Effect of the enrichment with natural antioxidants obtained by maceration or ultrasound-assisted extraction from olive leaves on organic extra virgin olive oil

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Improvement of nutritional quality and oxidative stability of Tunisian organic extra virgin olive oil (OEVOO) from "Chetoui" variety were studied after the enrichment with natural antioxidants obtained by maceration and ultrasound-assisted extraction of phenols from organic olive leaves. Results showed that the enrichment conserved the organic criteria and did not affect the OEVOO quality. The ultrasound assisted extraction of phenols in OEVOO induced a significantly higher oleic acid content (67.75) and lower linoleic acid (18.67). This sample showed the highest biophenols (269 mg/kg), chlorophylls (4.67ppm) and carotenoids (1.67ppm) contents and the lowest PV (14.52 meq-O2/kg-oil) and K_{232} (22.2) values, at the end of storage. However, Tocopherols content increased by maceration during storage. These findings explained the significant anti-radical activity of macerated and ultrasound enriched samples (29.82% and 35.5%) at the end of storage compared to control (8.03%). Thus, enrichment by ultrasonic extracts was more reliable against oxidation compared to macerated oil and control. Moreover, ultrasound assisted extraction improved the nutritional quality and sensorial properties of olive oil which was devoid of defects with a slight bitterness when compared to macerated oil having an unacceptable taste.

Keywords: Organic extra virgin olive oil, organic olive leaves, ultrasound-assisted extraction, maceration, stabilisation, natural antioxidants.

INTRODUCTION

In recent years, the global economy has shifted from mass production to quality production. This leads to new perceptions encouraging demand for" functional foods". It is a relatively new term used to describe food products that have been enriched with natural substances improving their quality and resistance to some phenomena such as the oxidation. To avoid or delay this phenomenon, recent studies have focused on the valorisation of natural plants extracts such as, tocopherols and polyphenols**.** These are known, by their fight against the oxidation of foods, their ability to reduce the risk of cancer, optimisation of infertility treatments [1] heart disease and diabetes [2], as well as their antibacterial, antioxidant, antiviral, anti-inflammatory and anti-allergenic activities [3]. Some fats such as olive oil are partially protected against oxidation by their natural antioxidant content but remain sensitive to photo-oxidation. In this approach, the stabilisation of vegetable oils (olive oil, sunflower, soybean, etc.) has been the subject of a great number of research, having opted for the enrichment of these oils with vegetable matrices. Among the matrices found abundantly in nature and little exploited olive leaves, which present 10% of the total mass of harvested olives [4]. It contains between 15 and 70 mg of phenolic compounds per gram of fresh mass, from which they can be exploited for food, pharmaceutical and cosmetic purposes [5]. Olive leaves phenols have a strong antioxidant capacity [6], broad antimicrobial

activity and several health benefits such as anti-inflammatory, cardio-protective and anti-diabetic effect [7]. Therapeutic interest of olive leaves was correlated also to its richness in Oleuropein whose concentration can reach 6.8% [8]. Several studies have proven antioxidant [6], antimicrobial [9], and anti-tumour [10] activities of oleuropein. Classical methods of extracting secondary metabolites from plants have been used such as maceration but innovative techniques such as supercritical fluid extraction, microwave assisted extraction and ultrasound assisted extraction, may increase production efficiency, and contribute to environmental preservation by reducing the use of water and solvents [11]. Although global demand for organic products has recently increased, studies on organic olive oil are still limited.

In this study, the stability of Tunisian organic extra virgin olive oil of the "Chétoui" variety enriched by olive leaves extracts, was studied. The enrichment assisted by ultrasound was tested and compared to the control and to the macerated oil. The quality criteria, tocopherols and polyphenols contents, antioxidant activity and sensorial properties were performed, during six months of storage at room temperature.

1. MATERIALS AND METHODS

1.1 PREPARATION OF RAW MATERIALS

The basic product used for this study was organic extra virgin olive oil from the "Chétoui" variety, extracted using a continuous three-phase system in the Al-jazzira-Morneg oil mill located in the north of Tunisia during 2019/2020 drop. Organic olive leaves (*Olea Europaea*) of the same variety were collected from an organic farm located in the same area. After washing the harvested leaves, they were dried in the open air until a constant mass was obtained, which makes possible the preservation of thermo-labile substances such as polyphenols and vitamins.

