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# Identification and characterisation of phenolic compounds extracted from Moroccan olive mill wastewater

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**Abstract**

Olive mill wastewater, hereafter noted as OMWW was tested for its composition in phenolic compounds according to geographical areas of olive tree, i.e. the plain and the mountainous areas of Tadla-Azilal region (central Morocco). Biophenols extraction with ethyl acetate was efficient and the phenolic extract from the mountainous areas had the highest concentration of total phenols' content. Fourier-Transform-Middle Infrared (FT-MIR) spectroscopy of the extracts revealed vibration bands corresponding to acid, alcohol and ketone functions. Additionally, HPLC-ESI-MS analyses showed that phenolic alcohols, phenolic acids, flavonoids, secoiridoids and derivatives and lignans represent the most abundant phenolic compounds. Nüzhenide, naringenin and long chain polymeric substances were also detected. Mountainous areas also presented the most effective DPPH scavenging potential compared to plain areas; $IC_{50}$ values were 11.7 ± 5.6 µg/ml and 30.7 ± 4.4 µg/ml, respectively. OMWW was confirmed as a rich source of natural phenolic antioxidant agents.

***Keywords:*** phenolic compounds; olive mill wastewater; HPLC-ESI-MS; antioxidant activity.

## 1 Introduction

The olive oil industry in the Mediterranean region rejects annually up to 8.4 million $m^3$ of OMWW, of which 250 000 $m^3$ are produced in Morocco (Ben Sassi et al., 2006). This has become a major environmental problem in the countries of this region. Tadla-Azilal is an important olive oil production region of Morocco and the resulting OMWW was directly discharged in soils without any treatment, thus causing a negative environmental impact (Ruiz-Rodriguez et al., 2010). OMWW is a mildly acidic, red-to-black coloured, liquid of high conductivity. It is particularly rich in organic matter and toxic fatty acids (Mekki et al., 2006; Sayadi et al., 2000). The high-molecular-weight polyphenols, similar in structure to lignin, give OMWW their characteristic brownish black colour (Assas et al.., 2002; D'Annibale et al., 2004). Moreover, the high pollution potential of this effluent is commonly attributed to its high phenolic content of monomeric phenols, toxic to plants, water and some microorganisms (Capasso et al., 1992). OMWW may contains up to 10 g of phenols per liter (D'Annibale et al., 1998) while in the European Union, the accepted maximum phenol concentration in wastewaters is 1 mg/L (Urban waste water treatment Directive 91/271/EEC) (European Commission, 1991). Therefore, environmental survey authorities encourage the producers to change their production systems. However, OMWW can be considered as a rich source of natural antioxidant phenolic compounds 100 fold concentrated than in olive oil (Lesage-Meessen et al., 2001). Its composition varies both qualitatively and quantitatively according to the olive variety, climate conditions, cultivation practices, the olive storage time, and the olive oil extraction process (Borja et al., 1997; Ergun Ergül et al., 2009; Fiorentino et al., 2003; Davies et al., 2004). Apart from water (83-92%), the main components of OMWW are phenolic compounds, sugars and organic acids. OMWW contains also valuable resources such as mineral nutrients, especially potassium, which could potentially be reused as a fertilizer (Aranda et al., 2007; Dermeche et al., 2013). Thus, the scientific community is still in search of effective processes for reducing these contaminants (Mantzavinos & Kalogerakis, 2005). In this way, several techniques have been used to recover phenolic compounds from olive by-products, including enzymatic preparation, solvent extraction, membrane separation, centrifugation, and chromatographic procedures (Dermeche et al., 2013). Solvent extraction is the most commonly employed technique to extract phenolic compounds, and ethyl acetate is the most effective solvent for the treatment of OMWW under acidic conditions (Allouche et al., 2004). Different processing practices such as biological, chemical and physical methods have been used for the valorisation of olive residues while the development of these processes is deterred by their expensive costs. Furthermore, the renewed interest in such natural products has been supported by advances in chromatographic and spectroscopic techniques that have greatly facilitated drug discovery from plants (Obied et al., 2005).

The aim of the present work was to identify and quantify the phenolic compounds, occurring in Moroccan OMWW originating from different areas, in view to understanding their molecular bioactivities of further applications such as antioxidant potentialities.

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## 2 Materials and methods

### 2.1 Chemicals

All solvents and chemicals were of HPLC grade and were obtained from Sigma Chemical Co. Saint Quentin (France).

### 2.2 Samples

Four OMWW samples were obtained as a liquid by-product (vegetation waters) by discontinuous three-phase olive processing mill and conserved at 4 °C. Samples were generated from *Moroccan Picholine* olives variety (Moroccan Picholine olive variety was identified and authenticated by Pr. A. Boulli, Department of Biology, Sultan Moulay Slimane University, and stored as a voucher specimen in the Faculty of Science and Technologies, Beni Mellal, Morocco). The studied samples were collected at the end of the olive harvest season (January to March 2010) – at the maturation stage of red-black olives from four areas of Tadla-Azilal region (central Morocco): Beni Mellal and Krazza (for plain areas samples), Azilal and Afourar (for mountainous areas samples).

