Journal de la Société Chimique de Tunisie, **2005**, *7*, 231-238 231

ISOLATION OF HYDROXYTYROSOL 4-β-D-GLUCOSIDE AND 3,4-DIHYDROXYPHENYLGLYCOL WITH ANTIOXIDANT ACTIVITY FROM OLIVE MILL WASTEWATERS

N. Allouche [a] , M. Feki [b], M. Damak [c], S. Sayadi [a] [*]

[a] *Laboratoire des Bio-procédés, Centre de Biotechnologie de Sfax: B.P "K". 3038, Sfax, Tunisie.*
[b] *Unité de Chimie Industrielle, Ecole Nationale dIngénieurs de Sfax, BP « W », 3038 Sfax, Tunisie.*
[c] *Laboratoire de Chimie des Substances Naturelles, Faculté des Sciences de Sfax, B.P. "802". 3018, Sfax, Tunisie.*

(Reçu le 19 Juin 2004, accepté le 8 Juin 2005)

RESUME : Le but du présent travail est le développement et la validation d'une méthode d'analyse multirésidus rapide et simple de l'Oxytétracycline, la Tétracycline, la Chlortétracycline et la Doxycycline dans la viande bovine par couplage HPLC-MS. L'extraction de ces antibiotiques a été réalisée sur une cartouche d'extraction SPE de type C_{18} qui a été préalablement activée avec 3 ml de méthanol et 6 ml d'eau. L'ensemble est élué par la suite avec 2 ml d'une solution hydroalcoolique (5 % de méthanol). Les antibiotiques sont adsorbés sur la phase stationnaire de la cartouche, puis élués avec 5 ml de méthanol (2 fois 2,5 ml). La séparation a été obtenue en utilisant une phase inversée Omnisher C_{18}, la phase mobile étant constituée d'eau (ajustée à pH = 2,7 avec 20 mM d'acide trifluoroacétique (TFA)) et d'acétonitrile. Nous avons appliqué un gradient d'élution de 20 % pendant 1 min puis de 20 à 100 % d'acétonitrile en 8 min, le débit de la phase mobile étant fixé à 0,25 ml/min. La détermination quantitative des résidus d'antibiotiques a été réalisée par couplage HPLC-MS avec une détection en mode SIM. Cette méthode ainsi mise au point nous a permis de récupérer l'Oxytétracycline avec un rendement de 81,9 ± 2,5 %, la Tétracycline à 89,6 ± 1,5 %, la Chlortétracycline à 80,1 ± 1,6 % et la Doxycycline à 69,6 ± 2,9%.

Mots clés : résidus, antibiotiques, viande bovine, HPLC-MS

ABSTRACT : The aim of this work is to develop and validate a multi residue analytical method for a rapid and simple determination of Oxytetracycline, Tetracycline, Chlortetracycline and Doxycycline in bovine meat by HPLC-MS. Solid phase extraction of these antibiotics was made on a C_{18} cartridge that was first activated with 3 mL of methanol and 6 mL of water. This mixture was then eluted with 2 mL of a hydroalcoholic solution (5 % methanol). The antibiotic mixture was adsorbed on the stationary phase of the activated cartridge and eluted twice with 2.5 mL of methanol. The chromatographic separation was obtained on a RP-C_{18} Omnisher type column and using water (adjusted at pH 2.7 with a 20 mM solution of TFA) and acetonitrile in a gradient elution mode (20 % for 1 min and from 20 to 100 % with acetonitrile in 8 min). The flow was maintained constant at 0.25 mL/min. Quantitative analysis of antibiotics residues was made by HPLC-MS in SIM detection mode. This method allowed the determination of the four antibiotics with good yields: 81.9 ± 2.5 % for Oxytetracycline, 89.6 ± 1.5 % for Tetracycline, 80.1 ± 1.6 % for Chlortetacycline and 69.6 ± 2.9 % for Doxycycline.

Key words: residue, antibiotics, bovine meat, HPLC-MS

INTRODUCTION

L'agriculture moderne a recours à une très large gamme de médicaments vétérinaires. A l'inverse des médicaments vétérinaires destinés aux animaux de compagnie, l'utilisation de médicaments chez les animaux producteurs de denrées alimentaires peut avoir des conséquences néfastes sur la santé publique. En effet, ces pratiques peuvent induire la présence de résidus dans les produits alimentaires tels que le lait, les œufs et les tissus animaux, les viandes et les abats. Des limites maximales de résidus (LMR) compatibles avec le respect de la santé publique ont été fixées par les pouvoirs publics. Les valeurs des LMRs varient de 1 à 1000 µg/kg selon les denrées alimentaires et

---

[*] corresponding author, e-mail : sami.sayadi@cbs.rnrt.tn

the husks and the olive mill wastewaters (OMW). This liquid effluent results from the olive paste washing with warm water. This procedure generates large volumes of OMW [1]. The Mediterranean region, which serves as the major international olive growing area [2], produces annually more than 3 x 10^7 m^3 of OMW [3].