1.2 PREPARATION OF ENRICHED OLIVE OIL

1.2.1 Maceration

The olive leaves have been incorporated into the organic extra virgin olive oil in whole form at raison of 2% [12]. Enriched olive oil was stored in black glass bottles at room temperature and sampled during six months of storage.

1.2.2 Ultrasound assisted extraction

According to Achat et al. [13], the ultrasound-assisted extraction method was used to incorporate phenolic extracts directly from olive leaves into olive oil without solvents.

Twenty grams of dried and crushed organic olive leaves were added to 1L of organic extra virgin olive oil, which was used as a solvent. The whole was transmitted in a 3 L ultrasound tank. The optimal parameters used for more efficient extraction in terms of concentration of total phenols, oleuropein, tyrosol and hydroxytyrosol were power (60W), temperature (16°C) and time (45 min) [13]. The resulting mixture was filtered. All control and enriched samples were stored in black glass bottles in the dark at room temperature for six months.

1.3. HUMIDITY, PESTICIDES, AND IMPURITIES CONTENTS

For the characterization of the organic olive oil subject of this study, impurities contents and humidity were carried out, respectively according to ISO 663 [14] and ISO 662 [15]. The level of pesticides was analysed at the beginning and at the end of storage using the HPLC method to verify if the flavoring affects the biological criterion of the studied oil. The described analytical procedure was validated according to the SANCO/10684/2009 [16] validation protocol for analytical techniques for pesticide residues analysis in food and feed. This procedure fulfils the European Decision 2002/657/EC requirements [17]. The molecules sought for this analysis were: Dimethoate, Malation, ChlopyrifosEthyl, Methidathion, Phosmet, Féniquazin, Lamdacyhalothin, Acrinathin, Permethrin, Cypermethrin, Definiconazol, Deltamthrin.

1.4. QUALITY INDEXES

The acidity was determined according to ISO 660 [18] amending the regulations EEC (EEC n° 2568/91) [19], the peroxide value (PV) following the international standard ISO 3960 [20] and extinction coefficients (K_{232}, K_{270}) were performed according to the standard ISO 3656 [21].

1.5. FATTY ACIDS COMPOSITION

The determination of the total fatty acids composition of all studied olive oil samples was carried out at the beginning of storage by preparing the methyl esters according to the international standard ISO 5509 [22]. These esters were, then, analysed by gas chromatography (GC) according to the ISO 5508 [23].

1.6. PIGMENTS CONTENTS MEASUREMENTS

The pigments contents were performed using spectrophotometric method as described by Haddada et al. [24] using the following formulas:

Chlorophyll (mg/kg): $(A670 \times 10^6) / (E1 \times 100 \times d)$;
Caroténoïd (mg/kg): $(A470 \times 10^6) + (E2 \times 100 \times d)$
Where:
d: optical path = 1 cm;
A 670: absorbance at 670 nm;
A 470: absorbance at 470 nm;
E1: coefficient linked to the spectrophotometer = 613;
E2: coefficient linked to the spectrophotometer = 2000.

1.7. BIOPHENOL CONTENT

The determination of biophenols content was carried out by referring to the standard recommended by the

International Olive Council (IOC) [25]. The method was based on an extraction of minor polar compounds of biophenolic nature directly from olive oil using methanolic solution, followed by their assay by HPLC using an UV developer at 280 nm. The internal standard consists of syringic acid. The content corresponding to natural and oxidized derivatives of oleuropein and ligstroside, lignans, flavonoids and phenolic acids was expressed in mg / kg of tyrosol.

1.8. DETERMINATION OF TOCOPHEROLS

Tocopherols composition was determined according to the ISO 9936 [26]. The compounds were identified by chromatographic comparisons with authentic standards by co-elution and by their UV spectra.

1.9 SENSORY EVALUATION

The sensorial evaluation of the studied olive oils was carried out according to the IOC [27] by 8 expert panellists of the Tunisian National Oil Office. The test conditions were chosen according to the same standard. Panellists smelled and tasted each oil and carried out on the profile sheet made available to him the intensity at which they perceived each of the negative (fusty/muddy, musty, winey, metallic, rancid, Frost-bitten olives (wet wood)) and positive (fruity, bitter, pungent) attributes. The head of the jury, after having collected the profile sheets completed by each of the tasters, checked the assigned intensities and calculated the medians of the various attributes.

1.10 STATISTICAL ANALYSIS

Results of different parameters were expressed as the mean ± standard deviation. An analysis of variance (ANOVA) was performed at a significance level of 5%. Duncan multiple range test (DMRT) is a multiple comparison method in which group means were ranked in ascending order. This method was performed using IBM SPSS Statistics version 23. All analytical determinations were performed at least in triplicate.

