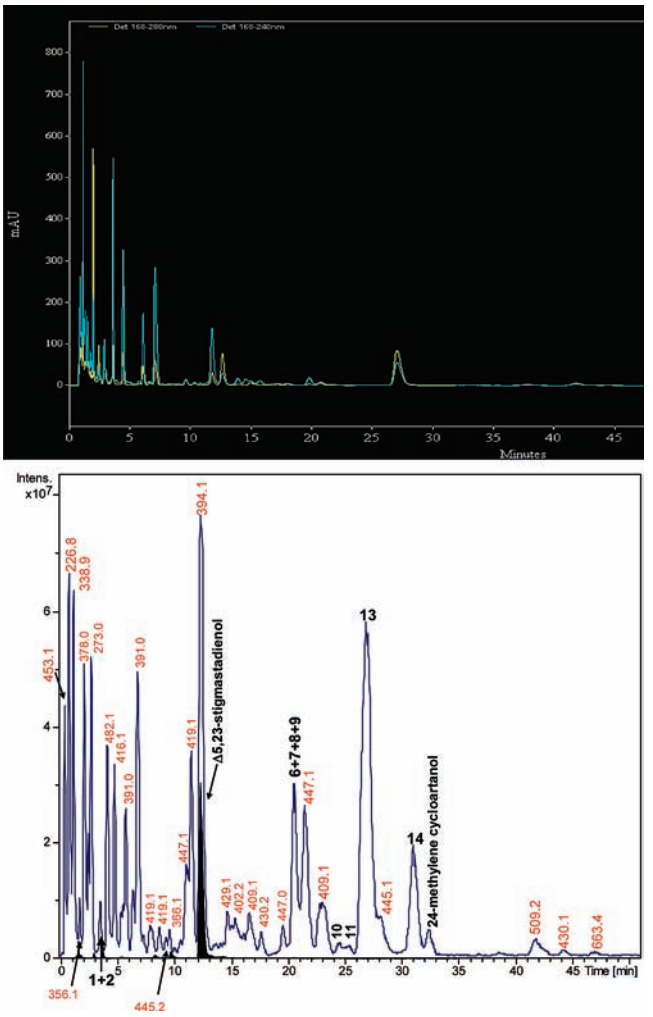

Figure 2. UV chromatograms (240 and 280 nm (signal was saved in the whole range 190−600 nm)) and base peak chromatogram of the unsaponificable fraction of Chemlali Zarzis VOO obtained under the optimal conditions. Peak identification is as described in **Figure 1**.

(r^2) were higher than 0.97 for all of the compounds quantified. The limits of detections (DL, based on a signal-to-noise ratio of 3) achieved using these experimental conditions were within the range 1.21 and 10.31 μg/kg for sitostanol and β-sitosterol, respectively.

Repeatability was studied by performing a series of separations using the optimized method on one of the samples on the same day (intraday precision, $n = 6$) and on three consecutive days (interday precision, $n = 36$). The intraday repeatabilities on the retention time (expressed as RSD) were found to be inside the range from 0.73 to 1.99%, whereas the interday repeatabilities on the migration time were between 3.22 and 6.37%.

The parameters of the regression equations, DL, QL, repeatability, and reproducibility are summarized in **Table 3**.

Analysis of Tunisian Virgin Olive Oils from the Region of Medenine. Using the described extraction protocol and the optimum HPLC-APCI-IT MS/MS method, we analyzed six monovarietal olive oil samples (Chemlali Zarzis, Dhokar Ben Guerdane, Indouri Jerba, Jemri Ben Guerdane, Zalmati, and Zarrazi Zarzis). As mentioned before, the positive full scan mass spectra of standard analytes showed intense fragment ions at *m*/*z* 369.2 (for cholesterol), 381.2 (for brassicasterol), 383.2 (for campesterol), 395.2 (for Δ5-avenasterol and stigmasterol), 397.2 (for β-sitosterol), 399.2 (for sitostanol), and 409.2 (for lupeol and