OMW are black effluents that consist of mineral and organic matters [4]. Their organic fraction includes sugar, pectins, lipids and mainly polyphenols [5]. Phenolic fraction is made of phenolic compounds with high molecular-mass and free phenolic monomers with low molecular-mass such as tyrosol, hydroxytyrosol, para-coumaric acid and caffeic acid [6].

OMW constitute a significant source of potential environmental pollution in the Mediterranean countries [7-9]. The treatment difficulties of OMW are related to the presence of organic compounds which are hard to biodegrade such as long-chain fatty acids and mainly phenolic compounds [10, 11]. This low biodegradability of polyphenols is caused, among others parameters, by their phytotoxic effects [12] and their antimicrobial activity [13]. Nevertheless, some of the OMW phenolic monomers and namely the ortho-diphenolic compounds are well-known antioxidants, protecting foods from oxidation [14]. They also are challenging biological antioxidants protecting against oxidative degradation in living cells which may contribute to the prevention of human disease [1]. Consequently, these ortho-diphenols are of interest not only to food scientists but also to health professionals [15].

Therefore, several investigations have been performed to recover products of high added value from OMW as well as to discover new natural phenolic compounds with interesting biological activities [16]. In our laboratory, a new process for the continuous liquid-liquid extraction of polyphenols from OMW and the production of hydroxytyrosol has been developed [17]. Recently, we have purified tyrosol, hydroxytyrosol, caffeic acid, protocatechuic acid, para-coumaric acid, apigenin and luteolin from OMW. The antiradical and antioxidant activities of these phenolic compounds were determined [18].

The present work represents the continuity of our previous investigation dealing with the development of experimental procedures for extracting and isolating phenolic compounds from OMW. This investigation aims also at evaluating the antioxidative and the antiradical activities of the isolated compounds.

MATERIALS AND METHODS

1- Materials:

Fresh olive mill wastewaters (OMW) were supplied by mills from a Cooperative in Sfax (Tunisia). This sample was stored in dark, at low temperature (4 °C) and in anaerobic conditions to prevent polymerization and/or hydrolysis reactions. 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.

2- Extraction and isolation procedures:

OMW (200 mL) were lyophilized. The lyophilizate was then extracted with methanol:water (4:1, V/V) (3 × 100 mL for 24 h) with magnetic stirring, at room temperature, in dark and under N_2. The mixture was filtered on a Buchner funnel. The methanol was evaporated under reduced pressure and the water was eliminated by lyophilization. The lyophilized residue (3 g) was lowed on a (2.5 × 75 cm) Bio-gel P-2 (provided by Bio-rad Laboratories) chromatography column. Aromatic compounds were eluted with water at a flow rate of 0.2 mL/min. 2 mL fractions volume were collected and measured spectrophotometrically at 280 nm. The resulted chromatogram (optical density versus fraction number) was represented and analyzed (Figure 1).

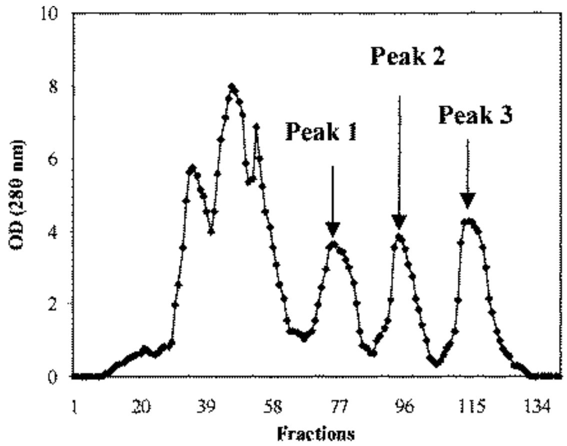


Figure 1: Bio-gel P-2 chromatography of the methanol-water (4:1 ; V/V) extract of OMW.

