

Usefulness of phenolic profile in the classification of extra virgin olive oils from autochthonous and introduced cultivars in Tunisia

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Abstract The aim of this work was to characterize extra virgin olive oils (EVOOs) from four cultivars (Arbequina, Chétoui, Neb Jmal and Picholine) cultivated in Northern Tunisia (Borj El Amri) in terms of their phenolic profile. Determination of total phenol content was first performed spectrophotometrically using the Folin–Ciocalteu reagent. The total phenol content of the analyzed EVOOs varied from 77.33 mg GAE kg⁻¹ in ‘Arbequina’ oil to 405 mg GAE kg⁻¹ in ‘Picholine’ oil. Moreover, the polar extracts were analyzed by a rapid and effective Rapid-resolution liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry (RRLC–ESI-TOF/MS) method, and 48 phenolic compounds belonging to different classes were identified. The results showed significantly quantitative differences observed for all phenolic compounds except for oleuropein that was present only in two cultivars. In all olive oil samples studied, secoiridoids were the most abundant. Deacetoxyoleuropeinaglycone remains the most abundant complex phenol in Picholine (250.39 mg kg⁻¹), Neb Jmal (104.34 mg kg⁻¹) and Arbequina (19.93 mg kg⁻¹) EVOOs, while in Chétoui oil, the oleuropeinaglycone isomer 5 content is the highest with mean value of 381.63 mg kg⁻¹. The application of principal component analysis permitted a good classification of the EVOOs according to their phenolic profiles.

Keywords Extra virgin olive oil · Chemometrics · Phenolic compounds · Cultivar · RRLC–TOF MS

Introduction

The olive tree (*Olea europaea* L.) is one of the oldest known cultivated trees in the world, and olive oil has been used by human for many tasks since the days of antiquity [1]. This tree is one of the most important crops in the Mediterranean countries, especially in Spain, Italy, Greece, Tunisia, Morocco and Algeria for climatic reasons. About 90 % of the olive fruit world production is used for obtaining olive oil according to the International Olive Council [2]. In 2014, the olive oil world production reached 2320 thousand tons [2], thus placing the olive tree as the sixth most relevant oil crop in the world.

In Tunisia, the commercial production of olive oil is an important economic activity. Olive trees cover an area of 1700 thousand ha [3] and account for more than 6 % of the olive oil produced in the world. Indeed, Tunisia is the second largest exporter of olive oil in the world (270,000 tons), after the European Union [2]. However, at present, the Tunisian olive cultivation is dominated by two main cultivars: Chemlali and Chétoui. To increase our knowledge on local and introduced cultivars and improve the quality of olive oil produced in Tunisia, research on additional cultivars needs to be conducted.

Virgin olive oil (VOO) is obtained from the fruits of olive trees by mechanical processes and therefore is a ‘fruit juice’ vegetable oil ready for direct human consumption without further treatment. Moreover, VOO is considered a prime component of the Mediterranean diet, due to its nutritional and sensory properties [4]. The nutritional qualities of VOO have been attributed exclusively to its high

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monounsaturated fatty acid (MUFA) content, mostly in the form of oleic acid and its minor components, particularly phenolic compounds [5, 6]. Moreover, the growing interest in the dietary consumption of virgin olive oil was attributed to its potential beneficial effect to human health like the prevention of some types of cancer, coronary diseases, diabetes and cell aging [7, 8].

Polyphenols are important group of natural compounds, which are produced in the secondary metabolism of many plants in nature. Phenolic compounds presented in olive oil contribute to the sensory characteristics, like its bitter, astringent and pungent taste [9] and shelf life. The main classes of phenolic compounds described in VOO are phenolic acids, phenolic alcohols, secoiridoids, lignans and flavonoids. These compounds affect the sensory and health properties of VOO [10–12]. The most abundant phenolic compounds in VOO are mainly aglycones which are derived from secoiridoid compounds that exist in olives [13].

Previous studies on the phenolic profiles of VOOs produced in different countries showed that the factors that most affect the content of phenolic compounds are the olive cultivar and the geographical area where the olive trees were cultivated [14–16]. In another study, El Riachy et al. [17] observed a dual effect of genotype and fruit ripening on the phenolic profile of olive oils from different crossing cultivars growing in Spain, with more pronounced genetic influence in both total and individual phenolics.

Similar results were obtained by Morello et al. [18] when they investigated the effect of the maturation process of the olive fruit on the phenolic fraction of drupes and oils from three Spanish cultivars, their results showed a general decreasing trend in the phenolic content of olive oils during the ripening process in the cultivars studied.

The studies on the effect of irrigation focused on the total phenolic compounds showed that irrigation decreased the concentration of phenolic compounds. Thus, Aparicio et al. [19] working on parameters affecting olive oil quality showed that the total content of phenols is lower in the virgin olive oil harvested from irrigated zones. In other work, Bengana et al. [20] showed no qualitative differences in polar phenolic profile were detected among EVOO samples from different harvest dates. However, significant quantitative differences were found in a wide number of phenolic compounds.

The development of methodologies for the identification and quantification of phenols in VOO has been discussed extensively in scientific reviews; separative techniques (LC, GC or, in some applications, CE) coupled to different detectors have been used, being LC–MS one of the most popular coupling nowadays. LC–MS combines relatively short analysis times and high separation efficiency for the analysis of VOO phenolic compounds [8,

10]. Besides, the application of multivariate statistical techniques for the treatment of the chromatographic data opens up great expectations for the assessment of VOO phenolic fraction. The combination of innovative LC–MS methodologies together with statistical approaches stands out as an appropriate tool for the traceability and classification of VOO considering its phenolic composition [21–24].

Taking into account these considerations, the aim of this work was designed to identify and quantify the phenolic compounds in olive oil from four cultivars (Neb Jmal, Chétoui, Arbéquina and Picholine) using an advanced analytical technique for their classification.

Materials and methods

Chemicals and reagents

All solvents used were analytical or HPLC grade (Panreac, Barcelona, Spain) and used as received. Double-deionized water was obtained with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Standard compounds such as hydroxytyrosol, tyrosol, luteolin, apigenin, *p*-coumaric acid, ferulic acid and quinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA), and (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland) and oleuropein from Extra synthèse (Lyon, France). The stock solutions containing these analytes were prepared in methanol/water (50/50, v/v).

Olive samples and oil extraction

The monovarietal VOO samples used in this work were obtained from olive fruits collected in November 2014 at the same ripeness stage from four cultivars cultivated in Borj El Amri in the North of Tunisia. Healthy fruits were harvested without any kind of infection or physical damage. The olives were washed and deleafed and then crushed with a hammer crusher using an Abencor analyzer (MC2 Ingenierias y Sistemas, Sevilla, Spain). The paste was mixed at 25 °C for 30 min and centrifuged, and the oil was transferred into dark glass bottles and stored at 4 °C till analysis.

Determination of ripening index

A sample of 100 olive fruits was randomly taken to calculate the ripening index (RI), according to the method developed by Experimental Station Venta del Llano- IFAPA de Mengibar (Jaén, Espagne) and described by the International Olive Council [25]. This method defines the RI as a function of fruit color in both skin and pulp. RI values

range from 0 (100 % intense green skin) to 7 (100 % purple flesh and black skin).