Table 2. *m/z* Signal of the New Compounds Detected by the Developed HPLC-APCI-IT MS Method, Their Fragments in MS/MS Mode (auto MS/MS) and Their Presence/Abscence in the Different VOOs Studied[a]

				presence/absence in the different VOOs studied					
m/z signal	retention time (min)	MS/MS fragments	other ions detected (same t_r)	Chemlali Zarzis	Dhokar Ben Guerdane	Indouri Jerba	Jemri Ben Guerdane	Zalmati	Zarrazi Zarzis
453.1	0.5	226.8/292.0/208.8/191.8		+	+	+	+	+	+
226.8	0.9	183.7/200.8	307.0	+	+	+	−	+	−
338.9	1.2	304.9/226.8/197.8	474.1	+	+	+	−	+	−
356.1	1.8	338.1/274.9/226.8	243.1/648.4	+	+	+	−	+	+
378.0	2.2	326.0/338.0/121.8	423.1	+	+	+	−	+	
378.0	2.5	335.0/282.0/148.7		+	+	+		+	−
273.0	2.8	226.8/216.9	378.0/445.1	+	+	+	+	+	+
482.1	4.2	363.0/148.7/232.7/407.1		+	+	+	−	+	−
416.1	4.8	−	482.0	+	+	+	−	+	−
423.1	5.4	407.1/395.1/338.0		+	+	+	+	+	+
338.1	5.6	160.8/121.8	423.1	+	+	+	−	+	−
391.0	5.8	338.1/278.8	467.2	+	+	+	−	+	−
467.2	6.0	425.2/446.1	551.3	+	−	+	−	+	+
445.1	6.4	364.1/425.2/293.0/162.7		+	+	+	+	+	+
391.0	6.8	148.7/260.8	419.1	+	+	+	−	+	−
419.1	7.3	312.1/391.0/338.1	445.1	+	+	+	−	+	+
419.1	7.9	403.0/391.0/338.1		+	+	+	+	+	+
421.1	8.8	306.9/338.1		−	+	+	+	+	+
445.2	9.3	403.1/419.1	463.1/559.2	+	+	+	+	+	+
366.1	9.7	135.8/121.7/217.8		+	+	+	−	+	−
407.2	10.2	367.1/397.1	425.2/445.2/486.3	+	−	−	−	−	+
447.1	10.6	419.1/403.1/391.1	481.1	+	+	+	+	+	−
447.1	11.1	405.1/429.1/121.7	527.2	+	+	+	+	+	+
419.1	11.5	288.9/148.7	543.2	+	+	−	+	+	−
429.1	14.7	409.1/286.7	509.2	+	+	+	+	+	−
402.2	15.4	164.7/135.8/202.8	430.1/475.1	+	+	+	−	+	+
409.1	16.6	178.8/135.7	431.1/447.1	+	+	+	+	+	+
430.2	17.7	403.1/419.1/121.7		+	+	+	+	+	+
447.0	19.5	409.1/178.7		+	+	+	−	+	−
447.1	21.4	429.1/405.1	527.2	+	+	+	+	+	+
409.1	23.0	216.8/121.8/295.8		+	+	+	+	+	+
445.1	28.1	409.1/137.8/201.7		+	+	+	+	+	+
509.2	41.6	429.2/484.2/469.1		+	+	+	+	+	+
430.1	43.9	135.7/162.8	486.2	+	+	+	−	+	−
663.4	46.7	611.4/409.1		+	+	−	+	+	−

[a] (+) Presence; (−) absence.

β-amyrin), indicating the loss of water $[M + H - H_2O]^+$ (**Table 1**). For the tocopherols, we observed that APCI generated in all cases major fragments ions at *m/z* 402.2 (for δ-tocopherol), 416.2 (for β- and γ-tocopherols), and 430.2 (for α-tocopherol). The formation of the observed ions in the case of tocopherols could be explained by the facile one-electron oxidation of tocopherol by the corona discharge in the APCI source (*19*). The main ions in the mass spectra of erythrodiol and uvaol were *m/z* 407.2 $[M + H - 2H_2O]^+$ and 427.2 $[M + H - H_2O]^+$.

Quantification of the analytes was carried out using the transitions in MRM mode, which are shown in **Table 1**. Lupeol, brassicasterol, cholesterol, Δ5-avenasterol, campesterol, β-amyrin, stigmasterol, α-tocopherol, β-sitosterol, and sitostanol were quantified individually in mg/kg (**Table 4**), while erythrodiol and uvaol, and β- and γ-tocopherols were quantified together because they appeared at the same time (with the same retention time) and provided the same *m/z* in MS; their MS/MS signals were very useful in distinguishing between them, but individual quantification was not made in these two cases because of the influence of ionization suppression phenomena.

