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MASTER

Thème

Enrichissement de l'huile d'olive vierge avec le
péricarpe du fruit de Myrtus communis

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Introduction

Virgin olive oil is widely consumed in the Mediterranean basin. It is well known for its nutritional properties due to its high content in volatile and non-volatile compounds of interest. The first group of interesting compounds is the fatty acid composition: olive oil is rich in monounsaturated fatty acids (oleic acid) and polyunsaturated fatty acids (linoleic, and linolenic acid) that are known to show protective effects against cardiovascular diseases by preventing oxidation of low density lipoproteins (LDL). Other compounds of interest are the phenolic compounds, which are secondary plant metabolites that show antioxidant properties and thus have been reported to induce lower rate of cancers and Alzheimer's disease. The protective role of olive oil also comes from its content in vitamins, especially vitamin E which has been reported to be one of the most effective antioxidant (Servili and Montedoro 2002; Veillet, Tomao et al. 2010).

The content of phenolic compounds is an important factor to be considered in evaluating virgin olive oil (VOO) quality (Servili and Montedoro 2002; Veillet, Tomao et al. 2010).

The daily intake of polyphenols differs according to the multiple agronomic and technological factors, being the cultivar and the ripening index some of the most important ones. Thus, a good strategy to ensure an optimal intake of polyphenols through habitual diet would be to produce enriched VOO with well-known bioactive polyphenols (Alma, Mavi et al. 2003).

The enrichment of VOO with natural antioxidants contained in various herbs was studied, and in the present study, for the first time, an enrichment of virgin olive oil with phenolic compounds of *Myrtus communis* (pericarp) extracted by ultrasound assisted by microwave is realized.

Myrtus communis L. is known as a medicinal plant to anti-hyperglycemic, antiseptic and anti-inflammatory activities. It's considering a native plant of the Mediterranean and Asia region, which is reported to have antibacterial and antifungal activity different parts of the plant find various uses in food and cosmetic industries (Elfellah, Akhter et al. 1984; Al-Hindawi, Al-Deen et al. 1989; Chalchat, Garry et al. 1998; Mansouri, Foroumadi et al. 2001).

The main objective of this study was to optimize conditions (time, temperature, and powder diameter) of enrichment of virgin olive oil by *Myrtus communis* pericarp powder, and evaluate the potential of enrichment of olive oil obtained at optimal conditions.

1. Definition:

Olive tree (*Olea europaea* L.), a native of the Mediterranean basin and parts of Asia, is now widely cultivated in many other parts of the world for production of olive oil and table olives. The genus *Olea* L. is represented by the single species *Olea europaea* L. and two varieties, var. *sylvestris* (wild olive), which forms part of the natural vegetation, and var. *europaea* cultivated primarily for oil (Díaz de la Guardia et al., 2003; Ghanbari et al., 2012).

Virgin olive oil (VOO), extracted mechanically from the fruit, is also very popular for its nutritive and health-promoting potential, especially against cardiovascular disorders due to the presence of high levels of monoinsaturates and other valuable minor components such as phenolics, phytosterols, tocopherols, carotenoids, chlorophyll and squalene. The cultivar, area of production, harvest time, and the processing techniques employed are some of the factors shown to influence the composition of olive fruit and olive oil (Ghanbari et al., 2012).

Olive oil production is an important agro-industrial sector (in terms of both production and consumption) in many Mediterranean regions. Furthermore, the olive groves and olive production are increasing yearly and, recently, the importance of olive oil has also been growing in new producing countries located in America, Africa and Australia (Salomone et al., 2015).

2. Olive oil extraction technology:

Virgin olive oil (VOO) is exclusively extracted from fruits by means of mechanical techniques that include crushing, malaxation and extraction steps. Each of these technological operations, in addition to the olive fruit characteristics, affects the quality of the product (Clodoveo, 2012). There are mainly two extraction methods:

2.1. Traditional method:

The traditional method of olive oil extraction is by using an olive press. The olive press works by applying pressure to olive paste to separate the liquid oil and vegetation water from the solid materials. In this method, after separating the oil and water from the fruit paste, the water and the oil themselves are then separated from one another by a

standard decantation procedure. This method has been in use since the Greeks first began pressing olives over 5000 years ago and it is still widely used today with some modification.

2.2. Modern method:

The modern method of olive oil extraction uses an industrial decanter to separate all the phases by centrifugation. In this method, olives are crushed to a fine paste by a hammer crusher, a disk crusher, a depitting machine or a knife crusher. The paste is then malaxed for 30-40 minutes to allow the small olive droplets to agglomerate and the fruit enzymes to create aromas. After this the paste is pumped into an industrial decanter where the phase will be separated. Depending upon the system, water might be added with the paste to facilitate the extraction process (Zeev Wiesman, 2009).

3. Classification of virgin olive oil:

The designation of *virgin* olive oil is solely recognized as the olive oil obtained from the fruit of the olive tree by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil, which has not undergone any treatment other than washing, decantation, centrifugation and filtration, virgin olive oils are classified into:

3.1. Extra virgin olive oil:

A higher quality olive oil with no more than 0.8 g per 100 g of free acidity (expressed as oleic acid) and a superior taste (fruitiness and no sensory defect). It must be produced entirely by mechanical means without the use of any solvents, and under temperatures that will not degrade the oil (lower than 30 °C) (Salomone et al., 2015).

3.2. Virgin olive oil:

Virgin olive oil which has a free acidity, expressed as oleic acid, of not more than 2 grams per 100 grams and the other characteristics of which correspond to those fixed for this category in this standard.

3.3. Lampante virgin olive oil:

It is the virgin olive oil which has a free acidity, expressed as oleic acid, of more than 3.3 grams per 100 grams and/or the organoleptic characteristics and other characteristics of which correspond to those fixed for this category in this standard (dairi, 2014).

Other classifications are related to the definition of *olive oil*, distinguishing:

- **refined olive oil**, obtained by the refining of virgin olive oil using methods that do not lead to alterations in the initial glyceridic structure; it has no more than 0.3 g per 100 g of free acidity;
- **olive oil**, which is a blend of refined oil and virgin oil (excluding the lampante virgin oil), fit for consumption as it is and having no more than 1 g per 100 g of free acidity (Salomone et al., 2015).

4. Olive oil composition:

The chemical composition of VOO is influenced by the olive variety, the climatic conditions, the geographical site and the maturity stage.

In the olive fruits, oil is mainly concentrated in the pericarp (96–98%). The formation and accumulation of oil in the drupe, a rich reservoir of many classes of lipids, is possibly the reason why the oil has a unique flavour and fragrance. The olive flesh components are transformed to the oil, which mainly consists of two components, namely saponifiables and unsaponifiables. The former, comprising triacylglycerols (TAG), partial glycerides, esters of fatty acids or free fatty acids and phosphatides, represent nearly 98% of the oil chemical composition, while the later, consisting of mainly minor components such as tocopherols, phytosterols, coloring pigments and phenolics, contribute around 1–2% of the oil composition. The oil triglycerides are mainly represented by monounsaturates (oleic acid), alongwith small amount of saturates and considerable quantity of polyunsaturates (mainly of linoleic acid) (Fuentes et al., 2015; Ghanbari et al., 2012).

5. Enrichment of olive oil:

Olive oil is a vital component in Mediterranean diet which is highly recognized for its health benefits, including but not limited to improving. Despite the fact that olive oil has been widely used across the globe (Sena and Juliastuti, 2017).

The daily intake of endogenous bioactive phenolics from VOO is variable due the influence of multiple agronomic and technological factors, being the cultivar and the ripening index some of the most important ones. Therefore, a large variety of VOO can be found in the market, containing not only quite different total polyphenol content but also quite different polyphenol compositions (Segade et al., 2016).

Thus, a good strategy to ensure an optimal intake of polyphenols through habitual diet would be to produce enriched VOO with well-known bioactive polyphenols (Achat et al., 2012).

A great number of different sources of natural biological active substances, also known as functional, can be potentially used to enrich VOO. Several published studies on VOO enrichment employed raw materials derived from the same olive tree, mainly leaves or residual olive pomace obtained after the mechanical extraction of the oil, being a cheap and good source of phenolic compounds to be used to increase their concentration in the developed enriched oil. Other studies used plants and vegetables, mainly herbs and spices. These sources of natural bioactive ingredients employed to develop novel functional oils are summarized in Table 1 (Suárez et al., 2010; Japón-Luján et al., 2008).

Table1: Summary of the recent work on enrichment effect of olive oil.