### 2.3 Extraction of phenolic compounds

The procedure was carried out using the analytical methodology described by De Marco et al. (2007) with some modifications. The Samples were washed with hexane [1:1, (v/v)] in order to remove the lipid fraction: 45 ml of OMWW were mixed with 45 mL of hexane; the mixture was shaken and then centrifuged during 15 min at 4000 rpm. The phases were separated and the washing was repeated successively two times. Extraction of phenolic compounds was then carried out with ethyl acetate: 100 mL of OMWW samples, preventively washed, were mixed with 100 mL of ethyl acetate; the mixture was vigorously shaken and centrifuged for 10 min at 4000 rpm. The phases were separated and the extraction was repeated successively three times. The ethyl acetate was evaporated and the residue was stored at - 20 °C for subsequent analyses.

### 2.4 Evaluation of the total phenolic compounds content

The total phenolic compounds content in each extract was evaluated by spectrophotometry using the Folin-Ciocalteu method (Singleton & Rossi, 1965; Scalbert et al., 1989) with some modifications. Briefly, 2.5 ml portion of Folin-Ciocalteu reagent 0.2 N was mixed with 0.5 ml of the sample. The reaction was kept in the dark for 5 min. Then, 2 ml of a sodium carbonate solution (75 g/l) was added to the mixture and the reaction was kept in the dark for 1 h. The absorbance was measured at 765 nm in Jasco V-630 spectrophotometer. Gallic acid was used as phenolic compound standard for calibration curve (10-90 mg/L; y = 0,0009 x - 0,0004, where x and y represent gallic acid concentration (mg/L) and absorbance at 765 nm, respectively; $r^2$ = 0,9981). Contents of total phenolic compounds in OMWW were expressed as gallic acid equivalents in gram per liter (g GAE/L residue).

### 2.5 Middle Infrared spectroscopy analysis

FT-MIR spectra of duplicate samples were obtained using a Bruker Vector 22 spectrometer possessing an integrated Michelson interferometer and Opus 5.5 software. The measurements were obtained with crude samples deposited on an attenuated reflection cell equipped with a diamond crystal. The generated spectra showed wavenumbers ranging between 400 and 4000 $cm^{-1}$.

### 2.6 HPLC/ESI-MS analyses (High performance liquid chromatography/Electro-Spray Ionization-Mass Spectrometry)

HPLC–MS analyses were performed at 279 nm and 30°C using a RP C18 column (150 × 4.6) × 5 µm possessing a Thermo Fisher apparatus equipped by a Surveyor quaternary pump coupled to a PDA detector (diode array detector: 200-600 nm) and an LCQ Advantage (ESI) ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). The injected volume was 20 µL. The mobile phase (0.5 mL/min) consisted of solvent A (water +0.05% Trifloroacetic acid) and solvent B (Acetonitrile +0.05% methanol). The six-step gradient was applied, for a total run time of 76 min, as follows: Starting from 80% solvent A and 20% solvent B increasing to 30% solvent B over 30 min, then isocratic for 10 min, increased to 30% solvent B over 10 min, to 40% over 30 min and to 20% solvent B over 2 min, and finally isocratic for 4 min. ESI ionization conditions were spray voltage 4 KV, capillary 350°C, 14 V. Pure nitrogen was the sheath gas and pure helium was the collusion gas. The full scan mass data m/z was obtained in negative mode and ranged from 100 to 2000 Da.

### 2.7 Antioxidant activity

The antioxidant activity was performed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. The test was carried out in a 96 well microtiter plate. The samples and positive control, vitamin C, were diluted with methanol to prepare the extract solutions equivalent to 200, 100, 50, 25, 12.5, 6.25, 3.125 µg of sample/ml concentrations. 150 µl of 0.004% DPPH solution was pipetted into each well of 96 well plate followed by 8 µl of the sample solutions. The plates were incubated at 37 °C for 30 min and the absorbance was measured at 540 nm, using ELISA microtiter plate reader. The experiment was performed in triplicate and percentage of the scavenging activity was calculated using the formula given below. $IC_{50}$ (Inhibitory concentration) is the concentration of the sample required to scavenge 50% of DPPH free radicals (Equation 1).

$$\% \text{ Scavenging} = (Ao - As/ Ao) * 100 \quad (1)$$

where $Ao$ is absorbance of the control and $As$ is absorbance of the sample at 540 nm.

### 2.8 Statistical analysis

The experimental results were performed in triplicate and the data were expressed as means ± standard deviation. The comparison of the averages was made by Student test (STATISCA software). Differences are considered significant at p<5%.