We proceeded to analyze the olive oil samples from Tunisia, and the results are shown in **Figure 3**. (We only included the HPLC-APCI-IT MS chromatograms of Dhokar Ben Guerdane and Jemri Ben Guerdane oils as examples to limit the size of the article.) The peak which eluted just after β-sitosterol is tentatively identified as 24-methylene cycloartanol on the basis of the MS signal obtained, its fragmentation, information reported before, the higher apolarity due to the additional methyl group in the molecular structure of this compound, and also taking into account the huge quantity of this compound found in olive oils as reported by Azadmard-Damirchi and Dutta (*20*). APCI-IT MS signal of this compound showed an intense fragment ion at *m/z* 423 $[M + H - H_2O]^+$ (**Figure 4**). MS/MS spectra of 24-methylene cycloartanol obtained after ion trap collision induced dissociation of the precursor ion at *m/z* 423 showing in our scanning conditions fragmentation patterns similar and characteristic to those of lupeol and β-amyrin, compounds which belongs to the same family as 4,4′-dimethyl sterols. Fragment ion peaks appeared mainly at *m/z* 259.0, 270.9, 285.1, 313.1, and 340.2.

The last eluted peak observed in the BPC of all samples was assigned as squalene, on the basis of good agreement with MS data of the literature (*21*). In fact, a protonated quasi-molecular ion at *m/z* 411 $[M + H]^+$ was observed for this compound in the positive measurement mode of the APCI (**Figure 4**) as reported by Hagiwara et al. (*21*). On MS/MS fragmentation of squalene, the product ions at *m/z* 69, 149, 109, 231, 259, 287, and 329 were present as major fragments. The concentration of that compound in all of the samples under study seemed to be very high considering

Table 3. Analytical Parameters of the LC-APCI-IT Tandem MS Method Developed[a]

analytes	MW	transition used for quantification	DL (μg kg^{-1})	QL (μg kg^{-1})	calibration equations	r^2	repeat. intraday [b]	repeat. interday [b]	reproducibility [c]	accuracy [d]
erythrodiol uvaol	442 442	407.2 >295.0	1.31	4.37	$y = 336569400.15x + 20023091.71$	0.993	1.03	5.16	5.98	95.7
δ-tocopherol	402	402.2 >136.8	10.11	33.7	$y = 220478211.41x + 49613335.23$	0.993	1.00	3.76	7.01	98.3
lupeol	426	409.2 > 271.0	3.51	11.7	$y = 187254812.022x + 117814952.42$	0.967	0.95	5.37	7.55	102.1
brassicasterol	398	381.2 > 255.1	2.10	7.01	$y = 114131555.36x + 492995.28$	0.997	0.73	5.05	6.50	101.6
β-tocopherol γ-tocopherol	416 416	416.2 > 150.8	1.99	6.63	$y = 1361751551.14x - 3445580611.01$	0.981	1.27	6.37	8.78	99.5
cholesterol	386	369.2 > 243.0	2.76	9.20	$y = 335027213.73x - 37710411.67$	0.981	1.65	4.74	7.04	97.6
Δ5-avenasterol	412	395.2 > 255.0	3.01	10.03	$y = 45388111.29x + 33381761.97$	0.983	1.87	3.52	5.55	96.5
campesterol	400	383.2 > 243.0	3.98	13.27	$y = 49307201.79x + 40002429.98$	0.985	1.65	4.01	6.01	98.5
β-amyrin	426	409.2 > 313.1	10.12	33.73	$y = 166260511.20x + 60390925.76$	0.981	1.99	4.11	5.51	97.1
stigmasterol	412	395.2 > 297.1	9.01	30.03	$y = 16000801.01x + 19682944.08$	0.977	1.54	3.54	5.01	99.0
α-tocopherol	430	430.2 > 164.8	5.91	19.70	$y = 337745711.62x + 92418057.67$	0.967	1.34	3.52	6.11	99.7
β-sitosterol	414	397.2 > 243.0	10.31	34.37	$y = 44793501.53x + 59036219.49$	0.987	0.97	3.22	6.22	99.2
sitostanol	416	399.2 > 245.0	1.21	4.03	$y = 80292222.14x - 205643.11$	0.967	1.02	3.99	5.33	96.6

[a] MW: molecular weight. DL: detection limit. QL: quantitation limit. [b] RSD values (%) for peak areas corresponding to each compound under study; measured from six consecutive injections of each analyte within the same day (intra-) and on three different days (inter-). [c] RSD values (%) from three consecutive injections with two different technicians and within two different days. [d] The accuracy of the assay is the closeness of the test value obtained to the nominal value. It is calculated by determining trueness and precision. (% recovery and % RSD).