Extraction of polar fraction

A 2.5 g of oil sample was weighted and dissolved in 5 mL of hexane. After that, 5 mL of methanol/water (60:40) was added. The mixture was vortexed and then centrifuged at 3500 rpm during 10 min [26]. The polar extract was evaporated to dryness in a rotary evaporator under reduced pressure and a temperature of 35 °C. The residue was dissolved in 0.25 mL of methanol/water (50:50 v/v) and finally filtered through a 0.45- μ m filter before the HPLC analysis.

Colorimetric determination of VOO total polar phenol content

An aliquot of the polar fraction (0.2 mL) isolated from VOO samples was transferred into a 10-mL volumetric flask, and subsequently, water (4.8 mL) and Folin–Ciocalteu reagent (0.5 mL) were added. At 3 min after the addition of the reagent, 1 mL of a sodium carbonate solution (35 %) was added to the reaction mixture. The solution was diluted with water to 10 mL, and after 1 h the absorbance at 725 nm was measured against a blank solution [27]. The instrument used was a Labomed Dual-Beam UV–Vis spectrophotometer (Los Angeles, CA, USA). The determination of the total polar phenol content was performed in triplicate. Results were expressed as gallic acid equivalents (mg gallic acid/kg oil).

Separation and determination of phenolic compounds

Quantitative determination of total phenols in olive oil is usually performed according to the Folin–Ciocalteu colorimetric method. However, this method is not specific as it gives no indication of the nature of the phenolic compounds present. Rapid-resolution liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry (RRLC–ESI–TOF–MS) was used to separate, identify and quantify the profile of phenolic compounds in the EVOOs under study.

The separation of the phenolic compounds was performed according to the method of Lozano-Sanchez [28] using an Agilent 1200-RRLC system (Agilent Technologies, Waldbronn, Germany) of the Series Rapid Resolution equipped with a vacuum degasser, autosampler, a binary pump and a UV–vis detector. The chromatographic separation was carried out on a Zorbax Eclipse Plus C₁₈ analytical column (4.6 mm \times 150 mm, 1.8 μ m particle size) from Agilent Technologies (CA, USA). The flow rate was 0.80 mL/min, and the temperature of the column was maintained at 25 °C. The mobile phases used were water with

0.25 % acetic acid as eluent A and methanol as eluent B. The chromatographic method consisted in the following multistep linear gradient: 0 min, 5 %B; 7 min, 35 %B; 12 min, 45 %B; 17 min, 50 %B; 22 min, 60 %B; 25 min, 95 % B, 27 min, 5 %B, and finally a conditioning cycle of 5 min with the same conditions for the next analysis. The injection volume in the RRLC was 10 μ L. The compounds separated were monitored in sequence first with DAD (240 and 280 nm) and then with a mass spectrometry detector.

MS was performed using the microTOF (Bruker Daltonik, Bremen, Germany) which was coupled to the RRLC system. At this stage, the use of a splitter was required to the coupling with the MS detector as the flow which arrived to the TOF detector had to be 0.2 mL/min in order to obtain reproducible results and stable spray. The TOF mass spectrometer was equipped with an ESI interface (model G1607A from Agilent Technologies, Palo Alto, CA) operating in negative ion mode. External mass spectrometer calibration was performed with sodium formate clusters (5 mM sodium hydroxide in water/2-propanol 1/1 (v/v), with 0.2 % of formic acid) in quadratic high-precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run, and all the spectra were calibrated prior to polyphenol identification. The optimum values of source parameters were: capillary voltage of +4 kV; drying gas temperature, 190 °C; drying gas flow, 9 L/min; nebulizing gas pressure, 2 bar and end plate offset, –0.5 kV. The values of transfer parameters were: capillary exit, –120 V; skimmer 1, –40 V; hexapole 1, –23 V, RF hexapole, 50 Vpp and skimmer 2, –22.5 V.

The accurate mass data for the molecular ions were processed using the software Data Analysis 3.4 (Bruker Daltonik), which comes provided with a list of possible elemental formulas by using the Generate Molecular Formula Editor. The latter uses a CHNO algorithm providing standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalent, as well as a sophisticated comparison of the theoretical with the measured isotopic pattern (Sigma value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm for most of the compounds.

The phenolic and other polar compound concentrations were determined using the area of each individual compound (three replicates) and by interpolation in the corresponding calibration curve. Phenolic compounds hydroxytyrosol, tyrosol, luteolin, apigenin, (+)-pinoresinol and quinic acid were quantified by the calibration curves obtained from their respective commercial standards. The other phenolic compounds, which had no commercial standards, were tentatively quantified on the basis of other compounds having similar structures. Hydroxytyrosol

acetate was quantified using hydroxytyrosol calibration curve, hydroxypinoresinol, (+)-1-acetoxypinoresinol and syringaresinol using a (+)-pinoresinol calibration curve. Regarding secoiridoid group, all these compounds were quantified with oleuropein standard.

Statistical analysis

All the statistical analyses (Anova test, Duncan post hoc test and principle component analysis PCA) were performed by means of SPSS 13.0 for Windows. Analyses were performed in triplicates, and the data are given as individual values \pm standard deviation (SD). The mean values obtained in the different groups were compared by One-way ANOVA (ANOVA, Duncan significant difference multiple comparison).

Results and discussion

Ripening index

All the olive cultivars under study presented a ripening index of 3.3 ± 0.28 . The ripening degree influences the phenolic composition in virgin olive oil. Thus, in our study all of the samples have the same ripening index to exclude the effect of olive fruit ripening factor. It has been reported that olives harvested within a ripening index frame between 2.5 and 3.5 give oils with a superior sensory and compositional quality [29].

Spectrophotometric determination of total phenols

As shown in Table 2, the amount of total phenols in the analyzed oils varies significantly among the studied cultivars. The highest phenolic content was detected in Picholine oil with $405 \text{ mg GAE kg}^{-1}$, whereas the lowest amounts were recorded in Arbequina oil with $77.33 \text{ mg GAE kg}^{-1}$, while Neb Jmal and Chétoui oils have values ranging from 254 to $316 \text{ mg GAE kg}^{-1}$, respectively. These results illustrate a significant difference in the content of total phenols among the cultivars. They are in agreement with those obtained by Mahjoub-Haddada et al. [30], who found that the amount of total phenols in the analyzed oils varies significantly among six Tunisian cultivars from the north of the country (260.7 and $321.68 \text{ mg kg}^{-1}$ in Neb Jmal and Chétoui oils, respectively). The same was observed by Krichene et al. [31] when they analyzed the olive oils of some Tunisian cultivars for which the content of total phenols in Neb Jmal oil ranged about 66.14 (as mg of syringic acid kg^{-1}).

In addition, our results illustrated in Table 2 showed that the concentration of these compounds in Arbequina

EVOO was less than $100 \text{ mg GAE kg}^{-1}$ which confirmed the studies of Chtourou et al. [32] working on Arbequina cultivar grown in Sfax ($90.27 \text{ mg GAE kg}^{-1}$). In another research [33], the amount of total phenols varied between 83.12 and 108.27 (as mg of syringic acid kg^{-1}) in VOO of Arbequina cultivar grown in the South and North of Tunisia; respectively.