## 3 Results and discussion

### 3.1 FT-MIR Infrared Spectra analysis

The FT-MIR spectra analyses of all OMWW extracts showed similar spectroscopic profiles although they originate from different geographical locations. The FT-MIR spectra of two OMWW extracts show similar characteristic features with some differences in bands intensity (Figure 1). The OH-stretching at 3430 $cm^{-1}$ from numerous sources (including water) is very similar and shows higher intensity (Ibarra, 1989). The band at 2900 $cm^{-1}$ from aliphatic C-H stretching (with the band at 1450 $cm^{-1}$ from single bond vibrations) shows important intensity and points to important aliphatic moieties. OMWW extract shows a distinct band at 1625 $cm^{-1}$, which is attributed to C-C bonds conjugated with C-O and COO– groups. In the region 1400-1000 $cm^{-1}$ the OMWW extracts show smaller intensity of the peak at 1400 $cm^{-1}$ corresponding to COO– vibrations but in the entire region 1300-1000 $cm^{-1}$ they show much stronger intensity including the peak at 1260 $cm^{-1}$ corresponding to C-O vibrations (Hoque, 1999). The detected functions presumably suggest the presence of the main constituents of olive mill wastewaters: fatty organic acids and phenolic compounds (Dermeche et al., 2013).

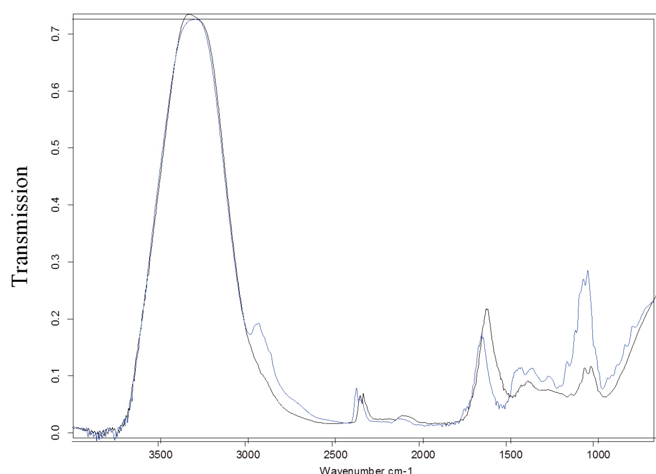


**Figure 1**. FT-MIR Spectra of two OMWW extracts originating from two geographical areas. For the extracts from the other areas, the TF-MIR spectra were similar. The band identities were so: 3430 $cm^{-1}$: (OH), 2940 $cm^{-1}$:(C-H),1625 $cm^{-1}$ : (C=C), 1400 $cm^{-1}$: ($COO^{-}$), and 1260 $cm^{-1}$: (C-O).

**Table 1**. Total phenolic content in OMWW extracts from four geographical areas of Tadla-Azilal region, expressed as gram of gallic acid equivalents per litre of OMWW extracts.

| | Mountainous areas | | Plain areas | |
|---|---|---|---|---|
| | Azilal | Afourar | Beni Mellal | Krazza |
| Concentration (g GAE/L) | 10.1±0.011[b] | 8.8±0.514[b] | 5.29±0.171[a] | 5.27±0.230[a] |

Each value is expressed as mean ± standard deviation (n = 3). Different letters mean significant differences (P < 0.05) (Student test).

### 3.2 Total phenolic content

As can be seen in Table 1, the amount of total biophenols content varied according to the production area. The OMWW extracts originating from the mountainous areas have the highest amount of phenolic content compared to the extracts from the plain areas.

These results suggest the climate and the geographical conditions impact of the phenolic composition. Several factors converge to determine the amount of total phenolic compounds in OMWW, including the olive cultivar, soil composition, ripeness of the fruit, climate and agronomic conditions, storage conditions prior to extraction, and the processing techniques. These factors affect the phenol profile in olive fruit, so too will they affect the profile in olive residues (Dermeche et al., 2013; Allouche et al., 2004; Obied et al., 2005).

In this perspective, Boscaiu et al. (2010) have found a positive correlation between the degree of environmental stress and the level of phenolic compounds accumulated in the plants, suggesting a role of these secondary metabolites in the defence mechanisms against stress. However, Tadla Azilal region is characterized by a continental climate with an intense cold winter and a very hot summer. The temperature varies from 3°-4° C to 48°-50° C with large variations of rainfall. The mountainous areas are characterized by poor soils with large periods of water shortage compared to plain areas with irrigated rich soils (Juili et al., 2013), which may suggest an important degree of environmental stress mainly in mountainous areas.

### 3.3 Identification of phenolic compounds extracted from OMWW

The identification of biophenols was performed by comparing retention times on HPLC with literature and confirmed by relevant molecular mass data from LC-MS (data not shown). HPLC provided the separation of the major biophenols in the OMWW extracts as illustrated in Figure 2 for detection at 279 nm where the differences between mountainous and plain areas OMWW extracts are observed. Mountainous areas' extracts had higher levels of biophenols classes (68.01%; 68.81%) than plain areas' extracts (52.59%; 58.98%). The HPLC-MS analyses revealed the presence of a high amount of hydroxytyrosol, flavonoids and secoiridoid derivatives. In addition, verbascoside, nüzhenide and, mainly, a higher amount of polymeric substances (P) were also detected. The phenolic composition of OMWW extracts is summarized in Table 2.