2. RESULTS AND DISCUSSION

2.1 CHARACTERISATION OF ORGANIC EXTRA VIRGIN OLIVE OIL

According to the standard IOC [28], obtained results on raw material shown in Table I, proved that the studied olive oil was of good quality and under the nomination “extra virgin”. Initial values of peroxide value (PV), free fatty acids (FFA), K_{232} and K_{270} were respectively 7.39 meqO_2/Kg±0.45; 0.24±0.03; 1.87±0.04 and 0.17±0.01 which correlate with those found by Ben Tkaya et al. [29] for an extra virgin olive oil (EVOO) from the same studied variety. Compared to the results found by Oueslati et al. [30] for various varieties of Tunisian EVOO, the initial chlorophylls and biophenols contents noted on the studied extra virgin olive oil were lower (4.51±0.05 et 255±0.05 respectively). However, it was observed that carotenoids and α-tocopherol contents were higher (1.55±0.06 et 417.32±5.6, respectively).

Table I - Characterization of organic extra virgin olive oil

Paramètre	Value
FFA (%)	0.24±0.03
K_{232}	1.87±0.04
K_{270}	0.17±0.01
PV (meq O_2/Kg)	7.39±0.45
Carotenoid content (ppm)	1.55±0.06
Chlorophyll content (ppm)	4.51±0.05
Humidity (%)	0.067
Impurities content (%)	0.0063
Biophénols content (mg/kg)	255±0. 05
α-tocopherols (ppm)	417.32±5.6
β-tocopherols (ppm)	n.d
Ɣ-tocopherols (ppm)	22.81±1.5
δ-tocopherols (ppm)	5.58±0.2

Table II - Initial fatty acids composition (%) of control and flavored oils

	Control	MOO	UOO	Limit (IOC, 2019)
$C_{16:0}$	10.78±0.28^a	10.78±0.32^a	11.47±0.42^a	7.20-20.00%
$C_{16:1}$	0.48±0.05^a	0.46±0.05^a	0.49±0.05^a	0.30-3.50%
$C_{17:0}$	0.05±0.00^a	0.05±0.01^a	0.05±0.00^a	≤0.40
$C_{17:1}$	0.05±0.01^a	0.05±0.00^a	0.06±0.02^a	≤0.60
$C_{18:0}$	3.06±0.0^a	3.05±0.01^a	3.12±0.00^a	0.5-5.00%
$C_{18:1}$	63.06±0.39^a	63.34±0.76^a	67.75±0.42^b	55.00-83.00%
$C_{18:2}$	20.75±0.63^b	20.67±0.37^b	18.67±0.24^a	2.50-21.00%
$C_{18:3}$	0.71±0.03^a	0.7±0.00^a	0.7±0.04^a	≤1.00
$C_{20:0}$	0.43±0.01^a	0.44±0.00^a	0.45±0.09^a	≤ 0.60%
$C_{20:1}$	0.36±0.08^a	0.36±0.01^a	0.39±0.03^a	≤0.50

Control: Unenriched olive oil; MOO: enriched olive oil by maceration UOO: Ultrasound enriched olive oil.
Data are mean ± standard deviation, n=3. Means with different superscripts are significantly different ($p < 0.05$). Letters represent the statistical difference between samples.

2.2 ASSESSMENT OF PESTICIDE LEVELS

The results related to the levels of pesticides analysed at the beginning and at the end of the storage period showed that the enrichment of EVOO by natural antioxidants using maceration or ultrasound-assisted extraction maintained the biological criterion of studied olive oil. In fact, all pesticides were absent in control and enriched EVOO samples during the whole of storage period.

2.3 CHANGES IN FATTY ACIDS COMPOSITION

The initial fatty acids composition of the studied olive oil is shown in Table II. The results demonstrated a predominance essentially of oleic acid (63.06%) and a richness in palmitic acid (10.78%) and linoleic acid (20.75%) in the control olive oil, compared to the Spanish, Italian and Greek olive oils as reported before by Boudiche et al. [31]. It was noted that the enrichment by maceration of organic olive leaves had no effect on the fatty acids composition. However, applying ultrasound-assisted extraction leaded to an EVOO with a significant ($p<0.05$) high amount in oleic acid (67.75%) and low level in linoleic acid (18.67%) compared to the control. This finding was attributed, first, to the richness of olive leaves from Chetoui variety in oleic acid (25.07%) [32]. Besides, according to Hang et al. [33] the amount of unsaturated fatty acids decreased in various olive oils after 1h of ultrasonic processing at 80°C that explain the decrease of linoleic acid level. Similar results were found by Jaber et al. [34] reporting that the enrichment of a refined olive oil by the chlorophyll extract of olive leaves increased the oleic acid content and decreased that of the linoleic acid.