Chromatographic profile and identification of phenolic compounds

The identification of phenolic compounds was carried out by comparing both retention times and MS spectral data from olive oil samples and standards. Remaining compounds, for which no commercial standards were available, were identified by the interpretation of their mass spectra provided by the TOF-MS and the information previously reported in the literature for VOO phenolic compounds [16, 21, 28]. The analysis of the true isotopic pattern by ESI-TOF-MS in combination with excellent mass resolution and mass accuracy is the perfect choice for molecular formula determination using the Generate Molecular Formula Editor. To identify the phenolic compounds, a low tolerance of 0.05 and a low error (5 ppm) were chosen. The position of the molecular formula in the table of possible compounds was also considered. Most of the identified compounds are in the first position. Table 1 summarizes the main compounds identified in the analyzed samples, including the information generated by TOF analyzer: retention time, experimental and calculated m/z, molecular formula, and error and sigma value. Finally, 48 compounds from different families (simple phenols, flavonoids, lignans and secoiridoids) were identified. Figure 1 presents the base-peak chromatograms (BPCs) of Chétoui; Arbequina; Picholine and Neb Jmal extra virgin olive oils.

Among the phenolic alcohols, it was possible to identify hydroxytyrosol (also known as 3,4-dihydroxyphenylethanol or 3,4-DHPEA) with two isomers and tyrosol (also known as p-hydroxyphenylethanol or p-HPEA). These compounds are present in all analyzed samples except for hydroxytyrosol isomer 1 which was detected only in Neb Jmal oils.

A hydroxytyrosol derivative, hydroxytyrosol acetate (3,4-DHPEA-AC), was found in Arbequina and Neb Jmal oils. According to previous studies [14, 24, 30, 34], this compound was reported for Chétoui oils [30, 34], but not for Picholine [14, 24].

Numerous studies have evaluated the antioxidant capability of hydroxytyrosol for its prevention role against tumoral and cardiovascular diseases [6, 35]. On the other hand, the simple phenol hydroxytyrosol could contribute to the preventive cancer activity attributed to VOOs due to the reduction of oxidative stress and oxidative DNA protection

Table 1 Phenolic compounds identified in olive oil cultivars by HPLC–ESI–TOF MS, including retention time, m/z experimental and calculated molecular formula and sigma value

Peak	R.T. (min)	m/z calculated	m/z experimental	Molecular formula	Error	m Sigma	Compound	Class	Ar	CH	NJ	Pi
1	2,06	191,0561	191,0563	C ₇ H ₁₂ O ₆	1,1	4,2	Quinic acid	Organic acids	+	+	+	+
2	3,95	151,0401	151,0401	C ₈ H ₈ O ₃	0,0	0,0	Vanillin isomer 1	Aldehydes	+	+	+	+
3	7,58	153,0559	153,0553	C ₈ H ₁₀ O ₃	0,9	7	Hydroxytyrosol isomer 1	Phenolic alcohol	-	-	+	-
4	7,96	151,0401	151,0402	C ₈ H ₈ O ₃	0,8	5,6	Vanillin isomer 2	Aldehyde	+	+	+	+
5	8,08	153,0559	153,0557	C ₈ H ₁₀ O ₃	1,3	3,6	Hydroxytyrosol isomer 2	Phenolic alcohol	+	+	+	+
6	8,50	407,1547	407,1559	C ₁₇ H ₂₈ O ₁₁	2,9	11,3	Oleoside derivative		+	-	+	-
7	9,08	151,0406	151,0402	C ₈ H ₈ O ₃	3,2	16,6	Vanillin isomer 3	Aldehyde	+	-	-	-
8	9,17	109,0295	109,0293	C ₆ H ₆ O ₂	2,0	4,9	1,2-Hydroxybenzene = catechol		-	+	-	-
9	9,87	241,0718	241,0708	C ₁₁ H ₁₄ O ₆	3,9	16,3	Elenolic acid isomer 1	Secoiridoid derivative	+	+	-	-
10	10,00	137,0608	137,0605	C ₈ H ₁₀ O ₂	2,5	4,9	Tyrosol	Phenolic alcohol	+	+	+	+
11	11,42	167,0353	167,0345	C ₈ H ₈ O ₄	3,0	9,8	Vanillic acid	Phenolic acid	+	+	+	+
12	11,62	225,0768	225,0759	C ₁₁ H ₁₄ O ₅	0,2	4,9	Desoxy Elenolic acid isomer 1	Secoiridoid derivative	-	-	-	+
13	12,09	225,0768	225,0764	C ₁₁ H ₁₄ O ₅	1,9	8,8	Desoxy elenolic acid isomer 2	Secoiridoid derivative	-	-	-	+
14	12,13	151,0401	151,0402	C ₈ H ₈ O ₃	0,8	17,4	Vanillin isomer 4	Aldehyde	+	+	+	+
15	12,29	225,0768	225,0766	C ₁₁ H ₁₄ O ₅	1,3	25,9	Desoxy elenolic acid isomer 3	Secoiridoid derivative	-	-	-	+
16	13,01	185,0455	185,0451	C ₈ H ₁₀ O ₅	2,5	2,3	NI		+	+	+	+
17	13,21	185,0455	185,0453	C ₈ H ₁₀ O ₅	1,5	7	NI		+	+	+	+
18	13,51	225,0768	225,0762	C ₁₁ H ₁₄ O ₅	2,9	3,5	Desoxy elenolic acid isomer 4	Secoiridoid derivative	+	+	-	+
19	14,08	163,0401	163,0395	C ₉ H ₈ O ₃	3,3	5,9	<i>p</i> -coumaric acid	Phenolic acid	+	+	+	-
20	14,25	225,0768	225,076	C ₁₁ H ₁₄ O ₅	3,7	11,3	Desoxy elenolic acid isomer 5		-	-	-	+
21	14,70	195,0663	195,0654	C ₁₀ H ₁₂ O ₄	4,8	6,4	Hydroxytyrosol acetate/3,4-DHPEA-AC	Secoiridoid derivative	+	-	+	-
22	15,81	241,0718	241,0714	C ₁₁ H ₁₄ O ₆	3,3	9,8	Elenolic acid isomer 2	Secoiridoid derivative	+	+	+	-
23	17,21	319,1187	319,1189	C ₁₇ H ₂₀ O ₆	0,5	3,9	Deacetoxyoleuropein aglycone	Secoiridoids	+	+	+	+
24	17,88	377,1242	377,1226	C ₁₉ H ₂₂ O ₈	5,1	6,5	Oleuropein aglycone isomer 1	Secoiridoids	-	+	+	+
25	18,28	377,1242	377,1223	C ₁₉ H ₂₂ O ₈	4,2	3,4	Oleuropein aglycone isomer 2	Secoiridoids	-	+	+	+
26	18,63	377,1242	377,1230	C ₁₉ H ₂₂ O ₈	3,1	6,7	Oleuropein aglycone isomer 3	Secoiridoids	-	+	+	+
27	18,92	539,1770	539,1749	C ₂₅ H ₃₂ O ₁₃	3,9	2,1	Oleuropein	Secoiridoids	+	+	-	-
28	19,62	417,1555	417,1533	C ₂₂ H ₂₆ O ₈	5,8	2,8	Syringaresinol	Lignans	+	+	-	-
29	20,02	387,1449	387,1422	C ₂₁ H ₂₄ O ₇	2,7	4,6	NI		+	-	-	-
30	20,37	357,1344	357,1322	C ₂₀ H ₂₂ O ₆	5,9	3,5	Pinoresinol isomer 1	Lignans	+	+	-	-
31	20,69	183,0663	183,0653	C ₉ H ₁₂ O ₄	5,6	7,3	Dialdehydic form of decarboxymethyl-elenolic acid	Secoiridoid derivative	+	-	+	+
32	20,72	415,1398	415,1403	C ₂₂ H ₂₄ O ₈	1,2	3,3	Acetoxypinoresinol	Lignans	+	-	-	-

