PHENOLIC COMPOUNDS WITH ANTIOXIDANT ACTIVITY FROM OLIVE MILL WASTEWATERS

N. ALLOUCHE [a,b], S. SAYADI[a], M. DAMAK [b *]

[a] *Laboratoire des Bio-procédés, Centre de Biotechnologie de Sfax, BP K, 3038 Sfax, Tunisie*
[b] *Laboratoire de Chimie des Substances Naturelles, Faculté des Sciences de Sfax, BP 802, 3018, Sfax, Tunisie*

(Reçu le 1er Décembre 2003, accepté le 5 Avril 2004)

ABSTRACT: In this investigation, different methods for the extraction and the purification of phenolic compounds from the olive mill wastewaters (OMW) were used. Consequently, seven phenolic monomers were isolated at pure grade. By means of spectroscopic methods (IR, UV, MS, ^{1}H NMR, HPLC-UV and HPLC-MS), their structures were established as tyrosol, hydroxytyrosol, caffeic acid, protocatechuic acid, para-coumaric acid, apigenin and luteolin. The antiradical activity of the purified phenolic compounds was evaluated by measuring the radical scavenging effect on 1.1-diphenyl 2-picrylhydrazyl (DPPH). Result showed that hydroxytyrosol presented the highest DPPH radical scavenging effect. The antioxidative activity of the isolated compounds was evaluated using a coupled oxidation of β-carotene-linoleic acid method. The antioxidant potency of the various tested polyphenols was in the following decreasing order: hydroxytyrosol > caffeic acid> protocatechuic acid > para-coumaric acid > tyrosol.

Key Words: olive mill wastewaters, polyphenols, isolation, antioxidants, β-carotene, 1.1-diphenyl 2-picrylhydrazyl.

RESUME: Au cours de cette étude, différentes méthodes d'extraction et de purification de composés phénoliques à partir des margines ont été adoptées. Par conséquent, sept monomères phénoliques purs ont été isolés. Leurs structures ont été établies moyennant les différentes techniques spectroscopiques (IR, UV, MS, ^{1}H NMR, HPLC-UV et HPLC-MS). Il s'agit du tyrosol, hydroxytyrosol, acide cafféique, acide protocatéchuique, acide para-coumarique, apigenine et luteoline. L'activité anti-radicalaire de ces composés phénoliques a été évaluée en mesurant leurs effets de chélation vis-à-vis le radical 1.1-diphenyl 2-picrylhydrazyl (DPPH). Le résultat obtenu a montré que l'hydroxytyrosol présente la plus forte activité anti-radicalaire. L'activité antioxydante des différents composés isolés a été déterminée en utilisant la méthode de la co-oxydation du système β-carotène-acide linoléique. Il s'est avéré que l'activité antioxydante des différents composés analysés décroît dans l'ordre suivant :
hydroxytyrosol > acide cafféique > acide protocatéchuique > acide para-coumarique >tyrosol.

Mots clefs : margines, polyphenols, isolement, antioxydants, β-carotene, 1.1-diphenyl 2-picrylhydrazyl.

* corresponding author: e-mail: mohamed.damak@fss.rnu.tn

INTRODUCTION

The mediterranean region, nowadays, serves as the major international olive growing area, accounting for almost 98% of the world's olive tree plantation [1]. The extraction of olive oil yields major quantities of water which is employed during the malaxation process, that is, the continuous washing of the olive paste (milled olives) with warm water prior to the procedure of separation of the oil from the paste [2]. This effluent is named "olive mill wastewaters" (OMW). In the mediterranean area, the annual OMW production is estimated to more than 3 x 10^7 m^3 [3].

OMW is an acidic, malodorous and black liquid that consists mainly of waters (80%) and contains 2% minerals and between 4 and 16% organic matter [4]. Its organic fraction includes sugar, tannins, polyalcohols, pectins, lipids and polyphenols [5]. Phenolic compounds, generically, include a great many organic substances that have the common characteristic of possessing an aromatic ring with one or more substituent hydroxyl group and a functional side chain [6]. Previous work carried out in our laboratory has shown that the molecular-mass of OMW polyphenols varied from simple phenolics to polymers of molecular-mass higher than 60 kDa. The simple phenolics fraction is made especially of free phenolic monomers mainly tyrosol, hydroxytyrosol, protocatechuic acid, syringic acid, gallic acid, para-coumaric acid, caffeic acid, ferulic acid, vanillic acid and vanillin [7].

Phenolic compounds in OMW make them highly toxic and ecologically noxious to the soil on which they are spreaded unprocesses [8, 9] as documented by its high COD and BOD values [10, 11], its phytotoxic effects [12] and its antibacterial activity [6, 13].

