Olive mill wastewater microconstituents composition according to olive variety and extraction process

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\textbf{A B S T R A C T}

Olive oil production yields a considerable amount of wastewater, a powerful pollutant that is currently discarded but could be considered as a potential source of valuable natural products due to its content in phenolic compounds and other natural antioxidants.

The aim of this work was to explore the variability in olive mill wastewater composition from Algerian olive oil mills considering extraction processes (traditional discontinuous press vs 3-phases centrifugal system) and olive varieties (Azerraj, Sigoise, Chemlal). Whereas pH, dry or organic matter content didn’t vary, there was a significant difference in ash content according to extraction process and olive variety. Carotenoid content was 2.2-fold higher with 3-phases than with press systems whereas tocopherol content was not significantly different. Among the phenolic compounds quantified, tyrosol was usually the most abundant whereas oleuropein concentrations were highly variable. Differences in phenolic compound concentrations were more pronounced between olive varieties than between processes.

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\textbf{1. Introduction}

Olive oil production is one of the most traditional agricultural industries with a great economic importance in most of the Mediterranean countries (Dermeche, Nadour, Larroche, Moutli-Mati, & Michaud, 2013). There are more than 800 million productive olive trees on the planet, occupying an area of 10 million hectares (Vossen, 2013). The Mediterranean region alone provides 98% of the total surface for olive tree cultivation and 97% of the world total olive oil production, which has been estimated at 2.74 million tons in the last six years (Vossen, 2013). Because of its excellent nutritional properties, the consumption of olive oil is increasing since 2005 and its production has grown by approximately 40% worldwide in the last decade (Dermeche et al., 2013).

The extraction of olive oil typically consists of three operational steps: (i) olive crushing, where fruit cells are broken down and the oil released; (ii) mixing, where the remaining paste is slowly mixed to increase the oil yield; and (iii) oil separation from the remaining
wastes. This latter step could be conducted according to one of the following processes: (i) traditional discontinuous press process, (ii) 3-phases centrifugal or (iii) 2-phases centrifugal extraction system (Klen & Vodopivec, 2012).

Water is added in some of these steps to squeeze out most of the oil from the olive. The mix between olive vegetation water and this under-process added water is called olive mill wastewater (OMWW) (El-Abbassi, Kiai, & Hafidi, 2012). Among the three processes of olive oil production, pressure and 3-phases centrifugation systems generate huge amounts (up to 30 million m$^3$ per year) of OMWW (El-Abbassi et al., 2012). Even though less ecologically-friendly, these two processes are still largely in use, especially around the Mediterranean area where they induce the production of large OMWW volumes during a very short period of the year (November to February). These two elements (concentration in time and in location) combined to the OMWW content in salts, heavy metals or phenolic compounds play a major part in the negative ecologic footprint of olive oil production that is reinforced by insufficient OMWW specific treatment plants and by bad practices like their illegal dumping to the soil or in surrounding rivers (McNamara, Anastasiou, O’Flaherty, & Mitchell, 2008). This waste is one of the most harmful effluent produced by agro-food industries because of its high polluting load and high toxicity to the whole ecosystem (plants, bacteria, aquatic organisms and air) owing to its acidic pH and to its content in organic substances such as phenols (Dermche et al., 2013; El-Abbassi et al., 2012). As an example of the extent of the environmental impact of OMWW, it should be noted that 10 million m$^3$ per year of this effluent are equivalent to the wastewater generated by about 20 million people (McNamara et al., 2008). Thus, OMWW treatment or valorization is a major environmental issue.

A great variety of processes have been investigated in the past to reduce OMWW toxicity or to facilitate their treatment, but research efforts turn nowadays toward other aspects. Indeed, OMWW could be considered as a source of bioactive compounds such as phenolics, tocopherols and carotenoids that can be extracted and applied as natural antioxidant for cosmetic, food and pharmaceutical industries (Dermche et al., 2013).

Phenolic compounds found in OMWW exert potent biological activities. For example, hydroxytyrosol has been recognized by the European Food Safety Authority as a protector of blood lipids from oxidation. Its interest for diseases prevention has been shown in numerous studies performed in vitro or in animal models (Achmon & Fishman, 2015; Azaizeh et al., 2012). It has also been demonstrated that hydroxytyrosol exerts in vitro an antimicrobial activity against both Gram-positive and Gram-negative bacteria (Obied, Bedgood, Prenzler, & Robards, 2007). In addition, bioavailability studies have shown that oleuropein and hydroxytyrosol from olive can be absorbed efficiently in human (de Bock et al., 2013). This would encourage their addition in the diet (Azaizeh et al., 2012) and consequently their purification from OMWW that could be a rich and inexpensive source.