Table 1 continued

Peak	R.T. (min)	<i>m/z</i> calculated	<i>m/z</i> experimental	Molecular formula	Error	<i>m</i> Sigma	Compound	Class	Ar	CH	NJ	Pi
33	20,97	361,1263	361,1269	C ₁₉ H ₂₂ O ₆	6,5	6	Ligstroside aglycone isomer 1	Secoiridoids	-	+	+	+
34	21,21	361,1293	361,1277	C ₁₉ H ₂₂ O ₆	4,3	1,9	Ligstroside aglycone isomer 2	Secoiridoids	-	+	+	+
35	21,88	361,1293	361,1278	C ₁₉ H ₂₂ O ₆	4,2	3,2	Ligstroside aglycone isomer 3	Secoiridoids	-	+	+	+
36	21,88	241,0718	241,0712	C ₁₁ H ₁₄ O ₆	0,6	2,5	Elenolic acid isomer 3	Secoiridoid derivative	+	+	-	+
37	22,18	361,1293	361,1283	C ₁₉ H ₂₂ O ₆	2,8	5,8	Ligstroside aglycone isomer 4	Secoiridoids	-	+	+	+
38	22,28	357,1344	357,1333	C ₂₀ H ₂₂ O ₆	2,9	1,4	Pinoreinol isomer 2	Lignans	+	-	-	-
39	23,10	377,1242	377,1235	C ₁₉ H ₂₂ O ₈	1,9	1,4	Oleuropein aglycone isomer 4	Secoiridoids	+	+	+	+
40	24,39	377,1242	377,1234	C ₁₉ H ₂₂ O ₈	2,1	1	Oleuropein aglycone isomer 5	Secoiridoids	-	+	+	+
41	24,94	285,0405	285,0407	C ₁₅ H ₁₀ O ₆	0,8	2,7	Luteolin	Flavones	+	+	+	+
42	25,19	377,1242	377,1233	C ₁₉ H ₂₂ O ₆	2,4	1,6	Oleuropein aglycone isomer 6	Secoiridoids	+	+	+	+
43	25,54	377,1242	377,1237	C ₁₉ H ₂₂ O ₆	1,3	1,8	Oleuropein aglycone isomer 7	Secoiridoids	+	+	+	+
44	25,86	377,1242	377,1227	C ₁₉ H ₂₂ O ₆	3,9	2,4	Oleuropein aglycone isomer 8	Secoiridoids	-	+	+	+
45	26,12	361,1293	361,1279	C ₁₉ H ₂₂ O ₆	3,7	3,8	Ligstroside aglycone isomer 5	Secoiridoids	+	+	+	+
46	26,31	269,0455	269,0445	C ₁₅ H ₁₀ O ₅	3,7	2,4	Apigenin	Flavones	+	+	+	+
47	26,36	299,0557	299,0561	C ₁₆ H ₁₂ O ₆	1,3	2,6	Diosmetin	Flavones	+	-	-	-
48	26,41	377,1242	377,1220	C ₁₉ H ₂₂ O ₈	5,7	3,8	Oleuropein aglycone isomer 9	Secoiridoids	-	+	+	+
49	26,49	361,1293	361,1272	C ₁₉ H ₂₂ O ₆	5,8	3,9	Ligstroside aglycone isomer 6	Secoiridoids	+	+	+	+
50	26,66	377,1242	361,1282	C ₁₉ H ₂₂ O ₆	3,5	3,4	Oleuropein aglycone isomer 10	Secoiridoids	-	+	+	+
51	27,01	361,1293	361,1274	C ₁₉ H ₂₂ O ₆	5,2	3,5	Ligstroside aglycone isomer 7	Secoiridoids	-	+	+	+

Ar Arbequina, CH Chétoui, NJ Neb Jmal, Pi Picholine, + Detected, - Not detected; NI Not identified

in normal breast cells at physiological concentrations [36]. Furthermore, the European Food Safety Authority [37] has recently claimed that ‘the consumption of olive oil rich in polyphenols (hydroxytyrosol, 5 mg/day) contributes to the protection of oxidative damage to lipids in blood.’

Regarding secoiridoids, oleuropein and ligstrosideaglycones together with their isomers were detected as shown in Fig. 2 (ten oleuropeinaglycone isomers and seven ligstrosideaglycone isomers). Concerning desoxyelenolic acid, another secoiridoid derivative, five isomers were identified but only in Picholine oils, except for isomer 4 which was detected in Arbequina and Chétoui oils. Furthermore, oleuropein was detected in Arbequina and Chétoui oils.

It has been unambiguously demonstrated that oleuropein is present in virgin olive oils and that its concentration

could be related likely to the different types of cultivars [38, 39]. Researchers have shown that oleuropein was present in a number of virgin olive oil samples coming from different geographical areas, produced either in the laboratory scale or obtained from different commercial sources. Oleuropein was also always present, even in the 1-year-aged samples stored at 5–10 °C [38]. The presence of oleuropein may be due to the extraction process of olive oil. In fact, the activation of endogenous β-glucosidases during crushing can catalyze the hydrolysis of oleuropein, demethyloleuropein and ligstroside leading to the generation of their aglyconic forms. When mill stones are used, the obtained oils have a lower intensity of bitterness and pungency because this crushing method helps to produce oil with a lower content of phenolic substances. When metallic crushers are used,

oils have, due to the violent action, a higher content of phenolic compounds and are more bitter and pungent [40].

In addition to these phenolic compounds, elenolic acid and deacetoxyoleuropeinaglycone were also detected in the analyzed oils. As reported by different authors, these compounds are widely associated with the sensory properties of VOO as they are the main contributors to its bitterness and pungency [10], and with the health properties such as the inhibition of blood platelet aggregation and protection of erythrocytes from oxidative damage [41]. Among these compounds, oleuropein was endowed with several biological properties, particularly antioxidant and anti-inflammatory activity [41] and might be good candidates for employment as antimicrobial agents against pathogenic bacteria in man [42].

Luteolin, apigenin and diosmetin, belonging to the flavonoids group, were present in VOO samples in this study, but, diosmetin was detected only in Arbequina EVOO. This compound was not found in Arbequina cultivated in Brazil [16] nor in Tunisia [32, 43]. However, it was detected before in Arbequina cultivated in Spain [44].