However, some of the OMW phenolic monomers are endowed with strong antioxidant activity which may contribute to the prevention of human disease [2, 14], and could be used in the food, cosmetic and pesticide industries as recycling products, as suggested by many researchers [12, 15]. Thus, increasing attention has been paid to recover products of biological interest from OMW as well as to discover new natural antioxidant molecules which could be used as alternatives to the well known synthetic antioxidants such as Butyl hydroxytoluene (BHT) and Butyl hydroxyanizole (BHA). In this way, some investigations were carried out to recover the major phenolic compounds from OMW [12, 16]. However, for procedures with industrial application, the used protocols and results are always patented [17-19].

In the present study, we investigate to develop experimental procedures for the extraction and isolation of some aromatic compounds from OMW. This investigation aims also at evaluating the antioxidative and the antiradical activities of the isolated compounds.

MATERIALS AND METHODS

Fresh olive mill wastewaters (OMW) were supplied by mills from a Cooperative in Sfax (Tunisia). The phenolic compounds used as standards were purchased from Fluka. The IR, UV, MS, and NMR analysis were performed using respectively a Shimadzu apparatus type "IR-470", a Shimadzu apparatus type "UV-2100", a spectrograph Kratos apparatus type "MS 25 magnetic sector" and a Geol apparatus 270 MHz.

1- Extraction and isolation procedures:

Two samples of OMW, stored in two different protocols, were used in this study:

- $(OMW)_a$: stored in natural conditions (ambient temperature, light and aerobic) where aromatics can undergo oxidative polymerizations.
- $(OMW)_b$: stored in dark, at low temperature (4°C) and in anaerobic conditions to prevent polymerization reaction.

a) First procedure:

$(OMW)_a$ (200 ml) were lyophilized. The lyophilizate was filtered on a (3.3 × 75 cm) Sephadex LH-20 gel chromatography column. Phenolic compounds were eluted with a mixture of water:methanol (5:5, V/V) at a flow rate of 0.33 ml/min. 5 ml fractions volume were collected and measured spectrophotometrically at 280 nm. The resulted chromatogram (optic density versus fraction number) was represented (figure 1).

Fractions corresponding to the separated peak were collected. The methanol was eliminated under reduced pressure, the water was lyophilized and the residue was redissolved in a small volume of methanol. Purification of polyphenols was carried out by prep. TLC eluted with chloroform:methanol (8.5:1.5, V/V) which allowed us to isolate two pure compouds identified as tyrosol **(1)** (11 mg) and hydroxytyrosol **(2)** (30 mg) by means of spectroscopic methods (IR, UV, MS and ^{1}H NMR). The purity of these two compounds was confirmed by HPLC analysis which gave one single peak (figure 3).

Tyrosol **(1)**: UV λ_{max} nm : 210 and 280; IR ν_{max} cm^{-1} : 3360 (phenolic and alcoholic OH), 1607, 1518 and 1447 (benzene ring); EI-MS *m/z* (relative intensity) : 138 ($M^{\cdot+}$,24), 107 (100), 91 (3), 77 (11) and 31 (2); ^{1}H-NMR (270 MHz, CD_3OD) δ ; 2.7 (2H, t, J_{7-8} = 7.26 Hz, H_7), 3.6 (2H, t, J_{8-7} = 7.26 Hz, H_8), 6.7 (2H, d, $J_{3-2} = J_{5-6}$ = 7.92 Hz, H_5 and H_3), 7.1 (2H, d, $J_{6-5} = J_{2-3}$ = 7.92 Hz, H_6 and H_2).

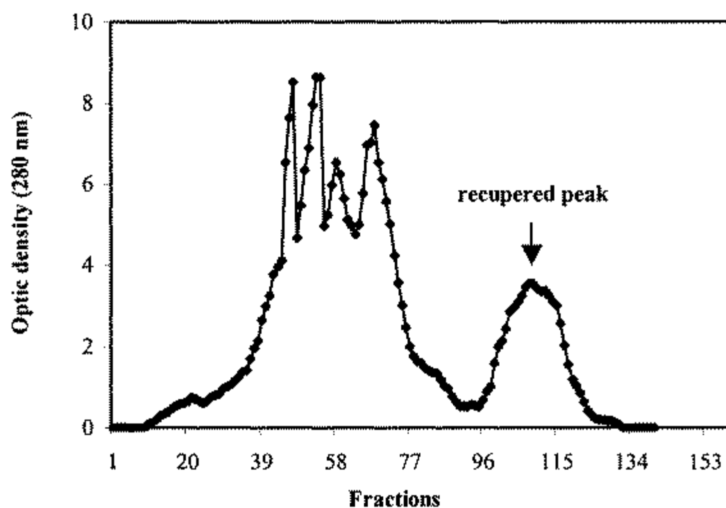


Figure 1: Chromatogram obtained from the Sephadex LH-20 gel filtration of the lyophilized $(OMW)_a$.

