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ORIGINAL ARTICLE

Assessment of conventional and microwave heating effects on the variation of the bioactive compounds of Chétoui VOO using HPLC-DAD-ESI-TOF-MS

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Phenolic compounds;
Microwaves;
Conventional oven

Abstract The goal of this work was to monitor the hydrophilic profile of the virgin olive oil (VOO) of the second main Tunisian variety, "Chétoui", in order to achieve a better understanding of the behavior of the bioactive phenolic compounds during 0, 2, 5, 10, and 15 min of microwave heating at medium power 800 W, and 0, 2.5, and 5 h of conventional heating at 180 °C. The extent of the oxidative and hydrolytic degradation of the different phenolic subclasses was evaluated using high-performance liquid chromatography (HPLC) coupled to electrospray time-of-flight mass spectrometry (TOF-MS) method. During heating process, the most represented component in Chétoui VOO was found to be isomer 1 and 2 of deacetoxy oleuropein aglycone, and hydroxy decarboxy oleuropein aglycon. These compounds may be considered as direct markers for the degree of transformation of secoiridoids during heating process. Among the studied phenolic compounds, hydroxytyrosol, tyrosol, luteolin and apigenin displayed the highest heating resistance in the whole time range of microwave and conventional applications. However, the main secoiridoids quantified in the fresh VOO, isomer 2 of oleuropein aglycone, and ligstroside aglycone, decreased in concentration with the thermal treatment and this decrease was drastic under conventional heating.

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1. Introduction

In the last decades, heating oils to frying temperatures is widely practiced in both culinary and industrial applications. However, it is known that oils degrade with heating and that this degradation is greater when the heating temperature is higher and when the time spent at these temperatures is longer.

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The effects of microwave and conventional heating on the oil components are, therefore, expected to be completely different. Recently, microwave heating has been introduced in order to improve the quality of the fried food (Oztop et al., 2007), since oil and fat have low specific heat constants and heat quickly (Jowitt, 1983).

The structure and concentration of the compounds present in the oil as a consequence of its thermo-oxidation during heating is a matter of great interest as some of these compounds are toxic and can be ingested directly from the degraded oil or through fried food.

Numerous researchers have reported that frying with virgin olive oil (VOO) instead of other vegetable oils can reduce the risk for colon cancer (Bosetti et al., 2002) and that its rich antioxidant composition is one of the major factors responsible for its positive effect on colon cancer prognosis and protection (Galeone et al., 2007). The main antioxidants of VOO are polyphenols represented by lipophilic and hydrophilic phenols. The VOO hydrophilic phenols constitute a group of secondary plant metabolites showing peculiar organoleptic and healthy proprieties that are not generally present in other oils and fats (Boskou, 1996).

Phenolic compounds in VOO are represented by phenolic acids, phenolic alcohols, hydroxy-isochromans, flavonoids, secoiridoids and lignans, which are of great importance on the quality of VOO. Among these secondary metabolites, secoiridoids and lignans are the most abundant (El Riachy et al., 2011). Furthermore, these compounds may generate derivatives, during VOO thermo-oxidation, which have not been completely characterized. The main reactions tested in the phenolic fraction over heating time was hydrolysis of secoiridoids and oxidation of some phenolic molecules (Brenes et al., 2001). So, the sustenance of olive oil quality and health attributes after thermal processing is frequently questioned.

With the aim of understanding the possible pathways, several investigations have been carried out for the determination of the type changes that phenols undergo during heating. Some researchers have subjected VOO, in presence of food such as potatoes, to thermal treatments under temperatures (160–190 °C) and heating times typical of domestic frying conditions (0.5–2 h) (Gomez-Alonso et al., 2003); others have performed thermal oxidation from 60 to 100 °C in an oven for a long periods (Nissiotis and Tasioula-Mahari, 2002). Many other investigations have been conducted on the influence of microwave heating on the thermo-oxidative stability of common oils and fats subjected to various heating times and microwave powers (Albi et al., 1997). While less attention has been focused on comparing the effects of microwave and conventional heating on the changes in the bioactive compounds of VOO (Brenes et al., 2002; Albi et al., 1997).

Olive oil is considered an economically important product, especially for producing countries in the Mediterranean area. Tunisia, which is among the olive growing countries in this region, is considered as a very important country in the olive oil producing world. It is the main African producer and the fourth largest exporter worldwide after Spain, Italy and Greece in 2015/2016 crop season (IOC, 2016). Many olive varieties are grown in Tunisia, but there are two that stand out: Chemlali and Chétoui (Dabbou et al., 2009). Chemlali variety spread from central to southern Tunisia producing ≥70% of national olive oil output. The preliminary study of Oueslati et al. (2009) showed that Chemlali VOO is characterized by a weak resistance to thermo-oxidation because of its low levels of oleic acid and total phenolic compounds, and therefore, its low stability against oxidation. The Chétoui variety is the second main Tunisian variety; it is widespread in the North of the country, and occurring in plains as well as in mountain regions, being highly adaptable to various pedo-climatic conditions. It accounts for more than 20% of the olive oil produced in Tunisia. Beside its remarkable high oxidative stability, high total phenolic compounds, and its great radical scavenging activity, Chétoui VOO is characterized by an exceptional bitter, fruity and pungent taste (Manai et al., 2007) highly appreciated by Tunisian consumers.

Several works were carried out to characterize the Chétoui variety by the study of the growing area effect (Ben Temime et al., 2008), olive oil and fruits storage conditions (Krichene et al., 2015), ripeness and processing conditions (Baccouri et al., 2007), on the biochemical composition of its VOOs. Nevertheless, up to now there is no study was carried out to evaluate the commercial potential of Chétoui VOO under microwave and conventional heating process.

The purpose of the present work was to characterize the phenolic profile of fresh Chétoui extra virgin olive oil (EVOO), and to evaluate the changes related to hydrolytic and oxidative degradation of its hydrophilic phenols induced by different times of microwave and conventional heating. A comparative study was performed between the two heating process. Monitoring was performed by a high-performance liquid chromatography (HPLC) coupled to electrospray time-of-flight mass spectrometry (TOF-MS) method. The effect of microwave and conventional heating on free radical–scavenger effectiveness of Chétoui VOO as well as their extracts was also investigated.