Three lignans, pinoresinol, acetoxypinoresinol and syringaresinol, were identified in this study. Pinoresinol and syringaresinol were found in Arbequina and Chétoui oils. On the other hand, 1-acetoxypinoresinol was detected only in Arbequina EVOO. However, none of these lignans were detected in olive oils from Neb Jmal and Picholine cultivars, and these results are in disagreement with previous studies. In this sense, Haddada et al. [30] found pinoresinol in Neb Jmal oil. In other study, pinoresinol and acetoxypinoresinol were detected in olive oil of Picholine introduced in Tunisia [14].

Among the other polar compounds, vanillin, vanillic acid, *p*-coumaric acid and quinic acid were also found in all samples, except for *p*-coumaric acid which is not present in the olive oil from Picholine cultivar. Four isomers of vanillin were detected in this study.

Three compounds were detected but could not be identified in the analyzed samples, and they correspond to peaks 16, 17 and 29 (Table 1).

Quantitative determination of phenolic compounds

Table 2 summarizes the quantitative results obtained by RRLC–MS according to analytic condition of the present method for each individual phenolic compound as well as the total content (mg of phenolic analyte per kg of oil) for each family of phenolics in each cultivar. Significant quantitative differences were observed in phenolic compounds according to the cultivar, except for oleuropein that was present only in two cultivars (Arbequina and Chétoui) without significant difference.

The main phenolic alcohols in the studied olive oils were tyrosol and hydroxytyrosol. The concentration of tyrosol ranged from 1.32 mg kg⁻¹ in Arbequina oils to 23.2 mg kg⁻¹ in Chétoui oils. The amount of hydroxytyrosol isomer 2 varied from 2.70 to 22.56 mg kg⁻¹ for Arbequina and Picholine oils, respectively.

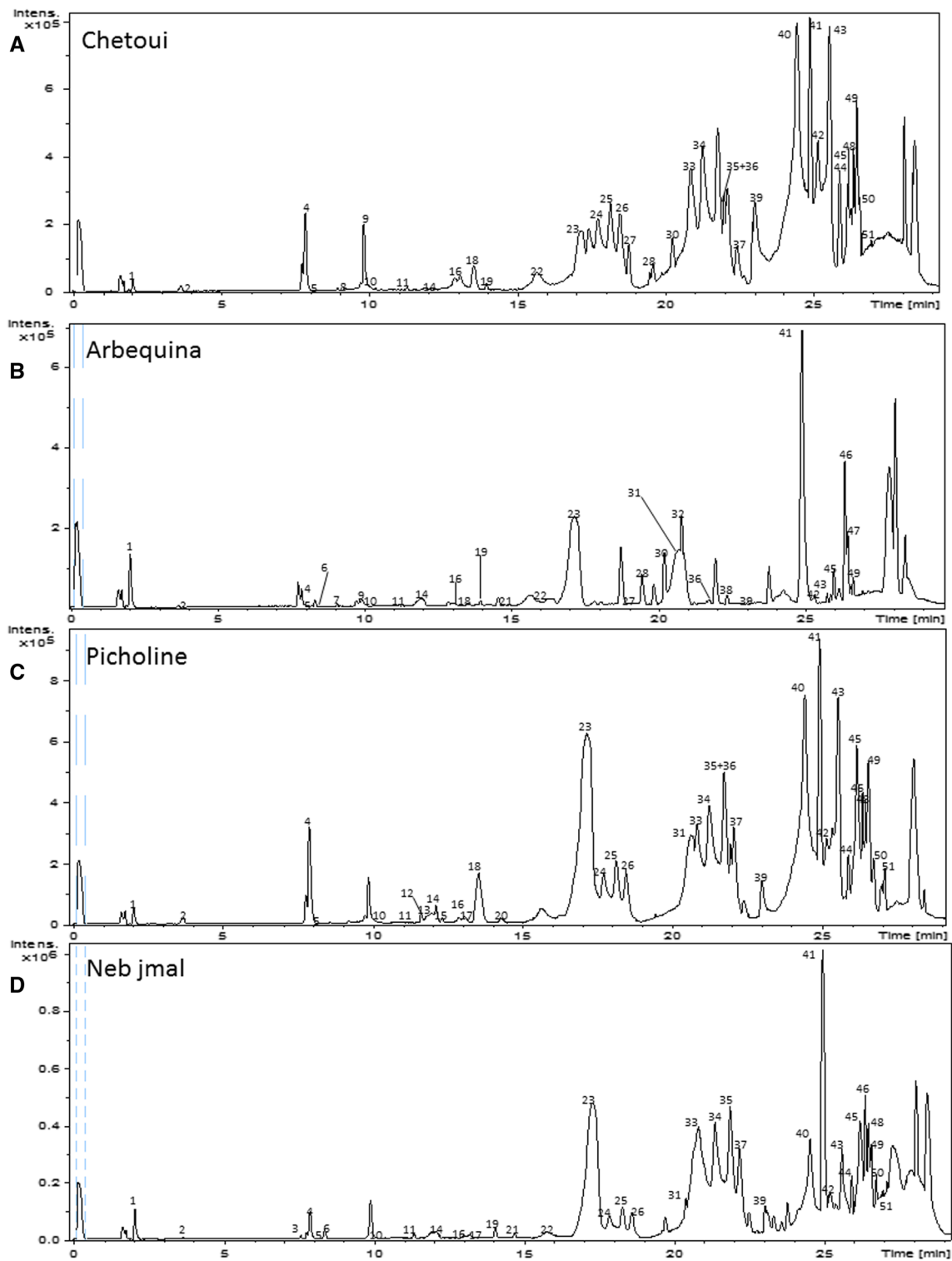
The concentration of tyrosol in Chétoui and Neb Jmal oils was higher than that of hydroxytyrosol. These results are similar to those reported by several authors for Tunisian cultivars [30, 45]. In contrast, tyrosol concentrations were relatively lower than those of hydroxytyrosol for Arbequina and Picholine olive oils which is in accordance with results reported by several authors [24, 46].

In all analyzed olive oil samples, secoiridoids were by far the most abundant group of phenolic compounds. This dominance of secoiridoids in the phenolic fraction of VOO is also reported in literature [21, 24, 47, 48]. On the one hand, the higher mean concentration of secoiridoids was observed in VOO from Chétoui cultivar with 414.58 mg kg⁻¹, whereas the lowest mean concentration was detected in Arbequina VOO with 46.01 mg kg⁻¹. On the other hand, the effect of cultivar on the quantitative content of secoiridoids can be clearly observed with the variation of the concentration of the predominant compounds in this group. Deacetoxyoleuropeinaglycone remains the most abundant complex phenol in VOO of Picholine (250.39 mg kg⁻¹), Neb Jmal (104.34 mg kg⁻¹) and Arbequina (19.93 mg kg⁻¹), while in Chétoui oil, oleuropeinaglycone isomer 5 content is the highest with a mean value of 381.63 mg kg⁻¹.