Hydroxytyrosol **(2)**: UV λ_{max} nm : 210 and 281; IR ν_{max} cm^{-1} : 3387 (phenolic and alcoholic OH), 1607, 1518 and 1447 (benzene ring); EI-MS *m/z* (relative intensity) : 154 ($M^{\cdot+}$,61), 153 (20), 136 (48), 123 (100), 105 (63), 87 (64) and 77 (66); ^{1}H-NMR (270 MHz, CD_3OD) δ : 2.66 (2H, t, J_{7-8} = 7.3 Hz, H_7), 3.66 (2H, t, J_{8-7} = 7.3 Hz, H_8), 6.52 (1H, dd, J_{6-5} = 8, J_{6-2}= 2.1 Hz, H_6), 6.64 (1H, d, J_{2-6} = 2.1 Hz, H_2), 6.67 (1H, d, J_{5-6} = 8 Hz, H_5).

b) Second procedure:

$(OMW)_b$ (1000 ml) were lyophilized. The lyophilizate was then extracted as in figure 2 to obtain four different extracts. Each extraction was carried out with shaking at 120 rpm, at room temperature, during 24 hours, in dark and under N_2. In a primary analysis, only the acetone and methanol extracts showed phenolic compounds.

The acetone extract (4 g) was chromatographed on a silica gel column (4 × 80 cm) eluted with a solvent gradient: hexane-dichloromethane-acetone-methanol. The chromatographic behavior was followed by TLC analysis.

