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Research Article

Separation of Polyphenols from Jordanian Olive Oil Mill Wastewater

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This research aims at separation of polyphenols from Jordanian olive mill wastewater which have possible applications in pharmaceutical industry. The phenolic compounds were isolated using silica column chromatography based on using different solvents after extracting the acidified solution with n-hexane and ethyl acetate. The structural elucidation of the separated compounds was achieved using ^{1}H-NMR, ^{13}C-NMR and mass spectrometry. The concentrations of these compounds were determined by GC-MS after derivatization with N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The concentrations of the main isolated phenolic compounds in the Jordanian olive mill wastewater were ferulic acid (93.6 mg/L), *trans*-cinnamic acid (105.3 mg/L), *p*-coumaric acid (117.0 mg/L), vanillic acid (128.7 mg/L), caffeic acid (140.4 mg/L), tyrosol (210.6 mg/L), and hydroxytyrosol (315.9 mg/L).

1. Introduction

Olive mill wastewater (OMWW) is a dark red-to-black-colored, mildly acidic liquid of high conductivity, obtained from mechanical olive processing during olive oil production [1]. Three phase-extraction systems involve the addition of large amounts of water (up to 50 L/100 kg olive paste), resulting in the worldwide production of more than 30 million m^3 per year of OMWW [2]. This represents a great environmental problem, since this by-pro-duct is characterized by a high inorganic and organic load. Organic substances found in OMWW include sugars, tannins, phenolic compounds, polyalcohols, pectins, and lipids [3]. The toxicity, the antimicrobial activity, and the consequent difficult biological degradation of OMWW are mainly due to the phenolic fraction [2, 4].

The treatment of OMWW is extremely difficult due to its large volume and the high concentration of organic matter. The major factor of the environmental problems imposed by the OMWW is the high concentration of polyphenols. These compounds are difficult to decompose [5, 6] and present phytotoxicity [7, 8], toxicity against aquatic organisms [9], or suppression of soil microorganisms [10].

The olive fruit is very rich in phenolic compounds, but only 2% of the total phenolic content of the olive fruit passes in the oil phase, while the remaining amount is lost in the OMWW (approx. 53%) and in the pomace (approx. 45%) [11].

In general, polyphenols are thought to deliver health benefits by several mechanisms, including: (1) direct free radical quenching, (2) protection and regeneration of other dietary antioxidants, (3) chelation of metal ions [12]. So they act as antioxidant [13], antibiotic/antiviral [12], anti-inflammatory [12], and protection from diseases [14].

One of the most abundant polyphenol present in OMWW and very interesting from the nutritional point of view is hydroxytyrosol, which has been widely studied demonstrating its antioxidant and health-beneficial properties as well as its good bioavailability: hydroxytyrosol scavenges free radicals during the oxidation process [15], inhibits human LDL oxidation [16], inhibits platelet aggregation [17] and

the production of leukotriene for human neutrophils [18], and shows *in-vitro* antimicrobial activity [4]. It is present both in the free and conjugate forms.

Hydroxytyrosol is not commercially available in large amounts as food additive; it is an expensive costly for scientific/experimental purposes [6]. Several methods have been proposed for the production of hydroxytyrosol by means of chemical [19] or enzymatic synthesis [20], but the protocols are usually slow and expensive, resulting in a small number of commercially available products containing pure hydroxytyrosol. By-products from processing materials of biological origin, such as wastewater from olive mills, may then become important sources of high added value compounds, such as hydroxytyrosol or other antioxidant polyphenols.

Various studies were conducted to isolate phenolic compounds from OMWW by using liquid-liquid extraction (LLE). A large number of solvents were tried, but it has been shown that ethyl acetate exhibits a higher extraction power compared to other solvents, such as methyl isobutyl ketone, methyl ethyl ketone, and diethyl ether [21]. The components of the extract were identified and quantified using different chromatographic methods. However, these studies were concerned with the separation and identification of each individual phenolic compound from the extract.