Olive oil is known to contain substantial amounts of carotenoids and vitamins E, which also play an important role in human health. They both have an antioxidant capacity with which they protect biological systems sensitive to oxidizing damage induced by free radicals. Furthermore, as precursors of vitamin A, carotenoids occupy at present an important place among food components of interest with respect to human health. As for vitamin E, it is known to promote immunity (Graulet, Martin, Agabriel, & Girard, 2013). However, the characterization of tocopherol and carotenoid contents in OMWW has never been performed.

For a long time, it has been reported that the low pH, the high organic load and the high salt concentrations are common characteristics of OMWW (Amaral et al., 2008). However, little is known on how OMWW composition is affected by the cultivar and the milling technology. According to Obied, Bedgood, Maier, Prenzler, and Robards (2008), phenol content in OMWW is mainly a function of the olive cultivar. In contrast, Ben Sassi, Boularbah, Jaouad, Walker, and Boussaid (2006) demonstrated that OMWW from traditional process units had the highest concentrations of total phenols and total ash compared to OMWW from centrifugal process. El-Abbasi et al. (2012) showed that OMWW from traditional discontinuous press process had a higher phenolic content compared to that obtained from 3-phases centrifugal system. In this work, phenolic profiles also were affected by olive oil processing. On the contrary, Klen and Vodopivec (2012) stated that the process induced significant differences in phenol content, but no qualitative difference in phenol profiles.

The determination of the effects of such factors on OMWW’s composition is expected to enhance the valuation at large scale as well as the development of sustainable OMWW management strategies.

Thus, the objective of the present study was to investigate the effect of oil extraction process (traditional discontinuous press vs. 3-phases centrifugal system) and olive variety on OMWW content and composition in minerals, organic matter, phenolic compounds, carotenoids and vitamin E.

2. Materials and methods

2.1. Chemicals

Zeaxanthin, lutein, β-cryptoxanthin, echinocarone, 13Z,β-carotene and 9Z,β-carotene were purchased from Carotenature (Lupsingen, Switzerland). All-E,β-carotene, α-tocopherol, γ-tocopherol and α-tocopherol were purchased from Sigma (Saint-Quentin-en-Yvelines, France). Gallic acid, caffeic acid, chlorogenic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, p-coumaric acid, ferulic acid, tyrosol, hydroxytyrosol, luteolin and luteolin-7-O-glucoside, apigenin, oleuropein were obtained from Extrasynthese (Genay, France).

All solvents used for standard and sample preparation and for chromatography (acetonitrile, dichloromethane, methanol, tetrahydrofuran (THF), ethyl alcohol, ethyl acetate, n-hexane, diethyl ether, acetone) and 99% formic acid were purchased from VWR (Fontenay-sous-Bois, France). All solvents were of HPLC grade and ultrapure water was prepared using a milli-Q system (Millipore Corp., Bedford, MA, USA).

2.2. Experimental design and OMWW sampling in oil mills

OMWW samples were collected from 15 olive oil mills located in four areas of the north-eastern Algeria (Batna, Constantine, Guelma and Skikda) during the milling campaign 2011–2012. Prior to sampling, a survey was conducted on the general characteristics of the oil mills (location, process used for olive oil production, staff size, average production at the plant…). The questionnaire, tested on 50 olive oil mills comprised 20 issues under 3 headings: (i) general characteristics of olive mill; (ii) characterization of the process at the day of sampling, olive varieties and maturity, storage mode and duration, salt addition, leaf removal, washing, treatment (volume and temperature of the added water), production yield (OMWW delivery), and environmental conditions (general tendency of the climate during the season before olive harvest); (iii) general questions: this section was designed to collect information about the awareness and sensitivity of the producers about the problems of OMWW and their management.

Thirty-five OMWW samples were collected at the beginning of the olive harvest season (October to December 2011). OMWW samples corresponded to olive oil production from 4 pure
varieties: Chemlal (probably the most famous olive variety of Algeria used for oil production, \( n = 11 \) samples), Azzerraj (very local variety used for both oil production or direct eating, \( n = 11 \)), Bouricha (local variety cultivated for oil production in rainy areas; \( n = 4 \)) and Sigoise (also called olive of Tlemcen, olive of the Tell or Picholine from Morocco, it is used both for oil production or direct eating, \( n = 9 \)). The mills ranged in two groups according to oil extraction process: traditional discontinuous press (\( n = 15 \)) or 3-phases centrifugal system (\( n = 20 \)). It should be noted that it was not possible to find several plants producing olive oil from the Bouricha variety using the traditional press process, and this situation was consequently not presented in the experimental design. All the samples were collected at the olive mills, one or more times during the period of oil production. Fresh samples of OMWW were collected into plastic bottles, quickly transported at dark to the laboratory where they were subdivided homogeneously into sub-samples and frozen at \(-20^\circ\text{C}\) until analyses.