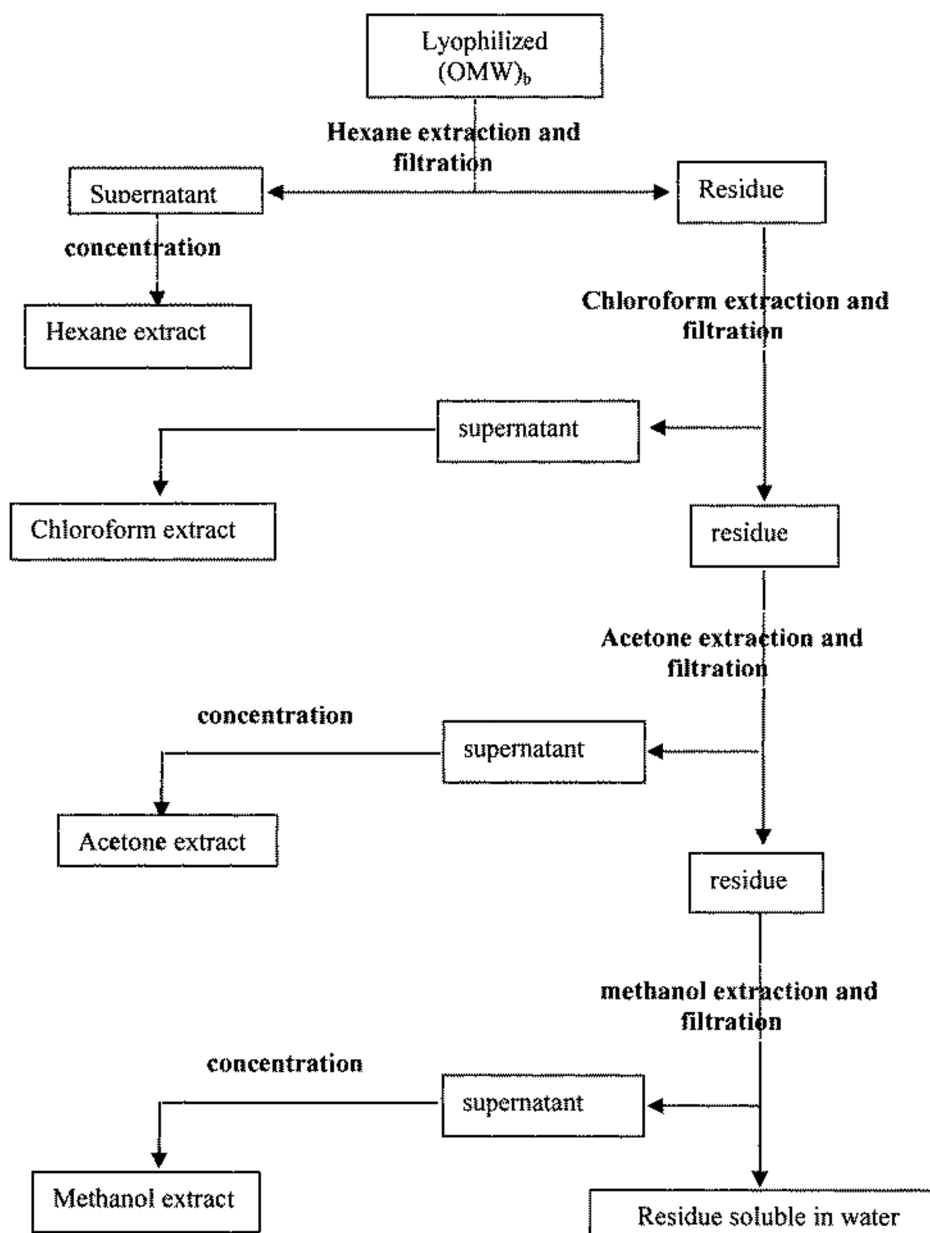


Figure 2: Extraction protocol used with $(OMW)_b$.

Nine groups of homogeneous fractions were collected and analyzed by HPLC-UV. Only three groups showed phenolic compounds. The first group was purified by prep.TLC, eluted witb chloroform:methanol (9:1, V/V), which afforded one pure compound with R_f = 0.6. This compound was identified as apigenin **(6)** (4 mg) by means of HPLC-UV and the obtained structure was confirmed using HPLC-MS technique. The second group was treated by prep. TLC eluted with toluene:ethyl acetate:acetic acid (7:2:1, V/V/V). As a result, one pure compound with R_f = 0.35 was isolated. Using HPLC-UV and HPLC-MS analysis, the later compound was identified as luteolin **(7)** (5 mg). The third group was analysed by prep. TLC eluted with toluene:ethyl acetate:methanol (6:3:1, V/V/V). This purification provided one pure compound

with R_f = 0.43 identified as para-coumaric acid **(3)** (5 mg) by means of HPLC-UV and HPLC-MS analysis.

The methanol extract (6 g) was filtered on a (4 × 80 cm) Sephadex LH-20 gel chromatography column eluted with a water-methanol gradient. The flow rate was adjusted to 1 ml/min. The group of fractions eluted with water:methanol (5:5, V/V) showed phenolic constituents according to the HPLC-UV analysis. This later group was treated by a further prep. TLC technique eluted with toluene:ethyl acetate:methanol (6:3:1, V/V/V). Consequently, two pure compounds with were isolated and then identified as protocatechuic acid **(4)** (3 mg, R_f = 0.5) and caffeic acid **(5)** (6 mg, R_f = 0.8) by HPLC-UV and HPLC-MS analysis. The purity of the products isolated in this paragraph was confirmed by HPLC analysis (figure 3).

Apigenin **(6)**: HPLC-UV retention time (min) : 19.3 ; UV λ_{max} nm : 237.9, 266.3 and 337.8 ; HPLC-MS retention time (min) : 16.5 ; EI-MS *m/z* (relative intensity) : 270($M^{\cdot+}$, 42), 261(9), 247(26), 243(11), 229(18), 225(22), 203(7), 185(23), 153(100), 145(15) and 119(10).

Luteolin **(7)**: HPLC-UV retention time (min) : 17.7 ; UV λ_{max} nm : 256.8 and 352.2 ; HPLC-MS retention time (min) : 15 ; EI-MS *m/z* (relative intensity) : 286($M^{\cdot+}$, 71), 277(13), 169(19), 263(23), 259(16), 245(9), 241(26), 231(5), 219(9), 185(24), 170(7), 161(20), 153(100), 137(6) and 135(15).

Para-coumaric acid **(3)**: HPLC-UV retention time (min) : 11.1 ; UV λ_{max} nm : 233.1 and 309.1 ; HPLC-MS retention time (min) : 9.4 ; EI-MS *m/z* (relative intensity) : 164($M^{\cdot+}$, 81), 149(100), 105(44), 77(19) and 59(25).

Protocatechuic acid **(4)**: HPLC-UV retention time (min) : 4.2 ; UV λ_{max} nm : 228.4, 261.5 and 294.8 ; HPLC-MS retention time (min) : 3.6 ; EI-MS *m/z* (relative intensity) : 154($M^{\cdot+}$, 100), 139(58), 95(35), 77(24), 59(19) and 45(23).

Caffeic acid **(5)**: HPLC-UV Retention time (min) : 7.51; UV λ_{max} nm : 242.6 and 323.4; HPLC-MS retention time (min) : 6.1 ; EI-MS *m/z* (relative intensity) : 180($M^{\cdot+}$, 100), 165(78), 91(40), 77(22) and 45(31).