The used chromatographic methods for the determination of the various phenolic compounds in OMWW are high-performance liquid chromatography (HPLC) [22], thin-layer chromatography (TLC) [23], and gas chromatography (GC) [24–27]. De Marco et al. [28] suggested a method for fractionation of the phenolic raw extract from OMWW using a C_{18}-solid phase cartridge (C_{18}-SPE) and ethyl acetate as eluent. The fractionated extracts were then analyzed using HPLC method. HPLC is simple and economical to perform but is limited by the low specificity of the reagent toward phenolic compounds; furthermore, it does not provide qualitative information of single phenolics. Conversely, HPLC is very sensitive and specific, but it is time consuming (one run lasts for about 1 hour) and does not provide information on phenolic molecules for which reference standards are unavailable using traditional detectors [29]. Finally, gas chromatography (GC) has been widely used to determine phenolic compounds [30]. In general, phenols are amenable to GC without derivatization [31] but at lower concentrations peak tailing might occur. To overcome this problem, phenols have to be derivatized with a suitable derivatizing agent. GC is the method of choice because of its superior separation efficiency. Additionally, it allows much lower detection limits and chemical structural information if it is coupled to a mass-selective detector (GC-MS) [32].

The aims of the present study are (1) to separate polyphenols from Jordanian OMWW, (2) to identify the isolated phenolic compounds by nuclear magnetic resonance spectrometry (NMR) and high resolution mass spectrometry (HRMS), and (3) to identify and quantify the isolated phenols by GC-MS after derivatization with BSTFA and to determine the limit of detection and the limit of quantification of each isolated compound.

2. Experimental

2.1. Chemicals. m-Methoxy-acetophenone (internal standard-I.S.) and N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) used for derivatization were purchased from Across Chemical (USA).

The following solvents of HPLC-grade and GC-grade were purchased from Riedel-de Haën (Germany): n-Hexane, ethyl acetate, methanol, chloroform, dichloromethane, ammonia, benzene, and pyridine.

The used silica gel was Silica Gel 60 (for column chromatography) and Silica Gel G/UV_{254} (for thin layer chromatography) which were purchased from Riedel-de Haën (Germany). The type of plates used for separation was silica gel glass precoated TLC plates SILG/25 with a thickness of 0.5 mm, purchased from Riedel-de Haën (Germany).

2.2. Olive Mill Wastewater Samples. The olive mill wastewater sample of 5 L volume was collected from the olive mill in Al-Balqa area, 20 km north of the capital Amman, and stored in aspirators at low temperature until required for experimental use. The storage of OMWW at low temperature is necessary because of the time-variable composition.

2.3. Extraction and Separation of Phenolic Compounds (see Figure 1). A sample of OMWW was filtrated, in order to get rid of any solid materials, followed by acidification to pH 2 with 2 M HCl. Four liters of the acidified sample was extracted three times with 150 × 4 mL n-hexane in order to remove the lipid fraction. The aqueous layer was extracted with 100 mL ethyl acetate (seven times) in order to collect the phenolic compounds. The pooled ethyl acetate extracts were dried over anhydrous sodium sulfate and evaporated using a rotary evaporator at 75°C to obtain 4.68 g of the crude extract sample.

The crude sample (4.68 g) was dissolved in chloroform/methanol solvent mixture, and 5 g of silica gel was added to the mixture. The solvent was evaporated in the fume hood, and then the mixture was loaded on a silica gel column (155 g silica type: MN Silica Gel 60, 3.5 cm in diameter), which was packed in chloroform. The column was eluted with chloroform, and then the polarity was gradually increased using methanol. The content of each collected fraction was evaporated and followed by TLC. Fractions of similar compositions were collected together to give a total of three pooled fractionated groups [A (I)–A (III)].

Group A (I) contained one compound that was eluted in a system of 100% chloroform.

Group A (II) contained five compounds which were eluted in a system of 5% methanol: 95% chloroform.

Group A (III) contained one compound that was eluted in a system of 10% methanol: 90% chloroform.