### 2.3. Physico-chemical characterization of OMWW

All measurements were performed in duplicate. The pH value of OMWW samples was measured using a pH meter pH538 (Wissenschaftlich - Technische Werkstätten, Weilheim, Germany). Dry weight and moisture content were determined by weighing (Wissenschaftlich - Technische Werkstätten, Weilheim, Germany). Samples of OMWW was measured using a pH meter pH538 and echinenone( and echinenone) and vitamins E (\( \alpha-, \delta- \) and \( \gamma- \)tocopherols) were detected at 450 and 292 nm, respectively, and identified by comparison of their retention times and spectra with those of pure standards.

### 2.4. Carotenoid and vitamin E content and composition in OMWW

#### 2.4.1. Extraction procedure

In OMWW, carotenoids and tocopherols are associated to the remaining fat of the OMWW. Consequently, their extraction requires a saponification step to hydrolyze triacylglycerols and release these compounds.

All sample OMWW preparation and treatment were performed in duplicate under yellow light at room temperature. The extraction method was based on the method developed for milk by Chauveau-Duriot, Doreau, Nozière, and Graulet (2010). Two milliliters of OMWW were deproteinized by adding the same volume of ethyl alcohol containing echinenone and \( \delta- \)tocopherol, as internal standards for carotenoids and vitamins E, respectively. After 1 min vortexing and 10 min agitation on a platform shaker, the same volume of \( n \)-hexane/ethyl acetate (9/1, \( \text{v/v} \)) was added to extract lipophilic components. After another 1 min vortexing and 10 min agitation on the platform shaker, samples were centrifuged at 1000g for 5 min, and the resulting organic phases were collected. This extraction step was repeated once, and the two organic phases were pooled. The resulting organic phase was re-extracted twice using two milliliters of ethanol/water (9/1, \( \text{v/v} \)) to recover xanthophylls and vitamins E and preserve them from saponification. After 1 min vortexing and centrifugation at 1000g for 5 min at room temperature, the lower ethanolic phase was collected in a new tube. This step was repeated and the resulting lower phase was pooled with the previous one and evaporated under nitrogen at 28 °C. The hexane phase was also evaporated under nitrogen, and the dry residue was saponified in 2 mL of a solution of 10% KOH in ethyl alcohol (\( \text{w/v} \)) in closed tubes placed in a shaking water bath for 1 h at 60 °C under darkness. The reaction was stopped by adding 2 mL of water in tubes transferred into an ice bath. Then, 2 mL of \( n \)-hexane/ethyl acetate 9/1 \( (\text{v/v}) \) were added for final carotenoids purification. This latter step was repeated twice, and the 3 successive hexane phases were pooled with the ethanol phases evaporated previously. After complete evaporation under nitrogen, the final dry residue was dissolved in 30 \( \mu\text{L} \) THF and 270 \( \mu\text{L} \) of acetonitrile/dichloromethane/methanol (75/10/15, \( \text{v/v/v} \)) and transferred into a 2 mL glass screw-top vial for chromatographic analysis.

#### 2.4.2. Quantification of carotenoids and tocopherols by UPLC analysis

The chromatographic analysis of the extracts was performed with a Waters UPLC Acquity system (Waters Corp., Milford, MA) equipped with a photodiode array detector scanning at between 210 and 600 nm. Empower Pro software from Waters was used for instrument control, data acquisition and processing. A 150 × 2.1 mm Acquity UPLC HSS T3, 1.8-\( \mu\text{m} \) column (Waters Corp., Milford, MA) was used with a gradient made of acetonitrile/dichloromethane/methanol (\( A \)) (75/10/15, \( \text{v/v/v} \)) and 0.05 M ammonium acetate in water (\( B \)). The linear gradient consisted of 75:25 (\( A:B \)) at initial conditions, 75:25 (\( A:B \)) from 0 to 20 min, 100:0 (\( A:B \)) from 20 to 21 min, 98:2 (\( A:B \)) from 21 to 30 min, 98:2 (\( A:B \)) from 30 to 44 min, and finally returned to initial conditions. Flow rate was 0.4 mL/min. Column temperature was maintained at 35 °C using a column oven. Sample extracts and standards were maintained at 10 °C into the autosampler during the complete run.

Carotenoids (zeaxanthin, lutein, \( \beta- \)cryptoxanthin, \( \beta- \)carotenes and echinenone) and vitamins E (\( \alpha-, \delta- \) and \( \gamma- \)tocopherols) were detected at 450 and 292 nm, respectively, and identified by comparison of their retention times and spectra with those of pure standards.

