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Abstract The analysis of minor compounds (minor fatty acids, squalene, phenols and tocopherols) of eight autochthonous Tunisian varieties of virgin olive oils (VOOs) (*Chemchali*, *Chemlali Sfax*, *Chemlali Zarzis, Chetoui*, *Oueslati*, *Sayali, Zalmati* and *Zarrazi*) allows for the varietal origin authentication. The compositions of minor ω9 and ω7 fatty acids, especially 16:1 and 18:1 isomers, are important criteria for distinction among eight varieties of VOOs. The squalene content was ranged between 1.39 and 5.37 g/kg. Total phenol and the sum of α-, β- and γ-tocopherol contents were, respectively, ranged between 81–691 and 147–585 mg/kg. This minor fraction is not only highly dependent on the variety, but also the maturity index. *Chemchali, Zarrazi* and *Sayali* are characterized by having high content in total phenols, whereas *Chemlali Sfax, Chemlali Zarzis and Zalmati* are rich in total tocopherols. The inter-varietal variation in the studied minor compounds was confirmed by chemometric treatment through PCAs. The potential of using the minor compounds in the authentication of the Tunisian olive oil varietal origin was tested through PLS-DA performed for the two most representative varieties (*Chemlali Sfax* and *Chetoui*). The obtained percentages of correct classification (superior to 97 %) proved the potential of the used method in varietal origin authentication.

Keywords Autochthonous Tunisian varieties · Minor fatty acids · Squalene · Total phenols · α-, β-, γ- and δ-Tocopherols · Chemometric

Introduction

Olive oil is one of the most important elements of the diet in the Mediterranean basin and is playing a major role in preserving a healthy and relatively disease-free population. Its unique profile of antioxidants (phenolic compounds and squalene notably) and monounsaturated fatty acid (oleic acid) contributes to its health-promoting properties [1]. Tunisia is the leading producer and exporter of olive oil in the southern Mediterranean. It ranks directly after the European Union with 20 % of world olive acreage [2]. Tunisian orchards are spread on almost 1.8 million hectares dominated by two main varieties: *Chetoui* in the northern part and *Chemlali Sfax* in the center and the southern part. However, there are also other secondary local varieties specific to smaller regions such as "*Sayali*" in the northern regions, "*Oueslati*" in Kairouan, "*Chemlali Zarzis,*" "*Zalmati*" and "*Zarrazi*" in Zarzis, and "*Chemchali*" in Gafsa. Trigui and Msallem [3] have listed fifty-six different varieties, whereas Grati-Kamoun and Khlif [4] have identified more than seventy varieties of olive trees in the country. *Chemlali Sfax* cultivar accounts for nearly 85 % of cultivated olive trees [2]. It is a productive variety, self-fertile, drought tolerant and well adapted to the local tough environmental conditions [5]. Nevertheless, this variety is criticized for its lipid

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profile characterized with high levels of palmitic and linoleic acids and sometimes low levels of oleic acid which can lead to decreasing the olive oil global market value [4]. With the aim of increasing the quality of Tunisian virgin olive oils (VOOs), many investigation researches have focused on the foreign and the introduced varieties in Tunisia such as *Coratina*, *Koroneiki* and *Arbequina* [6]. However, varieties of other countries could show a problematic behavior under the Tunisian pedoclimatic conditions. In addition, it can lead to a loss of the Tunisian identity and specificity. Selecting and spreading the cultivation of the local varieties in the Tunisian orchard would be a solution for the diversification of the composition and the organoleptic profile of VOOs in Tunisia, especially that many of them are well adapted to the hard climatic conditions of the southern Tunisian soil.

Thus, this work focuses on the (1) characterization of eight Tunisian autochthonous varieties by analyzing their minor fatty acid (FA) compositions and their minor fractions of squalene, total phenols and tocopherols, and (2) evaluation of varietal origin authentication by chemometric analysis of minor compounds.

Materials and methods

Virgin olive oil samples

Sampling was carried out during two successive crop years (2011/2012 and 2012/2013). Oil extractions were carried out using a laboratory extraction system called oleodoseur (composed of crusher, vertical malaxator and centrifuge) from handpicked fresh olives (2.5 kg) without storage time before the extraction. The produced oils were filled in dark bottles and stored at 3 °C.

Every five oil samples obtained in the same day from the same locality and the same variety were mixed together, in equal proportion, in order to have more locality representative samples and to be as close as possible to the industrial production. Seventy-eight VOO samples were obtained and analyzed for this study: *Chemchali* ($n = 1$) (Ch), *Chemlali Sfax* ($n = 42$) (Cm Sfax), *Chemlali Zarzis* ($n = 3$) (Cm Zr), *Chetoui* ($n = 15$) (Ct), *Oueslati* ($n = 8$) (Ou), *Sayali* ($n = 1$) (Sa), *Zalmati* ($n = 3$) (Zl) and *Zarrazi* ($n = 5$) (Zr) varieties.

Moreover, three samples of *Chemlali Sfax,* originated from Chaal area (Sfax), were randomly picked (2.5 kg for each sample) at 3 ripening stages according to their color, in order to control the changes in the olive oil composition during the maturity progress.

Maturity index

The maturity progress was controlled by calculating maturity index of olives before each extraction. The maturity index (MI) of olives was established by visual appreciation of the color samples of 100 fruits according to a color scale varying from green-intense to a black skin and an entirely violet pulp. The maturity index values range from 0 to 7 [8].

Reagents

All chemicals and reagents were of analytical grade. Potassium hydroxide (≥99.8 %) was obtained from Prolabo (Fontenay-sous-Bois, France). Squalene (≥98 %), syringic acid (Syr) (≥95 %), tyrosol (Tyr) (≥98 %), α-tocopherol (≥98 %) and α-, β-, γ- and δ-tocopherol mixture were purchased from Sigma-Aldrich (Steinheim, Germany). Acetic acid glacial (≥99.5 %) and 2,2,4-trimethylpentane (≥99 %) were obtained from Carlo Erba Reactifs SDS (Val de Reuil, France). Hexane, acetonitrile and methanol (HPLC grade, ≥99.9 %) were purchased from Sigma-Aldrich (Steinheim, Germany). Milli-Q ultrapure water was purified in the laboratory by an ultrapure water purification system (Millipore-Merck KGaA, Darmstadt, Germany).

Quality criteria

Free acidity (A), conventionally expressed in oleic acid (g/100 g), peroxide value (PV) (meqO_2/kg) and UV absorption characteristics (K_{232} and K_{270}) were determined according to International Olive Council standard (IOC) [9].