Source/Ingredient	Extraction Method and Conditions	Observed Functional Effect in Enriched Olive Oil	Reference
Olive leaves	SLE: directly in the oil (organic-solvent free), dynamic extraction	Increased oxidative stability	(Japón-Luján et al., 2008)

	chamber, USAE (25 °C, 20 min)		
	SLE and LLE with ethanol, MAE (8–10 min, 200–400 W)	Increased oxidative stability	(Japón-Luján and Luque de Castro, 2008)
Red pepper	Infusion: 10%–20% up to 30 days	Reduced oxidative stability	(Caporaso et al., 2013)
	SFE at 40 °C and 15–23 MPa	Reduced oxidative stability	(Gouveia et al., 2006)
Basil	Infusion: 15% with USAE (1 W/cm ²) for 15 min	Not determined	(Veillet et al., 2010)
Lemon and thyme	Infusion: 20% during 2 months	Unchanged oxidative stability	(Issaoui et al., 2011)
Oregano	Addition of 0.05% of essential oil	Increased oxidative stability	(Asensio et al., 2013)
	Infusion by stirring at 1000 rpm for 3 h	Increased oxidative stability	(Perestrelo et al., 2017)

1. Definition and distribution:

Myrtus communis L, belonging to the *Myrtaceae* family with some 145 genus and over 5500 species. It is mainly spread around the Mediterranean, including the Middle East and many other countries in Southern Europe. It is also found in Asia, New Zealand, America, Southern Russia and Australia. This shrub with leathery hermaphrodite, persistent and with aromatic leaves has a longevity of over 300 years. Generally, it is found at an altitude not exceeding 800 m. When in flower, it emits a characteristic aroma of mixed incense and honey. It is often associated with forests of oak, mastic and Aleppo pine. It grows equally well on limestone or silica, and easily adapts to many soils. It is fairly resistant to cold and readily acclimatizes to hot weather (Wannes, Mhamdi et al. 2010; Berka-Zougali, Ferhat et al. 2012).

The plants produce abundant, solitary, white flowers during the long, hot mediterranean summer. The fruits are blue-black, spherical, multi-seeded berries, which are bird dispersed; yellowish-white-fruited forms are also rarely found. *Myrtus* is the only European genus of the large tropical and subtropical family *Myrtaceae*, which includes other familiar plants such as a cloves, allspice and eucalyptus (Migliore, Baumel et al. 2012)

In Algeria, the wild plant known as “Al-Rihan” or “el-halmouche” grows very well in many areas, on mounds or hills, in coastal or in more remote areas. The Myrtle plant is currently generating real interest regarding its use as a medicinal plant in Algeria (Berka-Zougali, Ferhat et al. 2012).

2. Applications:

Myrtle is an aromatic plant rich in essential oils used in pharmaceutical, cosmetic, and food industries. Its characteristics have long been known; in the past, it is used in the treatment of urinary infections, digestive problems, vaginal discharge, bronchial congestion, sinusitis, and dry coughs (Amensour, Sendra et al. 2010), also, myrtle was used as flavoring agent and in folk medicine for its anti-inflammatory and antiseptic properties. The medical literature have confirmed the antimicrobial properties and assessed the apoptotic activity against cancer cells of myrtucommulone, a nonprenylated acylphloroglucinol present in myrtle leaves. Furthermore, recent data highlighted the good

antioxidant properties of myrtle berry extracts and their use as food preservatives was also suggested. In the last years, myrtle has been appreciated as an ornamental plant as a result of the new interest of consumers in Mediterranean flora (Melito, Fadda et al. 2014).

3. Etymology and classification of the myrtle plant:

Myrtle has closely associated names in most European and even some non-European languages; besides English myrtle, German myrte, Estonian mürt, Spanish and Italian mirto, French myrte, modern Greek mirtia, Russian myrt, Armenian mrdeni, Farsi mourd and Turkish murt. All these names relate to the Old Greek myrtos. In Algeria, the wild plant known as Al-Rihan or el-halmouche.

Taxonomically *Myrtus* genus belong to the *Myrtaceae* family growing in temperate, tropical and subtropical regions. *Myrtus communis* is the only *Myrtaceae* species native to Europe and it is classified according to: (Quezel 1963)

Reign: Planta

Sub-kingdom: Tracheobionta

Embranchment: Magnoliophyta

Deputy embranchment: Magnoliophytino

Subclass: Rosidae

Order: Myrtales

Family: *Myrtaceae*

Genus: *Myrtus*

Species: *Myrtus communis* L. (Goetz and Ghédira 2012).

4. Composition:

Seed, pericarp and whole berry of *Myrtus communis* were compared in terms of oils, glycerolipid classes and fatty acids. The fruit is composed of pericarp and approximately 9 seeds which constituted 63.5 and 36.5% of the whole ripe fruit, respectively. The latter presented a weight of 8.8 g% fruits while seed had only 0.5 g%

seeds. The moisture contents were 80.1% in pericarp, 72% in whole fruit and 39.7% in seed. The oil yield of seed (11.7%) was significantly higher than that of whole fruit (5.9%) and pericarp (2.1%). Total lipid amounts were 61.26 mg/g in seed, 28.97 mg/g in whole fruit and 4.14 mg/g in pericarp. The amounts of polar glycerolipids were lower than those of neutral glycerolipids in all samples. Triacylglycerol constituted the main neutral glycerolipid with 57.47 mg/g in seed, 25.68 mg/g in whole fruit and 1.67 mg/g in pericarp. The predominant fatty acids of total lipids and different glycerolipid classes were linoleic, palmitic, oleic and linolenic acids in all samples but with different proportions (Wannes, Mhamdi et al. 2010).

1. Samples:

The material used in our study is pericarp of *Myrtus communis L.* and virgin olive oil. The methods used are related to the following experiments:

1.1. Preparation of *Myrtus communis L* pericarp powder:

Mature *Myrtus communis L* fruits were collected in December 2016 from 30 to 50 plants growing wild in Addekar of Bejaia region (Bejaia, north-east of Algeria). The fruits were recovered manually from the aerial parts, and were washed with distilled water to remove any adhering soil and dust.

1.2. Characterization of *Myrtus communis L* fruit:

1.2.1 Determination of moisture content:

Principle:

To determine the water content, the moisture test carried out for the fruit of *Myrtus*, three samples of 8g are dried at $105\pm 2^{\circ}\text{C}$ in an oven (Ecocell). The weight of the samples is taken each 30min until its stabilization. The result is average of three samples according to Bourkhiss, Hnach et al. (2009). Water content is given according to the following equation:

$$H\% = \left(\frac{W_i - W_f}{W_i} \right) \times 100$$

Where:

H%: moisture.

W_i: represent the initial weight of the sample.

W_f: represent the dry weight of the sample.

2. Preparation of the powder:

2.1. Ultrasound pre-treatment:

An ultrasonic bath (Ctra.NII:585 Abrera (Barcelona) Spain, Ultrasound H-D, frequencies: 20 to 60 KHZ, Power: 80 to 600W) was used (figure n°1).



Figure n°1: Ultrasound bath

An amount of myrtle fruit was added to the distilled water in the bath. The ratio of raw material to water was set at $\frac{1}{4}$. The ultrasound energy was applied for 90min at $25\pm 2^{\circ}\text{C}$. After the treatment, the fruit was wiped with absorbing paper (figure n°2).



Figure n°2: Dried myrtle fruits

2.2. Microwave drying method:

After ultrasound pretreatment, the fruits were dried in a microwave (500W) for 20 ± 2 min.

After ultrasound and microwave treatment, pericarp and seed of the fruit were isolated manually, than, the pericarp portion was ground and sieved using two diameters for the sieves $125\mu\text{m}$ and $250\mu\text{m}$.

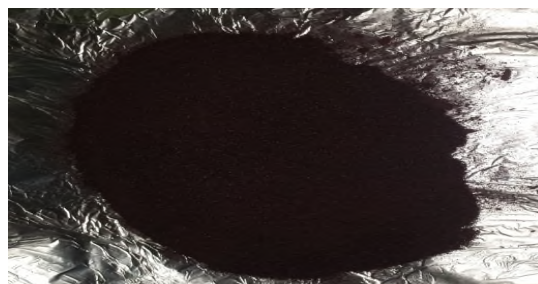


Figure n°3: *Myrtus communis* L pericarp powder

3. Preliminary study:

Prior to optimization, experiments were carried out to determine the appropriate range of conditions for the extraction of phenolic compounds from the powder of *Myrtus communis L* pericarp, namely, extraction temperature, extraction time, and the diameter of pericarp powder of *Myrtus communis L* by changing one independent variable at a time while maintaining the other constants (Hossain, Strezov et al. 2011).