### 2.5. Quantification of phenolic compounds in OMWW by MS analysis

#### 2.5.1. Extraction of phenolic compounds from OMWW

Phenolic compounds were extracted in triplicate according to the method described by De Marco, Savarese, Paduano, and Sacchi (2007) with some modifications. Four grams OMWW were acidified with 200 \( \mu\text{L} \) of 2 N HCl to pH 2 and washed 3 times with 4 volumes \( n \)-hexane in order to remove the lipid fraction. The mixture was vigorously shaken for 1 min then centrifuged for 15 min at 1000g and room temperature. The upper EA phases were pooled and evaporated to dryness under nitrogen flow at 35 °C. The dry residue was solubilized in 200 \( \mu\text{L} \) of THF and 800 \( \mu\text{L} \) of methanol then diluted 5 times with 0.1% formic acid in water before injection for quantitative analysis of phenolic compounds by HPLC–MS.

#### 2.5.2. OMWW phenolic compounds quantification by HPLC–MS

HPLC was run on a Waters Alliance system, equipped with an ESCI-multi mode ionization enabled Quattro-micro™ API Waters detector (Waters, Saint-Quentin-en-Yvelines, France). MassLynx software (Waters) was used for instrument control and data acquisition. The separation was carried out using a Sphinx column (125 × 2 mm, 3 \( \mu\text{m} \); Macherey–Nagel, Hoerdt, France) at room temperature. Elution solvents were A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile, for a total run time of 30 min. The gradient changed linearly as follows: 93:7 (A:B) from 0 to 20 min, 100:0 (A:B) from 20 to 30 min, 98:2 (A:B) from 30 to 44 min, and finally returned to initial conditions. Flow rate was 0.3 mL/min. Column temperature was maintained at 35 °C using a column oven. Sample extracts and standards were maintained at 10 °C into the autosampler during the complete run.

Using data from the literature, a set of fifteen phenolic compounds that had been described and/or quantified in OMWW, was selected for MS detection. It included some phenolic compounds currently encountered in plants and herbs like the following phenolic acids (vanillinc, caffeic, 4-hydroxyphenylacetic, 3,4-dihydroxyphenylacetic, gallic, ferulic, p-coumaric and...
Analytical conditions used in the analysis of the phenolic compounds of OMWW.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT (min)</th>
<th>Ion Precursor ion (m/z)</th>
<th>Products ions (m/z)</th>
<th>Source potential (V)</th>
<th>Collision energy (eV)</th>
<th>Transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vanillyl acid</td>
<td>6.37</td>
<td>[M + H]^+ 169</td>
<td>93</td>
<td>26.0</td>
<td>13.0</td>
<td>169 &gt; 93</td>
</tr>
<tr>
<td>Hydroxytyrosol</td>
<td>2.81</td>
<td>[M + H]^+ 137</td>
<td>91</td>
<td>34.0</td>
<td>18.0</td>
<td>137 &gt; 91</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>5.73</td>
<td>[M + H]^+ 163</td>
<td>88</td>
<td>38.0</td>
<td>25.0</td>
<td>163 &gt; 88</td>
</tr>
<tr>
<td>Luteolin</td>
<td>11.23</td>
<td>[M + H]^+ 287</td>
<td>153</td>
<td>60.0</td>
<td>28.0</td>
<td>287 &gt; 153</td>
</tr>
<tr>
<td>Tyrosol</td>
<td>4.81</td>
<td>[M + H]^+ 121</td>
<td>103</td>
<td>34.0</td>
<td>15.0</td>
<td>121 &gt; 103</td>
</tr>
<tr>
<td>4-Hydroxyphenylacetic acid</td>
<td>6.15</td>
<td>[M + H]^+ 106</td>
<td>76</td>
<td>46.0</td>
<td>17.0</td>
<td>106 &gt; 76</td>
</tr>
<tr>
<td>3,4-Dihydroxyphenylacetic acid</td>
<td>4.34</td>
<td>[M + H]^+ 123</td>
<td>76</td>
<td>48.0</td>
<td>19.0</td>
<td>123 &gt; 76</td>
</tr>
<tr>
<td>Oleuropein</td>
<td>9.65</td>
<td>[M + H]^+ 361</td>
<td>137</td>
<td>30.0</td>
<td>18.0</td>
<td>361 &gt; 137</td>
</tr>
<tr>
<td>Apigenin</td>
<td>12.55</td>
<td>[M + H]^+ 271</td>
<td>133</td>
<td>58.0</td>
<td>29.0</td>
<td>271 &gt; 153</td>
</tr>
<tr>
<td>Oleuropein aglycone</td>
<td>18.69</td>
<td>[M + H]^+ 377</td>
<td>137</td>
<td>58.0</td>
<td>29.0</td>
<td>377 &gt; 157</td>
</tr>
<tr>
<td>β-Coumaric acid</td>
<td>8.16</td>
<td>[M + H]^+ 163</td>
<td>119</td>
<td>28.0</td>
<td>15.0</td>
<td>163 &gt; 119</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>5.80</td>
<td>[M + H]^+ 353</td>
<td>191</td>
<td>30.0</td>
<td>19.0</td>
<td>353 &gt; 191</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>8.47</td>
<td>[M + H]^+ 193</td>
<td>134</td>
<td>32.0</td>
<td>18.0</td>
<td>193 &gt; 134</td>
</tr>
<tr>
<td>Luteolin-7-O-glucoside</td>
<td>8.44</td>
<td>[M + H]^+ 447</td>
<td>285</td>
<td>118.0</td>
<td>26.0</td>
<td>447 &gt; 285</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>2.09</td>
<td>[M + H]^+ 169</td>
<td>125</td>
<td>34.0</td>
<td>14.0</td>
<td>169 &gt; 125</td>
</tr>
</tbody>
</table>