The HPLC-MS analyses showed the presence of:

**Phenolic alcohols**: The ion spectra of tyrosol (m/z 137) and hydroxytyrosol (m/z 153), identified as the main phenolic compounds detected in OMWW, were found in all the investigated samples. The concentration of total phenol content ranged from 13 to 22 % depending on the geographical area. They were characterized by their highest antioxidant activity, especially hydroxytyrosol, and some other biological effects such as antimicrobial and anti-inflammatory activities (Allouche et al., 2004; De Marco et al., 2007; Capasso et al., 2002; Suarez et al., 2009; Visioli et al., 2002).

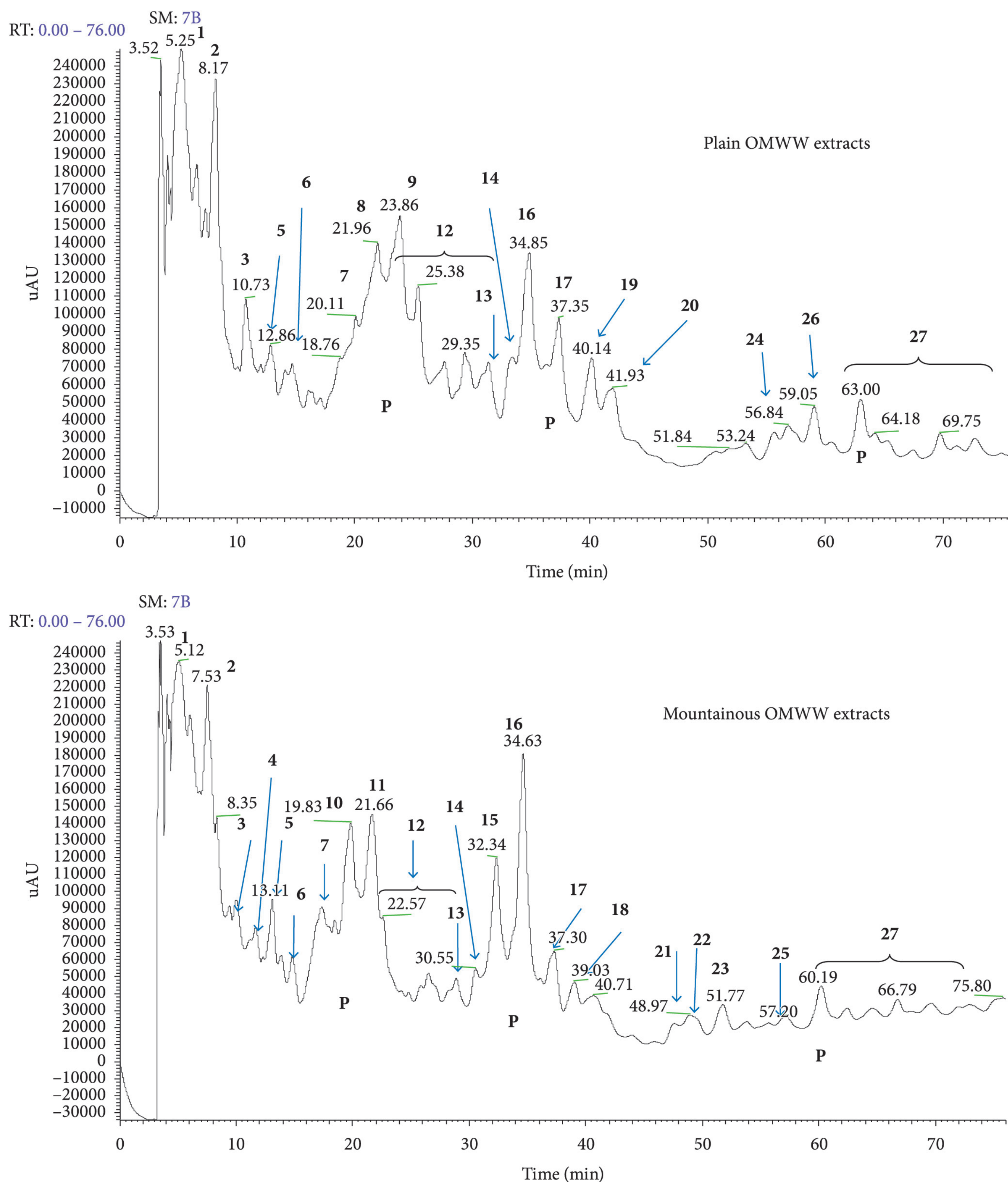


**Figure 2**. Representative HPLC chromatogram of OMWW phenolic extracts. Peaks identities: (1) Hysdroxytyrosol glucoside, (2) Hydroxytyrosol, (3) Tyrosol, (4) Vanilic acid, (5) Sinapic acid, (6) Syringic acid, (7) Caffeic acid, (8) *p*-coumaric acid, (9) Dihydroxymanelic acid, (10) Vanillin, (11) 3,4,5 Trimethoxybenzoic acid, (12) Secoiridoids derivatives, (13) Verbascoside, (14) Rutin, (15) Luteolin-7-rutinoside, (16) Luteolin-7-glucoside, (17) Luteolin, (18) Apigenin, (19) Nüzhenide, (20) Quercetin, (21) Apigenin-7-rutinoside, (22) Apigenin-7-glucoside, (23) Oleuropein,(24) Oleuropein aglycon, (25) Ligstroside, (26) Ligstroside aglycon, (27) Secoiridoids derivatives, (P) polymeric substances.

**Table 2**. Phenolic composition of Moroccan OMWW extracts analysed by HPLC-MS[a].

| Compounds | [M-H]- (m/z)[b] | Plain areas | | Mountainous areas | | References |
|---|---|---|---|---|---|---|
| | | Béni Mellal | Krazza | Azilal | Afourar | |
| Hydroxytyrosol | 153 | 9.38 | 15.19 | 9.27 | 15.04 | (Obied et al., 2005; Lesage-Meessen et al., 2001; De Marco et al., 2007; Allouche et al., 2004; Ramos et al., 2013; Suarez et al., 2009) |
| Tyrosol | 137 | 3.92 | 4.89 | 8.73 | 6.37 | (Lesage-Meessen et al., 2001; De Marco et al., 2007; Allouche et al., 2004; Romero et al., 2002 |
| **Total phenolic alcohols** | | **13.3** | **20.08** | **18** | **21.41** | |
| Vanillic acid | 167 | nd | nd | 4.25 | nd | (Ramos et al., 2013; Cardoso et al., 2005; Suarez et al., 2009; Aranda et al., 2007; Dermeche et al., 2013; Romero et al., 2002) |
| Caffeic acid | 179 | nd | 3.64 | nd | 4.49 | (Lesage-Meessen et al., 2001; De Marco et al., 2007; Allouche et al., 2004; Juarez et al., 2008; Suarez et al., 2009; Dermeche et al., 2013) |
| *p*-coumaric acid | 163 | nd | 9.98 | nd | nd | (Lesage-Meessen et al., 2001; Juarez et al., 2008; Dermeche et al., 2013) |