In addition, notable variations have been observed in the content of other determined secoiridoids derivatives; two main compounds were identified at relatively high concentrations. The first one was the dialdehydic form of decarboxymethylelenolic acid present in mean concentration ranged between 16.12 mg kg⁻¹ (Arbequina) and 81.28 mg kg⁻¹ (Neb Jmal). The second one was elenolic acid isomer 3 present with contents ranging from 1.62 to 6.30 mg kg⁻¹ for Arbequina and Chétoui oils, respectively.

Several authors, by using HPLC–MS for the characterization of the phenolic fraction of Spanish VOOs [8, 21] and Tunisian VOOs [48, 49], have observed that the deacetoxyoleuropeinaglycone content is higher than elenolic acid content, which is similar to our results. But, studies on Picholine cultivated in northern Morocco showed contrary behavior [24].

Moreover, oleuropein was detected only in Chétoui and Arbequina oils with small contents and varied between 2.18 and 2.58 mg kg⁻¹, respectively. The variations of the content of secoiridoids depending on the cultivar were also observed for the isomers of oleuropein and ligstrosideaglycones. In the first case, isomer 5 was the most abundant isomer found



only in Neb Jmal and Chétoui oils with concentrations that ranged between 30.62 and 381.63 mg kg^{-1} , respectively. Picholine oil was distinguished among the other oils by the abundance of isomer 8 of oleuropeinaglycone with mean concentration of 24.45 mg kg^{-1} . Whereas isomer 4 was found only in Arbequina oil presenting 3.79 mg kg^{-1} .

Ligstrosideaglycone, its isomer 5 was present in Arbequina (0.23 mg kg^{-1}), Neb Jmal (12.24 mg kg^{-1}) and Picholine (17.32 mg kg^{-1}) oils. Isomer 2 was quantified only in the Chétoui oil with a mean concentration of 9.72 mg kg^{-1} .

Regarding lignans, Owen et al. [50] have reported pinoresinol, acetoxypinoresinol and hydroxypinoresinol

◀ **Fig. 1** Base peak chromatograms (BPCs-All MS-50-1000 m/z) of Chétoui(A); Arbequina(B); Picholine(C) and Neb Jmal (D) extra virgin olive oils (1 quinic acid, 2 vanillin isomer 1, 3 hydroxytyrosol isomer 1, 4 vanillin isomer 2, 5 hydroxytyrosol isomer 2, 6 oleoside derivative, 7 vanillin isomer 3, 8 1,2-Hydroxybenzene, 9 elenolic acid isomer 1, 10 tyrosol, 11 vanillic acid, 12 desoxyelenolic acid isomer 1, 13 desoxyelenolic acid isomer 2, 14 vanillin isomer 4, 15 desoxyelenolic acid isomer 3, 16 NI, 17 NI, 18 desoxyelenolic acid isomer 4, 19 *p*-coumaric acid, 20 desoxyelenolic acid isomer 5, 21 hydroxytyrosol acetate, 22 elenolic acid isomer 2, 23 deacetoxyoleuropeinaglycone, 24 oleuropeinaglycone isomer 1, 25 oleuropeinaglycone isomer 2, 26 oleuropeinaglycone isomer 3, 27 oleuropein, 28 syringaresinol; 29 NI, 30 pinosresinol isomer 1, 31 Dialdehydic form of decarboxymethyl-elenolic acid, 32 acetoxypinosresinol, 33 ligstrosideaglycone isomer 1, 34 ligstrosideaglycone isomer 2, 35 ligstrosideaglycone isomer 3, 36 elenolic acid isomer 3, 37 ligstrosideaglycone isomer 4, 38 pinosresinol isomer 2, 39 Oleuropeinaglycone isomer 4, 40 Oleuropeinaglycone isomer 5, 41 luteolin, 42 Oleuropeinaglycone isomer 6, 43 Oleuropeinaglycone isomer 7, 44 oleuropeinaglycone isomer 8, 45 ligstrosideaglycone isomer 5, 46 apigenin, 47 diosmetin, 48 Oleuropeinaglycone isomer 9, 49 Ligstrosideaglycone isomer 6, 50 Oleuropeinaglycone isomer 10, 51 Ligstrosideaglycone isomer 7)

as the most frequent lignans in VOO. In this study, lignans concentrations in Chétoui and Arbequina oils varied between 8.09 mg kg⁻¹ and 18.18 mg kg⁻¹, respectively. Pinosresinol isomer 1 was present at concentrations ranging from 4.93 mg kg⁻¹ in Arbequina oil to 6.49 mg kg⁻¹ in Chétoui oil. Acetoxypinosresinol content in Arbequina oil was of 9.77 mg kg⁻¹ being more abundant than pinosresinol. These results are in accord with those reported by Ballus et al. [16] and Bajoub et al. [24], but disagree with those obtained by Allalout et al. [43] in Arbequina oils where very low content of acetoxypinosresinol was observed in comparison with pinosresinol. The low content of acetoxypinosresinol has been proposed recently as a method for the authentication of oils [51].

As far as flavonoids are concerned, two main compounds have been found in all samples: luteolin and apigenin. Luteolin, which is the most abundant flavonoid occurring in the analyzed VOOs, was found in mean concentration ranging