2. Materials and methods

2.1. Chemicals and reagents

Standard compounds such as hydroxytyrosol, tyrosol, luteolin, apigenin, and vanillin were purchased from Sigma-Aldrich (St. Louis, MO, USA), and oleuropein from Extrasynthèse (Lyon, France). 1,1-diphenyl-2-picrylhydrazyl radical purchased from Sigma-Aldrich (St. Louis, MO, USA), HPLC-grade acetonitrile and acetic acid (assayed at >99.5%) used for preparing mobile phases were from Labscan (Dublin, Ireland) and Fluka (Switzerland), respectively. Methanol reagent was from Panreac (Barcelona, Spain). Ethyl acetate and hexane reagents were from Sigma-Aldrich (St. Louis, MO, USA). Distilled water with a resistance of 18.2 MΩ was deionized in a Milli-Q system (Millipore, Bedford, MA, USA). The stock solutions containing these analytes were prepared in methanol. All chemicals were of analytical reagent grade and used as received. All the solutions were stored in dark flasks at −20 °C until analysis.

2.2. Sample preparation

The VOO samples were extracted, using an Abencor analyzer, from Chétoui variety (Olea europaea, Oleaceae) grown in northern Tunisia. The studied VOOs showed values of quality parameters (Acidity value, peroxide index, and ultra violet absorbency) comprised within the ranges established for "extra

virgin olive oil" category, high oxidative stability (Rancimat method) (73 h), high oleic acid (64%) and low palmitic acid (11%) percentages. Each VOO sample (70 mL) was weighed into glass beaker of dimension 7 cm (h) × 5 cm (id) and then heated separately.

For the microwave heating, the open beaker was placed alone in the middle of the rotating plate of the microwave oven (Midea, Model AG820ASI, China) and exposed to a frequency of 2450 Hz at medium power (800 W). Oil samples were subjected to microwaving for 0, 2, 5, 10, and 15 min, which simulate the usual times used to cooking. Oil temperature was determined after every microwave exposure period with a digital thermometer (Ama-digit ad 14th, Germany).

For the conventional heating, temperature was regulated at 180 °C, and the beakers were heated for 0, 2.5, and 5 h in a Binder FD series heating oven (BINdER GmbH, Tuttlingen, Germany). The temperature was controlled during the whole process.

It is interesting to mention that heating olive oil for up to 10 h at 180 °C was carried out to simulate the prolonged use of oil in some commercial activities (i.e. restaurants, etc.).

After heating, VOOs were allowed to cool at room temperature and then stored at −20 °C into sealed dark glass vials under nitrogen atmosphere until analysis.

Three independent series of experiments were carried out under the same conditions. After heating and cooling, samples were combined in order to achieve a homogeneous samples that could be used for chemical analysis.

2.3. Extraction of polar fraction

VOO samples (2.5 g) were dissolved in 5 mL *n*-hexane and extracted with 5 mL of MeOH/H_2O mixture (60:40, v/v). The resulting mixture was shaken vigorously by means of a mechanical shaker (Vortex) and centrifuged at 2054×*g* for 10 min. Then, the supernatant was removed, and the polar fraction was retained and evaporated in a rotary evaporator. The residue was dissolved in 0.25 mL of MeOH/H_2O (50:50).

2.4. HPLC-DAD-TOF-MS separation - identification - quantification

HPLC analyses were carried out using an Agilent 1200 series Rapid Resolution Liquid Chromatograph (Agilent Technologies, Santa Clara, CA, USA). The phenolic fractions were separated in a Poroshell 120 EC-C18 analytical column (4.6 mm × 100 mm, 2.7 μm). The gradient eluent was used at flow rate of 0.8 mL/min, following the method described by Talhaoui et al. (2014). The column temperature was maintained at 25 °C and the injection volume was 2.5 μL. In addition, the HPLC system was coupled to a microTOF (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer, using an electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The effluent from the HPLC column was split using a T-type phase separator before being introduced into the mass spectrometer (split ratio = 1:3). Analysis parameters were set using a negative-ion mode with spectra acquired over a mass range from *m*/*z* 50 to 1000. The optimum values of the ESI-MS parameters were: capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 9.0 L/min; and nebulizing gas pressure, 2 bar. The accurate mass data on the molecular ions was processed through Data Analysis 4.0 software (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formulae via the Smart Formula Editor. The Smart Formula Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (Sigma Value) for increased confidence in the suggested molecular formula. The quantification was carried out using Bruker Compass Target Analysis 1.2 software for compound screening (Bruker Daltonics, Bremen, Germany).

Quantification was made according to the linear calibration curves of standard compounds. Different calibration curves were prepared using the following standards: oleuropein, hydroxytyrosol, tyrosol, apigenin, and luteolin. All calibration curves showed good linearity among different concentrations. The calibration plots revealed good correlation between peak areas and analyte concentrations, and the regression coefficients in all cases were higher than 0.97.

2.5. Fractionation of unheated and heated olive oil samples

The fractionation of the oil samples was performed according to the method described by Kalantzakis et al. (2006). Unheated and heated VOO samples (2.5 g) were dissolved in 5 mL of *n*-hexane and extracted with 5 ml of a methanol-water mixture (60:40, v/v). The resulting mixture was shaken vigorously by means of a mechanical shaker (Vortex) and centrifuged at 2054×*g* for 10 min. The lipidic fraction (LF) (apolar) was obtained after evaporation of *n*-hexane. The methanolic fraction (MF) (polar) was also retained and used as it was without any further manipulation. The total fraction (TF) oil is without fractionation.

2.6. Radical scavenging activity (RSA)

VOO samples were analyzed for their capacity to scavenge the stable 1,1-diphenyl-2-picrylhydrazyl radical [DPPH·] (Kalantzakis et al., 2006). One mL of the oil solution in ethyl acetate (10%, w/v) was added to 4 mL of a freshly prepared DPPH· solution (10^{-4} M in ethyl acetate) in a screw-cap 10 mL test tube. The reaction mixture was vortexed for 10 s and the tube was held in the dark for 30 min, which allowed the reaction to reach a steady state. The mixture's absorbance at 515 nm was measured with respect to a blank solution (without radical). A control sample (without oil) was prepared and measured daily.

The DPPH· concentration in the reaction medium was calculated from the following calibration curve, which was determined by linear regression:

$$A_{515\,nm} = 11.2\,[DPPH\cdot] + 0.0124\ (r^2 = 0.99)$$

where [DPPH·] is the concentration of DPPH·, expressed as M (mol/L).

The RSA of the TFs toward DPPH was expressed as the percent reduction in DPPH· concentration by the constituents of the oils:

$$\%\,[DPPH\cdot]_{red} = 100 \times (1 - [DPPH\cdot]_{30}/[DPPH\cdot]_0),$$

where $[DPPH^{\cdot}]_0$ and $[DPPH^{\cdot}]_{30}$ are the concentrations of DPPH$^{\cdot}$ in the control sample (t = 0) and in the test mixture after the 30 min reaction, respectively.

The same procedure was followed for the LFs of the studied VOO samples.