| Sinapic acid | 223 | 2.69 | 2.94 | nd | 4.13 | (Obied et al., 2005; Dermeche et al., 2013) |
| Syringic acid | 197 | 1.07 | nd | 3.5 | nd | (Obied et al., 2005; Juarez et al., 2008; Dermeche et al., 2013) |
| Dihydroxymandelic acid | 183 | nd | nd | nd | 3.4 | (Aranda et al., 2007) |
| 3,4,5 trimethoxybenzoic acid | 211 | nd | 0.4 | 4.37 | nd | (Juarez et al., 2008) |
| **Total phenolic acids** | | **3.76** | **16.96** | **7.87** | **12.02** | |
| Oleuropein | 539 | nd | nd | nd | 2 | (Suarez et al., 2009; Aranda et al., 2007; Dermeche et al., 2013; Romero et al., 2002; Ramos et al., 2013; Visioli et al., 2002) |
| 3,4-DHPEA-EA | 377 | nd | 1.04 | nd | nd | (Ramos et al., 2013; Cardoso et al., 2005; Suarez et al., 2009) |
| 3,4-DHPEA-EDA | 319 | 1.01 | nd | nd | nd | (Ramos et al., 2013; Suarez et al., 2009; De Marco et al., 2007) |
| ME 3,4 DHPEA-EA | 409 | 1.59 | nd | nd | 2.66 | (Suarez et al., 2009) |
| Oleuropein derivative | 333 | nd | nd | nd | 4.44 | (Ramos et al., 2013; De La Torre-Carbot et al., 2005; Suarez et al., 2009) |
| Oleuropein derivative | 365 | nd | nd | nd | 4.05 | (Suarez et al., 2009) |
| Ligstroside | 523 | nd | nd | 0.89 | 1.26 | (De La Torre-Carbot et al., 2005; Suarez et al., 2009; Aranda et al., 2007; Dermeche et al., 2013; Obied et al., 2007) |
| *p*-DHPA-EA | 361 | 3.41 | nd | nd | nd | (De Marco et al., 2007; Ramos et al., 2013; De La Torre-Carbot et al., 2005; Suarez et al., 2009) |
| Ligstroside derivative | 335 | 3.19 | nd | nd | 5.23 | (De La Torre-Carbot et al., 2005; Suarez et al., 2009) |
| Ligstroside derivative | 393 | nd | 0.71 | nd | nd | (De La Torre-Carbot et al., 2005) |
| Elenolic acid | 241 | nd | nd | 5.7 | nd | (Suarez et al., 2009; Aranda et al., 2007) |
| Elenolic acid glucoside | 405 | nd | 6.37 | nd | nd | (Obied et al., 2005) |
| Hydroxytyrosol glucoside | 315 | 11.47 | 4.4 | 13.11 | 7.01 | (Ramos et al., 2013; Cardoso et al., 2005; Aranda et al., 2007) |
| Verbascoside | 623 | 1.81 | 1.07 | 0.31 | nd | (De Marco et al., 2007; Cardoso et al., 2005; Aranda et al., 2007; Dermeche et al., 2013; Romero et al., 2002; Obied et al., 2007) |
| **Total Secoiridoids** | | **22.48** | **13.47** | **20.01** | **26.65** | |
| Apigenin | 269 | nd | nd | nd | 0.38 | (Dermeche et al., 2013; Obied et al., 2005; Suarez et al., 2009) |
| Apigenin-7-glucoside | 477 | nd | 3.64 | nd | 1.49 | (Dermeche et al., 2013; Obied et al., 2005; Suarez et al., 2009; Obied et al., 2007) |