RT = retention time.

Table 1: Analytical conditions used in the analysis of the phenolic compounds of OMWW.

The sum of carotenoids and vitamin E composition of OMWW

The sum of carotenoids was different depending on the process since the contents were 2.22-fold higher with 3-phases than with press (9.44 ± 1.46 vs 4.25 ± 1.75 g per kg DM, p = 0.001), this being...
mainly due to the zeaxanthin, lutein, and the 13-Z and all-E isomers of β-carotene (Table 3). The highest total carotenoid concentration found in OMWW from Azerraj treated by the 3-phases centrifugal process, was 4-fold higher than the lowest, found in OMWW from Sigoise treated by the press process.

The carotenoid composition was 50% lutein, 32% β-cryptoxanthin, and 13% β-carotene (Table 3). The highest total carotenoid concentration found in OMWW from Azerraj treated by the 3-phases centrifugal process, was 4-fold higher than the lowest, found in OMWW from Sigoise treated by the press process.

Two forms of vitamin E were observed and quantified: α- and γ-tocopherols. Their mean concentrations were 25.63 ± 4.46 and 3.68 ± 0.52 10–3 g per kg DM respectively and did not vary according to the process or the variety.

### 3.4. Phenolic compounds of OMWW

Among the phenolic compounds selected for analysis in OMWW (Table 1), ferulic acid, p-coumaric acid and oleuropein aglycone concentrations in the samples were lower than the limit of quantification or detection of our assay conditions. Of the twelve quantified phenolic compounds, tyrosol was the most abundant (195.87 g per kg DM) although oleuropein reached largely superior values in some samples (Table 4). In the decreasing order, concentrations of luteolin (96.57 ± 12.43 μg per g DM), caffeic acid (68.70 ± 9.53 μg per g DM), luteolin-7-O-glucoside...
(51.38 ± 8.56 μg per g DM), vanillic acid (43.05 ± 6.52 μg per g DM), and 3,4-dihydroxyphenylacetic acid (38.87 ± 5.62 μg per g DM) were followed by those of 4-hydroxyphenylacetic acid (5.62 ± 0.96 μg per g DM), gallic acid (4.06 ± 0.47 μg per g DM), apigenin (3.45 ± 0.55 μg per g DM), hydroxymyristol (1.55 ± 0.96 μg per g DM) and finally chlorogenic acid (0.19 ± 0.03 μg per g DM) that were very low.

The OMWW samples showed different content in phenolic compounds according to the variety and/or the process (Table 4). Indeed, considering comparisons between OMWW from different olive variety, concentrations of caffeic, 4-hydroxyphenylacetic and 3,4-dihydroxyphenylacetic acids were significantly different in the DM of OMWW and a tendency was observed for vanillic acid and luteoline-7-O-glucoside. For these compounds, the highest values were observed for the Sigoise variety, by comparison to Azerraj or Chemlai, reaching significance for caffeic acid (4.3-fold higher; \( p < 0.05 \)), 4-hydroxyphenylacetic (3.4-fold; \( p < 0.05 \)) and 3,4-dihydroxyphenylacetic acids (2.6-fold; \( p < 0.05 \)).