The RSA of the MFs from VOO samples were determined as follows: 0.5 mL of each MF was added to 3 mL of a DPPH$^{\cdot}$ methanolic solution (10^{-4} M) and the resulting mixture was shaken vigorously. Absorbance at 515 nm was measured after 20 min.

2.7. Statistical analysis

Significant differences in the RSA of the Chétoui VOO during heating were determined by one-way analysis of variance, followed by Duncan's test ($p < 0.05$). Calculations were performed with SPSS 16.0 software for Windows (SPSS, Inc., Chicago, IL).

3. Results and discussion

3.1. Temperature variation

Chétoui VOOs subjected to conventional and microwave heating were analyzed, and a comparison was made between the two heating processes. Conventional heating was set to 180 °C along the experimentation, whereas, the temperature was registered at the end of each microwave heating period (Fig. 1). Heating induced an increased temperature, in the samples, that was proportional to the treatment time, as expected. Temperatures of all samples increased from 139 °C, after two minutes of treatment, to 246 °C at 10 min, to finally reach 268 °C at 15 min of treatment.

3.2. Chromatographic profile, and identification of phenolic compounds

The identification of phenolic compounds was carried out by comparing both retention times and spectral data provided by the TOF–MS from olive oil samples and standards. The identification was confirmed by a comparison with the information previously reported in the literature for VOO phenolic compounds (Lozano-Sánchez et al., 2010).

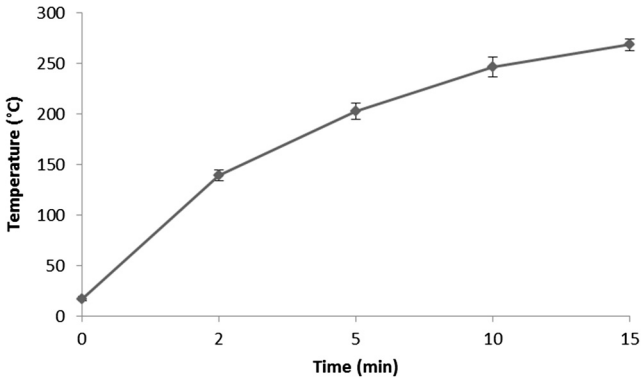

Fig. 1 Mean temperature for Chétoui VOO samples at different microwave treatment times.

The base-peak chromatograms (BPCs) of the studied phenolic extracts of the fresh Chétoui EVOO and the heated ones, in negative ionization mode, are shown in Figs. 2 and 3. The identified phenolic compounds, retention time, experimental and calculated m/z, molecular formula with their proposed identities, error and sigma value generated by TOF analyzer are summarized in Table 1.

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 and a low error 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.

A total of 14 phenolic compounds were characterized in fresh and heated Chétoui VOOs. Among these, two were characterized as phenolic alcohols, eight were from secoiridoids, one from lignan, two flavones, and one aldehyde (Table 1).

With regard to phenolic alcohols, hydroxytyrosol (also known as 3,4-dihydroxyphenylethanol or 3,4-DHPEA) (peak 3) and tyrosol (also known as *p*-hydroxyphenylethanol or *p*-HPEA) (peak 5), were identified in both fresh and heated Chétoui VOOs. From the secoiridoid group, isomer 1 and 2 of oleuropéine aglycone (also known as 3,4-DHPEA-EA or hydroxytyrosol linked to elenolic acid) (peak 11, 17), and ligstroside aglycone (also known as *p*-HPEAEA or tyrosol linked to elenolic acid) (peak 18), were the most representative complex phenols identified in fresh EVOO, whereas, their derivatives isomer 1 and 2 of deacetoxy oleuropein aglycone (peak 7, 9), isomer 1 and 2 of 10-hydroxy oleuropein aglycone (peak 14, 15), and hydroxy decarboxy oleuropein aglycon (peak 8) were the most representative complex phenols identified in heated VOOs. Additional secoiridoids, such as desoxy elenolic acid derivative (peak 4) and decarboxylated form of hydroxy-elenolic acid (peak 6) were detected only in oxidized VOOs. Regarding the lignans group, only the derivative of pinoresinol, acetoxy-pinoresinol (peak 13), was detected. The flavones luteolin and apigenin (peak 10, 16), belonging to the flavonoids group, were present in both fresh and oxidized samples. Other polar compound was also identified in all the studied VOOs, vanillin with its two isomers (peak 1, 2).

3.3. Quantification of phenolic compounds

In almost all the phenolic groups, the main phenolic compounds hydroxytyrosol, tyrosol, luteolin, apigenin, and vanillin were quantified using the equation of the calibration curves of their respective commercial standards. The lignan acetoxy-pinoresinol was quantified using luteolin. Concerning secoiridoid group, all these compounds were quantified with oleuropein standard. Quantification data of the phenolic compounds of all the studied samples appear in Table 1.

3.4. Characterization of polar compounds in fresh Chétoui EVOO

The analysis of the polar fraction of the unheated Chétoui EVOO, allowed the separation and the identification of 12 phenolic compounds accounting 478 mg analyte/kg oil, as the sum of the individual phenolic compound concentrations.