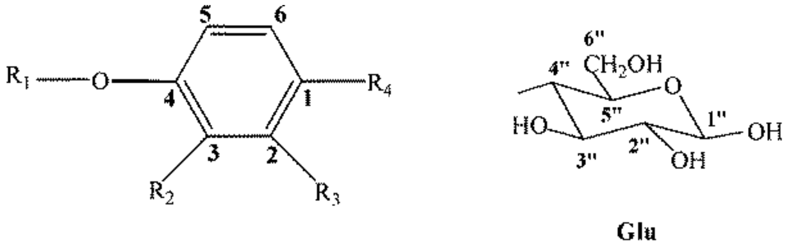
Fractions corresponding to the separated peaks were collected into three groups. The first group was purified by prep. TLC eluted with chloroform:methanol (8:2, V/V) which resulted in the isolation of a pure compound identified as hydroxytyrosol 4-β-D-glucoside (4-β-D-glucosyl 3-hydroxyphenylethanol) **(1)** (10 mg) with $R_f = 0.6$. From the second group, two compounds were purified by prep. TLC eluted with chloroform:methanol (8:2, V/V). By means of spectroscopic methods (IR, UV, MS and ^1H NMR), these compounds were identified as tyrosol **(2)** (11 mg) and hydroxytyrosol **(3)** (30 mg). The purification of the third group was carried out by prep. TLC eluted with toluene:ethyl acetate:methanol (6:3:1, V/V/V) which afforded one pure compound with R_f = 0.65. This compound was identified by means of GC-MS analysis as 3,4-dihydroxyphenylglycol **(4)** (3 mg). This structure was established according to the comparison of the isolated compound GC-MS data with GC-MS apparatus library.

Hydroxytyrosol 4-β-D-glucoside **(1)**: UV λ_{max} nm : 220 and 280; IR ν_{max} cm^{-1} : 3377 (phenolic and alcoholic OH), 1600, 1520 and 1465 (benzene ring); ^1H-NMR (270 MHz, CD_3OD) δ (ppm) :2.7 (2H, t, J_{7-8} = 7.1 Hz, H_7), 3.69 (2H, t, J_{8-7} = 7.1 Hz, H_8), 3.4 (5H, m, glucosidic hydrogen), 6.58 (1H, d, J_{2-6} = 1.98 Hz, H_2), 6.73 (1H, d, J_{6-5} = 8 Hz, H_6), 7.1 (1H, d, J_{5-6} = 8 Hz, H_5), ^{13}C NMR (CD_3OD) δ (ppm) : 32.1 (C_7), 62.5 (C_8), 64.3 ($C_{6'}$), 78.8 ($C_{5'}$), 72.2 ($C_{4'}$), 78.9 ($C_{3'}$), 75.7 ($C_{2'}$), 104.2 ($C_{1'}$), 119.7 (C_5), 137.9 (C_6), 124.1 (C_1), 117.2 (C_2), 145.9 (C_3) and 148.2 (C_4).

Tyrosol **(2)**: 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); ^1H-NMR (270 MHz, CD_3OD) δ (ppm) : 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).

Hydroxytyrosol **(3)**: 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); ^1H-NMR (270 MHz, CD_3OD) δ (ppm) : 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).

3,4-Dihydroxyphenylglycol **(4)**: GC-MS retention time (min) : 21.37; EI-MS *m/z* (relative intensity) : 458 ($M^{\cdot+}$, 7), 369 (8), 355 (100), 280 (15), 147(20), 73 (51).



(**1**) R_1 = Glu, R_2 = OH, R_3 = H, R_4 = $\overset{7}{C}H_2-\overset{8}{C}H_2-OH$: **Hydroxytyrosol 4-β-D-glucoside**

(**2**) R_1 = H, R_2 = H, R_3 = H, R_4 = $\overset{7}{C}H_2-\overset{8}{C}H_2-OH$: **Tyrosol.**

(**3**) R_1 = H, R_2 = OH, R_3 = H, R_4 = $\overset{7}{C}H_2-\overset{8}{C}H_2-OH$: **Hydroxytyrosol.**

(**4**) R_1 = H, R_2 = OH, R_3 = H, R_4 = $\overset{7}{C}HOH-\overset{8}{C}H_2-OH$: **3,4-dihydroxyphenylglycol.**

Figure 2: Chemical structures of the isolated polyphenols from OMW.