3.1. Enrichment of olive oil:

Principle:

Optimization of Enrichment of virgin olive oil using ultrasound has been proposed to improve the efficiency and/or duration of this step. Enrichment of virgin olive oil with bioactive substances (polyphenols) from pericarp of *Myrtus comminus L* by using ultrasound solid liquid extraction, in which the pericarp is dried under appropriate conditions and the powder obtained is partially dissolved into the oil as a function of the solubility in order to increase nutritional quality and oxidative stability in the olive oil.

Procedure:

For the enrichment, 5 g of the *Myrtus communis L* pericarp powder was added to 50 ml of virgin olive oil in the beaker, which was placed in ultrasound for 30min at different temperatures (15, 25, 35, 45°C). After setting the temperature at 35°C, time was varied (5, 15, 30, 45min), after that, the efficiency of two particle size (125µm, 250µm) was tested at fixed temperature and time.

3.2. Extraction and characterization:

3.2.1 Extraction procedure of phenolic compounds from the control oil and enriched olive oil:

Principle:

It is a liquid / liquid extraction which consists to dissolving the oil in an organic solvent and mixed with a polar solvent for the recovery of the phenolic compounds.

Procedure:

A test portion of 2.5 g of oil was added to 5 ml of hexane (99%) and 5 ml of methanol / water (6/4, v / v), then vortexed for 2 min, The mixture was centrifuged at 3500 rpm for 10 min. The polar fraction (methanol phase) was recovered, while the apolar

(hexanic) phase was depleted. The procedure was repeated three times. The three fractions obtained are mixed and stored at 4 °C and protected from light (Kalantzakis, Blekas et al. 2006).

3.3. Phytochemical analyzes:

3.3.1. Determination of total phenolic compounds (TPC):

Principle:

The concentration of the total phenolic compound content was estimated by the Folin-Ciocalteu assay. The reagent consists of a mixture of phosphotungstic acid (H₃ PW12 O₄₀) and phosphomolybdic acid (H₃ PMo12 O₄₀), reduced during the oxidation of the phenols, into a blue mixture of tungsten and molybdenum. The coloration produced is proportional to the quantity of polyphenols present in the plant extracts.

Procedure:

The determination of the total phenolic compounds is carried out according to the protocol described by George and Bennett (2005). In test tubes, a volume of 500 µL of enriched oil extract was mixed with 2.5 mL of the Folin-Ciocalteu reagent (diluted 1/10) and after 2 min in the dark, a volum of 2mL of sodium carbonate (Na₂ CO₃) (75g / L) was added. After 15 minutes of incubation at 50°C, the absorbance is measured at 760 nm against a control where the extract is replaced by the same volume of the solvent used. The concentrations are expressed in mg equivalent of gallic acid per kg of dried pericarp *Myrtus communis L* powder.

2.3.2. Determination of carotenoids and chlorophylls content:

According to Minguez Mosquera et al (1991), the protocol for the determination of carotenoids and chlorophylls is as follows:

A sample of 7.5 g of oil is adjusted to 25 ml of cyclohexane in a graduated flask. The maximum absorbance at 670 nm and at 470 nm gives information on the chlorophyll fraction and the carotenoids respectively. The value of the specific extinction coefficient used:

- E₀ = 613 for Pheophytine (major component of chlorophylls).
- E₀ = 2000 for Lutein (major component of carotenoids).

And the results are calculated according to:

$$\text{Chlorophyll} \left(\frac{\text{mg}}{\text{kg}} \right) = \frac{A_{670} - 10^{-6}}{613 - 100 \times T}$$

$$\text{Caroténoids} \left(\frac{\text{mg}}{\text{Kg}} \right) = \frac{A_{470} - 10^{-6}}{2000 - 100 \times T}$$

A: absorbance.

T: optical path (thickness of the vessel 1 cm)

4. Experimental design:

After preliminary study where we fixed the optimal condition for particle size and the range of value of the time and temperature of the enrichment, experimental design was made with twelfths experiments. Enrichment for each time / temperature condition from the twelfths is performed followed by an extraction and then after a dosage of the TPCs, chlorophylls, and carotenoids according to the same protocols already done (page 14).

5. Optimal conditions and model validation:

The TPCs, chlorophylls, and carotenoids results obtained from the experimental design are processed by JMP software to have the optimal condition, once the latter has been selected, we carried TPCs, chlorophyll and carotenoids dosages (protocol page 14) and measuring antioxidant powder test, and Physico-chemical characterization of the control oil and the enriched oil (protocols following in pages).

5.1. Test for measuring antioxidant power:

5.1.1 DPPH test:

Principle:

The chemical compound 2,2-diphenyl-1-picrylhydrazyl (α , α -diphenyl- β -picrylhydrazyl) was one of the first free radicals used to study the antioxidant structure-activity relationship of phenolic compounds. Unmatched on a nitrogen bridge atom, the molecules of the radical do not form dimers, so it remains in its relatively stable monomer form at ambient temperature. It is this delocalization which causes the dark violet

coloration characteristic of the solution of DPPH. Measurement of the decrease in DPPH staining makes it possible to measure the efficacy of an antioxidant, due to a recombination of DPPH radicals which has a maximum absorbance at 515 nm. Concerning phenolic compounds, the main mechanism of action is trapping of Free radicals by transfer of the H atom onto the DPPH ° then transformed into a stable DPPH molecule.

The results may be expressed as anti-free radical activity or as a percentage of free radical inhibition using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Control Abs} - \text{sample Abs}}{\text{Control Abs}} * 100$$

Procedure:

The DPPH test is carried out according to the method described in (Dudonné, Vitrac et al. 2009), 100 µl of extract at different concentrations are mixed with 3 ml of the DPPH solution (60 µM) C, the absorbance is read at 515 nm The results are expressed as percent inhibition, and an inhibition curve as a function of concentration is plotted% inhibition = f (concentration).

5.2. Physico-chemical characterization of the control oil and the enriched oil:

5.2.1 Determination of free fatty acids:

This is the percentage of free fatty acids expressed conventionally according to the nature of the fatty substance, in oleic or palmitic acid. It informs us on the degree of the freshness of the fat compounds.

Principle:

It is a neutralization of the FFAs (Free fatty acids) by a solution of NAOH (0.1 N) in the presence of the phenolphthalein as a colored indicator, according to the following reaction:



Procedure:

5 g of oil are weighed into a flask, 2.5 ml (ethyl ether / ethanol) are added and the mixture is stirred to thoroughly solubilize the oil in the solvent followed by the addition of a few drops of phenolphthalein and titrated with KOH 0.1N) until a persistent pink color is obtained (Regulation (EEC) No 2568/91), and the results are expressed according to the following equation:

$$\text{Acidité \%} = (V - V_0) (N \times M_{\text{eq}} / 10 \cdot m)$$

Where:

Meq: equivalent mass of oleic acid = 282meq

N: normality of KOH at 0.1N.

M: weight of the test portion.

V: volume of KOH used for the titration of the sample.

V0: volume in ml of KOH required for titration of the blank.

5.2.2 Determination of the peroxide index:

Principle:

It consists in treating an amount of oil in solution in acetic acid and chloroform with a solution of potassium iodide (KI). The titration of released iodine is carried out by means of a solution of sodium thiosulphate in the presence of starch paste as a colored indicator (Regulation (EEC) No 2568/91).

Procedure:

A quantity of 2 g of oil are weighed into a flask, 10 ml of chloroform, 15 ml of glacial acetic acid and 1 ml of KI (potassium iodide) are added. After stirring for 1 min, the light is shielded for 5 minutes and 75 ml of distilled water are added, 3-4 drops of the starch solution and titrated with sodium thiosulfate (0.01 N) until to the disappearance of the color. In parallel, a blank test is carried out.

Expression of results:

The determination of the peroxide index is made according to the following formula:

$$I_p = \frac{[N \cdot (V_1 - V_0) \cdot 100]}{m}$$

I_p: Peroxide index (Meq of O₂ / kg of oil).

N: Normality of Na₂S₂O₃ (N).

V₁: Volume of Na₂S₂O₃ used to titrate the sample (ml).

V₀: Volume of Na₂S₂O₃ used for the blank test (ml).

m: mass of the test portion (g).

5.2.3 Determination of the Reduced power:

Principle:

The yellow color of the test solution changes to green depending on the reducing power of test specimen. The presence of reductants in the solution causes the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by the measurement of the absorbance at 700 nm (Zou, Lu et al. 2004).

Procedure:

One mL of different extracts was mixed with 2.5 mL of a 0.2 M (m/v) sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% (m/v) Potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$). The mixture was incubated in a water bath at 50° C for 20 min. Then, 2.5 mL of 10% (m/v) trichloroacetic acid were added. Finally, 1mL of the obtained solution was added to 5 mL of distilled water and 1mL of 0.1% (m/v) ferric chloride (FeCl_3), the intensity of the blue green color was measured at 700 nm. Tests were carried out in triplicate.