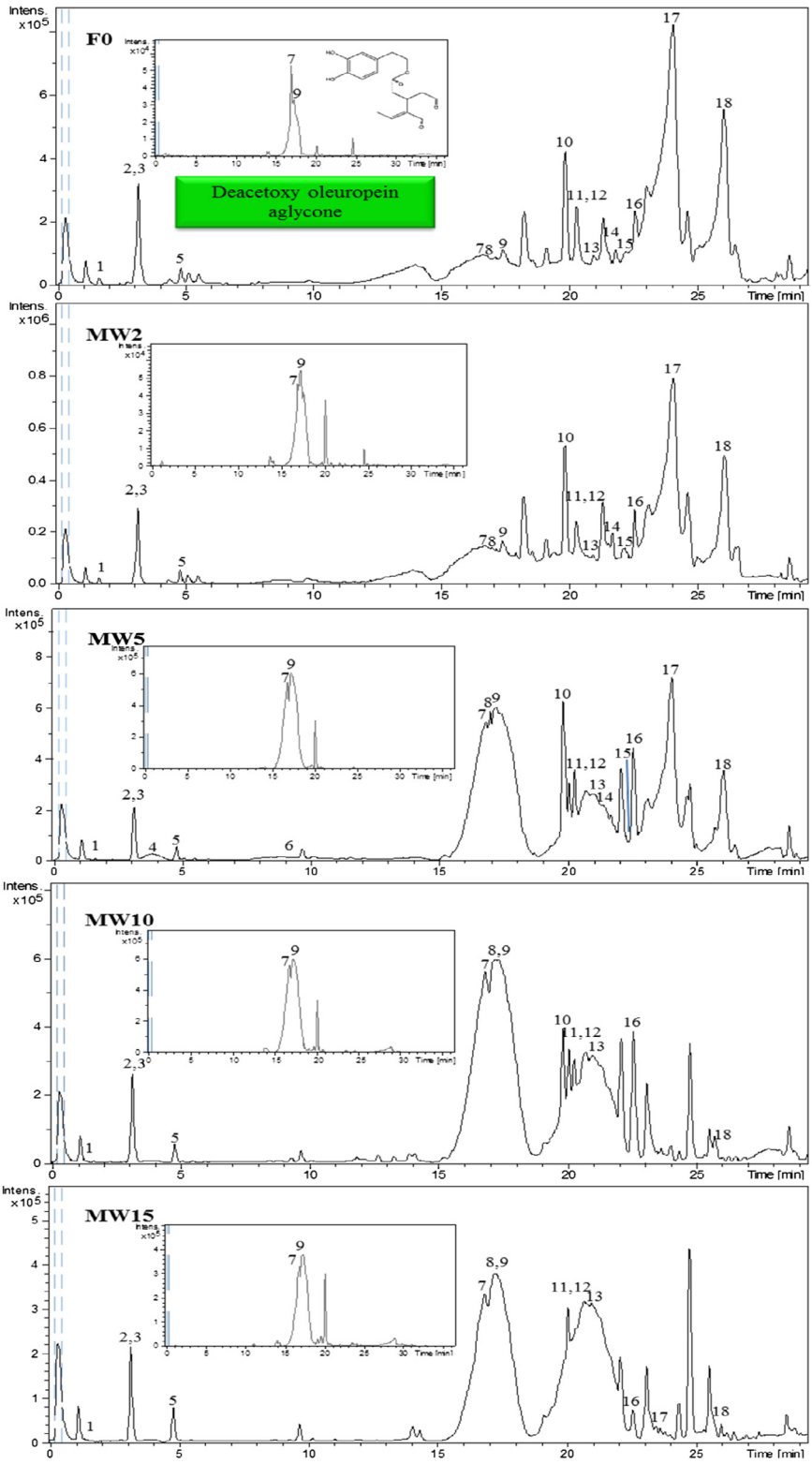

Fig. 2 Base peak chromatograms (BPCs) of 'Chétoui' VOO phenolic profiles, and extracted ion chromatograms (EICs) of the ions at *m/z* 319 (isomer 1 and 2 of deacetoxy oleuropein aglycone) during microwave heating, using HPLC-DAD-TOF-MS. Proposed phenolic compounds were numbered by elution order (See Table 1 for peak numbers).

This amount of total phenolic compounds is two to three folds higher than that found in some Spanish VOOs (Talhaoui et al., 2016) and similar to that found in some Tunisian VOOs (Loubiri et al., 2017; Ouni et al., 2011).

In the polar fraction of Chétoui EVOO, secoiridoids were by far the most abundant group of phenolic compounds accounting 457 mg analyte/kg oil, corresponding approximately to 96% of the total phenolic compounds (Table 3). This

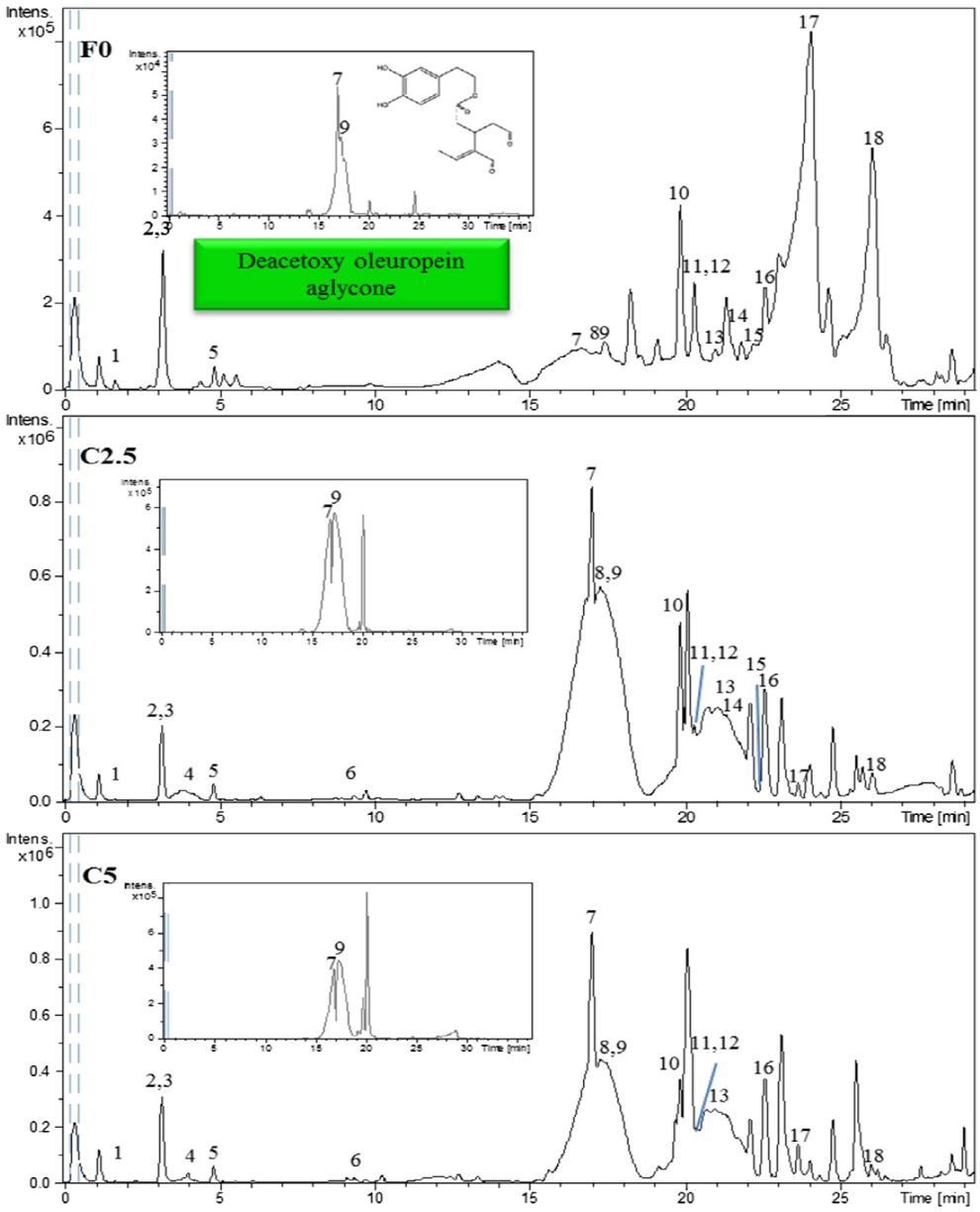

Fig. 3 Base peak chromatograms (BPCs) of 'Chétoui' VOO phenolic profiles, and extracted ion chromatograms (EICs) of the ions at *m*/*z* 319 (isomer 1 and 2 of deacetoxy oleuropein aglycone) during conventional heating, using HPLC-DAD-TOF-MS. Proposed phenolic compounds were numbered by elution order (See Table 1 for peak numbers).