[a]The Results are expressed as percentage of total phenols in OMMW extracts. [b]Mass charge value. nd = not detected.

| Compounds | [M-H]- (m/z)[b] | Plain areas | | Mountainous areas | | References |
|---|---|---|---|---|---|---|
| | | Béni Mellal | Krazza | Azilal | Afourar | |
| Apigenin-7-rutinoside | 577 | nd | nd | 0.82 | 0.88 | (Obied et al., 2005; Dermeche et al., 2013) |
| Luteolin | 285 | 0.86 | nd | 5.69 | nd | (Obied et al., 2005; De Marco et al., 2007; De La Torre-Carbot et al., 2005; Suarez et al., 2009; Dermeche et al., 2013) |
| Luteolin-7-glucoside | 447 | 4.76 | nd | 6.25 | nd | (De Marco et al., 2007; Cardoso et al., 2005; Aranda et al., 2007; Dermeche et al., 2013; Romero et al., 2002) |
| Luteolin-7-rutinoside | 593 | nd | nd | 3.35 | 0.9 | (Obied et al., 2005; Cardoso et al., 2005; Dermeche et al., 2013) |
| Naringenin | 271 | nd | 0.23 | nd | 1.77 | (Mantzavinos & Kalogerakis, 2005) |
| Nüzhenide | 685 | 1.89 | nd | nd | 1.67 | (Obied et al., 2005; Silva et al., 2006) |
| Quercetin | 301 | nd | 2.06 | nd | nd | (Obied et al., 2007; Dermeche et al., 2013) |
| Rutin | 609 | 2.9 | nd | 0.44 | nd | (Cardoso et al., 2005; Suarez et al., 2009; Dermeche et al., 2013; Romero et al., 2002) |
| Vanillin | 151 | nd | nd | 2.32 | nd | (Lesage-Meessen et al., 2001; Cardoso et al., 2005; Suarez et al., 2009; Dermeche et al., 2013) |
| **Total flavonoids** | | **10.41** | **5.93** | **18.87** | **7.09** | |
| Pinoresinol | 357 | nd | 0.05 | 0.96 | 0.93 | (Obied et al., 2005; Suarez et al., 2009) |
| 1 Acetoxypinoresinol | 415 | 2.32 | nd | 2.3 | 0.71 | (Obied et al., 2005; Suarez et al., 2009) |
| **Total lignans** | | **2.32** | **0.05** | **3.26** | **1.64** | |
| **Total phenolic content** | | **52.59** | **58.98** | **68.01** | **68.81** | |

[a]The Results are expressed as percentage of total phenols in OMMW extracts. [b]Mass charge value. nd = not detected.

**Phenolic acids:** Vanillic acid, sinapic acid, syringic acid, caffeic acid, and *p*-coumaric acid, (peaks: 4, 5, 6, 7 and 8, respectively) that have been frequently reported in OMWW could be found in most of the investigated extracts. Vanillic acid (m/z167) and caffeic acid (m/z 179) were found with an important content in the extracts from mountainous areas (4.25 and 8.52 % of total phenols). *p*-coumaric acid (m/z 163) was detected only in the extracts from plain areas, and its amount was significantly higher (9.98% of total phenols). Furthermore, new phenolic acids, namely dihydroxymandelic acid (m/z 183), tetrahydroxymandelic acid (m/z 215), and 3,4,5 trimethoxybenzoic acid, characterized by Aranda et al. (2007) and Capasso et al. (1992), were also identified.