Table 4. Mean Values and Standard Deviations of the Amount of Sterols, Tocopherols, and Triterpenic Dialcohols (mg kg $^{-1}$) Found in the Olive Oil Samples Analyzed by HPLC-APCI-IT MS[a]

	olive cultivars					
analytes	Chemlali Zarzis	Dhokar Ben Guerdane	Indouri Jerba	Jemri Ben Guerdane	Zalmati	Zarrazi Zarzis
erythrodiol + uvaol	3.22 ± 0.60 d	3.46 ± 0.29 d	4.90 ± 0.33 c	26.26 ± 0.78 a	9.21 ± 1.61 b	2.35 ± 0.16 e
lupeol	nd	nd	nd	nd	nd	nd
brassicasterol	nd	0.22 ± 0.21 b	nq	nd	nd	1.03 ± 0.21 a
cholesterol	0.59 ± 0.14 a	nq	nd	0.49 ± 0.17 b	0.48 ± 0.16 b	0.55 ± 0.10 ab
Δ5-avenasterol	69.40 ± 7.29 d	100.34 ± 16.90 b	66.16 ± 5.99 d	278.73 ± 5.16 a	72.90 ± 5.10 cd	79.63 ± 8.03 c
campesterol	9.48 ± 1.89 c	7.61 ± 1.29 d	2.86 ± 0.08 e	67.79 ± 1.36 a	1.67 ± 0.20 e	15.50 ± 3.13 b
β-amyrin	nq	2.35 ± 0.60 c	3.05 ± 0.35 bc	8.95 ± 2.32 a	3.72 ± 1.09 b	nq
stigmasterol	2.12 ± 0.61 a	2.02 ± 0.41 a	2.11 ± 0.21 a	1.99 ± 0.33 a	2.02 ± 0.35 a	1.95 ± 0.52 a
β-sitosterol	748.40 ± 33.45 c	695.39 ± 41.44 cd	636.40 ± 3.30 de	1445.45 ± 163.95 a	707.57 ± 36.28 cd	868.88 ± 67.59 b
sitostanol	nd	nd	0.24 ± 0.09 b	0.99 ± 0.22 a	nd	0.30 ± 0,10 b
β- and γ- tocopherols	2.15 ± 0.50 a	0.81 ± 0.10 d	1.43 ± 0.19 b	1.56 ± 0.14 b	0.46 ± 0.05 e	1.05 ± 0.19 c
α-tocopherol	121.42 ± 13.68 b	77.44 ± 7.34 d	130.72 ± 2.15 a	90.32 ± 2.86 c	70.14 ± 9.36 d	61.13 ± 8.51 e

[a] Different letters for the same analytical parameter indicate significant differences among cultivars ($n = 9$; $p < 0.05$). nd: nondetectable. nq: nonquantifiable.

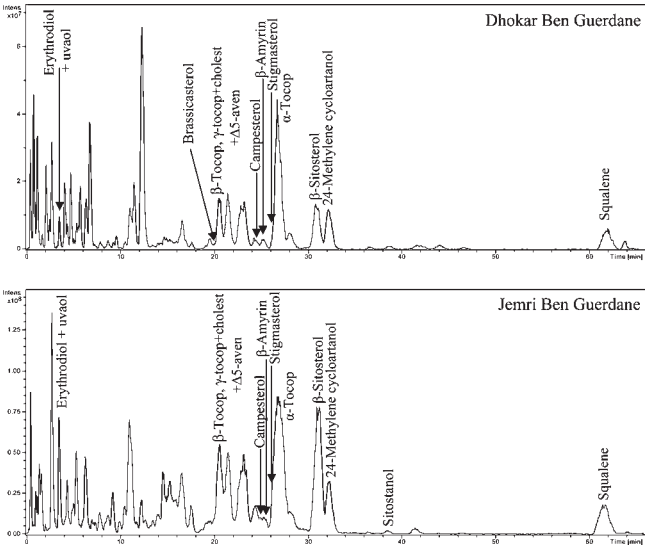

Figure 3. HPLC-APCI-IT MS chromatograms (base peak chromatograms) of Dhokar Ben Guerdane and Jemri Ben Guerdane oils.