6. Extraction procedure for phenolic compounds of the pericarp of *Myrtus communis*L powder:

Extraction of phenolic compounds from the *Myrtus comunis* powder was made with an ultrasonic apparatus (SONICS Vibra cell, VCX 75115 PB, SERIAL No. 2012010971 MODEL CV 334).

Procedure:

A quantity of 1g of the powder was placed in a 250ml amber glass bottle containing 28ml of 70% ethanol., irradiation time (7min 30s), and amplitude of 30%. The temperature ($27\pm 2^\circ\text{C}$) was controlled continuously by circulating external cold water. After the extraction, the solution was filtered through filter paper.

After that, test for measuring antioxidant power was done (protocol page 15).

7. Statistical analysis:

The analysis of variance (ANOVA) was performed using XLSTAT release 10 (Addinsoft, Paris, France), Tukey's multiple range test (HSD) was used for each extraction trial and all the analyses were carried out in triplicate, which allowed us to compare between TPC content and antioxidant activity of olive oil enriched by *Myrtus communis* pericarp.

1. Moisture content:

The determination of moisture is very important to predict performance after drying. Indeed, the humidity conditions the retention setting to avoid possible economic and nutritional losses caused by microbial deterioration and enzymatic activities of preserved fruit.

The result of the test of moisture shows that the myrtle has an average water content of $57 \pm 0.0814\%$, as represented in table 2:

Table2: Moisture content.

Moisture (%)	57
Dried matters (%)	43

2. Preliminary study:

This study was used to determine the most suitable time, temperature and diameter of parameters for TPCs, chlorophylls and carotenoids extracted from pericarp powder of *Myrtus comminus L* and thus were to be used in subsequent ultrasonic studies.

2.1. Total phenolic compounds, chlorophylls, and carotenoids:

The results obtained by the preliminary study as shown in different histograms that follow reveal the TPCs, Chlorophylls, and Carotenoids quantity of enriched olive oil extract obtained by ultrasound enrichment with different times, temperatures, and powder diameter.

The results were expressed as milligram (mg) Gallic acid equivalent (GAE) per kilogram of enriched olive oil for TPCs and as milligram (mg) per kilogram (kg) for chlorophylls and carotenoids.

2.1.1. Determination of total phenolic compounds in enriched olive oil extracted by varying the temperature:

The TPC yield obtained from virgin olive oil (control) is lower than that of enriched olive oil at different temperatures (figure n°4).

The effect of enrichment temperature parameter in TPCs yield was evaluated at different temperatures in comparison to the control. Figure 4 shows that the TPC yield increased

from 15°C (25mg GAE/kg) to 35°C (62mg GAE/kg). After that, the TPC yield decreased to 38 mg GAE/kg at 45°C. We suggested that the decreasing in the TPC yield at the temperature of 45°C was due to the degradation of myrtle phenolic compounds added in the olive oil as reported by Salehan et al., (2006).

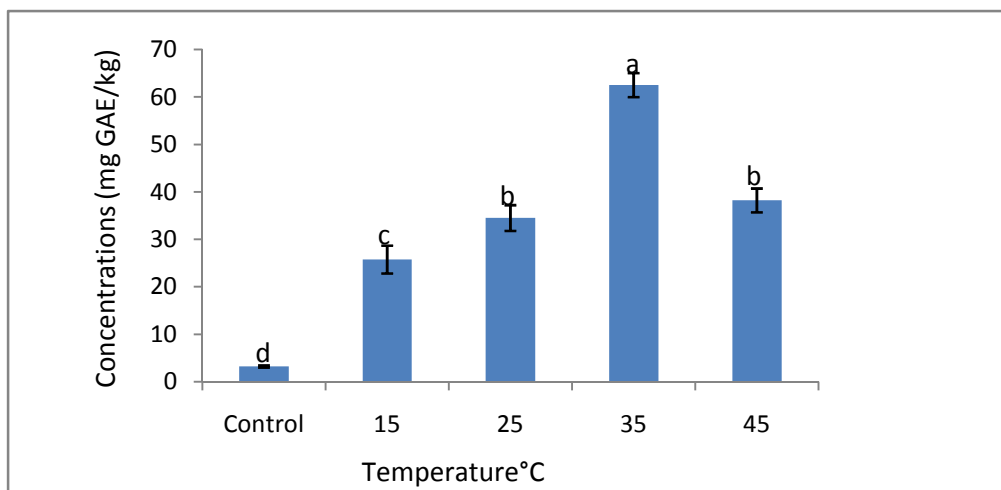


Figure n°4: TPCs content in enriched olive oil at different temperatures.

2.1.2. Determination of Chlorophylls and Carotenoids content in enriched olive oil extracted by varying the temperature:

The results obtained with chlorophylls and carotenoids were identical to those of TPC only for chlorophyll at the temperature of enrichment of 45°C, where there was not significant difference with 35°C (figure 5) ((MASON, PANIWNYK et al.).

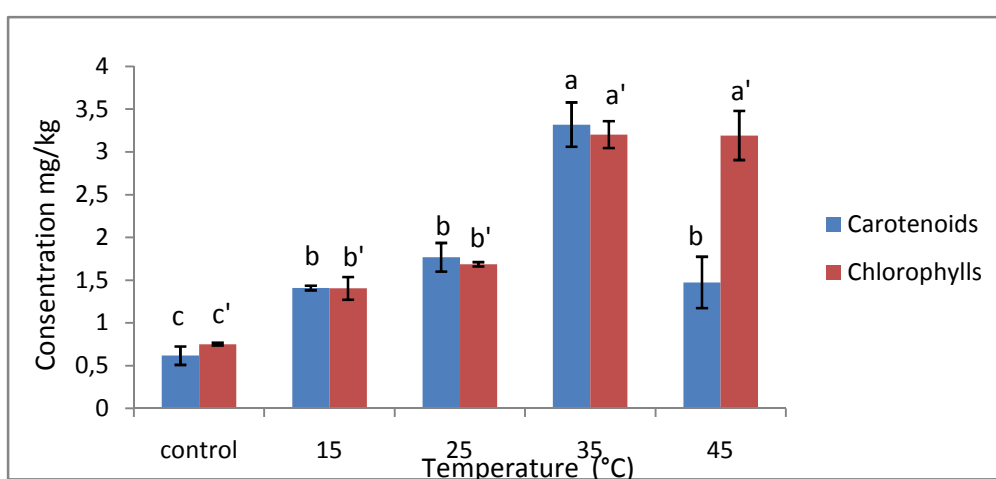


Figure n°5: Chlorophylls and carotenoids content in enriched olive oil at different temperatures.

2.1.3. Determination of TPCs content in enriched olive oil extracted by varying the time:

The effect of enrichment time parameter in TPCs yield was evaluated at different times. We observed an increasing in TPC yield from 5 min (10 mg GAE/kg) to 30min (61mg GAE/kg). After that, the TPC yield decreased to 50 mg GAE/kg at 45min (figure n°6).

However, the TPC yield obtained from virgin olive oil stay lower than that of enriched olive oil at different times of extraction again.

The lowest content in olive oil enriched at 45°C could be due to degradation of TPC by the high temperature (45°C) and the largest duration time (45min) in ultrasound (Ozkan, Akbudak et al. 2007).

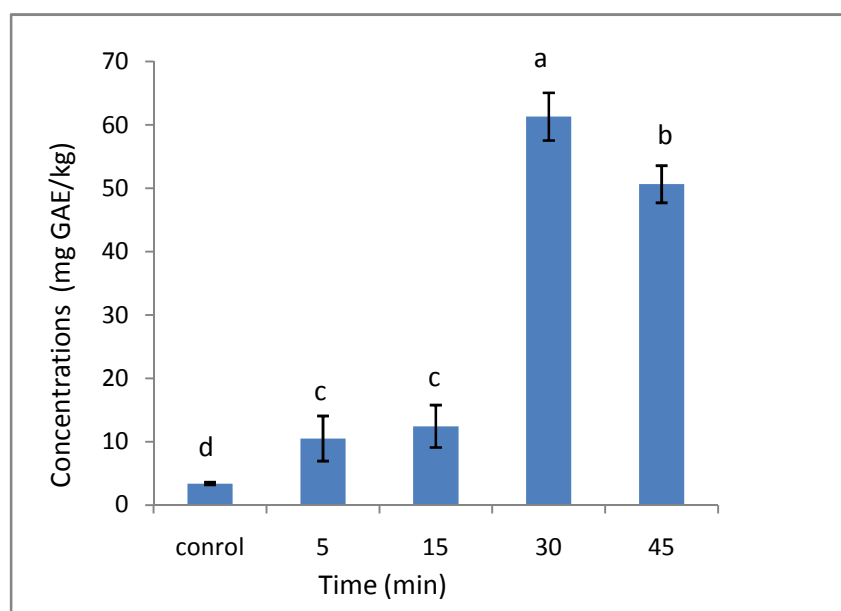


Figure n°6: TPCs content of enriched olive oil in different times.