2.4 CHANGES IN QUALITY INDICES

Acidity (FFA)

From the Table III, no significant differences ($p>0.05$) in terms of acidity were observed between control and enriched EVOO initially and during the six-month storage. However, a significant increase ($p<0.05$) of

Table III - Changes in quality parameters and pigments contents of control and enriched olive oils during six months of storage

	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150	Day 180
	Acidity (%)						
Control	$0.24\pm0.03^{a,1}$	$0.27\pm0.01^{a,1,2}$	$0.29\pm0.01^{a,1,2}$	$0.3\pm0.6^{a,1,2}$	$0.31\pm0.06^{a,1,2}$	$0.33\pm0.04^{a,1,2}$	$0.35\pm0.05^{a,2}$
MOO	$0.24\pm0.03^{a,1}$	$0.27\pm0.00^{a,1,2}$	$0.29\pm0.02^{a,1,2,3}$	$0.31\pm0.3^{a,1,2,3,4}$	$0.33\pm0.04^{a,2,3,4}$	$0.36\pm0.07^{a,3,4}$	$0.39\pm0.03^{a,4}$
UOO	$0.24\pm0.02^{a,1}$	$0.26\pm0,05^{a,1,2}$	$0.29\pm0.01^{a,1,2,3}$	$0.3\pm0.4^{a,1,2,3}$	$0.32\pm0.05^{a,2,3}$	$0.33\pm0.04^{a,2,3}$	$0.35\pm0.04^{a,3}$
	PV ($meqO_2/Kg$)						
Control	$7.39\pm0.45^{a,1}$	$8.82\pm0.49^{a,2}$	$9.76\pm0.43^{b,3}$	$10.77\pm0.46^{b,4}$	$12.89\pm0.43^{b,5}$	$14.58\pm0.50^{b,6}$	$16.84\pm0.52^{b,7}$
MOO	$6.69\pm0.39^{a,1}$	$8.51\pm0.48^{a,2}$	$9.16\pm0.41^{b,2,3}$	$9.65\pm0.24^{a,3}$	$10.81\pm0.35^{a,4}$	$12.72\pm0.53^{a,5}$	$14.85\pm0.54^{a,6}$
UOO	$7.24\pm0.35^{a,1}$	$8.52\pm0.48^{a,1,2}$	$9.77\pm0.26^{a,2}$	$9.24\pm0.25^{a,3}$	$11.41\pm0.12^{a,4}$	$12.27\pm0.47^{a,5}$	$14.52\pm0.48^{a,6}$
	K_{232}						
Control	$1.89\pm0.04^{a,1}$	$2.01\pm0.04^{a,2}$	$2.15\pm0.04^{b,3}$	$2.25\pm0.04^{c,4}$	$2.35\pm0.05^{c,5}$	$2.43\pm0.05^{c,6}$	$2.5\pm0.06^{c,6}$
MOO	$1.87\pm0.03^{a,1}$	$1.92\pm0.07^{a,1}$	$2.03\pm0.05^{a,2}$	$2.13\pm0.03^{b,3}$	$2.23\pm0.04^{b,4}$	$2.28\pm0.06^{b,4,5}$	$2.35\pm0.05^{b,5}$
UOO	$1.87\pm0.02^{4,a,1}$	$1.93\pm0.03^{a,1,2}$	$1.97\pm0.03^{a,2,3}$	$2.03\pm0.05^{a,3,4}$	$2.08\pm0.06^{a,4}$	$2.17\pm0.04^{a,5}$	$2.22\pm0.04^{a,5}$
	K_{270}						
Control	$0.17\pm0.01^{a,1}$	$0.18\pm0.02^{a,1}$	$0.18\pm0.02^{a,1}$	$0.19\pm0.03^{a,1}$	$0.2\pm0.03^{a,1}$	$0.2\pm0.03^{a,1}$	$0.21\pm0.03^{a,1}$
MOO	$0.17\pm0.01^{a,1}$	$0.19\pm0.04^{a,1}$	$0.18\pm0.04^{a,1}$	$0.20\pm0.02^{a,1}$	$0.20\pm0.04^{a,1}$	$0.2\pm0.03^{a,1}$	$0.22\pm0.05^{a,1}$
UOO	$0.17\pm0.03^{a,1}$	$0.18\pm0.02^{a,1}$	$0.19\pm0.04^{a,1}$	$0.19\pm0.04^{a,1}$	$0.19\pm0.03^{a,1}$	$0.21\pm0.04^{a,1}$	$0.21\pm0.04^{a,1}$
	Chlorophyll contents (ppm)						
Control	$4.5\pm0.05^{a,7}$	$4.27\pm0.06^{a,6}$	$4.09\pm0.05^{a,5}$	$3.84\pm0.06^{a,4}$	$3.48\pm0.06^{a,3}$	$2.90\pm0.06^{a,2}$	$1.95\pm0.06^{a,1}$
MOO	$4.5\pm0.08^{a,5}$	$4.44\pm0.05^{b,5,4}$	$4.32\pm0.06^{a,b,4,3}$	$4.18\pm0.03^{b,3,2}$	$4.07\pm0.04^{b,2}$	$3.4\pm0.05^{b,1}$	$3.26\pm0.05^{b,1}$
UOO	$4.67\pm0.03^{b,4}$	$4.57\pm0.03^{b,4}$	$4.43\pm0.09^{b3,}$	$4.32\pm0.02^{b,3,2}$	$4.25\pm0.03^{c,2}$	$4.00\pm0.03^{c,1}$	$3,97\pm0,04^{c,1}$
	Carotenoids contents (ppm)						
Control	$1.55\pm0.06^{a,5}$	$1.45\pm0.02^{a,5,4}$	$1.36\pm0.05^{a,4}$	$1.17\pm0.09^{a,3}$	$1.09\pm0.00^{a,3,2}$	$0.96\pm0.04^{a,2}$	$0,54\pm0.04^{a,1}$
MOO	$1.56\pm0.03^{a,5}$	$1.47\pm0.04^{a,b,5}$	$1.39\pm0.04^{a,b,4}$	$1.25\pm0.04^{a,b,4,3}$	$1.18\pm0.04^{a,b,3,2}$	$1.07\pm0.03^{b,2}$	$0,86\pm0.06^{b,1}$
UOO	$1.67\pm0.03^{b,5}$	$1.55\pm0.02^{b,4}$	$1.5\pm0.02^{b,4}$	$1.34\pm0.04^{b,3}$	$1.25\pm0.04^{b,2}$	$1.17\pm0.05^{c,2}$	$1,04\pm0.05^{c,1}$