Fatty acid and squalene determinations

Approximately 0.120 g of olive oil (accurately weighed, ±0.001 g) in 2,2,4-Trimethylpentane (isooctane, 2 mL) was trans-methylated with a cold solution of KOH (2 M) (200 µL) according to the European Standard NF EN ISO 12966-2 [10]. Fatty acid methyl esters (FAME) were analyzed according to the European Standard NF EN ISO 5508 [11]. Analyses were performed on an Agilent Technology gas chromatograph 7890A (GC) equipped with a split/split-less injector ($T = 250$ °C) and flame ionization detector (FID) ($T = 250$ °C). A silica capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness) coated with polyethylene glycol (Supelcowax, Supelco, France) was used. The carrier gas was hydrogen (column flow 1 mL/min), and the split ratio was 1:60. The oven temperature was programmed as follows: 20 min at 210 °C, from 210 to 245 °C at 6 °C/min, 20 min at 245 °C. The identification of FAs was performed by the comparison of retention times with those of olive oil FAs which composition is known [12]. FA percentages were determined by internal standardization without taking into account mass response factors. The coefficients of variation in FA percentages were

lower than 5 % for the most important FAMEs and lower than 10 % for some minor ones.

Squalene was analyzed by gas chromatography, at the same time than the fatty acid methyl esters. The quantification method is easy to implement and requires no additional sample preparation. Squalene is well separated from lignoceric acid (24:0) (retention times 38.48 and 37.55 min, respectively). It was determined along with the FAs using an external standard calibration curve (Eq. 1).

$$A = 214.32\,C - 0.21 \quad (1)$$

where A is the peak area of squalene and C is the concentration expressed in mg/mL (linearity 0.030–0.60 mg/mL, $R^2 = 0.999$). Final results, calculated on the basis of the analyzed oil weight, were expressed in mg/kg with a coefficient of variation equal to 1.5 % according to the experimental error.

Nomenclature

Fatty acids: 16:0, palmitic acid (hexadecanoic acid); 16:1ω9, hypogenic acid (7-hexadecenoic acid); 16:1ω7, palmitoleic acid (9-hexadecenoic acid); 17:0, margaric acid (heptadecanoic acid); 17:1ω8, margaroleic acid, 18:0, stearic acid, (9-heptadecenoic acid); 18:1ω9, oleic acid (9-octadecenoic acid); 18:1ω7, z-vaccenic acid (11-octadecenoic acid); 18:2ω6, linoleic acid (9,12-octadecadienoic acid); 18:3ω3, linolenic acid (9,12,15-octadecatrienoic acid); 20:0, arachidic acid (eicosanoic acid); 20:1ω9, gondoic acid (11-eicosenoic acid); 22:0, behenic acid (docosanoic acid); 24:0, lignoceric acid (tetracosanoic acid).

Phenolic compound determinations

Total phenolic content

Determination of total phenolic content was based on the IOC testing methods [13]. Some optimizations were made in the HPLC analysis as previously described [14]. Briefly, approximately 2.000 g of olive oil sample (accurately weighed, ±0.001 g) was introduced in a centrifuge tube, and 1 mL of solution of syringic acid (0.015 mg mL^{-1} in methanol/water (80/20, v/v), used as internal standard) and 3 mL of methanol/water (80/20, v/v) were added. The mixture was stirred 5 min with a test tube agitator and centrifuged at 3900 rpm for 12 min. The solvent phase was carried out, and oil residue was extracted again with 2 mL of methanol/water. Both organic solvent phases were mixed and evaporated in a rotary evaporator at 40 °C under vacuum. The residue was dissolved in 1 mL of methanol and was analyzed (20 µL injected) on a LC Agilent 1200 series system equipped with an autosampler, a quaternary pump, column heater module (25 °C) and a photodiode array detector operated with Chemstation software. Two coupled Chromolith RP18e (100 × 4.6 mm) (Merk, Darmstadt, Germany) columns were preceded by a guard column (Chromolith RP18e, 5 × 4.6 mm). Separation was achieved by elution gradient (1 mL min^{-1}) using an initial composition of 96 % water with 0.2 % acetic acid (A) and 4 % methanol/acetonitrile (50/50, v/v) (B). The concentration of (B) was increased to 50 % in 40 min, and then, it was raised to 60 % in 5 min and to 100 % in 15 min. This composition was maintained for 10 min before decreasing to 4 % (initial composition) in 2 min and then maintained for 10 min. Detection was performed at 280 nm. Total phenol contents were expressed as mg eq Tyr kg^{-1} oil [13].

Tocopherol content

Determination of tocopherols was based on the analysis by normal phase HPLC of oil samples with an optimization of the standard method [15]. As α-tocopherol is much more abundant in the samples than β- and γ-tocopherols, two olive oil sample solutions were prepared, one to quantify α-tocopherol (approximately 0.250 g accurately weighed, ±0.001 g, dissolved in a 10-mL volumetric flask with hexane/2-propanol (99/1, v/v) and a second to quantity β- and γ-tocopherols (approximately 0.700 g accurately weighed, ±0.001 g, in 5 mL). Twenty microliters of each solution was injected on a LC Agilent 1200 series system, operated with Chemstation software, equipped with an autosampler, a quaternary pump, column heater module (25 °C), and a photodiode Array (DAD) detector connected along with a multiwavelength fluorescence detector (FLD). Separation was achieved on LiChrospher-Si 60 column (250 × 4 mm, 5 µm) (Merk, Darmstadt, Germany) with a hexane/2-propanol (99/1, v/v) mobile phase at 1 mL min^{-1} flow rate. Fluorescence detection was performed at excitation and emission wavelengths of 295 and 330 nm, respectively. Two calibration ranges were used. α-tocopherol content was determined using the following external standard calibration curve (Eq. 2):

$$A = 255056\,C - 135 \quad (2)$$

where A is the peak area of α-tocopherol and C is its concentration expressed in mg mL^{-1} (linearity 0.002–0.012 mg mL^{-1}, $R^2 = 0.999$).

β- and γ-tocopherol contents were determined using the external standard calibration curve (Eq. 3) (linearity 0.0005 to 0.0020 mg mL^{-1}, $R^2 = 0.987$):

$$A = 187578\,C - 59 \quad (3)$$

Final results, calculated on the basis of α-, β- and γ-tocopherol areas and the oil weight, were expressed in mg eq α-tocopherol kg^{-1} oil with a coefficient of variation equal to 1.5 % according to the experimental error.