dominance of secoiridoids may extend VOO shelf-life due to the positive relationship between the ratio of secoiridoid derivatives/elenolic acid derivatives and oxidative stability of the VOO (Pinelli et al., 2003). The most predominant secoiridoid quantified in the studied EVOO was isomer 2 of oleuropein aglycone (3,4-DHPEA-EA: Elenolic acid mono-aldehyde). It showed a remarkable high concentration (307 mg analyte/kg oil) by comparison with that found in some Tunisian and European EVOOs (Loubiri et al., 2017, Talhaoui et al., 2016). Oleuropein aglycone can be used as marker of VOO freshness (Bonoli et al., 2004). Ligstroside aglycone (HPEA-EA: *p*-HPEA-Elenolic acid mono-Aldehyde) was the second abundant secoiridoid (144 mg analyte/kg oil) occurring in the Chétoui EVOO phenolic compounds. It is interesting to underline that both oleuropein aglycone and ligstroside aglycone are the most potent bioactive compounds in decreasing breast cancer cell viability (Menendez et al., 2007) and also efficient radical scavengers (Bonoli et al., 2004). Through chain breaking and radical scavenger mechanisms, these molecules can play important role in VOO stability by slowing down the induction step of the oxidative process.

The amounts of the remaining detected secoiridoids, especially isomer 1 of both oleuropein aglycone and the complex phenol deacetoxy oleuropein aglycone (also known as 3,4-DHPEA-EDA: 3,4-DHPEA-elenolic acid di-aldehyde, or decarboxymethyl oleuropein aglycone), detected in the Chétoui EVOO extracts were lower (4.30 and 2.64 mg analyte/kg oil, respectively) than those previously reported, and, this

Table 1 Identification of phenolic compounds of Chétoui VOOs during heating treatments by HPLC-ESI-TOF-MS; including retention time (R.T), *m/z* experimental and calculated, tolerance, error, sigma value and molecular formula.

Peak	Compound	R.T (min)	*m/z* calculated	*m/z* experimental	Tolerance (ppm)	Error (ppm)	m Sigma	Molecular formula
1	Vanillin isomer1	1.67	151.0401	151.0396	15	3.3	9.3	$C_8H_8O_3$
2	Vanillin isomer2	3.06	151.0401	151.0396	15	3.4	6.7	$C_8H_8O_3$
3	Hydroxytyrosol	3.19	153.0557	153.0559	15	−1.1	5.6	$C_8H_{10}O_3$
4	Desoxy elenolic acid derivative	4.01	199.0612	199.0597	15	7.7	10.3	$C_{11}H_{14}O_5$
5	Tyrosol	4.85	137.0608	137.0603	15	0.5	3.5	$C_8H_{10}O_2$
6	Decarboxylated form of hydroxy-Elenolic Acid	9.18	213.0768	213.0767	15	0.5	8.6	$C_{10}H_{14}O_5$
7	Deacetoxy oleuropein aglycone isomer 1	16.79	319.1187	319.1198	15	−3.4	4.8	$C_{17}H_{20}O_6$
8	Hydroxy-Decarboxy Oleuropein aglycon	16.95	335.1136	335.1147	15	−3.1	9.8	$C_{17}H_{20}O_7$
9	Deacetoxy oleuropein aglycone isomer 2	17.20	319.1187	319.1199	15	−1.2	5.7	$C_{17}H_{20}O_6$
10	Luteolin	19.79	285.0405	285.0411	15	−2.4	10.7	$C_{15}H_{10}O_6$
11	Oleuropein aglycone isomer 1	20.22	377.1242	377.1243	15	−0.2	9.0	$C_{19}H_{22}O_8$
12	Elenolic acid methyl ester	20.38	255.0874	255.0873	15	0.1	2.9	$C_{12}H_{16}O_6$
13	AcetoxyPinoresinol	21.07	415.1398	415.1388	15	2.4	6.3	$C_{22}H_{24}O_8$
14	10-Hydroxyoleuropein aglycone isomer 1	21.59	393.1191	393.1177	15	3.7	8.9	$C_{17}H_{20}O_7$
15	10-Hydroxyoleuropein aglycone isomer 2	22.17	393.1191	393.1188	15	0.8	18.2	$C_{17}H_{20}O_7$
16	Apigenin	22.50	269.0455	269.0432	15	8.6	37.2	$C_{15}H_{10}O_5$
17	Oleuropein aglycone isomer 2	23.89	377.1242	377.1226	15	4.3	5.2	C19H22O8
18	Ligstroside aglycone	25.96	361.1293	361.1287	15	1.7	5.9	C19H22O7

was also the case for some Spanish EVOOs (Talhaoui et al., 2016).

The phenolic alcohols were by far the second main phenolic group accounting 13.4 analyte/kg oil, corresponding to 2.81% (Table 3). As mentioned previously, they are represented by hydroxytyrosol (3,4-DHPEA) and tyrosol (HPEA). Hydroxytyrosol is one of analytes that exert a strong antioxidant activity, contributing to the effective inhibition of oxidation (Carrasco-Pancorbo et al., 2005). Generally, it is higher than that of tyrosol. In the present study, the level of such compounds was almost comparable to each other (7.06 and 6.34 mg analyte/kg oil, respectively). These results are similar to those reported by Loubiri et al. (2017), and Ouni et al. (2011).

Concerning the flavonoids, which showed a low concentration (6.06 mg analyte/kg oil, corresponding to 1.28% of the total profile) by comparison with phenolic alcohols, luteolin is quantified as the most abundant flavonoid (4.94 mg analyte/kg oil) occurring in Chétoui EVOO, whereas apigenin was detected at lower level (1.15 mg analyte/kg oil). These results are similar to those reported by several authors for Tunisian (Chtourou et al., 2013) and introduced varieties (Loubiri et al., 2017). Mateos et al. (2003) reported an antioxidant activity for luteolin similar to that of hydroxytyrosol; however, apigenin did not show any antioxidant activity.