3- Gas chromatography coupled with mass spectrometry (GC-MS):

GC-MS was performed with a HP Model 5872A, equiped with a capillary HP5MS column (30 m length ; 0.32 mm i.d. ; 0.32 µm film thickness). The carrier gas was He used with a 1.7 mL/min flow rate. The oven temprature program was as follows: 1 min at 100°C, from 100 °C to 260 °C at 4 °C/min, 10 min at 260 °C. Before analysis, the trimethylsilyl derivative of each compound was prepared to enhance volatility and thermal stability. For this purpose, 100 µl of BSTFA (bis (trimethylsilyl)-acetamid) were added to 100 µl of each compound dissolved in ethyl acetate. The obtained solution was incubated within 20 min at 80 °C. Ethyl acetate and BSTFA were evaporated under N_2 current. The residue was redissolved in cthyl acetate (1 ml) and analysed by GC-MS.

4- β-Carotene bleaching method:

Antioxidant activity was determined in emulsion by the β-carotene bleaching method of Chevolleau et al. [19] 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. [20]. 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.

RESULTS AND DISCUSSION

1- Polyphenols isolation from OMW:

From a point of view of olive mill wastewaters (OMW) valorization, a chromatographic procedure has been carried out in order to isolate and identify polyphenols with antioxidant activity. This procedure which combined bio-gel P-2 chromatography with prep.TLC, gave four pure compounds: 4-β-D-glucosyl 3-hydroxyphenylethanol (hydroxytyrosol 4-β-D-glucoside), tyrosol, hydroxytyrosol and 3,4-dihydroxyphenylglycol with relative concentrations 50 mg/L, 55 mg/L, 150 mg/L and 15 mg/L respectively.

In a previous study, Bianchi and Pozzi [21] have isolated 3,4-dihydroxyphenylglycol from olive fruits. After two years, this phenolic compound was identified for the first time in OMW using GC-MS technique [22]. Our GC-MS data of the isolated 3,4-dihydroxyphenylglycol are in full agreement with those recorded by Knupp et al. [22]. Later, this ortho-diphenolic compound was isolated from OMW [9]. In this study, the phytotoxicity of OMW and their phenolic components was tested on the green alga *Ankistrodesmus brauni*. It was established that 3,4-dihydroxyphenyl glycol exhibited any toxicity towards the tested plant. Recently, it was demonstrated that 3,4-dihydroxyphenylglycol is the major metabolite of Noradrenaline drug (Norepinephrine) which acts as an inotropic stimulator of the heart and dilator of coronary arteries [23].

Concerning hydroxytyrosol 4-β-D-glucoside, Romero et al. [24] have noted that this phenolic compound is co-eluted with hydroxytyrosol under reversed phase conditions in the phenolic chromatograms of olive pulp, pomace of olive oil processing and OMW. In another investigation, researchers have isolated hydroxytyrosol 4-β-D-glucoside besides two other hydroxytyrosol glucosides from olive fruits, olive leaves, olive oil and OMW [25]. Our ^{13}C NMR data of hydroxytyrosol 4-β-D-glucoside agree with those cited by Bianco et al. [25].

Although the several studies carried out for isolating phenolic monomers from OMW, the isolation of hydroxytyrosol 4-β-D-glucoside from OMW has been reported only in few investigations. This can be explained by two major reasons.

The first reason is the extraction procedure. In the previous investigations, the most useful solvent for polyphenols extraction from OMW is ethyl acetate [26]. It was established that this solvent did not extract hydroxytyrosol 4-β-D-glucoside which remained in the aqueous residue because this compound is much more polar than hydroxytyrosol. In the other hand, it was reported that hydroxytyrosol 4-β-D-glucoside can be extracted only with a mixture of (alcohol-water) which is more polar than ethyl acetate. In contrast, using other solvents, this compound was not extracted from OMW [24]. This idea is well confirmed in the extraction procedure developped in our study where polyphenols extraction was performed with a mixture of (methanol-water) (4:1 , V/V).

The second reason is the hydrolysis phenomena. Indeed, it was reported that glucosides of hydroxytyrosol are present in the leaves and in the fruits of olive. As a consequence of the production process of olive oil, they are partially hydrolyzed either by β-glycosidase, witch is released in the olive past, or by heating affording glucose and simple hydroxytyrosol [25]. In addition, hydroxytyrosol glucosides can be hydrolysed when OMW are stored in light, at ambient and aerobic conditions. In this way, Ryan et al. [27] 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. This fact is well confirmed in our study which resulted in the hydroxytyrosol glucoside isolation and where cautions (anaerobic, in dark, low temperature) have been paid during OMW storge and extraction procedure to avoid hydrolysis process. Thus, it is worthy to take into account in the future that the amount of hydroxytyrosol in OMW comes not only from the hydrolysis of oleuropein and verbascoside [28] but also from the hydrolysis of hydroxytyrosol 4-β-D-glucoside and the other form of hydroxytyrosol glucoside.