Regarding the effect of the process, the differences mainly concerned chlorogenic acid concentration that was 4-fold higher with the 3-phases centrifugal process. This difference was due to the Sigoise samples, since for Azerraj, chlorogenic acid concentration was higher with the press process. However, this effect on chlorogenic acid can be considered marginal because of its very low concentration level. By contrast, the concentration of oleuropein that was only 30.6 g per kg DM with the press process reached 995.3 with the 3-phases centrifugal process. Nonetheless, due to the strong variability, the difference only reached the trend of significance. Conversely, the press system gave higher caffeic acid concentrations than the 3-phases centrifugation system, with mean significance. Conversely, the press system gave higher caffeic acid concentrations than the 3-phases centrifugation system, with mean significance. Conversely, the press system gave higher caffeic acid concentrations than the 3-phases centrifugation system, with mean significance. Conversely, the press system gave higher caffeic acid concentrations than the 3-phases centrifugation system, with mean significance. Conversely, the press system gave higher caffeic acid concentrations than the 3-phases centrifugation system, with mean significance.

Finally, for hydroxymyristol, luteolin, tyrosol, apigenin and gallic acid, neither the process nor olive variety induced any significant difference.

3.5. Relationships between production conditions and OMWW characteristics

The three first axes of the PCA explained 66.4% of the total variability in the population (Fig. 1). The first axis PC1 (28.6%) seemed to make a distinction according to the solid content in OMWW (DM, OM and ash to a lower extent; Fig. 1B and D). Several phenolic compounds (gallic, chlorogenic and 3,4-dihydroxyphenylacetic acids; tyrosol mainly) were inversely related to the OMWW solid content. The second axis PC2 (Fig. 1B) explained 20.9% of the total variability inside the population for the variables considered and it would discriminate the OMWW according to the olive maturity. The PCA let show a strong relationship between this parameter and the tocopherol content of OMWW. It also illustrated the link between olive cultivar and several phenolic compounds (vanillic, caffeic, 4-hydroxyphenylacetic and 3,4-dihydroxyphenylacetic acids, luteoline-7-O-glucoside). Finally, the third axis PC3 of the PCA (Fig. 1D) explained 17.0% of the variability and illustrated mainly the relationship between the process and the carotenoids content in OMWW.

4. Discussion

4.1. Physico-chemical characteristics of OMWW

The investigation reported in this paper was undertaken to compare the physico-chemical and micronutrient composition of OMWW of four olive varieties according to two different oil mill processing systems: press process versus 3-phases centrifugal system. An acidic pH is a common characteristic in OMWW reported in the literature, owing to the presence of acid compounds such as phenolic acids (Chaari et al., 2015; Fakhredine, El Hajjouji, Ait Baddi, Revel, & Hafidi, 2006). In our study, pH mean value was close to 5 and not significantly different between varieties or production processes, as well as DM and OM contents. The observed values for these parameters were within the range previously reported in the literature (El-Abbassi, Hafidi, Khayet & Garcia-Payo, 2013; Jeguirim, Chouchène, Réguiou, Trouvé, & Le Buzit, 2012). However, ash content showed significant difference according to the olive variety when using the press process. This result is in accordance with El-Abbassi et al. (2012) who found that ash concentration in OMWW generated by press process was more than 3-fold that in OMWW generated by 3-phases centrifugal system. However, opposite results have been also reported previously (Ben Sassi et al., 2006), in the context of traditional Moroccan farmers’ practices. In the present investigation performed in Algeria, no salt was added for the preservation of olive fruits used prior to the extraction. Therefore, the variations recorded truly reflect differences between olive varieties, whether intrinsic or due to the maturity stage for example, that are expressed with the traditional press process but not with the more standardized 3-phases centrifugal system.

4.2. Carotenoids and vitamin E composition of OMWW

This paper presents the first qualitative and quantitative investigation of the composition in carotenoids and tocopherols in monovarietal OMWW from the three main olive varieties (Azerraj, Chemlai, Sigoise) cultivated in Eastern Algeria. Six different carotenoids and two tocopherols were identified and quantified in all OMWW. For both compound groups, the analysis revealed a common pattern whatever the variety or the process. For carotenoids, the composition was consistent with reports indicating that lutein was the main carotenoid in olive oils from various Spanish varieties (Gandul-Rojas & Mínguez-Mosquera, 1996) followed by all-\( \beta \)-carotene. Zeaxanthin was not identified by these authors but this compound and lutein are usually very difficult to separate by classic chromatography. Conversely, the minor xanthophylls found in olive oil (anthexanthin, violaxanthin, neoanxanthin...) were not observed in the present work although the method was able to extract and quantify them (Chauveau-Duriot et al., 2010). This major difference between oil and OMWW reflects the lipophilic behavior of these compounds that preferably follow oily than aqueous matrices.