2- High-performance liquid chromatography coupled with UV-spectrometry (HPLC-UV):

A Waters apparatus composed of a LC 600 pump, a 996 photodiode array detector and a Lichrospher 100 RP-18 column (4 × 250 mm) was used to identify some polyphenols by comparing their retention time and UV spectrum to some standards analysed in the same conditions. The elution conditions were as follows: linear gradient 25-100% solvent B from 0 to 20 min. After that, elution was conducted in the isocratic mode with 100% solvent B within 5 min.

Solvent A = water:acetic acid (9.8:0.2, V/V).

Solvent B = methanol:acetic acid:water (9:0.5:0.5, V/V/V).

3- High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS):

A Waters apparatus composed of a 600 E pump, a Merck-Hitachi L-4000 UV detector and a Merck Lichrosphere 100 RP-18 column (4 × 250 mm) was used to confirm chemical structures determined by HPLC-UV technique. This system is coupled with a Finnigan-MAT LCQ mass spectrophotometer. The elution conditions were as follows: linear gradient 25-100% solvent B from 0 to 15 min with a 1 ml/min flow rate.

Solvent A = water:acetic acid (9.9:0.1, V/V).

Solvent B = methanol

Figure 3: HPLC spectra of : (a) standards and the isolated products: (b) hydroxytyrosol **(2)**, (c) tyrosol **(1)**, (d) protocatechuic acid **(4)**, (e) caffeic acid **(5)**, (f) para-coumaric acid **(3)**, (g) Apigenin **(6)** and (h) Luteolin **(7)**.

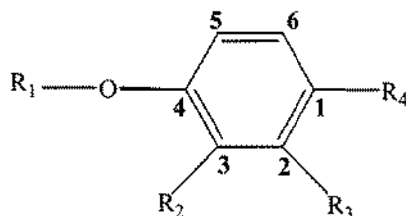
4- β-carotene bleaching method:

Antioxidant activity was determined in emulsion by the β-carotene bleaching method of Chevolleau et al. [20] consisting in a coupled oxidation of linoleic acid and β-carotene at 50°C. This method was slightly modified. Briefly, an aliquot (1 ml) from β-carotene (1 mg) dissolved in chloroform (10 ml) was pipetted into a flask containing linoleic acid (20 mg) and tween 20 (200 mg). The solvent was evaporated, deionized and oxygenated water (50 ml), and an amount of polyphenolic sample were then added. Emulsification was performed by vigorous agitation. The mixture was incubated in a rotatory shaker at 50°C and 150 rpm.

The antioxidant activity was evaluated by absorbance measurement at 470 nm against a blank containing emulsified linoleic acid without β-carotene. To avoid contamination by heavy metals, all experiments were carried out in glass equipments previously immersed for at least 24 hours in EDTA (0.5% ; w/v), rinsed several times with deionized water and dried at 150°C.

5- DPPH radical scavenging method:

The DPPH radical scavenging effect was evaluated according to Na Mee et al. [21]. Methanolic solution (4 ml) of varying sample concentrations was added to a 10 ml DPPH methanol solution (1.5×10^{-4} M). After mixing the two solutions gently and leaving for 30 min at room temperature, the absorbance was measured at 520 nm. The antioxidant activity of each sample was expressed in terms of IC_{50} microgram per ml, concentration required to inhibit DPPH radical formation by 50% and calculated from the log-dose inhibition curve.



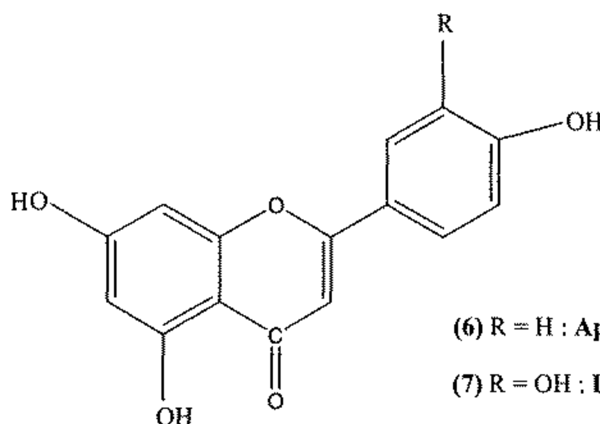
(1) $R_1 = H, R_2 = H, R_3 = H, R_4 = \overset{7}{CH_2}—\overset{8}{CH_2}—OH$: **Tyrosol.**

(2) $R_1 = H, R_2 = OH, R_3 = H, R_4 = \overset{7}{CH_2}—\overset{8}{CH_2}—OH$: **Hydroxytyrosol.**

(3) $R_1 = H, R_2 = H, R_3 = H, R_4 = \overset{7}{CH}=\overset{8}{CH}—COOH$: **Para-coumaric acid.**

(4) $R_1 = H, R_2 = OH, R_3 = H, R_4 = COOH$: **Protocatechuic acid.**

(5) $R_1 = H, R_2 = OH, R_3 = H, R_4 = CH=CH—COOH$: **Caffeic acid.**



(6) R = H : **Apigenin**

(7) R = OH : **Luteolin**

RESULTS AND DISCUSSION

1- Polyphenols isolation from OMW:

From a point of view of the olive mill wastewaters (OMW) valorization, two chromatographic procedures have been carried out in order to isolate and identify some polyphenols with antioxidant activity.