2.1.4. Determination of Chlorophylls and Carotenoids content of enriched olive oil extracted by varying the time:

The enrichment duration influence chlorophyll and carotenoid extraction. The adequate duration to maximize chlorophyll and carotenoid extraction from enriched olive oil is at 30 min. and that is higher than chlorophyll and carotenoid content extracted from olive oil (figure n°7) (He et al.,2014; He, Yang et al 2012).

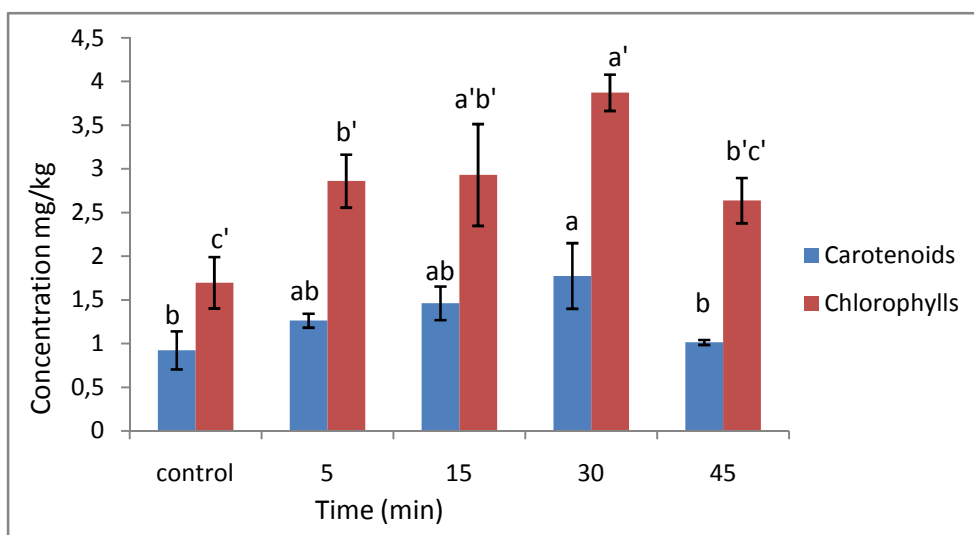


Figure n°7: Chlorophylls and Carotenoids content of enriched olive oil at different times.

2.1.5. Determination of total phenolic compounds of enriched olive oil extracted by varying the diameter:

The results observed in figure n°8 show that the difference of TPC content obtained from olive oil enriched by different diameter of myrtle powder is not significant.

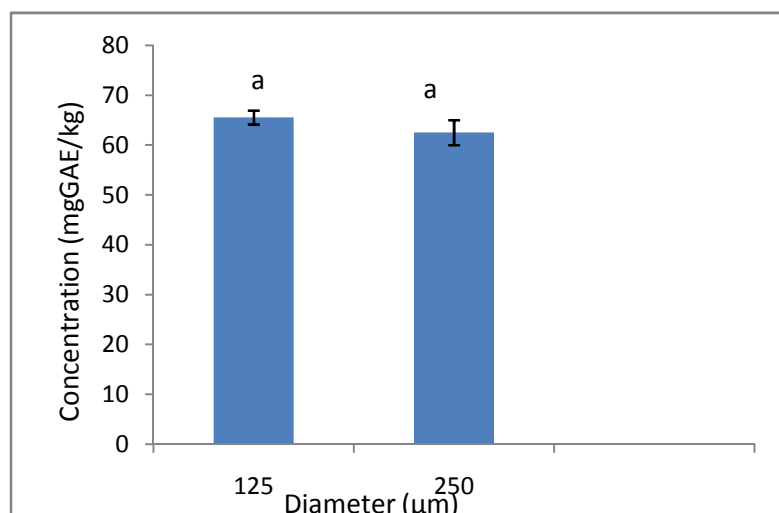


Figure n°8: Yield of TPCs in the olive oil enriched by different diameter of myrtle powder.

2.1.6. Determination of chlorophylls and carotenoids content of enriched olive oil extracted by varying the diameter:

The main significant differences were found in chlorophylls content, the diameter of myrtle powder influenced the chlorophylls content, so the highest content was that in olive oil enriched by myrtle powder with diameter of 125 μm (figure n°9).

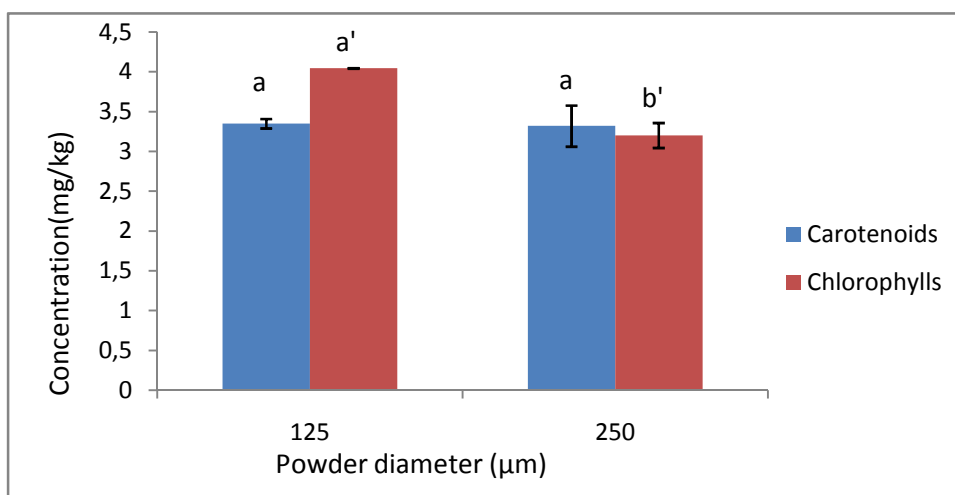


Figure n°9: Yield of Chlorophylls and Carotenoids in the olive oil enriched by different diameter of myrtle powder.

3. Experimental design:

Many parameters can influence the efficiency of TPC extraction, such as extraction method, solvent nature and concentration, extraction temperature, and extraction time (Banik et Pandey, 2007; Silva et al, 2007; Pinelo et al, 2005).

Two factors (time X_1 , and temperature X_2) have been applied for optimize extraction of TPCs, Chlorophylls, and Carotenoids from the enriched olive oil. The conditions selected in this study are:

25/45, 25/30, 5/30, 5/45, 45/15, 5/15, 45/30, 25/15, 25/30, 25/30, 25/30, and 45/45 time (min)/temperature ($^{\circ}\text{C}$) respectively.

Table n°3: Experimental and expected yields by JMP software of the experimental design containing 12 manipulations.

Rows	Pattern	X ₁ Time (min)	X ₂ Temperature (°C)	TPC (mg/kg)	Chlorophylls (mg/kg)	Carotenoids (mg/kg)	Predicted TPC	Predicted Chlorophylls	Predicted Carotenoids
1	0A	25	45	109,01	6,22	2,56	107,07	6,91	2,87
2	00	25	30	99,95	10,16	2,92	96,58	9,70	3,28
3	a0	5	30	94,72	6,63	4,63	96,38	6,75	4,72
4	++	5	45	89,68	4,65	4,76	90,47	4,41	4,49
5	+-	45	15	101,14	10,16	4,21	99,04	10,09	4,62
6	--	5	15	109,23	4,99	3,55	106,78	5,12	3,74
7	A0	45	30	120,49	10,31	5,63	121,44	10,83	5,26
8	0a	25	15	86,02	8,56	3,07	90,58	8,52	2,47
9	00	25	30	94,02	11,07	2,92	96,58	9,70	3,28
10	00	25	30	93,83	9,45	3,61	96,58	9,70	3,28
11	00	25	30	101,14	8,78	3,39	96,58	9,70	3,28
12	++	45	45	147,16	8,03	4,73	148,31	7,58	4,68

During the optimization of extraction conditions, we obtained between:

- 86,02 and 147,16 g GAE/kg of TPC
- 4,65 and 11,07 mg /kg of Chlorophylls
- 2,56 and 5,63 mg/kg of Carotenoids

3.1. Fitting models:

The statistical software JMP was used for model fitting and statistical analysis such as regression analysis of experimental data to fit the empirical mathematical equation, analysis of variance (ANOVA, TUKEY) and 3D plots of response surface. Response surface methodology (RSM) was utilized to fit a surface to a set of data.