OMWW processed with the 3-phases centrifugal system showed a significantly higher carotenoid content that was due to zeaxanthin, lutein, 13Z-\( \beta \)-carotene and all-\( \beta \)-carotene. This result is in agreement with the results previously observed in olive oil. Indeed, Giusfrida, Salvo, Salvo, La Pera, and Dugo (2007) reported that higher pigment contents are found in oils produced by the continuous system technology, compared to oils produced by the traditional pressure system. García, Yousf, Oliva, García-Díaz, and Pérez-Camino (2005) stated that virgin olive oils processed under stronger extraction conditions (hot water) have a significantly higher carotenoid content, probably due to the better breakage of the olive tissue and the higher paste temperature reached, that also could induce the inactivation of the enzymes responsible for pigment degradation.

In all OMWW samples analyzed, two tocopherols were detected and quantified: \( \alpha \)- and \( \gamma \)-tocopherols, the alpha isomer representing about 90% of total vitamin E, in accordance with data on virgin olive oil (Ballus et al., 2014; Limón et al., 2015; Manai-Djebali et al., 2012). The present study showed no significant differences in OMWW \( \alpha \)-tocopherol content between olive varieties. This was surprising since the report by Deiana et al. (2002) that tocopherol content was highly variety-dependent in olive oil, was likely to be
true in OMWW. However, it could be hypothesized that if the malaxing step was well performed before oil separation (through press or centrifugation), the lipid droplets coalesced well, favouring the extraction of tocopherols to the oily fraction and leaving only a minor fraction in OMWW, not quantitatively related to quantities initially present in the fruit (Jiménez, Sánchez-Ortiz, & Rivas, 2014). The PCA gave complementary information on tocopherols content variability in OMWW since it would suggest a relationship with the ripeness level of the olive used for the oil production. Ripeness level in the present study was evaluated by the miller during the survey performed at the oil factory at the moment of the production and OMWW sampling. Three levels of olive ripeness were given: level 1 when olives were ripe, level 2 when olives were rather ripe and level 3 when olives were very ripe. These results were used as illustrative variable for the PCA. Surprisingly, the PCA would suggest a positive relationship between the ripeness degree of olives and tocopherols content in OMWW (confirmed by the specific correlation analysis between these two measures, data not shown) whereas data from the literature suggested globally the opposite when considering olive oils (Baccouri et al., 2008). However, when considered with caution, results obtained by Baccouri et al. (2008) were contrasted with regards to the ripeness degree, the olive cultivar and the way of water provision to olive trees. Indeed, variations in tocopherols content in olive oils observed by these authors according to fruit ripening were absolutely not linear or quadratic. It is not possible to define the positioning of the ripening degree we observed in the present work to the ripening scale used by Baccouri et al. (2008); though, it is not possible to compare exactly the results of the 2 studies.

4.3. Phenolic compounds of OMWW

Studies in the literature focusing on the quantification of olive oil phenolic compounds can be easily encountered, but are less frequent when considering OMWW. It is known that OMWW...
phenolic compounds quantitatively comprise 98% of those initially present in olive fruits; however, the identified phenolic compounds in OMWW as well as their concentrations vary from a study to another (reviewed by Obied et al., 2005). A major bias comes from the fact that data are the most often expressed as a volume concentration whereas water is sometimes added during the oil production process (0–60 L per 100 kg olives in the present study). Therefore, we preferably expressed our results related to the DM content of the OMWW. Qualitatively, it is generally accepted that the most abundant phenolic compounds in OMWW are hydroxytyrosol, tyrosol, and oleuropein (Obied et al., 2005). The present study confirmed tyrosol as a major component in OMWW samples, but very low hydroxytyrosol concentrations were observed and oleuropein aglycone was undetectable. Beside, oleuropein amounts varied strongly (more than 2 hundred-fold between tested conditions). It is reported that the content and composition in phenolic compounds in OMWW depend on the maturity and the cultivar of the fruit, on the climatic conditions, storage time, malaxing time and process of milling (Jiménez et al., 2014; Obied et al., 2005). Visioli et al. (1999) identified oleuropein as a major phenolic, but it was detected in very low concentration by Fakharedine et al. (2006) and by Lafka, Lazou, Sinanoglu, and Lazos (2011), and not found by Lesage-Meessen et al. (2001). In the present study, OMWW samplings were always performed just after the processing and the ripening degree was evaluated through the survey. This helped to suggest that some of our observations could be partially ascribed to the olive ripeness degree. Indeed, in the present study, the amount of oleuropein was largely higher (mean value higher than 1700 μg/g DM) in OMWW collected from olives that had a low ripening status by comparison to others which could suggest that its concentration would be related to the maturity stage. Unfortunately, our experimental design that was deliberately exploratory didn't let characterize the maturity stage as a main factor that could be done more easily in controlled conditions at the laboratory. This observation has also been performed by Dağdelen, Tümen, Özcan, and Dündar (2013), and explained by oleuropein enzyme degradation to elenolic acid and hydroxytyrosol during fruit ripening. In OMWW, hydroxytyrosol was reported as the major phenolic compound, achieving approximately 70% of the total phenolic content (El-Abassi et al., 2012). Some authors also found hydroxytyrosol as the main phenolic compound in olive pomace, reaching concentrations between 1.6 and 2.9 g/kg (Rubio-Senent, Rodríguez-Gutierrez, Lama-Munoz, & Fernandez-Bolanos, 2012). The level of this phenolic alcohol increased as a result of the degradation of oleuropein during fruit ripening (Amiot, Fleuriet, & Macheix, 1989; Esti, Cinquanta, & La Notte, 1998), but also of hydroxytyrosol glucoside during crushing/malaxing (Klen & Vodopivec, 2012). Our investigation demonstrated that hydroxytyrosol or oleuropein concentrations in OMWW are highly variable and suggests that they could be major or minor compounds depending of several factors (process, ripening degree . . .).