Unsupervised pattern recognition

Principal component analysis (PCA) is an unsupervised method describing data set contained in a multidimensional table (where each row represents a sample, and each column represents a variable) without a priori knowledge of the data structure [16]. PCA models lead to score plots and loadings plots. Scores describe the variation in the samples compared with the data set, while loadings describe the correlations among the variables.

PCA was performed on all the chemical values (minor FAs, squalene, total phenols and α-, β- and γ-tocopherol contents) without excluding samples, by using full cross-validation and dividing variable values by standard deviation.

Partial least squares-discriminant analysis (PLS-DA)

With the aim of discriminating between cultivars, partial least squares-discriminant analysis (PLS-DA) was performed for the two most representative varieties *Chemlali* and *Chetoui*. PLS-DA method has been previously described as classification method [17 and cited references]. Two models (one for each cultivar) were built by block cross-validation method during the calibration developments. Because of natural variation in compound contents, a threshold at 0.5 has been specified. Samples with membership values higher than 0.5 were identified as belonging to the cultivar corresponding to the model and samples with membership values lower than 0.5 as not belonging to it.

To constitute the "calibration set," two-thirds of the samples of each variety were randomly selected, i.e., 47 samples. The prediction set has counted 29 samples. All the variables (contents of 11 minor FAs, squalene, total phenols and tocopherols) were standardized by dividing them by the standard deviation of all samples.

The relevant statistics used were the correlation coefficient and the standard error for calibration (Rc and SEC) and for prediction (Rp and SEP).

For PLS-DA, the confusion matrices allow accessing the percentages of correct classification (%CC) (Eq. 4) which is the criterion used to compare the classification results obtained with chemometric methods [17]:

$$\%\mathrm{CC} = N_\mathrm{c} \cdot 100/(N_\mathrm{c} + N_\mathrm{ic}) \tag{4}$$

where N_c is the number of samples correctly classified and N_ic is the number of samples incorrectly classified.

Software

The chemometric applications are performed by the Unscrambler software version 9.8 from CAMO, Norway.

Results and discussion

Quality parameters and maturity indexes

The quality criteria, determined at the beginning of this study in order to identify the oil category, are presented in Table 1. It shows that there were no significant differences in the quality parameters between the eight varieties. According to the measured parameters, all oil samples were classified in the category "extra virgin olive oil" [9].

In order to limit the effect of ripening, sampling was realized during a short period. The olive maturity was faster in the southern and the central regions when compared to the northern areas. The optimal harvesting period for *Chemlali Sfax* olives would be from the end of November to the middle of December, which corresponds to a maturity index between 2.5 and 3.5 [18]. However, according to Baccouri et al. [19], this period could last until a maturity index was equal to 4.5. In fact, maturity indexes were around 3 for *Chemchali, Chemlali Sfax, Chemlali Zarzis, Zalmati and Zarrazi* varieties and around 2 for *Chetoui, Oueslati* and *Sayali* cultivated in cold and mountainous zones.

Fatty acids

For all the 78 Tunisian VOO samples, 14 FAs were identified and quantified. The FA compositions of three samples from the same trees (*Chemlali Sfax* variety) at three ripening stages are given in Table 2. For each variety, the mean, minimum and maximum of each FA are shown in Table 2. The FA composition varies according to the stage of maturity (*Chemlali Sfax*). In fact, the percentage of the palmitic acid, the main saturated FA of olive oil, decreases remarkably from 18.29 % at MI of 2.03 to 15.81 % at MI of 4.26. A small decline was also observed for all the saturated FAs. This trend confirms previous studies in the literature [20]. However, Dag et al. [21] affirm that the stearic acid (18:0) accumulates during the ripening process. Oleic acid increases slightly with the maturation process (MI = 2.03–3.50) up to 60.17 %; then, it starts to decrease (59.43 % at MI = 4.26). Linoleic acid increased during the maturation process from 13.59 % (MI = 2.03) to 15.76 % (MI = 4.26). These variations are explained by the fact that oleate desaturase enzyme transforms oleic acid into linoleic acid [22]. Regarding the minor FA contents, they are low impacted by maturity. Besides maturation, variety and geographical location also impact the FA composition.

Chemlali Sfax, cultivated in the south, showed high contents of palmitic acid (16:0 = 15.52–20.68 %) and linoleic acid (18:2ω6 = 11.47–20.34 %), with some problematic samples because their percentages were above the IOC standard [9]. *Chemlali Sfax* showed also high contents of

Table 1 Maturity indexes and quality parameters of studied Tunisian VOO samples

	Location	MI	Acidity[a]	K_{232}	K_{270}	PV[b]
IOC 2015			≤0.8	≤2.5	≤0.22	≤20
Ch *n = 1*	South	3.7	0.24	1.799	0.164	2.04
Cm *Sfax* *n = 45*	South and center					
	Mean	3.26	0.24	1.88	0.12	9.16
	Min	1.35	0.14	0.23	0.01	1.00
	Max	4.41	0.40	2.41	0.18	17.00
Cm Zr *n = 3*	South					
	Mean	2.93	0.29	1.86	0.14	10.25
	Min	1.86	0.20	1.57	0.10	10.00
	Max	4.00	0.50	2.07	0.19	10.50
Ct *n = 15*	North					
	Mean	1.85	0.32	1.78	0.08	8.17
	Min	0.93	0.17	0.58	0.01	1.70
	Max	3.44	0.52	2.37	0.18	15.50
Ou *n = 8*	Center					
	Mean	2.27	0.39	1.68	0.13	9.33
	Min	1.50	0.32	1.51	0.11	4.29
	Max	2.68	0.46	1.82	0.14	15.00
Sa *n = 1*	Northwest	2.84	0.26	1.66	0.12	13.00
Zl *n = 3*	Southeast					
	Mean	3.22	0.27	1.78	0.12	14.56
	Min	1.49	0.25	1.73	0.12	3.75
	Max	5.60	0.34	1.86	0.14	16.50
Zr *n = 5*	Southeast					
	Mean	3.38	0.29	1.50	0.11	8.89
	Min	2.29	0.16	1.14	0.07	1.81
	Max	5.10	0.45	2.41	0.16	13.00

Ch Chemchali, *Cm Sfax Chemlali Sfax*, *Cm Zr Chemlali Zarzis*, *Ct Chetoui*, *Ou Oueslati*, *Sa Sayali*, *Zl Zalmati*, *Zr Zarrazi*

[a] Free acidity (as g oleic acid/100 g of oil)

[b] Peroxide value (meq. O_2/kg)

two minor FAs: palmitoleic acid (16:1ω7 = 1.62–2.77 %) and cis-vaccenic acid (18:1ω7 = 2.77–3.63 %). The cis-vaccenic acid level was upper than the stearic acid (18:0) level for *Chemlali Sfax*. The ω7 FAs are important criteria to authenticate this cultivar.