PV: Peroxide value; K_{232} and K_{270} : specific extinctions Control: Unenriched olive oil; MOO: enriched olive oil by maceration UOO: Ultrasound enriched olive oil. Data are mean ± standard deviation, n=3. Means with different superscripts are significantly different ($p < 0.05$). Lowercase letters (a, b, c) represent the statistical difference between samples; Numbers (1-7) represent the statistical difference between the same sample during storage period.

acidity was noted for all studied olive oils that can be explained by the enzymatic activity caused by lipolytic reactions in olive oil [35]. This evolution was faster especially in macerated olive oil (MOO). Acidity increased to reach values of about 0.35±0.05, 0.39±0.04 and 0.35±0.03, respectively for the control, MOO, and ultrasound enriched olive oil (UOO). These results agreed with those of several studies showing that adding extracts from aromatic and medicinal plants to olive oil leads to an increase in acidity. Indeed, the addition of the aqueous extract of olive leaf during the mixing step resulted in an increase in acidity [36]. Similar results were found by Sousa et al. [35] reporting that adding garlic significantly increased acidity values compared to the control.

Peroxide value

Obtained initial peroxide values (PV) showed that studied EVOO were of good quality (Tab. III). These values increased significantly ($p<0.05$) during storage for all analysed olive oils particularly from the third month of storage. This result could be assigned to the increase of storage temperature as described by Ben Tekaya et al. [29] suggesting that an increase of temperature by about 10 degrees could accelerate oil oxidation leading to high PV values. Besides, it was confirmed in the literature that once auto-oxidation is started, it does not stop until all the free radicals that are formed are inactivated [37]. Results showed that enriched EVOOs were more stable than control over time favouring olive oil enriched by ultrasonic extracts having the lowest PV (14.52±0.48 meqO_2/kg), at the end of the sixth month of storage, when compared to MOO (14.85±0.54 meqO_2/kg) and the control (16.84±0.52 meqO_2/kg) (Tab. III). These findings confirmed that the enrichment with olive leaves rich in antioxidants and oleuropein reduced the formation of lipid hydroperoxides and the oxidation of the olive oil compared to the control. These results agreed with those of Jaber et al. [34] reporting that the addition of chlorophylls extracted from Chemlali olive leaves resulted in an appreciable resistance to oxidation. Likewise, Shahin et al. [38] found that olive oil enriched with oleuropein has a lower PV (7.07) than that of pure oil (9.09).