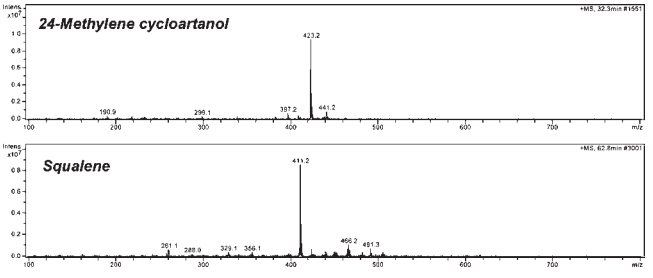

Figure 4. APCI-IT MS spectra of 24-methylene cycloartanol and squalene.

the large peaks areas observed in every chromatogram. In the literature, squalene is reported to occur in concentrations between 0.8 and 12 g/kg in virgin olive oil and is characterized by high stability under autoxidation conditions, while it was found to contribute to virgin olive oil stability under light exposure (*22*).

Table 4 shows the results obtained for the composition regarding tocopherols, sterols, and tritertepic dialcohols of the Tunisian olive oils. Several 4-desmethylsterols were identified, among which β-sitosterol was by far the predominant one. It was found from a minimum of 636.4 (Indouri Jerba) to 1445.45 mg/kg

(Jemri Ben Guerdane). β-Sitosterol is the most abundant sterol in olive oil and has a recognized effect on lowering cholesterol levels by opposing the absorption of cholesterol in the intestinal tract (*23*). The second and third phytosterols in terms of concentration were always Δ5-avenasterol and campesterol with mean values ranging from 66.16 to 278.73, and from 1.67 to 67.79 mg/kg, respectively. All other phytosterols isomers, consisting of brassicasterol, stigmasterol, cholesterol, and sitostanol, were present in lower amounts or were not even detected in the analyzed samples. As far as sterol content is concerned, we can confirm that Jemri Ben Guerdane oil was distinguishable from the others for its higher levels of all of the detected sterols components. Stigmasterol is related to various parameters of the quality of virgin olive oil. High levels correlate with high acidity and low organoleptic quality (*24*). Trace levels and not detected amounts of this sterol were observed in the analyzed olive oils, indicating that the oil samples came from healthy fruits not obtained by systems of forcing (*25*). The higher content of campesterol in relation to stigmasterol, described in the literature as a characteristic parameter of virgin olive oils (*26*, *27*), was confirmed in our research. With respect to brassicasterol, it was possible to quantify this sterol in Zarrazi Zarzis oil, while trace levels were found in Dhokar Ben Guerdane and Indouri Jerba oils. Nonetheless, it has been thoroughly proven that brassicasterol has a negligible presence in Spanish virgin olive oils (*28*). Cholesterol content was very low or even nonexistent, as in Indouri Jerba oil. The highest sitostanol content was found in the Jemri Ben Guerdane sample (0.99 mg/kg). Besides the mentioned compounds, two other compounds belonging to the 4,4′-dimethylsterol class, such as lupeol and β-amyrin, were also used in our study to characterize the olive cultivars. To the best of our knowledge, this is the first time that these pentacyclic triterpene compounds have been used to characterize monovarietal virgin olive oils. Our results showed that lupeol was not detected in all of the samples, a fact which is in good agreement with other results previously reporting any detected amount of lupeol in olive oil (*20*). Contrary to the minute amount of lupeol in the samples, β-amyrin was well represented, and its level varied widely according to the cultivar: from trace amounts for Chemlali Zarzis and Zarrazi Zarzis oils to 8.95 mg/kg for Jemri Ben Guerdane oil. β-Amyrin pentacyclic triterpene, which occurs naturally and is widespread in plants, has been reported as possessing anti-inflammatory, antinociceptive, hepatoprotective, and antiallergic properties (*29*). Its valuable pharmacological acitivity is due to the presence of perhydroaromatic ring systems in the molecule structure, which render the molecule very similar to steroid drugs (*29*).