In the center, *Oueslati* showed a high mean content of ω9 monounsaturated FAs especially with a high rate of 18:1ω9 (71.75 %) as well as 20:1ω9 (0.36 %).

In the north, *Chetoui* was characterized especially with higher mean contents of 16:1ω9 (0.13 %) and 20:1ω9 (0.35–0.44 %) and lower rate of 18:1ω7 (1.06–1.64 %), by comparison, respectively, with *Chemlali Sfax* and *Oueslati*.

Regarding minor varieties, in the south and center, *Zalmati* showed a very similar lipid profiles than *Chemlali Sfax*. It had a high content of omega 3 polyunsaturated FA (18:3ω3 = 0.71 %). *Chemlali Zarzis*, *Chemchali* and *Zarrazi* present lipid profiles different from *Chemlali Sfax* cultivar. *Chemlali Zarzis* and *Zarrazi* showed high levels of 18:1ω9 (≈70 %) and 18:0. The richness of these two varieties in oleic acid was also noticed by other authors [7]. *Zarrazi* and *Chemchali* had the highest contents in 20:1ω9. In the north, *Sayali* presents a very interesting lipidic composition with a high content of 18:1ω9 (76.69 %) and low content of 16:0 (10.78 %). *Sayali*, compared to *Chetoui*, was characterized with a low content of 18:2ω6 (6.12 %) and high contents of 17:1ω8 (0.20 %) and 17:0 (0.12 %). The major FA composition of *Sayali* fits well with listed by Sakouhi et al., [23] (18:1ω9 = 77.4 %, C16:0 = 11.0 %, 18:2ω6 = 5.9 %).

Table 2 Fatty acid compositions (%) of VOOs of eight autochthonous Tunisian varieties

	Maturity *Cm Sfax* ($n = 3$)			*Ch* $n = 1$	*Cm Sfax* $n = 42$			*Cm Zr* $n = 3$			*Ct* $n = 15$			*Ou* $n = 8$			*Sa* $n = 1$	*Zl* $n = 3$			*Zr* $n = 5$			IOC 2015
MI	2.03	3.50	4.26		Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max		Mean	Min	Max	Mean	Min	Max	
16:0	18.29	16.15	15.81	14.18	17.85	15.52	20.68	14.59	13.12	16.37	11.37	9.75	14.18	11.48	10.70	12.89	10.78	16.77	16.15	17.09	10.50	8.99	13.35	7.5-20
16:1ω9	0.05	0.05	0.07	0.05	0.06	0.02	0.08	0.09	0.08	0.10	0.13	0.12	0.16	0.09	0.08	0.11	0.12	0.06	0.06	0.07	0.09	0.08	0.11	0.3-3.5
16:1ω7	2.13	1.98	1.92	1.09	2.28	1.82	2.77	1.41	1.25	1.57	0.29	0.21	0.42	0.62	0.52	0.74	0.48	1.70	1.62	1.75	0.38	0.21	0.74	
17:0	0.04	0.04	0.04	0.04	0.04	0.04	0.05	0.05	0.05	0.05	0.05	0.04	0.06	0.04	0.04	0.04	0.12	0.05	0.05	0.05	0.04	0.02	0.04	≤0.3
17:1ω8	0.06	0.07	0.08	0.06	0.07	0.06	0.08	0.05	0.04	0.06	0.05	0.04	0.06	0.05	0.05	0.06	0.20	0.07	0.06	0.07	0.04	0.04	0.04	≤0.3
18:0	2.71	2.59	2.54	2.78	2.48	2.09	2.95	3.79	3.76	3.85	2.86	2.45	3.20	2.31	2.20	2.57	2.33	2.90	2.64	3.04	3.17	2.51	3.71	0.5-5
18:1ω9	58.65	60.17	59.43	65.75	56.60	49.44	63.02	69.59	67.36	71.32	67.47	62.21	72.69	71.75	68.95	73.72	76.69	60.07	57.64	61.94	70.83	68.11	73.94	55-83
18:1ω7	2.96	2.89	2.92	2.49	3.12	2.77	3.63	2.18	2.13	2.21	1.30	1.06	1.64	1.82	1.62	2.10	1.67	2.66	2.56	2.78	1.25	0.97	1.75	
18:2ω6	13.59	14.64	15.76	12.10	15.99	11.47	20.34	6.78	6.64	6.98	14.67	10.84	18.66	10.19	8.99	11.55	6.12	14.09	12.93	16.22	11.93	10.45	13.56	2.5-21
18:3ω3	0.66	0.60	0.65	0.48	0.67	0.56	0.96	0.59	0.56	0.64	0.72	0.62	0.85	0.64	0.53	0.69	0.56	0.71	0.67	0.74	0.63	0.49	0.86	≤1
20:0	0.48	0.44	0.43	0.48	0.44	0.38	0.50	0.51	0.49	0.54	0.50	0.45	0.54	0.44	0.41	0.48	0.40	0.21	0.19	0.24	0.51	0.45	0.53	≤0.6
20:1ω9	0.18	0.18	0.18	0.28	0.19	0.16	0.26	0.18	0.18	0.18	0.40	0.35	0.44	0.36	0.31	0.38	0.35	0.51	0.49	0.53	0.44	0.40	0.47	≤0.4
22:0	0.13	0.12	0.12	0.14	0.13	0.11	0.15	0.12	0.11	0.14	0.13	0.11	0.15	0.14	0.13	0.15	0.11	0.14	0.14	0.15	0.12	0.11	0.14	≤0.2
24:0	0.08	0.07	0.06	0.07	0.07	0.05	0.09	0.06	0.06	0.06	0.06	0.04	0.07	0.07	0.06	0.08	0.05	0.08	0.07	0.08	0.07	0.05	0.09	≤0.2

Crops: 2011/2012–2012/2013; *Ch Chemchali*; *Cm Sfax Chemlali Sfax*; *Cm Zr Chemlali Zarzis*; *Ct Chetoui*; *Ou Oueslati*; *Sa Sayali*; *Zl Zalmati*; *Zr Zarrazi*; *MI maturity index*