The extraction system marginally modified the phenolic composition of the OMWW since only chlorogenic acid concentration was significantly increased with the 3-phases process (more notably for the Sigoise and Chemial varieties). However, the 3-phases process tended to increase oleuropein concentration and to decrease caffeic acid. The above results are in agreement with previous reports of Lesage-Meessen et al. (2001) and Klen and Vodopivec (2012), but not with El-Abassi et al. (2012) who demonstrated that the phenolic profiles of OMWW pressed by 3-phases centrifugal system were different from that of traditional press process. It is stated that the water addition prior to oil separation influences the phenol partition rates. Rodis, Karathanos, and Mantzavinou (2002) demonstrated that olive biophenols oil/water partition coefficients range from 6.10⁻¹⁴ in the case of oleuropein to 1.5, inducing most of them to be recovered in wastewater. Obied et al. (2005) reported that both the processing temperature and the quality of water significantly affect the partition. The higher the water temperature, the higher the concentration of biophenols into the oil phase, and the larger the added water volume, the higher the biophenol amounts recovered in OMWW. The oleuropein solubility in OMWW is much higher than in the oil phase (Rodis et al., 2002), explaining its high concentration in OMWW obtained by 3-phases centrifugal systems. In the other hand, Caponio and Catalano (2001) have shown that even the temperature of the olives before and during crushing strongly influences the solubilization of phenols and consequently their amounts in OMWW. Di Giovacchino, Sestili, and Di Vincenzo (2002) had already proven that metallic crushers of centrifugal systems ensure much better breakage of olive flesh than the millstone of the press, thus enhancing the liberation of phenols into paste, and hence, to oil and wastewater as well. In the present study, 3-phases olive oil mill owners added higher volumes of warmer water to improve extraction and lower energy input (40 L per 100 kg of olives at 66 °C vs 27 L per 100 kg of olives at 47 °C in the case of traditional press process) but these differences did not induce notable effect on phenols content.

Quantitative differences between olive varieties have been noted for several biophenols. Higher amounts of caffeic, 4-hydroxyphenylacetic and 3,4-dihydroxyphenylacetic acids (as well as a tendency for vanillic acid and luteolin-7-O-glucoside) were observed for the Sigoise variety whereas the pattern was the same for the 2 other tested varieties. In line with the previous reports of Mulinacci et al. (2001) about OMWW and Romani, Mulinacci, Pinelli, Vincieri, and Cirimot (1999) about oil, analyzing phenolic composition might usefully contribute to characterize of OMWW diversity and origin.

5. Conclusion

The data presented in this paper seem to indicate, despite the limited number of cultivars examined that phenolic composition might represent a useful contribution to chemical characterization of OMWW varieties. Moreover, OMWW of some cultivars could be interesting source of single active compounds, such as tyrosol and oleuropein. However, the differences in OMWW composition related in the present paper and in the literature would suggest that a study at a larger scale would be necessary to get a better comprehension of and validate the main factors of variation of OMWW composition. Especially, a multi-parameter comparison of OMWW composition between olive varieties from several countries should be performed to get a better description of the components of the OMWW variability. Moreover, it would be a major concern to consider precisely the olive repining status as a factor to explore besides the variety or the process. However, it would justify to ensure to dispose of a largely wider experimental design and use this opportunity to explore additional components of OMWW that could have valuable interests (tanins, sugars . . .). The results of the present study indicate that the need to found sustainable OMWW valorization pathways should be thought in agreement with their origin since it could increase their mineral content almost 3-times (that could be of major concern in the case of river or soil dumping), or several phenolic compounds concentration such as oleuropein, strongly enriched in specific OMWW. At the opposite, variabilities in pH value, DM or OM contents wouldn’t be a real concern for future applications.

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