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

2-1- 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 [29]. Each purified compound was added to linoleic acid oxidation system at 200 ppm. An experiment, using Butyl-hydroxy-toluene (BHT) (a useful synthetic antioxidant) 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 3 shows the rate of β-carotene bleaching in the different linoleate systems.

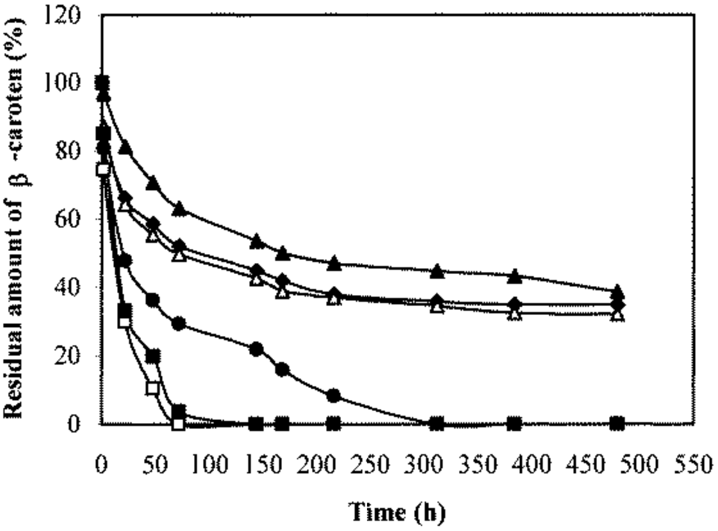


Figure 3: Time course of the β-carotene bleaching in model systems containing : hydroxytyrosol (▲); BHT (◆); 3,4-dihydroxyphenylglycol (△); hydroxytyrosol 4-β-D-glucoside (●); tyrosol (■); and without added compound (control) (□).

Comparing with the control sample and except tyrosol, all the tested compounds presented antioxidant activity. Hydroxytyrosol has the best antioxidative activity which is higher than BHT. Furthermore, 3,4-dihydroxyphenylglycol exhibited significant antioxidant activity which is comparable with BHT. 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 tyrosol, the curve slope β-carotene loss/time unit was broadly more intense comparing with hydroxytyrosol, BHT, hydroxytyrosol 4-β-D-glucoside and 3,4-dihydroxyphenylglycol. Moreover, in linoleate system containing tyrosol, β-carotene was completely bleached after 150 h incubation time. Accordingly, antioxidant potencies of the tested compounds were in the following decreasing order:

hydroxytyrosol > 3,4-dihydroxyphenylglycol ≈ BHT > hydroxytyrosol 4-β-D-glucoside > tyrosol.

2-2-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 1.

Table 1: 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
3,4-dihydroxyphenylglycol	0.78
BHT	0.89
Hydroxytyrosol 4-β-D-glucoside	2.1
Tyrosol	10.85

Comparing with BHT (IC_{50} = 0.89 µg/mL), hydroxytyrosol and 3,4-dihydroxyphenylglycol, with IC_{50} values of 0.57 and 0.78 µg/mL respectively, exhibited the highest radical scavenging activity. However, tyrosol showed no activity (IC_{50} = 10.85 µg/mL). This result correlates with that obtained using the β-carotene bleaching method.

According to the antioxidant activity decreasing order established in this study and the structures of the tested compounds (Figure 2), 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. Indeed, hydroxytyrosol, pocessing an ortho-diphenol function, has a scavenging effect on DPPH radical higher than tyrosol with one hydroxyl group. In this way, Dziedzic and Hudson [30], have found that at least two hydroxyl groups were required for antioxidative activity of phenolic acids and especially the ortho-diphenolic compounds. Furthermore, comparing the activities of hydroxytyrosol (IC_{50} = 0.57 µg/mL) and hydroxytyrosol 4-β-D-glucoside (IC_{50} = 2.1 µg/mL), it is evident that glycosilation of the hydroxyl group decreases the antioxidative activity. These results are in good agreement with that reported by Fukumoto and Mazza [29]. These researchers have demonstrated that antioxidative activity usually increases with an increase in the number of hydroxyl groups and a decrease in glycosilation.