Table 3 Squalene (g/kg), total phenol (mg/kg) and tocopherol (mg/kg) contents of eight autochthonous Tunisian varieties

		Squalene	Total phenols	Tocopherols			
				α	β	γ	Total
Ch n = 1		5.10	679	184	6	9	199
Cm Sfax n = 42	Mean	2.15	260	329	8	8	345
	Min	1.39	81	193	4	3	200
	Max	3.33	536	521	29	15	565
Cm Zr n = 3	Mean	2.00	206	425	11	31	467
	Min	1.98	152	420	11	30	461
	Max	2.02	259	429	12	31	472
Ct n = 15	Mean	3.65	488	374	10	16	400
	Min	3.20	143	271	5	6	282
	Max	4.91	691	557	26	33	616
Ou n = 8	Mean	4.18	185	185	13	6	204
	Min	3.50	84	136	5	5	146
	Max	4.51	257	305	25	8	338
Sa n = 1		5.37	613	264	5	13	282
Zl n = 3	Mean	2.10	225	336	6	9	351
	Min	1.98	173	250	5	7	262
	Max	2.24	285	412	6	11	429
Zr n = 5	Mean	3.55	307	193	7	8	208
	Min	3.11	242	145	5	4	154
	Max	4.24	473	305	10	12	327
Maturity Cm Sfax n= 3	MI 2.03	2.65	339	366	6	8	380
	MI 3.50	2.24	258	332	5	8	345
	MI 4.26	1.86	164	335	5	9	349

Ch Chemchali, *Cm Sfax Chemlali Sfax*, *Cm Zr Chemlali Zarzis*, *Ct Chetoui*, *Ou Oueslati*, *Sa Sayali*, *Zl Zalmati*, *Zr Zarrazi*, *MI* maturity index

The knowledge of olive oil FA composition has always been one of the main issues because of its importance in the characterization and the authentication of olive oil [12]. As all VOOs, the 78 samples contain three main FAs: 16:0, 18:1ω9 and 18:2ω6. The others are considered as minor FAs (<4 %). Usually, the global content 16:1 of and 18:1 is evaluated without distinction between the both structural isomers. However, although minor fatty acids, 16:1ω9, 16:1ω7 and 18:1ω7 are important for distinction among cultivars [24]. Compared to *Chemlali Sfax*, the principal varieties of the Country, *Chemlali Zarzis, Zarrazi* and *Sayali,* present a very interesting FA composition. Encouraging the cultivation of these varieties out of their localities, particularly in *Chemlali Sfax* zones and therefore the production of blending oils, should participate to ameliorate the FA composition of the oil produced in the south and the center of Tunisia and ensure their consistency with the IOC standard.

Squalene and phenolic contents

Table 3 gives the squalene and total phenolic contents of the eight autochthonous varieties and for three samples from *Chemlali Sfax* at three ripening stages to evaluate changes in these parameters during the maturity.

Squalene content

Squalene, a triterpenoid hydrocarbon, is the main compound of the unsaponifiable fraction and the main hydrocarbon of VOOs. In response to olive maturity process of *Chemlali Sfax*, the squalene content decreases progressively from 2.65 g/kg (MI = 2.03) down to 1.86 g/kg (MI = 4.26). This behavior has already been noticed by Baccouri et al. [19] for *Chemlali* and *Chetoui* varieties. Squalene acts as chain-breaking antioxidants scavenging

the peroxyl radicals and interrupting the chain propagation, but is in itself modified during this reaction. Squalene contributes to olive oil stability under light exposure, but has no significant effect on oil stability during its storage in the dark at room temperature [25]. The squalene loss increases with the ripeness degree, and this trend probably depends on oxidative reactions started in the ripe olives [26]. Furthermore, the squalene content measured in the oil results not only from its decomposition linked to its antioxidant role but also, according to Fernández-Cuesta et al. [27], from the dynamics of oil accumulation in the fruit (dilution effect) and of its participation in the biosynthesis of other compounds such as sterols and triterpenes.

For the eight studied autochthonous varieties, squalene contents were ranged between 1.39 g/kg (*Chemlali Sfax*) and 5.37 g/kg (*Sayali*) (Table 3). *Sayali* and *Chemchali* showed the highest average content of squalene with, respectively, 5.37 g/kg and 5.10 g/kg, whereas the lowest average contents of squalene were noticed in *Chemlali Sfax* (2.15 g/kg), *Chemlali Zarzis* (2.00 g/kg) and *Zalmati* (2.10 g/kg). *Chetoui, Oueslati* and *Zarrazi* showed an intermediate squalene average content (3.55–4.18 g/kg). The obtained results are lower than those found previously for *Chemlali*, and for *Chetoui* in a rain-fed control and an irrigation regime (10.48 and 8.27 g/kg, respectively) [19] but in the range commonly reported in the literature (0.8–12 g/kg) [28].

Total phenol content

The lipophilic phenols of VOOs (phenolic acids and derivatives, phenolic alcohols, secoiridoids, lignans and flavonoids) are related to sensory and healthy proprieties and showed a correlation with olive oil oxidative stability. According to Table 3, the total phenol contents of *Chemlali Sfax* oil decrease with fruit maturation from 339 mg/kg at MI of 2.03 to 164 mg/kg at MI of 4.26. Total phenol content is also highly dependent of the variety. It was ranged between 81 mg/kg (*Chemlali Sfax*) and 691 mg/kg (*Chetoui*) (Table 3). In fact, the highest average contents of total phenols were observed with *Chemchali* and *Sayali* (>600 mg/kg) followed by *Chetoui* and *Zarrazi* with, respectively, 488 and 307 mg/kg. The average contents of total phenols were between 200 and 300 mg/kg for *Chemlali Sfax*, *Chemlali Zarzis* and *Zalmati* oils. The lowest average content of total phenols was observed for *Oueslati* oil (185 mg/kg). These concentrations are in accordance with those reported in previous works. They may range between 40 and 900 mg/kg; nevertheless, higher concentrations (up to 1000 mg/kg) have also been reported in several oils [29]. In addition to the variety and the ripening, several agronomic parameters can modify the phenolic compounds of VOOs such as geographical origin, pedoclimatic conditions and irrigation [29]. These factors can explain the unusual high contents obtained in some localities for *Chemlali Sfax* variety (maximum value 536 mg/kg).