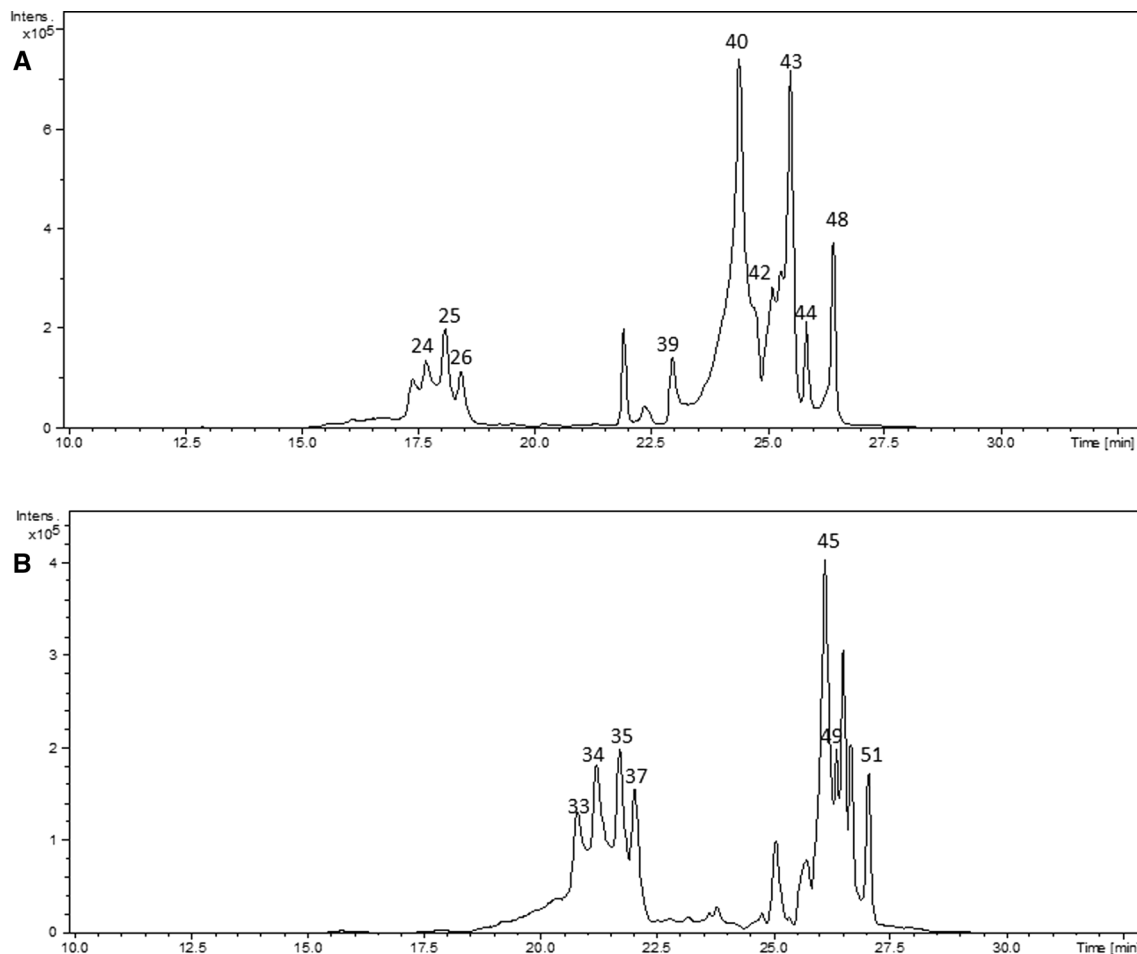


Fig. 2 Extracted ion chromatograms (EICs) of oleuropeinaglycone 377, 1242 m/z (A) and ligstrosideaglycone 361, 1293 m/z (B) compounds in Picholine extra virgin olive oil

Table 2 Phenolic contents in EVOO samples (expressed as mg of analyte/kg of oil)

Phenolic compounds	Arbequina	Chétoui	Neb Jmal	Picholine
Quinic Acid	3.12 ^a ± 0.04	0.24 ^d ± 0.01	2.37 ^b ± 0.02	1.10 ^c ± 0.01
Vanillin isomer 1	0.59 ^d ± 0.01	2.71 ^b ± 0.09	0.99 ^c ± 0.04	3.63 ^a ± 0.03
Hydroxytyrosol isomer 1	ND	ND	0.63 ± 0.01	ND
Vanillin isomer 2	7.02 ^c ± 0.06	10.21 ^b ± 0.07	2.37 ^d ± 0.05	11.5 ^a ± 0.01
Hydroxytyrosol isomer 2	2.71 ^d ± 0.06	15.13 ^b ± 0.17	6.22 ^c ± 0.12	22.57 ^a ± 0.02
Oléoside Derivative	NQ	ND	NQ	ND
1.2-hydroxybenzene (catechol)	NQ	0.43 ± 0.01	ND	ND
Tyrosol	1.32 ^c ± 0.03	23.2 ^a ± 0.35	16.55 ^b ± 0.14	17.02 ^b ± 0.15
Vanillic Acid	0.61 ^c ± 0.01	1.09 ^b ± 0.03	2.09 ^a ± 0.03	0.38 ^d ± 0.03
Desoxy Elenolic Acid isomer 1	ND	ND	ND	NQ
Desoxy Elenolic Acid isomer 2	ND	ND	ND	NQ
Vanillin isomer 4	13.1 ^c ± 0.18	NQ	15.75 ^b ± 0.24	18.95 ^a ± 0.07
Desoxy Elenolic Acid isomer 3	ND	ND	ND	NQ
Desoxy Elenolic Acid isomer 4	NQ	1.47 ^b ± 0.05	ND	6.45 ^a ± 0.02
<i>p</i> -Coumaric acid	0.3 ^c ± 0.03	0.81 ^b ± 0.04	1.82 ^a ± 0.03	ND
Desoxy Elenolic Acid isomer 5	ND	ND	ND	NQ
3,4-DHPEA-AC	1.75 ± 0.02	ND	1.16 ± 0.01	ND
Elenolic Acid isomer 2	NQ	NQ	NQ	ND
Deacetoxyoleuropeinaglycone	19.93 ^c ± 0.06	13.92 ^c ± 0.16	104.34 ^b ± 0.77	250.39 ^a ± 0.83
Oleuropein	2.58 ^a ± 0.05	2.18 ^a ± 0.05	ND	ND
Syringaresinol	2.81 ^a ± 0.01	1.61 ^b ± 0.03	ND	ND
Pinosresinol isomer 1	4.93 ^b ± 0.04	6.49 ^a ± 0.15	ND	ND
Dialdehydic form of decarboxymethyl- elenolic acid	16.12 ^{bc} ± 0.03	ND	81.28 ^a ± 11.54	32.56 ^b ± 0.17
Acetoxypinosresinol	9.77 ± 0.1	ND	ND	ND
Ligstroside aglycone isomer 2	ND	9.72 ± 0.37	NQ	NQ
Elenolic acid isomer 3	1.62 ^c ± 0.03	6.30 ^a ± 0.15	ND	5.22 ^b ± 0.02
Pinosresinol isomer 2	0.68 ± 0.01	ND	ND	ND
Oleuropein aglycone isomer 4	3.79 ± 0.02	NQ	NQ	NQ
Oleuropein aglycone isomer 5	ND	381.63 ^a ± 15.55	30.63 ^b ± 0.11	NQ
Luteolin	12.61 ^b ± 0.19	13.19 ^b ± 0.31	17.11 ^a ± 0.16	4.35 ^c ± 0.03
Oleuropein aglycone isomer 8	ND	NQ	NQ	24.45 ± 0.11
Ligstroside aglycone isomer 5	0.23 ^c ± 0.01	NQ	12.24 ^b ± 0.15	17.32 ^a ± 0.0
Apigenin	2.42 ^b ± 0.04	2.32 ^b ± 0.03	2.96 ^a ± 0.03	2.45 ^b ± 0.02
Diosmetin	1.21 ± 0.04	ND	ND	ND
∑ secoiridoids	46.01	414.58	229.65	336.38
∑ phenolic alcohols	5.78	38.32	23.40	39.57
∑ flavonoids	16.22	15.51	20.07	6.80
∑ lignans	18.18	8.09	0.0	0.0
Total HPLC	109.22 ^d ± 0.05	492.64 ^a ± 14.97	298.54 ^c ± 11.24	418.69 ^b ± 0.46
Total phenols Folin–Ciocalteu (mg GAE Kg ⁻¹)	77.33 ^d ± 0.44	316 ^b ± 4.89	254 ^c ± 1.33	405 ^a ± 9.11

Significant differences in a same row are showed by different letters ($p < 0.05$)

NQ not quantified, ND not detected, GAE gallic acid equivalent

from 4.35 mg kg⁻¹ (Picholine) to 17.11 mg kg⁻¹ (Neb Jmal). These results are similar to those reported by several authors for Tunisian [34, 52] and introduced varieties [14, 52].

Apigenin was detected at lower levels, fluctuating very little between 2.32 and 2.96 mg kg⁻¹ in Chétoui and Neb

Jmal oils, respectively. Another flavonoid, diosmetin, detected and quantified only in Arbequina oil, showed a content of 1.20 mg kg⁻¹.