As far as lignans are concerned, Owen et al. (2000) have reported pinoresinol, acetoxy-pinoresinol and hydroxyl-pinoresinol as the most frequent lignans in VOO. Carrasco-Pancorbo et al. (2005) showed that both lignans (pinoresinol and acetoxy pinoresinol) are antioxidants and pinoresinol exerted a stronger antioxidant activity than acetoxy-pinoresinol. In this study, lignans group was represented by only acetoxy-pinoresinol which showed a very low amount (0.18 mg analyte/kg oil) as observed in some Tunisian and Spanish varieties (Loubiri et al., 2017; Ouni et al., 2011; Brenes et al., 2002). The very low content of the lignan acetoxy-pinoresinol could be considered as a potential marker able to authenticate the oil obtained from olives of Chétoui variety. Gómez Caravaca et al. (2005), and Brenes et al. (2002) have used the weak amount of acetoxy-pinoresinol as tool to authenticate and to distinguish Picual variety.

3.5. Behavior of bioactive compounds in heated Chétoui VOOs over time

As a result of exposure to heating processes, several changes occurred to the phenolic profiles of the studied VOOs. As shown in Table 2, although almost all phenolic components detected in fresh EVOO showed a variable trend in their concentrations, the secoiridoids remain the predominant group (>90%) among the phenolic groups with an increase in the duration of the thermal treatment (Table 3). The variation in the Chétoui phenolic profiles is related to the chemical structure and the stability of the components.

During heating time, a gradual increase in the number of detected phenolic compounds was accompanied by an increase in the total phenols calculated as the sum of the individual phenolic compound concentrations (74 and 11% of increases at 5 min and 2.5 h of microwave and conventional treatments, respectively). Towards the end of the experimentation, a gradual decrease was observed in both the number of individual phenolic compounds and the total phenols (72 and 20% of decreases, respectively). These trends may be the consequence of the alteration of phenolic compounds due mainly to

Table 2 Quantification of phenolic compounds and total phenols (mg analyte/kg oil) of Chétoui VOOs during conventional (180 °C) and microwave heating by HPLC-ESI-TOF-MS; n.q. (not quantified); and n.i. (not identified).

Peak	Compound	Conventional heating (h)			Microwave heating (min)				
		F0	C2.5	C5	F0	MW2	MW5	MW10	MW15
1	Vanillin isomer1	0.17	0.02	0.03	0.17	0.13	0.05	0.05	n.q.
2	Vanillin isomer2	0.47	0.07	0.03	0.47	0.36	0.09	0.13	0.06
3	Hydroxytyrosol	7.06	3.92	6.35	7.06	5.90	4.15	5.26	4.15
4	Desoxy elenolic Acid derivative	nd	n.q.	n.q.	nd	nd	n.q.	nd	nd
5	Tyrosol	6.34	5.09	6.58	6.34	5.84	5.75	5.86	8.25
6	Decarboxylated form of hydroxy-Elenolic Acid	nd	n.q.	n.q.	nd	nd	n.q.	nd	nd
7	Deacetoxy oleuropein aglycone isomer 1	2.64	197	119	2.64	1.38	212	82.3	18.7
8	Hydroxy Decarboxy oleuropein aglycon	n.q.	4.16	25.2	n.q.	0.68	28.3	14.1	4.18
9	Deacetoxy oleuropein aglycone isomer 2	n.q.	312	216	n.q.	2.09	308	218	100
10	Luteolin	4.94	4.75	3.32	4.94	4.80	4.58	3.65	nd
11	Oleuropein aglycone isomer 1	4.30	1.64	n.q.	4.30	4.11	5.61	2.84	n.q.
12	Elenolic acid methyl ester	n.q.	0.25	0.15	n.q.	n.q.	n.q.	0.90	n.q.
13	Acetoxy-Pinoresinol	0.18	0.03	0.05	0.18	0.11	0.05	0.04	0.05
14	10-Hy-oleuropein aglycone isomer 1	n.q.	n.q.	nd	n.q.	1.69	1.09	nd	nd
15	10-Hy-oleuropein aglycone isomer 2	n.q.	n.q.	nd	n.q.	0.08	n.q.	nd	nd
16	Apigenin	1.15	0.92	3.31	1.15	1.56	4.43	3.58	0.14
17	Oleuropein aglycone isomer 2	307	n.q.	n.q.	307	281	219	nd	n.q.
18	Ligstroside aglycone	144	0.42	n.q.	144	110	38.4	n.q.	n.q.
	Total phenols (HPLC)	478	530	380	478	420	831	337	136

Table 3 Changes in phenolic groups (mg analyte/kg oil) of the hydrophilic profile of Chétoui VOOs during conventional (180 °C) and microwave heating.

	Conventional heating (h)			Microwave heating (min)				
	F0	C2.5	C5	F0	MW2	MW5	MW10	MW15
Phenolic alcohols	13.4	9.00	12.9	13.4	11.7	9.90	11.1	12.4
Secoiridoids	457	516	360	457	401	812	319	123
Lignans	0.18	0.03	0.05	0.18	0.11	0.05	0.04	0.05
Flavonoids	6.09	5.67	6.63	6.09	6.36	9.01	7.23	0.14
Aldehydes	0.64	0.09	0.05	0.64	0.49	0.14	0.18	0.06

hydrolysis, oxidation, and the increase of decarboxymethylated derivatives during thermo-oxidation. Similar trends were observed by Brenes et al. (2001), and Lozano-Sánchez et al. (2013) during the storage of EVOO in the industry or after sales. Contrary to the present study, the work of Cerretani et al. (2009) showed a high decrease of total phenolic content in VOOs after prolonged thermo-oxidation. It is interesting to mention that the increase of the total phenols during microwave heating is more pronounced than that of conventional treatment. Mainly phenolic compounds belonging to secoiridoids group were responsible for this behavior. In fact, this trend was the same as for the total secoiridoid amount, while they showed a remarkable increase at the first times of microwave and conventional thermo-oxidation (44 and 11% of increase at 2 min and 2.5 h of heating, respectively), followed by a drastically decrease (73 and 21% of losses from the initial values at 15 min at 5 h of heating, respectively). The drop of secoiridoids may be due to the hydrolysis of secoiridoids derivatives in the hydroxytyrosol, tyrosol and elenolic acid and formation of oxidized phenols (El Riachy et al., 2011).

Concerning the main secoiridoid, isomer 2 of the oleuropein aglycone showed a great resistance to microwave heating, only 8% of decrease (282 mg analyte/ kg oil) was proved during 2 min of thermo-oxidation. The loss was much more pronounced (29%) at 5 min of treatment followed by a complete disappearance until the end of heating. Conventional heating, was most destructive than microwave heating, in fact, isomer 2 of the oleuropein aglycone underwent a complete hydrolysis within 2.5 h of heating process. Isomer 1 of oleuropein aglycone follows the same trend as isomer 2. The decrease of oleuropein aglycone upon thermal treatment suggests that these isomers were degraded as a response to the high temperature. Moreover, oleuropein aglycone is with high antioxidant potential, so its sharp decrease during heating is to preserve VOO from oxidative reactions. Similar results were also observed by Brenes et al. (2002) and Allouche et al. (2007).