Several researchers have reported the relationships between the total phenol concentration and the “bitter” and the “pungent” sensation considering them as positive attributes at sensorial VOO tasting [29]. Consequently, thanks to their richness in total phenol fraction, *Chemchali, Zarrazi* and *Sayali* varieties have the potential to produce oils with sensorial positive attributes and long shelf life. Spreading the cultivation of these varieties, particularly in *Chemlali Sfax* zones, should participate to ameliorate the organoleptic profile of the oils produced in the south and the center of Tunisia.

Tocopherol contents

Tocopherols are natural antioxidants which protect membrane lipids (especially polyunsaturated FAs) from oxidative damage by scavenging lipid peroxy radicals. They are important compounds in olive oil because of their contribution in the final definition of the product quality [30]. Four forms of tocopherols (vitamin E) have been identified in vegetable oils and designed as α-, β-, γ- and δ-tocopherols. They differ in the number and position of the methyl groups on the chromanol ring. Some authors report the presence of the four tocopherols (α, β, γ and δ) in olive oils [31–33], whereas others affirm that there are only three tocopherols (α, β and γ) [34–36]. In order to resolve the established ambiguity, several analyses were realized.

Figure 1a presents a HPLC chromatogram of olive oil sample and Fig. 1b, a HPLC chromatogram of the α-, β-, γ- and δ-tocopherol mixture. In the analytical conditions described in experimental part, they are totally resolved. α-, β- and γ-tocopherol are easily detected in olive oil sample, whereas an ambiguity exists regarding the δ-tocopherol. So, olive oil in mixture with the standards (Fig. 1c) was injected. Two separate peaks (at RT 9.697 min and RT = 10.028 min) were obtained instead of one peak if olive oil contained δ-tocopherol. This result was verified and confirmed on the eighty-five analyzed samples. α-, β- and γ-tocopherols are the only three existing tocopherol congeners in VOOs.

In the analyzed samples, α-tocopherol levels varied from 136 to 557 mg/kg. β- and γ-tocopherol ranges were, respectively, between 4 and 29 and 3 and 33 mg/kg. This confirms data on oils from many geographic origins: The fat soluble α-tocopherol is the major tocopherol of VOOs, and β- and γ-tocopherols are found in smaller amounts [34–36]. Tocopherols are only synthesized by photosynthetic organisms in chloroplasts. The first intermediate in tocopherol synthesis, 2-methyl-6-phytylplastoquinol, is methylated to 2,3-dimethyl-5-phytyl-1,4-benzoquinone form. These two compounds are cyclized to yield, respectively, δ- and

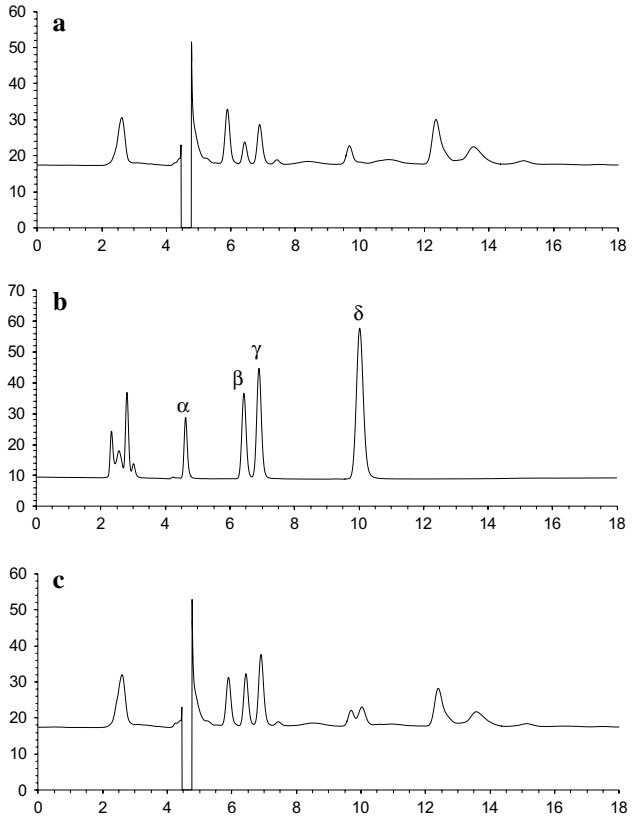

Fig. 1 Typical chromatograms of tocopherols: in **a** the studied olive oil; **b** tocopherol standard mixture; **c** olive oil in mixture with the standards. α, β, γ and δ: α-, β-, γ- and δ-tocopherols

γ-tocopherols which are finally converted by methylation into β- and α-tocopherols [30]. Thus, α-tocopherol is the main final product which could explain its much higher content. γ-tocopherol, its precursor, is much less abundant. β-tocopherol would be present in smaller amounts because it is derived from a parallel synthetic pathway from the initial intermediate.

The highest average content of total tocopherols was observed in *Chemlali Zarzis* (467 mg/kg) followed by *Chetoui* (400 mg/kg), *Zalmati* (351 mg/kg) and *Chemlali* oils (345 mg/kg). The content of total tocopherols in *Sayali* variety was equal to 282 mg/kg. *Zarrazi, Oueslati* and *Chemchali* showed almost the same average content of total tocopherols (around 200 mg/kg). The variety seems to be a source of variability for the total tocopherols as it was also observed in VOOs obtained from 29 olive cultivars grown in the World Olive Germplasm Bank of Córdoba, Spain [36]. Although *Chemlali Sfax, Chemlali Zarzis* and *Zalmati* are not characterized with a high fraction of total phenols, their richness in tocopherol contents give them the potential to produce VOOs with long self-life.

During the maturity of olives of *Chemlali Sfax*, α- and β-tocopherol levels decrease, whereas γ-tocopherol level increases slightly (Table 3). Total tocopherol content decreases from 380 mg/kg (MI = 2.03) to 349 mg/kg (MI = 4.26). These changes confirm results described by Beltran et al. [34]. The tocopherols scavenge lipid peroxy radicals by donation of a hydrogen atom from the phenolic ring hydroxyl. In cells, tocopherol radicals are recycling back, allowing each tocopherol to take part in many lipid peroxidation chain-breaking events before being degraded [30]. The tocopheroxyl radical formed could be reduced by squalene to regenerate tocopherols. Thus, tocopherols are slightly consumed first and only squalene disappears [37]. This could explain why in studied samples tocopherol losses were lower than squalene loss and why the tocopherols are reported to scavenge radicals faster than squalene [25].