The fitted quadratic models for TPCs, Chlorophylls, and Carotenoids in coded variables are given in equation (1), (2), and (3) respectively.

$$\text{TPCs} = 96.58 + 12.53X_1 + 8.24X_2 + 16.39X_1X_2 + 12.33X_1^2 + 2.24X_2^2 \quad (1)$$

$$\text{Chlorophylls} = 9.70 + 2.04X_1 - 0.80X_2 - 0.45X_1X_2 - 0.91X_1^2 - 1.99X_2^2 \quad (2)$$

$$\text{Carotenoids} = 3.28 + 0.27X_1 + 0.2X_2 - 0.17X_1X_2 + 1.71X_1^2 + 0.61X_2^2 \quad (3)$$

The statistical analysis of the regression model indicated that the proposed model was adequate, possessing no significant lack of fit ($p > 0.05$) (table X) and with satisfactory values of the R^2 for all responses. As shown in Figure n°9, the R^2 values for TPC, Chlorophylls, and Carotenoids were 0.97, 0.92, and 0.87 respectively. The use of an adjusted- R^2 is to evaluate the model adequacy and fitness. The adjusted- R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The values of adjusted- R^2 (0.94 for TPCs, 0.85 for Chlorophylls, and 0.76 for Carotenoids) are also high and advocate a high correlation between the observed and the predicted values (figure n°10).

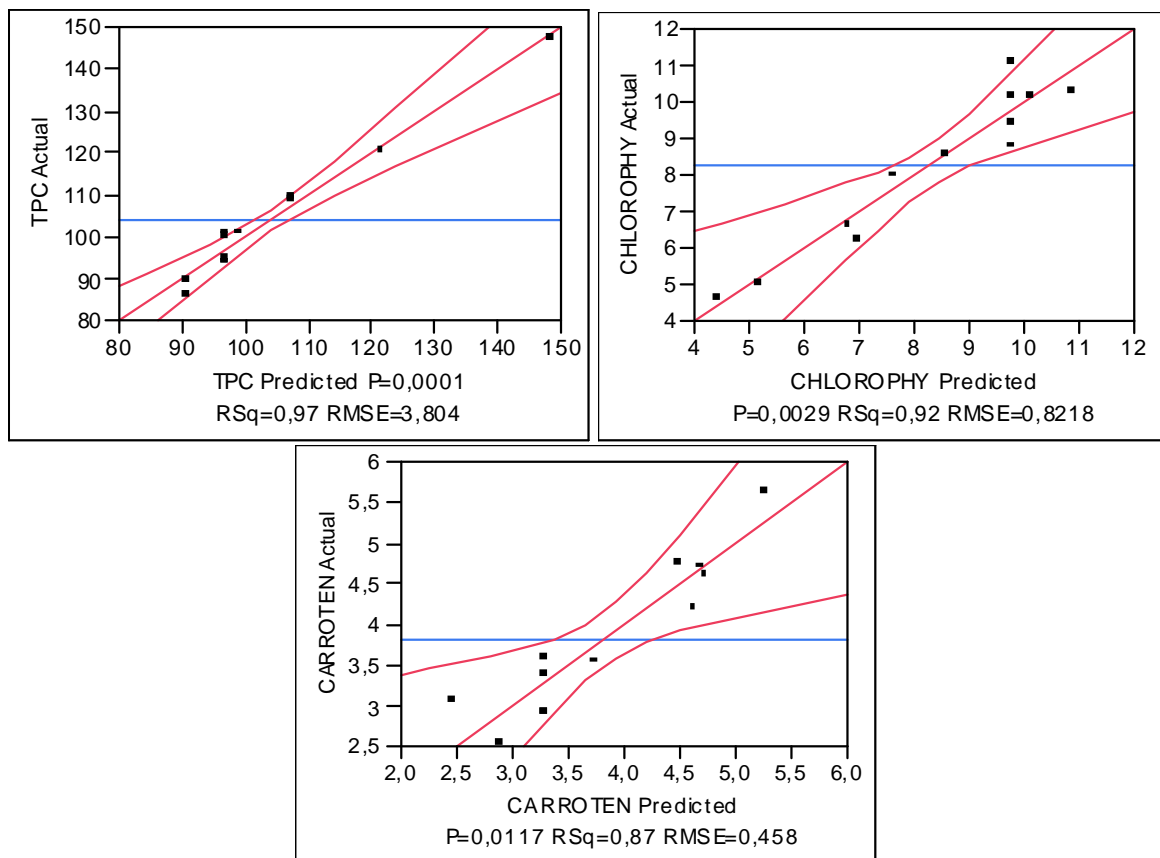


Figure n°10: Real prediction plan of the mixture par rapport TPCs, chlorophylls and Carotenoids.

3.1.1. Effect of factors (coefficients):

The analysis of the variance of the coefficients consists in analyzing the impact of the variables (X_1 and X_2) at $P < 0.05$, in order to determine the degree of influence of the factors (time and temperature) on the extraction of the bioactive substances (Uma et al, 2010).

The P-Values are used like a means to check the significance of each coefficient, which indicates also the intensity of interaction of each parameter. Smallest P-Value presents the greatest significance of the corresponding factor (Lieu e al, 2010).

3.1.1.1. Linear effect:

The results of this study show that the both factors (X_1 , X_2) had a highly significant linear influence on the enrichment of olive oil with TPC. However these two factors didn't have linear influence on the extraction of carotenoids. For chlorophylls only time influence its extraction (X_1) with a probability of $P = 0.0009$ (table n°4).

3.1.1.2. Quadratic effect:

The quadratic effect X_1^2 (time-time) is highly significant on the content of TPC and carotenoids extracted in the myrtle-VOO mixture with a probability $P = 0.0018$ and $P = 0.0009$ respectively. However, the quadratic effect X_2^2 (temperature-temperature) was the second effect influence the extraction of chlorophylls (table n°4).

3.1.1.3. Effect of interaction:

The results indicate that the interaction X_1X_2 (time-temperature) is significant on the TPC of the pericarp of myrtle-VOO mixture with a probability $P = 0.0001$ (table n°4).

Table n°4: ANOVA for the effect of time and temperature on the enrichment of VOO with TPCs, chlorophylls and carotenoids using a quadratic surface model.

Source	TPCs		Chlorophylls		Carotenoids	
	F Value	P-value prob>F	F Value	P-value prob>F	F Value	P-value prob>F
Model	40,75	0,0001	14,19	0,0028	8,15	0,0119
X_1 -Time	65,02	0,0002	37,1	0,0009	2,1	0,1976
X_2 -Temperature	28,15	0,0018	5,74	0,0536	1,18	0,3199
X_1X_2	74,22	0,0001	1,19	0,3168	0,56	0,4811

X_1^2	27,99	0,0018	3,31	0,1188	36,9	0,0009
X_2^2	0,92	0,3738	15,78	0,0073	4,64	0,0746
Lack of Fit	0,95	0,5162	0,4	0,7668	2,51	0,2347
R^2	0,97		0,92		0,97	
Adj R^2	0,94		0,85		0,76	
CV %	3,66		9,93		11,99	

3.2. Analyses of response surface:

The response surface methodology (RSM) was used to identify the relationship between the response function and the process variables as well as to determine the optimal conditions for extraction.

The 3D response surface is the graphical representation of regression equation. It provides a method to visualize the relationship between responses and experimental levels of each variable and the type of interactions between two test variables (Zhong and Wang, 2010).

The effects of the independent variables and their mutual interaction on the extraction yield of TPCs, Chlorophylls and Carotenoids can be seen on three dimensional response surface curves shown in Figure n°11.

Figure n°11-a shows that temperature and time had a positive interaction effect on the enrichment of VOO with TPCs. A positive effect was reached at high temperature (45°C) and long extraction time (45min). A similar effect of the temperature and the time were reported by Salehan et al. (2006).

Figure 11-b shows that temperature had a negative quadratic effect on the enrichment of VOO with chlorophylls. The extraction of this latter increase with the increasing of temperature and when the temperature reached 30°C the rate of extraction decreased.

Figure n°11-c shows a positive quadratic effect of the time on the extraction of carotenoids and negative quadratic effect of the temperature.