Ligstroside aglycone, which was described previously as the second main phenolic compound, showed a drastical decrease during heating time. The degradation of such secoiridoid was less pronounced when subjected to microwave heating (24 and 73% of losses at 2 and 5 min of heating, respectively) as compared with conventional heating which allowed the presence of ligstroside aglycone at trace level at 2.5 h of treatment (Table 2). However, findings reported by Cerretani et al.

(2009), and Brenes et al. (2002) showed substantially unvaried ligstroside aglycone concentration during heating treatment.

According to the data of Table 2, different reactions were taken into account to establish the change of the main secoiridoids over time, especially the oxidation of the main oleuropein aglycone derivatives: isomer 1 and 2 of deacetoxy oleuropein aglycone and hydroxy decarboxy oleuropein aglycon. In the hydrophilic fraction of Chétoui VOO, both isomer 1 and 2 of deacetoxy oleuropein aglycone showed a completely different behaviors by comparison with the previously studied bioactive compounds along heating processes. During 5 min of microwave treatment (202 °C), isomer 1 and 2 of deacetoxy oleuropein aglycone showed a remarkable increase reaching the sum of 519 mg analyte/kg of oil, followed by a progressive decrease reaching 119 mg analyte/kg of oil corresponding to 77% of loss after 15 min of thermo-oxidation. Similar to the case of microwave heating process, at the beginning of conventional heating, the behavior of isomer 1 and 2 of deacetoxy oleuropein aglycone follow the same trend. They increased to reach maximum of 509 mg analyte/ kg of oil at 2.5 h of heating, followed by a 34% of decrease at the end of the experimentation. Similar trends have been previously reported for this compound by Brenes et al. (2002), and Allouche et al. (2007) who showed a weak increase of deacetoxy oleuropein aglycone of VOOs after approximately 2 h of heating at 180 °C followed by a remarkable decrease. Brenes et al. (2002) and El Riachy et al. (2011), who analyzed the phenolic compounds using RP-HPLC, showed that the increase of deacetoxy oleuropein aglycone might be explained by a coelution of deacetoxy oleuropein aglycone oxidized forms. In the present study, TOF-MS was used for identification of VOO phenols by determination of elemental composition with excellent mass accuracy and confirmation of the correct isotopic pattern. As observed in peaks 7 and 9 (Figs. 3, 4), the most abundant oleuropein derivative had a deprotonated molecule at m/z = 319, corresponding to deacetoxy oleuropein aglycone. The decrease in the oleuropein aglycone concentration is reflected by an increase of deacetoxy oleuropein aglycone concentration due to consecutive hydrolytic reactions. In fact, the hydrolytic degradation pathway initially causes the formation of aglycons (3,4-DHPEA-EA, and *p*-HPEA-EA) as a result of the loss of a sugar molecule, which can be provoked by *ß*-glucosidase activity (Gutierrez-Rosales et al., 2010). The obtained aglycons can undergo isomerisation, which involves

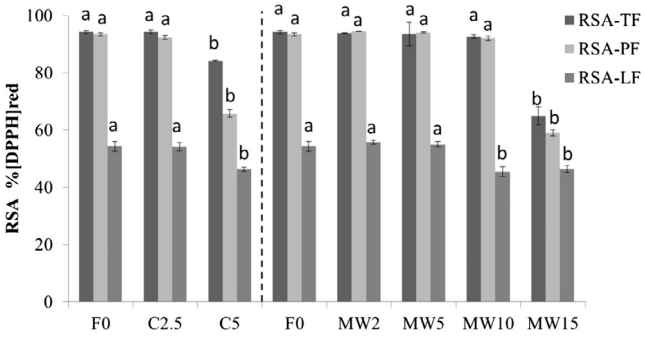

Fig. 4 Variation of the radical scavenging activity (RSA) of the total, methanolic and lipidic fraction (TF, MF, and LP) of Chétoui VOOs during microwave and conventional heating, expressed as percent reduction in concentration of DPPH•. Bars with the same letters showed no significant difference ($p < 0.05$).

opening of the secoiridoidic ring, followed by rearrangement into open dialdehydic form. Dialdehydic forms in turn decarboxylate into respective aglycons (3,4-DHPEA-EDA, and *p*-HPEA-EDA). This type of reaction was also described for oleuropein by Migliorini et al. (2012), and Limiroli et al. (1995) during crushing and malaxation of olives. It is important to highlight that the unusual high levels of deacetoxy oleuropein aglycone detected in the Chétoui VOOs after 5 min and 2.5 h of microwave and conventional heating, respectively, may be considered as a direct marker of the degree of the secoiridoids transformation in Chétoui VOO during heating processes. Some research (Loubiri et al., 2017) showed that deacetoxy oleuropein aglycone was the most abundant complex phenol in some fresh EVOOs. At the end of the experimentation, a remarkable decrease of deacetoxy oleuropein aglycone is observed due to the hydrolysis of the ester linkage giving rise to hydroxytyrosol and tyrosol (Migliorini et al., 2012).

With regard to the concentration of hydroxy decarboxy oleuropein aglycon (peak 8), it showed trend that was generally similar to that of deacetoxy oleuropein aglycone. A remarkable increase in such secoiridoid was observed after 5 min (T = 202 °C) and 5 h of microwave and conventional heating corresponding to 28.3 and 25.2 mg analyte/ kg of oil, respectively, followed by a drastically decrease corresponding to 85% of loss at the end of the microwave treatment. The detection of hydroxy decarboxy oleuropein aglycon during thermo-oxidation can be used as a marker of prolonged oxidation as observed in EVOO and in by-products during storage for 10 months (Lozano-Sánchez et al., 2013).