Varietal origin classification by chemometric analyses

In order to examine the data structure, principal component analyses (PCA) were performed on the 78 VOO samples belonging to 8 cultivars (*Chemchali*, *Chemlali Sfax*, *Chemlali Zarzis*, *Chetoui*, *Oueslati*, *Sayali*, *Zalmati* and *Zarrazi*) (Fig. 2). Three PCAs have been performed on minor FAs (Fig. 2a, b), on squalene and phenolic compounds (i.e., total phenols and α-, β- and γ-tocopherols) (Fig. 2c, d), and on minor FAs, squalene and phenolic compounds (Fig. 2e, f).

Figure 2a, b shows the score plot and the correlation loadings (PC1 = 46 % and PC4 = 9 % of explained variance) obtained with 11 minor FAs. These FAs allowed the discrimination of *Chemlali Sfax, Chetoui, Chemlali Zarzis* and *Oueslati* samples. *Chemlali Sfax* samples were characterized by 16:1ω7 and 18:1ω7 FAs (positive part of PC1) and *Chetoui* samples by 16:1ω9 and 20:1ω9 (negative part of PC1). *Chemlali Zarzis* samples were characterized by 18:0 and 20:0 (positive part of PC4), whereas *Oueslati* samples were characterized by low percentages of 18:0 and 20:0 FAs that explain their position on the negative part of PC4. *Zalmati* samples are not differentiated from *Chemlali Sfax,* and *Zarrazi* samples are mixed with *Chetoui* or *Oueslati* samples.

PCA on squalene, total phenols and α-, β- and γ-tocopherols (Fig. 2c, d) (PC1 = 32 % and PC2 = 28 % of explained variance) showed that squalene, total phenols and α- and γ-tocopherols bring information related to the varietal origin of VOOs. *Chemlali Sfax, Chemlali Zarzis, Chetoui and Oueslati* samples were correctly separated. *Zarrazi* samples are dispersed and overlay with *Oueslati, Chemlali Sfax* or *Chetoui* samples. *Zalmati* samples were classified into *Chemlali Sfax* group. This result was expected, knowing that these two varieties have very similar fraction of squalene and phenolic compounds. They are also similar at the morphological and agronomic

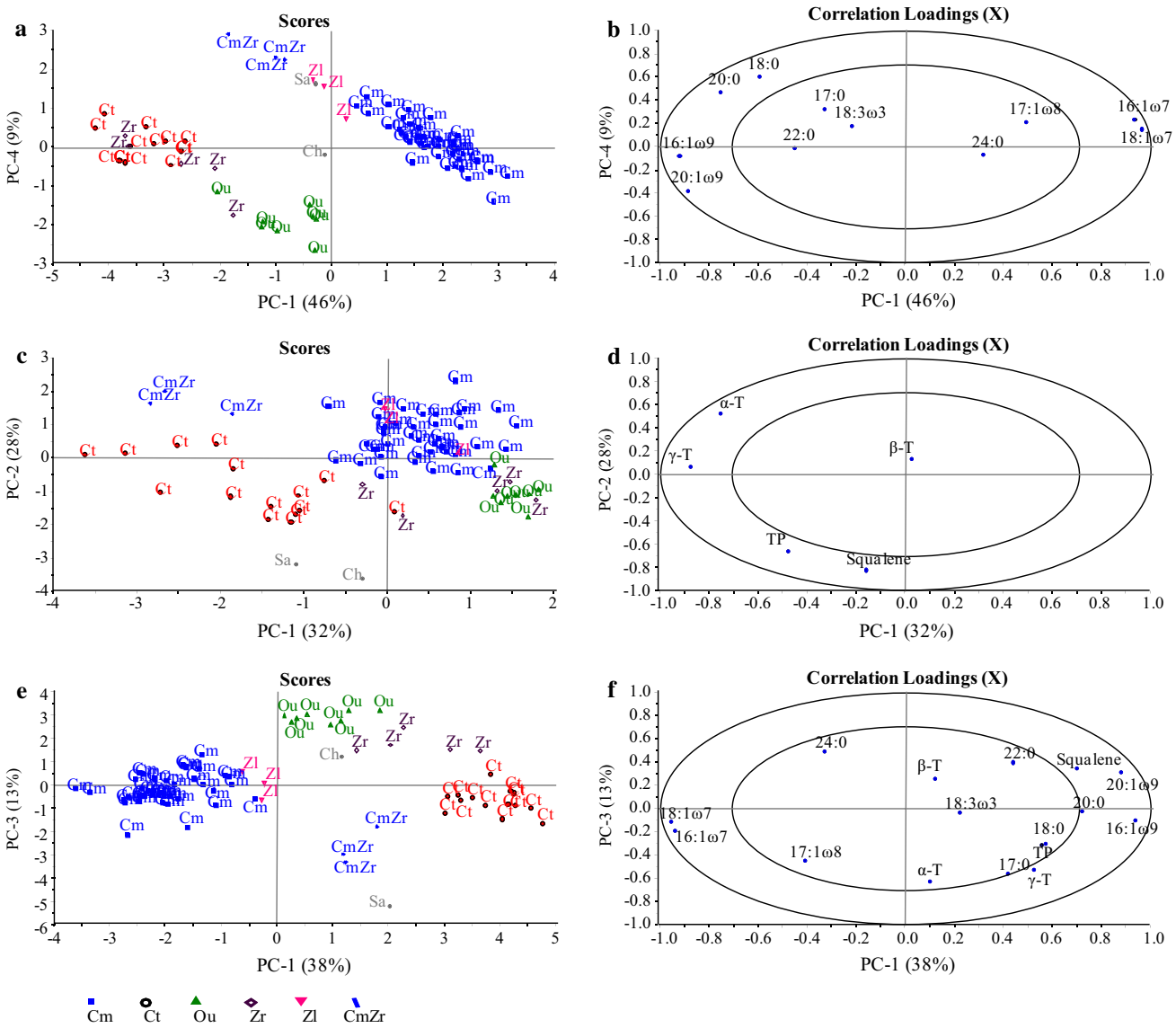

Fig. 2 Biplot of principal components for scores and correlation loadings of principal component analysis on **a**, **b** minor fatty acid contents, **c**, **d** squalene and phenolic compound (i.e., total phenols and α-, β- and γ-tocopherols) contents and **e**, **f** minor fatty acid, squalene and phenolic compound contents of 78 oil samples *Ch Chemchali*; *Cm Chemlali Sfax*; *CmZr Chemlali Zarzis*; *Ct Chetoui*, *Ou Oueslati*, *Sa Sayali*, *Zl Zalmati*, *Zr Zarrazi*, *α-T* α-tocopherol, *β-T* β-tocopherol, *γ-T* γ-tocopherol, *TP* total phenol

characteristics, and according to Fendri et al. [38], they are a synonym name of the same genotype. Hakim et al. [39] detected a 92 % of similarity by using the simple sequence repeats (SSR) on the leaves.