In the first procedure, which consisted of sephadex LH-20 chromatography followed by a further prep. TLC, two phenolic monomers (tyrosol and hydroxytyrosol) were completely isolated at a pure grade with corresponding yields 55 mg/l and 150 mg/l respectively. The obtained MS and ^{1}H NMR data are in good agreement with those reported by Capasso et al. [12]. These researchers have isolated tyrosol and hydroxytyrosol from OMW using several steps which consist in silica gel chromatography, C8-reverse phase chromatography, prep. TLC chromatography and crystallization technique. In contrast, our procedure consists in two simple chromatographic steps.

From the second procedure, which consisted of silica gel and sephadex LH-20 chromatography, three phenolic monomers (caffeic acid, para-coumaric acid and protocatechuic acid) and two flavonoids (luteolin and apigenin) were recovered with the following concentrations: 6 mg/l, 5 mg/l, 3 mg/l, 5 mg/l and 4 mg/l, respectively. Given the fact that these obtained amounts were not sufficient for NMR analysis, the corresponding isolated products were identified using HPLC-UV technique. The HPLC-MS technique was used in order to confirm the established structures.

For many years ago, several scientists have developed chromatographic methods to detect and purify some of the OMW polyphenols. In this way, Capasso [10] has reported that researchers have recovered tyrosol, hydroxytyrosol and caffeic acid as the main phenolic compounds from OMW and only small amount of protocatechuic acid, para-coumaric acid, vanillic acid, oleuropein, apigenin, luteolin, and quercetin. In the present paper, we have isolated, from the non fresh olive mill wastewaters $(OMW)_a$, only tyrosol and hydroxytyrosol. However, from the fresh olive mill wastewaters $(OMW)_b$, we have recovered caffeic acid, para-coumaric acid, protocatechuic acid, luteolin and apigenin.

In the light of these results, we can notify the importance of the OMW freshness and the cautions which must be paid during OMW storage, extraction procedure, and polyphenols purification to avoid polymerization and/or hydrolysis processes. In this way, Ryan et al. [22] have noted that the levels of some minor components, present in olive leaves, decreased after 2 h storage at ambient conditions and thus, extracts should be generally be examined with minimal delay or stored at low temperature in the dark.

2- Antioxidant activity determination of the purified polyphenols from OMW:

(a)- β-carotene bleaching method:

In this method, β-carotene was added to the model system as a monitor for linoleate oxidation. Antioxidant activity is measured by the ability of a compound to minimize the loss of β-carotene during the oxidation of linoleic acid in an emulsified aqueous system [23]. Each purified compound was added to linoleic acid oxidation system at 200 ppm. An experiment, using BHT at the same concentration, was conducted along with the other experiments in order to compare the antioxidative activity of the purified compounds with synthetic antioxidants currently used in the industry. Figure 4 shows the rate of β-carotene bleaching in the different linoleate systems.

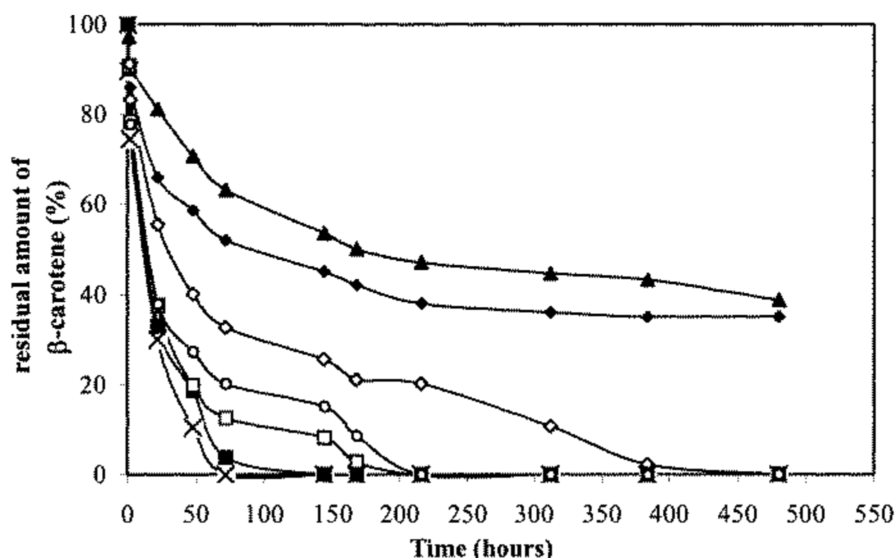


Figure 4: Time course of the β-carotene bleaching in model systems containing : hydroxytyrosol (▲); BHT (◆); caffeic acid (◇); protocatechuic acid (○); para-coumaric acid (□); tyrosol (■); and without added compound (control) (×).