**Flavonoids**: a fragment ion with m/z 285, diagnostic of luteolin (peak 17), and its derivative; luteolin-7-glucoside (peak 16) with m/z 447 (Ryan et al., 2002) were identified in both mountainous and plain areas extracts as the major flavones (5.69 % and 6.25 %, respectively). According to the molecular ion at m/z 593, luteolin-7-rutinoside could be proposed only for mountainous area (3.35 % of total polyphenols). According to the literature data, the flavone luteolin-7-rutinoside was previously detected in olive leaves and olive by-products, and its ESI-MS data were similar to those of the peak 15 eluted before luteolin-7-glucoside (Dermeche et al., 2013; Ryan et al., 2002). The comparison of the ESI-MS data with the literature data allowed the identification of ion at m/z 609 attributed to rutin molecule. This compound has previously been detected in OMWW and olive pulp (Dermeche et al., 2013). Another flavanone identified at the ions at m/z 271 (0.23; 1.77 % of total phenols) could be attributed to the naringenin. Nüzhenide, a compound identified by Silva et al. (2006) in the olive seed, was found at the ion m/z 685 at low quantities (below 2% of the total polyphenols). Both compounds exhibited a good antioxidant potential (Silva et al., 2006; McDonald et al., 2001). The MS analyses of mountainous extracts showed molecular ions at m/z 269, 431 and 577 suggesting the presence, respectively, of apigenin (0.38 % of the total phenols content) and its two derivatives: apigenin-7-glucoside and apigenin-7-rutinoside detected at variable amounts (Dermeche et al., 2013; Obied et al., 2005; Suarez et al., 2009).

**Secoiridoids:** Oleuropein, an ester of elenolic acid and hydroxytyrosol, identified as a major phenolic compound of OMWW (Dermeche et al., 2013; Visioli et al., 2002; Romero et al., 2002), was identified by its molecular ion at m/z 539 and by the presence of the hydroxytyrosol (m/z 153) ion fragment, only in the extracts derived from mountainous areas whereas we failed to identify this compound in the plain areas extracts. Oleuropein in these extracts had probably degraded into elenolic acid and hydroxytyrosol by an esterase during the mechanical olive oil extraction process (Visioli et al., 2002). This was probably the case with oleuropein in the studied samples, since abundant amounts in hydroxytyrosol were mainly recovered in olive oil residues. Another secoiridoid was identified at m/z 523, may correspond to the ligstroside produced by the loss of the glucose molecule (m/z 162) of nüzhenide and its characteristic fragment ion at m/z 335 (Suarez et al., 2009; De La Torre-Carbot et al., 2005). This compound was characterized by De Marco et al. (2007) as a well antioxidant phenolic compound in the Italian

olive oil mill wastewater. ESI-MS data of both mountainous and plain OMWW extracts indicated a molecular ion at m/z 623 (peak 13) and its characteristic fragments, which is in accordance with the fragmentation of verbascoside. These results were corroborated with the fragmentation profile of verbascoside described by Ryan et al. (2002). Peak 19 showed a deprotonated molecule at m/z 685 that could be attributed to nüzhenide previously identified in olive seed (Silva et al., 2006).

**Secoiridoids derivatives**: The spectra generated from plain OMWW extracts gave the deprotonated molecule at m/z 377 (peak 24) corresponding to the oleuropein aglycon (3,4-DHPEA-EA) (De Marco et al., 2007; Suarez et al., 2009). Another molecule identified as an oleuropein derivative shown by its deprotonated molecule $[M-H]^-$ at m/z 319 may be attributed to oleuropein aglycone isomer in aldehyde form (3,4-DHPEA-EDA) previously identified by Servili et al. (1999) in vegetation waters. The ion at m/z 241, corresponding to the elenolic acid fragment derivative of oleuropein, was identified only in mountainous areas with an important content (5.7 % of total phenols). Moreover, the spectra of all the extracts showed a product ion at m/z 315 (peak 1) with a higher content ranging from 4 to 13%, which could be attributed to hydroxytyrosol glucoside produced by the loss of a rhamnose unit of the verbascoside molecule (Ramos et al., 2013; Cardoso et al., 2005). More derivatives of ligstroside were detected both in mountainous and plain areas. The most abundant was the ligstroside aglycon at m/z 361 (3.19 % of total phenols) characterized by De La Torre Carbot et al. (2005) and Suarez et al. (2009).

**Lignans:** The product ion spectra showed an m/z 357 and m/z 415 which can be attributed, to the Pinoresinol and 1-acetoxypinoresinol, respectively, were identified in most of the extracts with smaller quantities (below 2.32% of the total polyphenols).

Furthermore, important levels of molecules with molecular mass above 1000 Da (800-2000 Da) corresponding to the polymeric phenols were detected as a very broad peak in the range between 16 and 22 min, 32 and 38 min, and 54 and 70 min, co-eluting with the previously mentioned secoiridoids and flavonoid glycosides. We hypothesize that these compounds are the supposed polymerin and metal polymeric organic compounds that were previously recovered from olive oil mill waste waters and proved to be composed of polysaccharides, melanin, proteins and metals (Aranda et al., 2007; Dermeche et al., 2013; Capasso et al., 2002; Hamdi, 1993).