PCA on all minor compounds (Fig. 2e, f) (PC1 = 38 % and PC3 = 13 % of explained variance) leads to a better separation of samples according to their varietal origin. Only *Zalmati* samples are still overlaid with *Chemlali Sfax* samples. *Zarrazi* samples are located between *Chetoui* and *Oueslati* samples.

Although the maturity highly affects the composition of minor compounds, the three samples of *Chemlali Sfax* produced at three maturity stages were classified into their corresponding group. Thus, minor FAs, squalene, total phenol and tocopherol compositions combined with chemometric treatment can be a reliable method for varietal origin authentication.

In order to verify the potential of using the minor compounds' data in the authentication of the Tunisian olive oil varietal origin, PLS-DAs on 76 samples were performed on minor FAs and on minor FAs, squalene, total phenols and α-, β- and γ-tocopherols for the classification of *Chemlali Sfax* and *Chetoui* samples, the most representative varieties in this study. The samples from the other varieties

Table 4 Statistics and confusion matrix of PLS-DA regression for *Chemlali Sfax* varietal prediction based on 11 minor fatty acids or 11 minor fatty acids and other minor compounds (squalene, total phenols and tocopherols) contents, threshold = 0.5

	Calibration (n = 47) (Cm= 25, Oth = 22)			Prediction (n = 29)						
									Predicted origin	
	Rc	SEC	LV	Rp	SEP	Bias	%CC		Cm	Oth
11 mFAs	0.914	0.204	2	0.906	0.213	-0.049	100%	Real origin		
								Cm (n=17)	17	0
								Oth (n=12)	0	12
11 mFAs+5 mC	0.938	0.174	2	0.936	0.178	-0.046	97%	Cm (n=17)	17	0
								Oth (n=12)	1 (*Zl*)	11

Cm: *Chemlali Sfax* origin; Oth: other origin; mFAs: minor fatty acids; mC: other minor compounds (squalene, total phenols and tocopherols); Rc: correlation coefficient of calibration; SEC Standard Error of Calibration; LV: number of latent variables, Rp: correlation coefficient of prediction; SEP: Standard Error of Prediction; CC %: correct classification (%)

Table 5 Statistics and confusion matrix of PLS-DA regression for *Chetoui* varietal prediction based on 11 minor fatty acids and on 11 minor fatty acids and other minor compounds (squalene, total phenols and tocopherols) contents, threshold = 0.5

	Calibration (n = 47) (Ct = 9, Oth = 38)			Prediction (n = 29)						
									Predicted origin	
	Rc	SEC	LV	Rp	SEP	Bias	%CC		Ct	Oth
11 mFAs	0.927	0.149	2	0.911	0.172	0.012	100%	Real origin		
								Ct (n=6)	6	0
								Oth (n=23)	0	23
11 mFAs+5 mC	0.947	0.129	2	0.940	0.142	0.011	100%	Ct (n=6)	6	0
								Oth (n=23)	0	23

Ct Chetoui origin, *Oth* other origin, *mFAs* minor fatty acids, *mC* other minor compounds (squalene, total phenols and tocopherols), *Rc* correlation coefficient of calibration, *SEC* standard error of calibration, *LV* number of latent variables, *Rp* correlation coefficient of prediction, *SEP* Standard Error of Prediction; *CC%* correct classification (%)

constitute the group "Others." As *Sayali* and *Chemchali* varieties are represented by a sole sample, they are not taking into account in this part.

The statistics obtained for the models of the two varietal origins and the corresponding confusion matrix are presented in Tables 4 and 5. All values under 0.5 (the threshold limit) conduce to nonrecognized samples and the ones superior to 0.5 to recognized samples as belonging to the cultivar corresponding to the model. According to their correlation coefficients (Rc) and standard errors of calibration (SEC), models are roughly as efficient for *Chemlali Sfax* (Rc: 0.914 and 0.938; SEC: 0.204 and 0.174) and for *Chetoui* (Rc: 0.927 and 0.947; SEC: 0.172 and 0.142) origins.

Chemlali Sfax model made on the 11 minor FA contents gives a good correlation coefficient and a low error of prediction, and 100 % of samples are correctly predicted. Using all the minor compound contents (squalene, total phenols, tocopherols and 11 minor FAs), the model is improved and the coefficients are better than those obtained in the model based on the minor FA contents. Nevertheless, a *Zalmati* sample is recognized as *Chemlali Sfax* sample (false positive). This result can be explained by the fact that, according to previously findings (PCA, Fig. 2e), *Zalmati* and *Chemlali Sfax* show a high similarity in their minor compound compositions. In this case, minor compounds do not allow to correctly predicting the varietal origins.

For *Chetoui* models, the correlation coefficients and the errors of prediction are good with 100 % of correct prediction. Moreover, the use of all the minor compounds improved the correlation coefficient (0.940 vs 0.911) and the standard error of prediction (0.142 vs 0.172).

These results confirm that using minor compounds (minor FAs or minor FAs + others minor compounds) combined with chemometric treatment can be considered as a reliable method for varietal origin authentication of *Chemlali* and *Chetoui* VOOs.

Conclusion

The compositions of minor ω9 and ω7 FAs, especially 16:1 and 18:1 isomers, are important for distinction among eight Tunisian VOO varieties. Contents of squalene, total phenols and tocopherols are involved in the characterization of these olive oils. Minor compound compositions

combined with chemometric treatment seem to be an efficient tool for the authentication of varietal origin of VOOs and can be used to control the traceability of the product. Furthermore, the cultivation of minor varieties should be spread out of their native zones in order to diversify the composition of the Tunisian VOOs, dominated up until now by two principal varieties *Chemlali Sfax* and *Chetoui.* This would allow making blends, which would lead fatty acid contents to comply with standard. Moreover, because of their specific content phenolic compounds, it would provide also an organoleptic diversity that should please consumers.

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Compliance with ethical standards

Conflict of interest None.

Human and animal rights This article does not contain any studies with human or animal subjects.

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