Comparing with the other tested compounds, hydroxytyrosol and BHT, exhibited high antioxidant activity. Indeed, in the corresponding linoleate systems, the amount of β-carotene decreased moderately and was not completely removed after 480 h incubation time. By contrast, for all the other tested compounds, the curves slopes β-carotene loss/time unit were broadly more intense comparing with hydroxytyrosol, and BHT. Moreover, in linoleate systems containing cafeic acid, protocatechuic acid, para-coumaric acid, and tyrosol, β-carotene was completely bleached after 480 h, 220 h, 220 h, and 150 h incubation times respectively. Accordingly, antioxidant potencies of the tested compounds were in the following decreasing order:
Hydroxytyrosol > BHT > caffeic acid> protocatechuic acid > para-coumaric acid > tyrosol.

Hydroxytyrosol has the best antioxidative activity which is higher than BHT. The luteolin and apigenin activities were not determined by the β-carotene bleaching method. In fact, they give a yellow color whicb prevents the β-carotene bleaching follow-up at 470 nm.

(b)- DPPH radical scavenging method:

1.1-diphenyl 2-picrylhydrazyl (DPPH) radical is a dye free radical. Due to its odd electron, DPPH radical gives a strong absorption band at 520 nm. The change in absorbance produced by reduced DPPH was used to evaluate the ability of the tested compounds to act as free radical scavenger. The radical scavenging effect on DPPH of the isolated phenolic monomers from OMW is shown in table I.

Accordingly, ortho-diphenol compounds (hydroxytyrosol, caffeic acid, luteolin, and protocatechuic acid) exhibited strong radical scavenging activity on DPPH radical. Indeed, they present low IC_{50} values. In this way, Dziedzic and Hudson [24] have found that at least two hydroxyl groups were required for antioxidative activity of phenolic acids and especially the ortbo-diphenol compounds. Comparing with BHT (IC_{50} = 0.89 µg/ml), hydroxytyrosol with IC_{50} value of 0.57 µg/ml exhibited the highest radical scavenging activity. However, para-coumaric acid and tyrosol, possessing only one hydroxyl group, showed no activity (IC_{50} = 9.5 and 10.85 µg/ml respectively). This result correlates with that obtained using the β-carotene bleaching method.

Table I: Radical scavenging effect on DPPH radical of the isolated phenolic monomers from OMW and synthetic antioxidant BHT.

Phenolic monomers	IC_{50} µg/ml
hydroxytyrosol	0.57
BHT	0.89
caffeic acid	0.91
luteolin	1.72
protocatechuic acid	2.42
apigenin	3.1
para-coumaric acid	9.5
tyrosol	10.85

According to the antioxidant activity decreasing order established in this study and the structures of the tested compounds, it is worthy that an increase in the number of hydroxyl group and the presence of an ortho-diphenol function led to higher antioxidative activity. For example, luteolin, pocessing an ortho-diphenol function, has a scavenging effect on DPPH radical higher than apigenin. This constatation agrees with the findings of Bocco et al. [25] who have showed that the antioxidant activity of flavonoids is mainly due to their hydroxyl groups on the B ring and that substances having an ortho-hydroxylation in this position are those with the highest antioxidant activity.

There is an increased preference for natural foods and food ingredients which are generally believed to be safer, more healthy and less subject to hazards than foods containing synthetic food additives [17]. Furthermore, many researchers have reported that these artificial antioxidants are carcinogenic and they are approved for food use within limits [26]. On the other hand, hydroxytyrosol is the most interesting and abundant ortho-diphenol occurring in the OMW and is commercially unavailable [27]. Due to the important antioxidant activity of hydroxytyrosol, it would be possible to take advantage of its prophylactic action in several human diseases induced by free radicals [14, 28]. In this sense, it would be used in diverse topical preparations, anti-aging, anti-inflammatory as suggested by many workers [29-30]. Since this polyphenol comes from a natural source, it can be integrated in the alimentary, agriculture and cosmetic industries as an alternative to the undesired synthetic antioxidant additives. Consequentely, polyphenols and especially hydroxytyrosol recovery from OMW constitutes a promising alternative to valuate this effluent and partly to solve some environmental problems caused by its complex polyphenols [2].