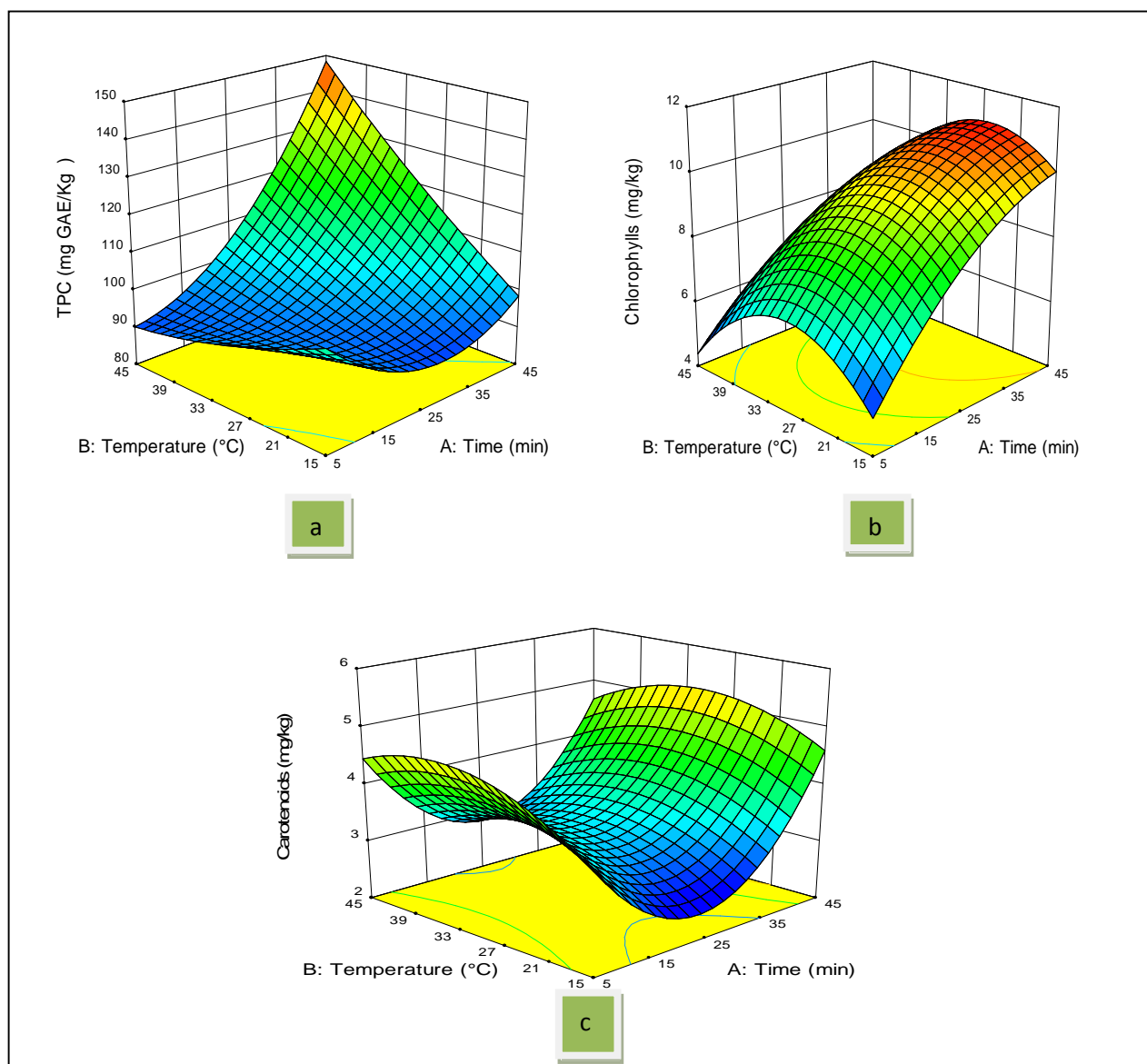


Figure n°11: 3D response surface: a: TPCs, b: chlorophylls, c: carotenoids

3.3. Optimal extraction conditions:

When comparing the optimal conditions based on TPCs to those obtained for Chlorophylls and those of Carotenoids, it was found that the optimums of time extraction were the same. However, there was a difference in extraction temperature required for optimal extraction of TPCs, Chlorophylls and Carotenoids. The optimal temperature of extraction was 45; 25.28; and 30.38°C for TPC, Chlorophyll and Carotenoid, respectively (table n°5).

Table n°5: Optimum conditions for the time-temperature extraction of TPCs (g GAE/kg oil), Chlorophylls (mg/kg) and Carotenoids (mg/kg).

	Time of extraction	Temperature of extraction	Prediction	Experimental
TPCs (g GAE/kg oil)	45	45	148,31±8,28	144,44
Chlorophylls(mg/kg)	45	25,28	11,02±1,41	10,75
Carotenoids(mg/kg)	45	30,38	5,25±0,79	5,82

4. Validation of the model:

The optimized conditions obtained by JMP were used to validate the predictive model of extraction for TPCs, chlorophylls and carotenoids from enriched olive oil. As shown in the table n°4, the adequacy of the predicted values confirming the validity and the adequacy of the predicted models.

4.1. Antioxidant activity:

The antioxidant properties are very important due to the deleterious role of free radicals in food and biological systems (Gülçin et al., 2006). The antioxidant activity of enriched virgin olive oil was evaluated by DPPH radical scavenging assay and reducing power test.

4.1.1. DPPH radical scavenging assay:

The inhibition effect of DPPH radical was most important in the enriched virgin olive oil (43.06%) followed by virgin olive oil (18.82%) (figure n°12).

Statistical analysis shows that there is a significant difference ($p < 0.05$) between the samples.

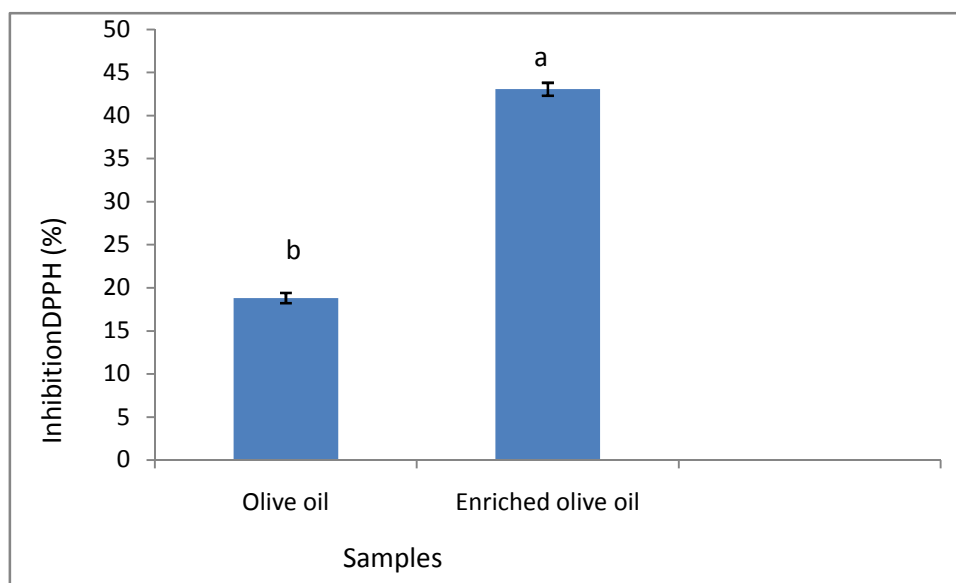


Figure n°12: Radical DPPH inhibition activity of extract of olive oil, enriched olive oil and myrtle pericarp powder.

4.1.2. Iron reducing power:

The reducing power was based on the capacity of the bioactive compounds to reduce the ferric iron to ferrous, the power of reducing is one of the antioxidant mechanisms (Karagozler et al., 2008).

The results in figure n°13 shows that the highest absorbance was observed in enriched olive oil. The difference was significant ($p < 0.05$) between the enriched olive oil and virgin olive oil.

These results suggest that phenolic compounds are the major contributors to the antioxidant activity of *enriched olive oil* (Amensour et al., 2010).

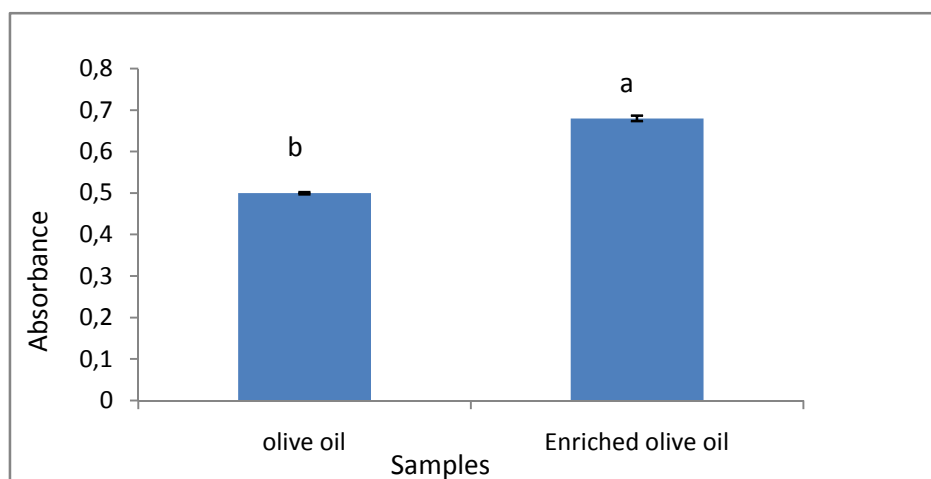


Figure n°13: Reducing power of olive oil and enriched olive oil.