Specific extinction coefficients

In this study, the obtained values of the K_{232} coefficient were in line with those of PV previously reported. Indeed, from the second month of storage, this coefficient noted for the control (2.15±0.4) became significantly higher ($p<0.05$) than those of enriched samples (Tab. III). Similar results were found in the literature [34, 36]. K_{232} values increased significantly ($p<0.05$), during storage, proving that flavouring by ultrasound-assisted extraction is more reliable against oxidation than that by maceration. The decrease in the quality of the oil during the maceration of the olive leaves may be due to oxidative enzyme (lipoxygenase in olive leaves).

Concerning the extinction coefficient K_{270}, no significant differences ($p>0.05$) were revealed between all analysed EVOOs from the beginning and until the end of storage period.

2.5 CHANGES IN PIGMENTS CONTENTS

From the first day of storage, initial chlorophylls contents were equal to 4.5±0.05 ppm; 4.5±0.08 ppm and 4.67±0.03 ppm; respectively for control, MOO and UOO showing a significant ($p<0.05$) higher level for the olive oil enriched by the ultrasonic extracts (Tab. III). Later, this also had a higher initial carotenoids content (1.67±0.03 ppm). These findings can be explained by the high time of contact (45 min) between EVOO and olive leaves, when using ultrasound-assisted extraction. Indeed, ultrasonic extraction is a very simple method that relies on the mechanical effect caused by the implosion of micro-bubbles that cause a rapid breakdown of the tissues allowing the release of compounds in the solvent [39] representing, in our study, the olive oil itself. During the six months of storage, the pigment contents decreased significantly ($p<0.05$) in all studied EVOO samples. At the end of storage, the highest chlorophylls (3.97±0.04) and carotenoids (1.04±0.05) contents were noted for ultrasonic enriched EVOO. These results were in accordance with those of Wang et al. [40] suggesting that the yellow pigment yield could be improved by an ultrasound-assisted extraction and will lead to antioxidant activity in treated olive oil.

2.6. CHANGES IN BIOPHENOL AND TOCOPHEROLS CONTENTS

2.6.1 Variations in tocopherols contents

Tocopherols are important components of olive oil because they have interesting properties due to their vitamin function and valuable antioxidant power, which makes their characterisation essential. As expected, the α-tocopherols were dominant in all studied EVOOs. As shown in Table IV, their levels varied between 416±8.1 ppm (MOO) and 418±5.3 ppm (UOO), at the beginning of storage without significant difference ($p>0.05$) between all analysed samples. The studied olive oils did not contain β-tocopherols. After six months of storage, α-tocopherol contents decreased significantly ($p<0.05$) to reach 383.57±3.2ppm and 381.57±8.2 ppm, respectively in UOO and control. However, the maceration leaded to a slight and significant increase of α-tocopherol content reaching a value of about 425.5 ppm, at the end of storage. For γ-tocopherols and δ-tocopherols contents evolution, it followed the same trends, during storage. The increase of tocopherols in macerated olive oil can be attributed to the migration of vitamin E from organic olive leaves, rich in tocopherols [41] to olive oil which played the role of a 'green' solvent. In fact, extracts from olive leaves had shown

Table IV - Changes in tocopherols and biophenols contents (ppm) of control and enriched olive oils during six months of storage

	Tocopherols contents (ppm)							
	Day 0				Day 180			
	α-tocopherols	β-tocopherols	Y-tocopherols	δ-tocopherols(mg/kg)	α-tocopherols	β-tocopherols	Y-tocopherols	δ-tocopherols(mg/kg)
Control	417.32±5.6^{a,2}	n.d	22.81±1.5^{a,2}	5.58±0.2^{a,2}	381.57±8.2^{a,1}	n.d	19,62±0.5^{a,1}	4,35±0.2^{a,1}
MOO	416.55±8.1^{a,1}	n.d	21.53±0.8^{a,1}	5.44±0.7^{a,1}	425.5±1.5^{b,2}	n.d	24,5±1.9^{b,2}	7,28±0.5^{b,2}
UOO	418.12±5.3^{a,2}	n.d	21.22±1.9^{a,2}	5.46±0.5^{a,2}	383.57±3.2^{a,1}	n.d	18,17±0.7^{a,1}	4,84^{a,1}
	Biophenols contents (mg/Kg)							
	Day 0			**Day 90**			**Day 180**	
Control	255±0.05^{b,3}			128±0.02^{a,2}			61±0.03^{a,1}	
MOO	233±0.06^{a,3}			138±0.06^{b,2}			84±0.04^{b,1}	
UOO	269±0.06^{c,3}			154±0.04^{c,2}			101±0.05^{c,1}	