Acknowledgements:

This research was supported hy EEC contract ICA3-CT2002-10033 and "Contrats Programmes SERST", Tunisia. The authors wish to thank Prof Monique Simmonds (Kew Gardens, Great Britain) for her help in HPLC-UV and HPLC-MS analysis.

REFERENCES

[1] D. Ryan, K. Robards; *Analyst.*, **1998,** *123*, 31R.

[2] F. Visioli, A. Romani, N. Mulinacci, S. Zarini, D. Conte, F. F. Vincieri, C. Galli; *J. Agric. Food Chemis.*, **1999,** *47*, 3397.

[3] S. Sayadi, R. Ellouz; *Appl. Environ. Microbiol.*, **1995,** *61*, 1098.

[4] J. Pérez, T. De La Rubia, O. Ben Hamman, J. Martinez; *Appl. Environ. Microbiol.*, **1998,** *64*(7), 2726.

[5] O. Yesilada, S. Sik, M. Sam; *World J. Microbiol. Biotech.*, **1998,** *14*, 37.

[6] C. A. Ramos, J. B. Juarez, P. M. P. Garcia, *Internat. Biodeter. Biodeg.*, **1996,** 283.
[7] S. Sayadi, N. Allouche, M. Jaoua, F. Aloui; *Proc. Biochem.*, **2000,** *35*, 725.
[8] A. D'Annibale, C. Crestini, V. Vinciguerra, G. G. Sermanni; *J. Biotech.*, **1998,** *61*, 209.
[9] G. J. R. Greco, G. Toscanoa, M. Cioffi, L. Gianfreda, F. Sannino; *Water Res.,* **1999,** *33*, 3046.
[10] R. Capasso; *Current Topics in Phytochem.*, **1997,** *1*, 145.
[11] S. Sayadi, R. Ellouz; *J. Chem. Techno. Biotech.*, **1993,** *57*, 141.
[12] R. Capasso, G. Cristinzio, A. Evident, F. Scognamiglio; *Phytochem.*, **1992,** *12*, 4125.
[13] E. Moreno, J. Perez, C. A. Ramos, J. Martinez; *Microbios*, **1987,** *51*,169.
[14] R. Capasso, A. Evidente, L. Schivo, G. Orru, M. A. Marcialis, G. Cristinzio; *J. Appl Bacteriol*, **1995,** *79*, 393.
[15] E. Galli, U. Tomati, A. Grappelli, R. Buffon; *Agrochimica*, **1988,** *5(6),* 451.
[16] R. Capasso, A. Evidente, C. Visca; *Agrochimica*, **1994,** *38*, 166.
[17] R. Crea; **2002,** Patent N°: WO0218310.
[18] R. Crea; **2003,** Patent N°: US2003108651.
[19] G. J. Fernandez-Bolanos, B. R. Guillen, A. R. Rodriguez, G. G. Rodriguez, M. A. Heredia, A.A. Jimenez; **2002,** Patent N°: WO02064537.
[20] S. Chevolleau, A. Debal, E. Ucciani; *Rev. Fra. Corps gras*, **1992,** *1(2)*, 3.
[21] K. Na Mee, K. Jong, C. Hae Young, C. Jae Sue; *Arch. Pharmacol. Res.*, **2000,** 23(3), 237.
[22] D. Ryan, H. Lawrence, P. D. Prenzler, M. Antolovich, K. Robards; (2001). *Analytica Chimica Acta*, **2001,** *445*, 67.
[23] L. R. Fukumoto, G. Mazza; *J. Agric. Food Chemi.*, **2000,** *48*, 3597.
[24] S. Z. Dziedzic, B. J. F. Hudson; *Food Chem.*, **1983,** *11*, 161
[25] A. Bocco, M. E. Cuvelier, H. Richard, C. Berset; *Scien. Aliments*, **1998,** *18*(1), 797.
[26] F. Visioli, A. Poli, C. Galli; *Medic. Res. Rev.*, **2002,** *22*(1), 65.
[27] R. Capasso, A. Evidente, F. Scognamiglio; *Phytochem. Analy.*, **1992,** *3*, 270.
[28] F. Visioli, D. Caruso, C. Galli, S. Viappiani, G. Galli, A. Sala; *Biochem. Biophys. Res. Commun.*, **2000,** *278*, 797.
[29] A. Petroni, M. Blasevich, M. Salami, M. Servili, G. F. Montedoro, C. Galli; *World Rev. Nutri. Diet*, **1994,** *75*, 169.
[30] M. Salami, C. Galli, L. De Angelis, F. Visioli; *Pharmacol. Res.*, **1995,** *31*, 275.