Nonetheless, in full scan mode several unidentified compounds were observed with a strong m/z whose MS fragmentation spectra indicated various product ions (data not shown).

Moreover, the Moroccan OMWW phenolic fraction represents a complex emulsion containing mainly phenolic compounds as confirmed by Bianco et al. (2003), who identified 20 phenolic compounds in OMWW using HPLC-MS-MS including the classes of hydrophilic phenols as identified in Table 2. As detailed above, its concentration and composition vary from a region to another depending on several parameters. Mountainous areas offered the highest phenolic content compared to plain areas, suggesting the impact of environmental stress factors and the variability of the geographical and the climatic conditions of Tadla-Azilal region. The olive variety and its maturity and the methods used to extract and analyse the phenolic compounds could also explain the variability of phenols in OMWW. Accordingly, the identified phenolic compounds in OMWW as well as their concentration are highly variable from a study to another. Several previous investigations have identified other phenolic compounds in OMWW extracts. As an example, Visioli et al. (2002) reported that oleuropein is a major phenolic compound in OMWW, whereas we failed to identify this compound in plain areas as Allouche et al. (2004) did in Tunisian OMWW. One explanation could be that the OMWW was sampled late in the olive harvest (mature olives), when the oleuropein and ligstroside had already been degraded into hydroxytyrosol, tyrosol and various derivative ions, which explains their high amount quantified in OMWW extracts (De Marco et al., 2007; Capasso et al., 2002; Bianco et al., 2003). Juarez et al. (2008) have identified in Spanish OMWW: ferulic acid, p-coumaric acid, 3,4,5 trimethoxybenzoic acid, and p-hydroxybenzoic acid, as major compounds. Dermeche et al. (2013), have detected other compounds in Algerian OMWW: gallic acid, vanillic acid, cinammic acid, 4-methylcatechol, 4-hydroxybenzoic acid, protocatechuic acid, and 3,4-dihydroxyphenylacetic acid. In other studies, Lesage-Messen et al. (2001) and Bouzid et al. (2005) have identified in French and Tunisian OMWW extracts: vanillic acid, ferulic acid and vanillin as major polyphenols. In Italian OMWW, the major polyphenols identified by Capasso et al. (1992) and Casa et al. (2003) were catechol, 4-methylcatechol, 4-hydroxybenzoic acid and trans-sinamic acid.

### *3.4 Antioxidant activity on ethyl acetate phenolic extracts*

Phenolic extracts were tested for their antioxidant activity (Table 3) using the stable free radical DPPH as test (Kumar et al., 2013). Mountainous extracts exhibited the highest antiradical potential with a value 3-fold greater than that of plain extracts. That could be attributed to the highest concentrations of antioxidant phenolic compounds, such as hydroxytyrosol, secoiridoids derivatives and flavonoids, quantified in large quantities of total phenols content. However, hydroxytyrosol proved, among the biophenols identified in olive mill waste, high antioxidant properties (Allouche et al., 2004; Obied et al.,

**Table 3**. Scavenging effects ($IC_{50}$ µg/ml) of OMWW extracts on DPPH free radicals.

| | Plain area | Mountainous area | Ascorbic acid[a] |
|---|---|---|---|
| $IC_{50}$ (µg/ml) | 30.7±4.4 | 11.7±5.6 | 3.2±0.6 |

Antiradical activity $IC_{50}$ (µg/ml) was defined as the concentration of extracts necessary to decrease the initial DPPH radical concentration by 50%. Values are expressed as the mean ± standard deviation (S.D.) of triplicate analyses. [a]Positive control

2005, 2007, 2008; De Marco et al., 2007; Visioli et al., 2002). Other studies (Suarez et al., 2009; Amro et al., 2002) concluded the most antioxidant compounds from olive mill residue were oleuropein, verbascoside, hydroxytyrosol glucoside and oleuropein aglycon (which are compounds related the secoiridoids derivatives). With regard to flavonoids, quercetin, luteolin, luteolin-7-glucoside, rutin, nüzhenide and naringenin, they were characterised by De Marco et al. (2007), Obied et al. (2005) and Silva et al. (2006) by their effective potentialin the antiradical activity.

## 4 Conclusion

For the first time, the olive wastewater was tested for its phenolic composition according to geographical origin areas. This work confirms the interest of olive mill wastewaters as a source of natural antioxidant biophenols, especially those derived from mountainous cultivar olive areas characterized by their pesticide free olives production and their highest polyphenols content. The high antioxidant potential of the OMWW phenolic extracts was related to their high contents of hydroxytyrosol, secoiridoids derivatives, and flavonoids. It was concluded that OMWW was a promising antioxidant phenolic source of further potential biological properties such as food antioxidant agents. Also, the understanding of the molecular activities of these natural compounds can lead to develop new applications in the pharmaceutical and biomedical domains.

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