Control: Unenriched olive oil; MOO: enriched olive oil by maceration UOO: Ultrasound enriched olive oil; n.d: None detected; Data are mean ± standard deviation, n=3. Means with different superscripts are significantly different ($p < 0.05$). Lowercase letters (a, b, c) represent the statistical difference between samples; Numbers (1-3) represent the statistical difference between the same sample during storage period.

their ability to improve the quality of olive oil regarding tocopherol contents in several studies [38].

2.6.2. Variations in biophenols contents

The initial values of biophenols contents registered for all studied extra virgin olive oils are shown in Table IV. The higher content was observed for UOO (269±0.06 mg/kg) followed by control (255±0.05 mg/kg) then macerated oil (233±0.06 mg/kg). This result can be explained by the absence of oleuropein in the control and the stability of natural phenols like tyrosol and hydroxytyrosol in EVOO, under sonication. As expected, a reduction of phenolic contents in all organic extra-virgin olive oils was registered, throughout the six months of storage (Tab. IV). At the end of storage, the lowest phenolic content was detected for control (61±0.03 mg/kg) and the highest contents were observed in enriched oils by ultrasonic extracts (101±0.05 mg/kg) and maceration (84±0.04 mg/kg). These findings agreed with the results registered in previous studies. Indeed, Jaber et al. [34] reported that the total phenol content decreased considerably by over 2% in refined olive oil enriched with chlorophyll pigments extracted from Chemlali olive leaves after 2 months of storage. The same authors showed that, the phenolic content decreased by about 5% compared to the initial content after 6 months of storage.

Contrastingly, these results disagree with those of Sousa et al. [35], who reported that the incorporation of different flavouring dried agents (garlic, laurel, oregano) did not show any protective effect against the oxidation of olive oil stored in the same conditions. This finding suggested a higher efficiency of ultrasound-assisted extraction compared to maceration explained by the fact that ultrasound waves after interaction with plant material alter its physical and chemical properties and that cavitation facilitates the release of extractable compounds and enhances the mass transport by disrupting the plant cell walls [12].

2.6.3 Variations in antioxidant activity

The antioxidant activity of various studied olive oils decreased significantly ($p<0.05$), during storage, to reach 8.03±0.02, 29.82±0.00 and 35.5±0.02, respectively for control, MOO and UOO (Tab. V). This result could be attributed to the fact that natural antioxidants such as tocopherols, chlorophylls, sterols, and polyphenols undergo auto-oxidation, leading to their degradation and a decrease in anti-radical activity [42]. It was noted that the antioxidant activity of enriched oils was higher than that of the control (Tab. V). Although the macerated olive oil exhibited the highest α-tocopherol content at the end of storage, UOO appeared to be more effective in scavenging the DPPH radical. This result can be attributed to the

Table V - Evolution of the antioxidant activity (RSA(%)) of control and enriched olive oils during six months of storage

	Day 0	Day 30	Day 60	Day 90	Day 120	Day 150	Day 180
	DPPH Essay						
Control	57.82±0.07^{a,4}	36.55±0.04^{a,3}	18.04±0.03^{a,2}	13.17±0.02a,2,1	11.87±0.01^{a,2,1}	9.19±0.02^{a,1}	8.03±0.02^{a,1}
MOO	64.75±0.09^{a,6}	52.98±0.04^{b,5}	48.94±0.04^{b,5,4}	45.05±0.02^{b,4,3}	40.70±0.01^{b,3,2}	33.90±0.02^{b,2,1}	29.82±0.00^{b,1}
UOO	60.60±0.03^{a,6}	58.74±0.02^{b,5}	51.44±0.01^{b,4}	47.75±0.03^{b,4,3}	44.21±0.05^{b,3,2}	39.00±0.04^{b2,1}	35.50±0.02^{c,1}

Control: Unenriched olive oil; MOO: enriched olive oil by maceration UOO: Ultrasound enriched olive oil;
Data are mean ± standard deviation, n=3. Means with different superscripts are significantly different ($p < 0.05$). Lowercase letters (a, b, c) represent the statistical difference between samples; Numbers (1-6) represent the statistical difference between the same sample during storage period.