4.2. Physico-chemical characterization:

4.2.1. Acidity.

It represents the proportion of free fatty-acids, which appear when the triglycerids of the olive oil are degraded. This rate is expressed in “grams of free oleic acid per 100 g of oil.

The rate of acidity is a marker of the degradation of the oil.

After enrichment, the free fatty acids content of olive oil was increased slightly from 1.86 to 2.05 g/100g as shown in figure n°14.

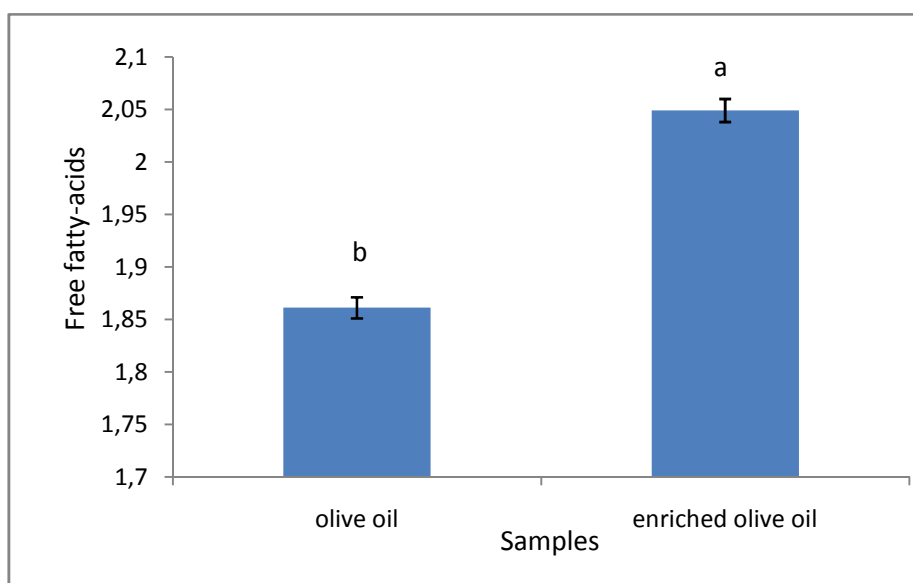


Figure n°14: Acidity of olive oil and enriched olive oil.

4.2.2. Peroxide index:

The rancidity of oil is due to an oxidation of the unsaturated fatty acids of the olive oil, this oxidation is done in several stages. The peroxide index is a control of the progress report of the first stage. More this index increased, more the oil is oxidized (figure n°15).

The Peroxide index of enriched olive oil is higher than that of olive oil, this oxidation may be due to the higher temperature in ultrasound bath during the enrichment.

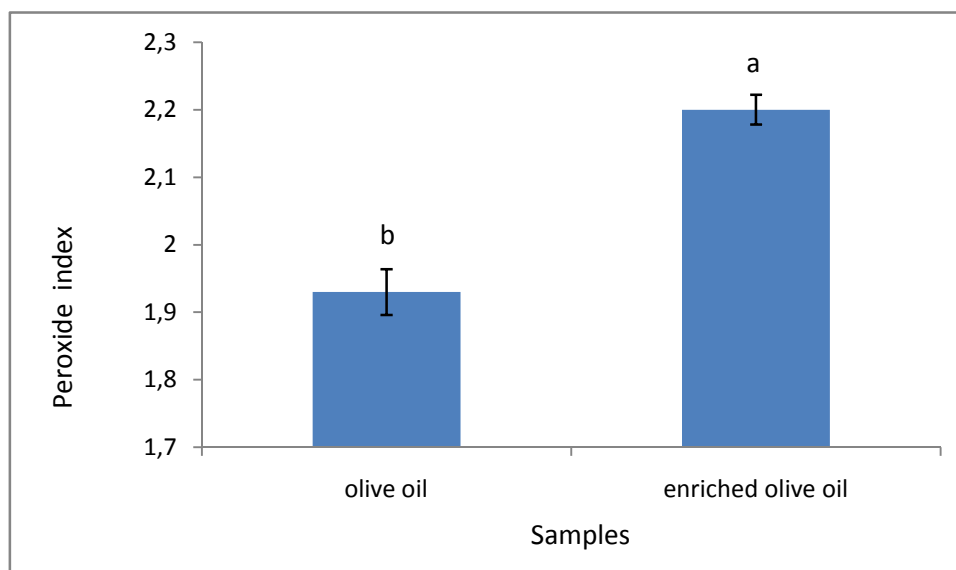


Figure n°15: Peroxide index of olive oil and enriched olive oil.

Conclusion

The polyphenols family contains many compounds of interest and valuables in the food and cosmetic industry due their reducing properties (antioxidant).

This work presents the first investigation on the effect of enrichment of virgin olive oil by phenolic compounds, chlorophylls and carotenoids extracted from the pericarp of myrtle fruit (*Myrtus comminus L*) and its antioxidant activity.

The ultrasonic apparatus was used to optimize the influence of the temperature and time on TPC, chlorophylls and carotenoids yield, extracted from pericarp myrtle fruit. The best conditions for extraction of TPCs, chlorophylls and carotenoids are 45°C/45min, 25°C/45min and 30°C/45min respectively, with rates of 144.44 mg GAE/kg for TPCs, 10.75mg/kg for chlorophylls and 5.82mg/kg for carotenoids.

In the light of this investigation, we can confirm that enriched virgin olive oil is richer in term of TPCs, chlorophylls and caroenoids than the virgin olive oil. Concerning the antioxidant activity, a good correlation has been found between bioactive compounds and antioxidant activity.

To concluded, myrtle plant has an interesting potential of bioactive compounds. However, it would be desirable to use myrtle plant as a valuable source of natural additive in food and in medicinal industry.

Abstract

The research on vegetable sources and the screening of raw materials for identifying new antioxidants has recently intensified.

Among the natural antioxidants, phenolic compounds, chlorophylls and carotenoids, are reported to play a key role in preventing oxidation and have been already correlated to the stability of virgin olive oils (VOO).

Among emergent new technologies, ultrasonic enrichment is very promising. The present work is carried out to optimize the enrichment of virgin olive oil with myrtle (*Myrtus comminus L.*) by using ultrasound apparatus. 5g powder pericarp of myrtle fruit with 50 ml of virgin olive oil was subjected to the ultrasonic waves on a water bath at a different condition temperature /time.

The studied kinetics showed that the highest level of TPCs is about 144.44 mg GAE/kg of enriched olive oil at 45°C/45min. The highest level of carotenoids is about 5.82mg/kg of enriched olive oil at 30.38°C/45min. Finally, the highest level of chlorophylls is about 10.75mg/kg of enriched olive oil at 25.28°C/45min.

Keywords: Virgin olive oil, *Myrtus comminus L.*, the enrichment, ultrasound, kinetics, antioxidant activity, phenolic compounds, carotenoids, chlorophylls.

Résumé

La recherche sur les composés bioactifs d'origine végétales et le criblage des matières pour identifier de nouveaux antioxydants a été récemment intensifié.

Parmi les antioxydants naturels, on rapporte que des composés phénoliques, chlorophylles et caroténoïdes, jouent une fonction clé en empêchant l'oxydation et il a été démontré leur effet sur la stabilité d'huile d'olive vierges (VOO).

Parmi des nouvelles technologies émergentes, l'enrichissement par ultrason est très prometteur. Le travail actuel est porté pour optimiser l'enrichissement d'huile d'olive vierge avec le myrte (*Myrtus comminus L.*) à l'aide de l'appareillage ultrason.

5g de poudre du péricarpe du fruit de myrte avec 50 ml de l'huile d'olive vierge, ont été soumises aux ondes ultrasoniques sur un bain d'eau à des différentes conditions température /temps. La cinétique étudiée a prouvé que le plus haut niveau de TPCs est de 144.44mg GAE/kg d'huile d'olive enrichie à 45°C/45min. le plus haut niveau de caroténoïdes est d'environ 5.82mg/kg d'huile d'olive enrichie à 30.38°C/45min. Enfin, le plus haut niveau de chlorophylles est d'environ 10.82mg/kg d'huile d'olive enrichie à 25.28°C/45min.

Mots-clés : L'huile d'olive vierge, *Mrtus comminus L.*, l'enrichissement, ultrason, cinétique, activité antioxydante, composés phénoliques, caroténoïdes, chlorophylles.