In this investigation, hydroxytyrosol and 3,4-dihydroxyphenylglycol exhibited interesting antioxidant activity. Therefore, their purification in a large scale from OMW would be a promising procedure to valuate these problematic effluents and to produce natural antioxidants as alternative to the undesired synthetic antioxidants. For this aim, a project (INCO-MED) supported by the European Community was undertaken for a large scale recovery of the active compounds from OMW and especially hydroxytyrosol [17].

Acknowledgements :

This research was supported by EEC contract ICA3-CT2002-10033 and "Contrats Programmes SERST", Tunisia. The authors wish to thank the members of "LARSEN" Laboratory (ENIS) for their help in GC-MS analysis.

REFERENCES

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

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

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

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

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

[6] S. Sayadi, N. Allouche, M. Jaoua, F. Aloui; *Process Biochem.*, **2000**, *35*, 725.

[7] A. D'Annibale, C. Crestini, V. Vinciguerra, G. G. Sermanni; *J. Biotechnol.*, **1998**, *61*, 209.
[8] G. Jr. Greco, G. Toscano, M. Cioffi, L. Gianfreda, F. Sannino; *Water Res.*, **1999**, *33*, 3046.
[9] M. DellaGreca, P. Monaco, G. Pinto, A. Pollio, L. Previtera, F. Temussi; *Bul. Environ. Contam. Toxicol.*, **2001**, *67*, 352.
[10] R. Capasso; *Current Topics in Phytochem.*, **1997**, *1*, 145.
[11] S. Sayadi, R. Ellouz; *J. Chemi. Technol. Biotechnol.*, **1993**, *57*, 141.
[12] R. Capasso, G. Cristinzio, A. Evident, F. Scognamiglio; *Phytochem.*, **1992**, *12*, 4125.
[13] C. A. Ramos, J. B. Juarez, P. M. P. Garcia; *Internat. Biodeter. Biodegrad.*, **1996**, 283.
[14] R. Capasso, A. Evidente, L. Schivo, G. Orru, M. A. Marcialis, G. Cristinzio ; *J. Appl. Bacteriol.*, **1995**, *79*, 393.
[15] F. Visioli, D. Caruso, C. Galli, S. Viappiani, G. Galli, A. Sala; *Biochem. Biophys. Res. Commun.*, **2000**, *278*, 797.
[16] R. Capasso, A. Evidente, C. Visca; *Agrochimica*, **1994**, *38*, 166.
[17] N. Allouche, I. Fki, S. Sayadi; *J. Agric. Food Chem.*, **2004**, *52*, 267.
[18] N. Allouche, S. Sayadi, M. Damak; *J. Soci. Chim. Tun.*, **2004**, *6(1)*, 33.
[19] S. Chevolleau, A. Debal, E. Ucciani ; *Rev. Fran. Corps gras*, **1992**, *1(2)*, 3.
[20] K. Na Mee, K. Jong, C. Hae Young, C. Jae Sue; *Arch. Pharm. Res.*, **2000**, *23(3)*, 237.
[21] G. Bianchi, N. Pozzi; *Phytochem.*, **1994**, *35*, 1335.
[22] G. Knupp, G. Rucker, A. R-Cormenzana, S. G-Hoyos, M. Neugebauer, T. Ossenkop; *Internat. Biodeter. Biodegra.*, **1996,** 277.
[23] W. Dong, X. Ni; *Origi. Res.*, **2002**, *23(1)*, 59.
[24] C. Romero, M. Brenes, P. Garcia, A. Garrido; *J. Agric. Food Chem.*, **2002**, *50(13)*, 3835.
[25] A. Bianco, R. A. Mozzei, C. Melchioni, G. Romeo, M. L. Scarpati, A. Soriero, N. Vccella ; *Food Chem.*, **1998**, *63*, 461.
[26] L. Lesage-Meessen, D. Navarro, S. Maunier, J-C. Sigoillot, J. Lorquin, M. Dellattre, J-L. Simon, M. Aster, M. Labat ; *Food Chem.*, **2001**, *75*, 501.
[27] D. Ryan, H. Lawrence, P. D. Prenzler, M. Antolovich, K. Robards; *Analytica Chimica Acta.*, **2001**, *445*, 67.
[28] M. Brenes, A. Castro; *J. Sci. Food Agric.*, **1998**, *77,* 353.
[29] L. R. Fukumoto, G. Mazza; *J. Agric. Food Chem.*, **2000**, *48*, 3597.
[30] S. Z. Dziedzic, B. J. F. Hudson; *Food Chem.*, **1983**, *11*, 161.