With respect to the levels of triterpenic dialcohols (erythrodiol + uvaol), which are usually analyzed together with the sterolic fraction of olive oil, a clear difference was observed between the high amount registered in Jemri Ben Guerdane oil (26.26 mg/kg) and those of the other olive oil varieties, which ranged between 2.35 (Zarrazi Zarzis) and 9.21 mg/kg (Zalmati).

All of the studied samples showed similar qualitative profiles of tocopherols with three identified isomers: α-, β-, and γ-tocopherols. As shown in **Table 4**, the major tocopherol was the α-isomer with concentrations in the range from 61.13 (Zarrazi Zarzis) to 130.72 mg/kg (Indouri Jerba), whereas only small amounts of β- and γ-tocopherols (not exceeding 2.2 mg/kg) were found in all of the samples studied. These contents are generally lower than those reported for tocopherol profiles of other Tunisian monovarietal olive oils (*30*, *31*) and European ones (*32*). Boskou et al. (*33*) mentioned that the concentration of α-tocopherol, reported in the literature for good-quality VOOs, is usually in the range of 100−300 mg/kg and that β- and γ-tocopherols are found in smaller amounts and δ-tocopherol only in traces. The lower quantities of tocopherols verified in our samples can be explained by the influence of the climatic conditions. In fact, it is well known and widely reported in the literature that virgin olive oil composition is greatly influenced by climatic conditions, mainly the cumulative rainfall and the minimum temperatures during the harvest period (*34*). This latter factor considerably affects the tocopherol content in virgin olive oil by a remarkable decrease in the amount of these antioxidant compounds as mentioned by Romero et al. (*34*). The minimum air temperature in the area of Medenine during the harvest period (mid December, 2007) remained around 4 °C (*35*) and could be related to the lower quantities of tocopherols found in the studied oils from southeastern Tunisia, which is characterized by an arid region climate with hot, dry summers and long, cold winters.

Table 5. Correlation among Original Variables and the First Three Principal Components (PCs) as Observed in the Studied Cultivars

	PC1	PC2	PC3
erythrodiol + uvaol	**0.952**	−0.067	−0.241
brassicasterol	−0.139	−0.462	**0.818**
cholesterol	0.401	0.244	**0.842**
Δ5-avenasterol	**0.982**	−0.069	−0.070
campesterol	**0.987**	−0.018	0.123
β-amyrin	**0.851**	−0.112	−0.494
β-sitosterol	**0.976**	−0.084	0.166
sitostanol	**0.946**	−0.041	0.112
β and γ- tocopherols	0.273	**0.883**	0.358
α-tocopherol	−0.040	**0.942**	−0.211

Duncan's test showed significant ($p < 0.05$) differences in the α-tocopherol content between the studied cultivars which were grown under the same pedoclimatic conditions as those mentioned above. Similar results have been found by other authors (*30*, *31*, *36*), suggesting that α-tocopherol content is highly variety-dependent (*30*, *37*).

Statistical Analysis. All collected data were submitted to principal component analysis (PCA) in order to transform a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. It was observed that three principal components were found to be significant and explained the 95.57% of data variance. Variables such as erythrodiol + uvaol, Δ-5-avenasterol, campesterol, β-sitosterol, sitostanol and β-amyrin (on PC1), β−γ-tocopherols and α-tocopherol (on PC2), and cholesterol and brassicasterol, (on PC3) explained the largest portion of the variance (**Table 5**). **Figure 5** shows a projection of the different cultivars in the factorial plan defined by the first three principal components. The three-dimensional representation of the first three PCs allows a clear separation for the analyzed samples, and bearing that in mind, we can say that PCA was an effective tool for discriminating between cultivars.