Table VI - Sensory evaluation values of control and enriched olive oils during storage

	Day 0			Day 180		
Attributes	**Control**	**MOO**	**UOO**	**Control**	**MOO**	**UOO**
Fusty	0	0	0	0,5^{b}	0^{a}	0^{a}
Musty	0	0	0	0^{a}	0,4^{b}	0^{a}
Winey	0	0	0	0^{a}	1,4^{b}	0^{a}
Wet wood	0	0	0	n.d	n.d	n.d
Metallic	0	0	0	n.d	n.d	n.d
Rancid	0	0	0	n.d	n.d	n.d
Fruity	3^{a,2}	3.5^{b,2}	4^{c,2}	1.5^{a,1}	2^{b,1}	3.2^{c,1}
Pungent	3^{a,2}	3.25^{b,2}	3.8^{c,2}	2.2^{b,1}	0.4^{a,1}	2.6^{c,1}
Bitter	3^{a,2}	3.25^{b,2}	3.5^{c,2}	2.2^{b,1}	1.8^{a,1}	2.8^{c,1}

Control: Unenriched olive oil; MOO: enriched olive oil by maceration UOO: Ultrasound enriched olive oil; n.d: None detected; Data are mean ± standard deviation, n=3. Means with different superscripts are significantly different ($p < 0.05$). Lowercase letters (a, b, c) represent the statistical difference between samples; Numbers (1-2) represent the statistical difference between the same sample during storage period.

richness of UOO sample with phenolic antioxidants (mainly oleuropein) and pigments, which confirmed the usual correlation between the antioxidant activity and total phenolic content. Therefore, Interesse et al. [43] reported that pigments had pro-oxidising power in oil samples exposed to light and an antioxidant power in the dark.

2.7 CHANGES IN SENSORIAL PROPERTIES

At the beginning of storage, the sensory profiles of all studied EVOOs were devoid of defects. Moreover, the difference was significant ($p<0.05$) between all studied samples in terms of positive attributes. In fact, enriched oils were more acceptable, especially ultrasound enriched one, with stronger fruity smell (4), bitter (3.8) and pungent taste (3.5) (Tab. VI). Similar results were found by [33] who proved that sensory attributes were enhanced with the addition of olive leaves, in term of green colour and fruity attributes.
At the end of storage, the fusty defect was identified for the control which had also the lowest fruity intensity (1.5). Macerated organic EVOO was musty (0.4), with an unacceptable taste. More, MOO showed a marked decrease in the bitterness probably explained by the reduction of phenolic content particularly oleuropein during storage. In fact, it was reported by Betran et al. [44] the significant positive correlation between bitterness intensity and the level of phenols. These findings showed the deterioration of the quality of whole fresh olive leaves in MOO, during storage, which leaded also, to the appearance of the 'winey' attribute (1.4) usually caused by the formation of acetic acid during storage. However, at the end of storage, the UOO sample had no defects and presented a slight increase in bitterness compared to maceration and unenriched oil due to the presence of the highest amount of phenols. This result was in line with those of Achat et al. [13]

CONCLUSION

During storage of organic extra virgin olive oils, at room temperature and in darkness, results revealed that enrichment using ultrasound-assisted extraction from olive leaves increased oleic acid content and reduced linoleic acid one compared to control and macerated oil. The enrichment of organic olive leaves extracts using the two methods improved the oxidation stability of virgin olive oil by reducing the PV and extinction coefficients. The antioxidant activity was higher when the ultrasound-assisted extraction was applied which can be explained by their highest content of pigment and biophenols, during the storage period. Sensorial analysis showed an improvement in taste and odour of olive oil enriched with ultrasonic extracts with the highest overall acceptability after six months compared to control and macerated olive oil. These results encourage the use of organic olive leaves as a source of natural antioxidants for improvement of quality and oxidative stability of olive oil

and mainly the application of an ultrasound-assisted extraction, which is a potential emerging technology that can accelerate heat and mass transfer with shorter processing times and reduced operating and maintenance costs leading to better quality. Thus, it should be mentioned on the label that it is an "enriched olive oil" and not "extra virgin olive oil" from a legislation point of view.

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