With regard to phenolic alcohols, hydroxytyrosol showed 17% of decrease (5.90 mg analyte/ kg of oil) during 2 min of microwave heating followed by a great drop, corresponding to 41% of loss (4.15 mg analyte/ kg of oil) after 15 min of treatment. The observed trend was consistent with the high antioxidant activity of hydroxytyrosol in virgin olive oil (Gomez-Alonso et al., 2003), since antioxidants act by reacting rapidly with lipid radicals and are thereby consumed. The level of hydroxytyrosol in virgin olive oil was reported to correlate well with the oxidative stability of the oil, as determined by the Rancimat method (Gomez-Alonso et al., 2003). Moreover, a similar rate of loss of hydroxytyrosol agree with the results reported by Brenes et al. (2002) who showed 20 and 30% of hydroxytyrosol loss in Spanish VOO after 5 and 10 min of microwave heating, respectively. Whereas, Cerretani et al. (2009) showed that 94% of hydroxytyrosol decreased after 15 min of microwave treatment. When subjected to conventional thermal treatment at 180 °C, hydroxytyrosol showed a pronounced decrease (45% of loss, 3.92 mg analyte/ kg of oil) at the first heating time (2.5 h), which is in compliance with the finding of D'Archivio et al. (2010), and Gomez-Alonso et al. (2003), who showed that the concentration of hydroxytyrosol in virgin olive oil rapidly decreased by the end of the first process of frying due to the thermo-oxidative deterioration. As shown in Table 2, hydroxytyrosol experienced a remarkable increase at the end of the experimentation (6.35 mg analyte/ kg of oil) likely due to an increase in hydrolytic activities on secoiridoids containing hydroxytyrosol in their molecular structures caused by the maintained high temperature for a long hours (Brenes et al., 2001). The observed trend is similar to that reported by Cinquanta et al. (1997) in virgin olive oil during storage at room temperature.

Tyrosol appeared to be more stable to heating treatment compared with hydroxytyrosol. This result is consistent with the much higher antioxidant activity of hydroxytyrosol as compared to that of the tyrosol, in virgin olive oil. The same observation was reported by other authors (Gomez-Alonso et al., 2003). Tyrosol is therefore not easily degradable during thermo-oxidation (Cinquanta et al., 1997). If tyrosol data of both heating methods are compared, microwave heating seems to be less degradative treatment. Tyrosol content at 10 min of microwave heating displayed a slight decrease (9% of loss, T = 246 °C) by comparison with conventional heating (20% of loss, 180 °C) for 2.5 h. The resistance of tyrosol during microwave and conventional heating confirmed its low effect in VOO protection for the oxidative reaction during thermal process (Servili and Montedoro, 1989). Other authors confirmed the stability of tyrosol; its reduction in the concentration during frying processes was much smaller than that observed for the hydroxytyrosol family (Gomez-Alonso et al., 2003). It is interesting to underline the remarkable increase of tyrosol at the end of both microwave and conventional heating processes reaching 6.58 and 8.25 mg analyte/kg of oil, respectively. The high level of tyrosol at 15 min and 5 h of the experimentation is a result of an increase in hydrolytic activities on ligstroside aglycon (characterized by the presence of the tyrosol as an aromatic alcohol), probably caused by the prolonged high temperatures (Di Maio et al., 2013). The same result was observed by Lozano-Sánchez et al. (2013), Cinquanta et al. (1997) and Brenes et al. (2001) who showed an increase in the tyrosol concentration during long VOO storage at room temperature.

The lignan acetoxy-pinoresinol, possess in vitro antioxidant activity, and it may be able to exhibit beneficial effects on human health (Allouche et al., 2007). In both heating treatments, acetoxy-pinoresinol displayed a drastically decrease at the beginning of microwave and conventional heating operations (85 and 40% of losses after 2 min and 2.5 h of heating, respectively), and remain with trace levels until the end of treatment suggesting its less stability against oxidation (Brenes et al., 2002). Similar results were reported, in frying conditions, for this compound by other authors (Baldioli et al., 1996).

Concerning flavones, luteolin remained almost constant over time, followed by a decline at the end of the study. Whereas, apiginin showed a remarkable increase in both heating process, followed by a decrease at 15 min of microwave heating.

3.6. Radical scavenging activity (RSA) of the Chétoui VOO during heating processes

The ability of antioxidant extracts to scavenge the free radical [DPPH·] across heating exposure times are given in Fig. 4. The RSA of Chétoui VOO was maintained with extremely high percentage (>92% for both TF and MF, and >54% for LF) during 10 min and 2.5 h of microwave and conventional treatments, respectively. At the end of the experimentation, the RSA showed a significant decrease ($p < 0.05$). The diminution was more pronounced for the TF and MF (31 and 37% of losses, respectively) of the microwave heating than that of conventional one (11 and 30% of losses). The loss of the capacity of the LP to quench the free radical [DPPH·] was similar in both heating process (15% of loss).

The extremely high RSA of the MF than that of the LP during thermo-oxidation can be explained by the fact that the antioxidant polyphenols in VOO as hydroxytyrosol, deacetoxy oleuropein aglycon and oleuropein aglycon, exhibit a strong radical scavenging activity, which has been proven to be more effective than vitamin E (Cossignani et al., 1998). The RSA of the LF, which contains mostly tocopherols, triglycerides, and phospholipids, is mainly due to the type and concentration of tocopherols, whereas phospholipids have a much lower RSA but affect the antioxidant capacity of vitamin E (Cossignani et al., 1998). Such results confirm that the regression of the capacities of the MF and as a results of TF to quench the free radical [DPPH·] at the end of heating processes, depend especially to the concentration of secoiridoid group.

4. Conclusion

Lipid oxidation does not only spoil the taste, smell and color of oil, but intake of the oxidized oil may also impose unmitigated threat to health. The obtained results can be of interest for understanding the degradation phenomena regarding phenolic compounds of virgin olive oil. Occurrence of hydrolytic transformation for secoiridoids during microwave and conventional heating of Chétoui VOO has been confirmed by the degradation of oleuropein aglycone and ligstroside aglycone, and the accumulation of dialdehydic form of oleuropein aglicon: isomer 1 and 2 of deacetoxy oleuropein aglycone, and hydroxy decarboxy oleuropein aglycon which may be considered as a direct marker of the degree of the secoiridoids transformation. Hydroxytyrosol and Tyrosol were shown to be more resistant to this kind of treatment due to the increase in hydrolytic activities on secoiridoids containing hydroxytyrosol and tyrosol. The behavior of flavonoids could be outstanding, as they belong to the most resistant phenolic group to thermal treatments. By comparison between the two heating possesses, conventional heating for 2.5 h at 180 °C was more destructive than 5 min (202 °C) of microwave heating. Therefore, the utilization of Chétoui VOO may be encouraged especially at short microwave treatment times for both domestic and food catering applications.

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Author contributions

Imen Oueslati prepared the samples and carried out a part of the analysis, was involved in the data interpretation and the manuscript redaction, Amani Taamalli carried out the experimental analyses, and was involved with Anis Loubiri

in the data interpretation. David Arraez Roman, and Antonio Segura-Carretero design the experimental plan and were involved in the data interpretation, and Mokhtar Zarrouk helped in the sample preparations and data interpretation.

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