In addition, all data were submitted to a classification test using an unsupervised pattern recognition technique, namely, cluster analysis. The associations obtained, on the basis of the similarity in Euclidian distances using the Ward algorithm, are shown as a dendrogram in **Figure 6**. The dendrogram indicates that at a rescaled distance of 19, the cultivars are distributed into four major clusters. Cluster 1 exclusively includes the Jemri Ben Guerdane cultivar, which is distinguished from the others by having the highest levels of Δ5-avenasterol, campesterol, β-amyrin β-sitosterol, sitostanol, erythrodiol, and uvaol. Cluster 2 regroups Indouri Jerba and Chemlali Zarzis oils, which are relatively rich in tocopherols in comparison to that in the other cultivars. Zarrazi Zarzis oil, which is characterized by higher rates of brassicasterol and cholesterol, forms cluster 3. Finally, cluster 4 includes Zalmati and Dhokar Ben Guerdane oils, showing the lowest levels of

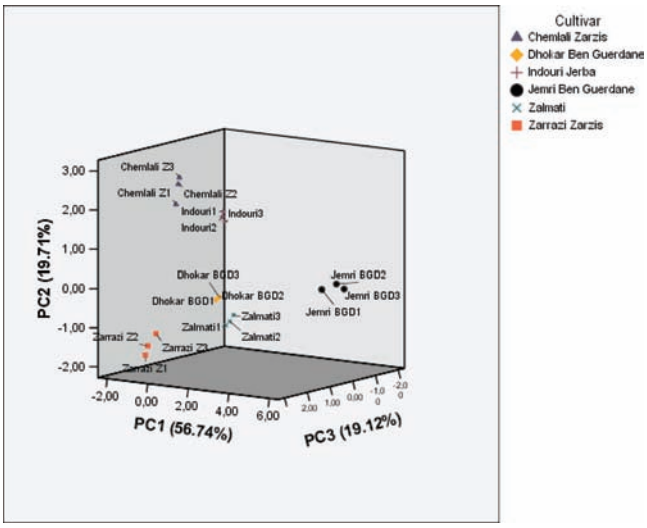

Figure 5. Three-dimensional PCA plot of the studied olive oils from different cultivars using the whole data set obtained.

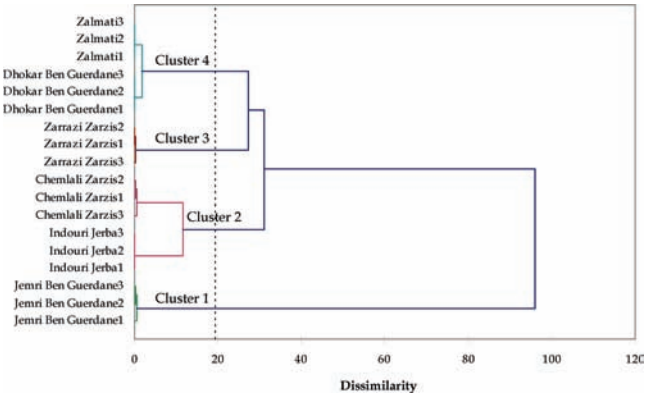

Figure 6. Dendrogram of the normalized data obtained using Euclidean distances and the Ward algorithm.

β- and γ- tocopherols, very low contents or even undetected amounts of brassicasterol and cholesterol, and lower contents in some sterol compounds belonging to PC1.

At a rescaled distance of 35, the cultivars analyzed are distributed into two major clusters: one cluster consists of only the Jemri Ben Guerdane cultivar, while the second cluster includes the remaining cultivars. This statistical analysis can explain the variability of oil composition according to the cultivar.

We noted good discrimination among olive varieties taking into consideration quantitative data from three different families of analytes. Thus, we can say that the developed multicomponent HPLC-APCI-IT MS^n method is a very sensitive tool for the characterization of olive oils, which allows the detection (within the same experiment) of some compounds belonging to different families of the unsaponifiable fraction of olive oil, information not reachable with any other single analytical procedure. The use of MS and MS/MS gave us the opportunity to carry out unequivocal identification and reliable quantification of several important compounds belonging to the sterol, tocopherol, and triterpenic dialcohol families and permitted us to collect very interesting qualitative information about other compounds from the unsaponifiable fraction.

This new HPLC-MS method could be used as a good alternative in order to assess the authenticity and quality control of olive oil, compared with other chromatographic methodologies and sample preparation protocols. Indeed, the proposed method is faster and simpler than the official method (*38*, *39*) for the analysis of sterols in olive oil since it avoids some tedious, time-consuming, and highly wasteful, in terms of reagents, steps.

From our point of view, this article is very promising and can help in the characterization of some important Tunisian virgin olive oils; however, we are aware that the study of a larger number of samples from various years of production would hopefully corroborate the results obtained by this first screening.

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