Regarding simple phenols, as shown in Table 2, these compounds varied according to the cultivar. Indeed, the

abundant isomer 4 of vanillin presents average values ranging from 13.10 to 18.95 mg kg⁻¹ in oils of Arbequina and Picholine cultivars, respectively. The same variation was observed for vanillin isomer 1 with concentrations oscillating between 0.59 and 3.63 mg kg⁻¹.

On the other hand, vanillic acid was found in all samples analyzed with a content that varied between 0.38 and 2.09 mg kg⁻¹ for Picholine and Neb Jmal oils, respectively. Among EVOO samples, *p*-coumaric acid concentration was the highest in Neb Jmal oil and reached 1.82 mg kg⁻¹.

In this study, phenolic compounds in Arbequina, Chétoui, Neb Jmal and Picholine EVOOs from the same region in Northern Tunisia were characterized by RRLC–ESITOF/MS. All the olive oils analyzed showed very low concentrations of flavonoids and higher concentrations of secoiridoid compounds and derivatives. All these compounds could be used as a fingerprint to characterize and differentiate these olive oils based on cultivars [16, 34, 49, 53].

Principal component analysis (PCA)

Principal component analysis model was applied to all VOO samples to try to classify them according to their phenolic. The PCA results are graphically displayed, and the sample scores are plotted to show the relationship between the samples (Fig. 3); the loadings of the original measured variables on successive principal components are plotted to aid the interpretation of components in terms of the original variables.

The score and loading plots can be interpreted together (Fig. 3). The first two PCs explained 70.63 % of variance. The first PC accounted for 40.57 % of total variance. The variables that contributed mostly to PC1 are vanillin isomer 1, hydroxytyrosol isomer 2 and hydroxytyrosol acetate (>7 %), total phenol HPLC, oleuropeinaglycone isomer 4 (between 6 and 7 %), acetoxypinoresinol (6.033 %), pinoresinol isomer 2 (6.027 %) and diosmetin (6.019 %), while the second PC (30.05 % of variance) was correlated to DFDEA and pinoresinol isomer 1 (>9 %), Oleuropein and vanillin isomer 4 (between 8 and 9 %), apigenin, catechol and oleuropeinaglycone isomer 5 (between 7 and 8 %), ligstrosideaglycone isomer 5 (6.745 %) and hydroxytyrosol isomer 1 (6.625 %).

The obtained biplot score showed that EVOO samples belonging to the same cultivar are clustered together. All VOOs are well represented in the plans defined by the F1-F2 axes. The cluster from Neb Jmal is characterized by its richness in DFDEA, apigenin, vanillin isomer 4 and hydroxytyrosol isomer 1. ‘Chétoui’ EVOOs were richer in oleuropeinaglycone isomer 5, pinoresinol isomer 1 and catechol and presented the lowest contents of apigenin and vanillin isomer 4.

Oils from ‘Arbequina’ presented the highest contents of hydroxytyrosol acetate, diosmetin, oleuropeinaglycone

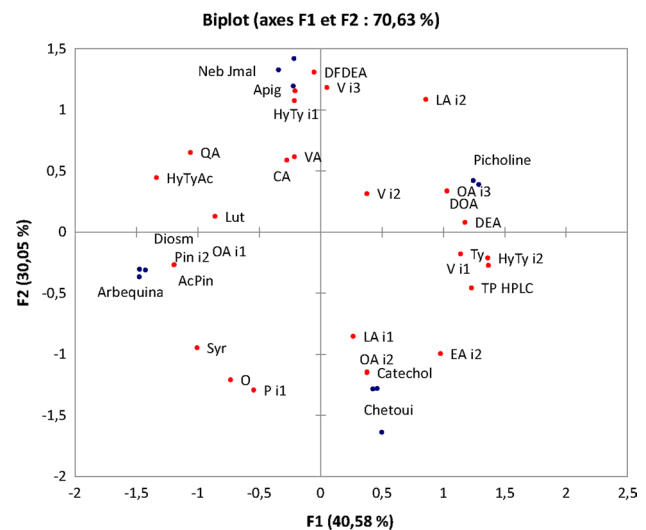


Fig. 3 Principal component analysis applied to the phenolic compound profile of the analyzed extra virgin olive oils (QA: quinic acid; v i1: vanillin isomer 1; HyTy i1: hydroxytyrosol isomer 1; v i2: vanillin isomer 2; HyTy i2: hydroxytyrosol isomer 2; Ty: tyrosol; VA: vanillic acid; V i4: vanillin isomer 4; DEA i4: desoxyelenolic acid isomer 4; CA: *p*-coumaric acid; HyTyAc: hydroxytyrosol acetate; DOA: deacetoxyoleuropeinaglycone; O: oleuropein; Syr: syringaresinol; Pi i1: pinoresinol isomer 1; LA i2: ligstrosideaglycone isomer 2; DFDEA: Dialdehydic form of decarboxymethyl-elenolic acid; AcPin: Acetoxypinoresinol; EA i3: elenolic acid isomer 3; Pin i2: pinoresinol isomer 2; OA i4: oleuropeinaglycone isomer 4; OA i5: oleuropeinaglycone isomer 5; Lut: luteolin; OA i8: oleuropeinaglycone isomer 8; LA i5: ligstrosideaglycone isomer 5; Apig: apigenin; Diosm: diosmetin; TP HPLC: total phenols HPLC)

isomer 4, acetoxypinoresinol and pinoresinol isomer 2 contents and the lowest contents of total phenol HPLC, vanillin isomer 1 and hydroxytyrosol isomer 2 in opposite to ‘Picholine’ oils which presented the highest contents of total phenol HPLC, vanillin isomer 1 and hydroxytyrosol isomer 2.

Conclusions

Rapid-resolution liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry (RRLC–ESI–TOF–MS) was employed in this work to characterize Tunisian EVOO phenolic compounds. It was possible to detect and estimate the concentrations of 48 phenolics belonging to different classes of those compounds already described for VOO samples. The highest phenolic content was detected in Chétoui and Picholine oils (>400 mg kg⁻¹), whereas the lowest amount was registered for Arbequina oil. Chétoui oil sample was characterized by its high amount in oleuropeinaglycone while Picholine oil was richer in deacetoxyoleuropeinaglycone.

In this study, the application of principal component analysis for VOOs from Tunisian and introduced cultivars was achieved to classify them according to their phenolic compounds composition. These compounds could be used as ‘markers’ to classify and differentiate these olive oils based on genotype. Indeed, Neb Jmal is characterized by its richness in DFDEA, apigenin and vanillin isomer 4. ‘Chétoui’ EVOOs were richer in oleuropein glycone isomer 5, pinosresinol isomer 1. Oils from ‘Arbequina’ presented the highest contents of hydroxytyrosol acetate, diosmetin, oleuropein glycone isomer 4, acetoxypinosresinol and pinosresinol isomer 2 contents, in opposite to ‘Picholine’ oils which presented the highest contents in vanillin isomer 1 and hydroxytyrosol isomer 2.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

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