The crude sample from group A (II) was dissolved in chloroform/methanol solvent mixture, and 4 g of silica gel was added to the mixture. The solvent was evaporated in

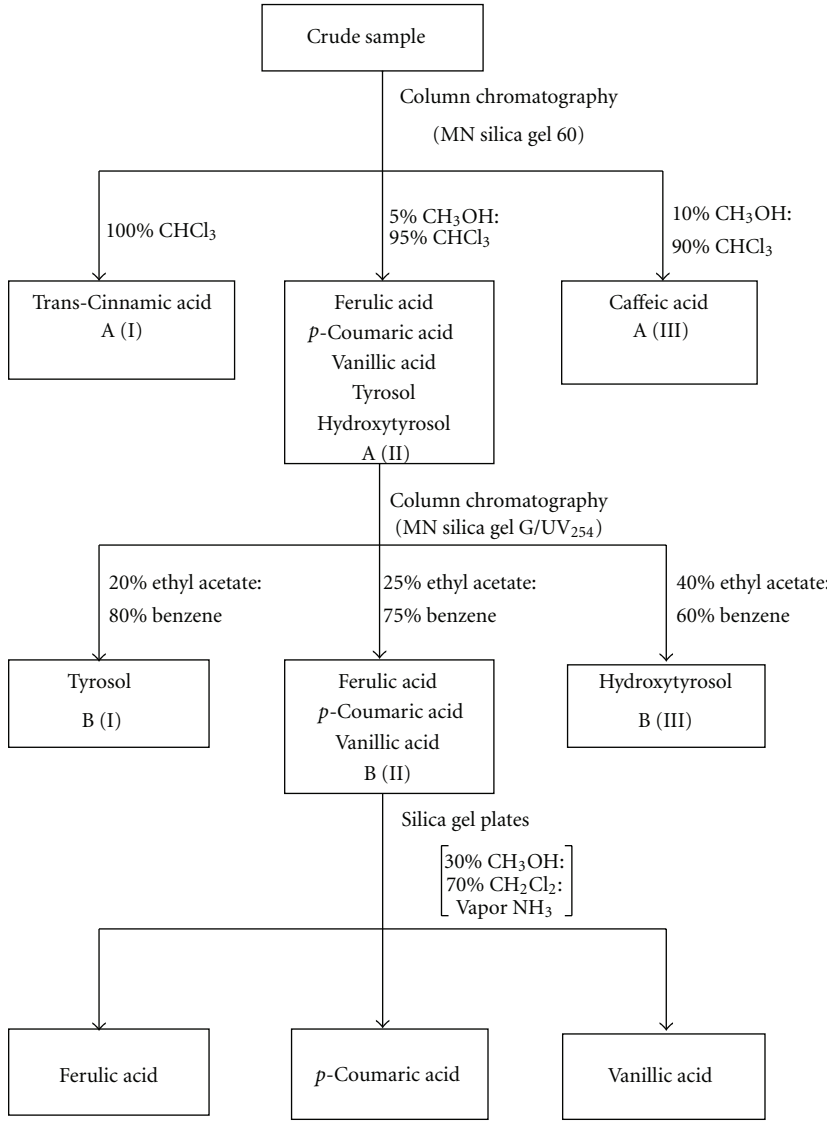

Figure 1: Scheme for the column and TLC separation of the phenolic compounds.

the fume hood, and then the mixture was loaded on a silica gel column (150 g silica type: MN Silica Gel G/UV_{254}, 3.5 cm in diameter), which was packed in benzene. The column was eluted with benzene, and then the polarity was gradually increased using ethyl acetate. The collected fractions were evaporated and separated by TLC. Fractions of similar compositions were pooled together to give a total of three collective groups [B (I)–B (III)].

Group B (I) contained one compound that was eluted in a system of 20% ethyl acetate: 80% benzene.

Group B (II) contained three compounds which were eluted in a system of 25% ethyl acetate: 75% benzene.

Group B (III) contained one compound that was eluted in a system of 40% ethyl acetate: 60% benzene.

Group B (II) was separated on silica gel plates using a system of 30% methanol: 70% dichloromethane in presence of ammonia vapor.

Each of the isolated compounds was purified by either preparative TLC or fractional crystallization.

2.4. Sample Preparation for GC/MS Analysis. Derivatization causes a nonvolatile sample to become volatile, or it improves the detectability upon derivatization. Furthermore, the derivatives may also be more thermally stable. N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) is mostly used as derivatizing agent and it has a high reactivity. The derivatization of phenolic compounds for GC-MS analysis was performed according to the procedure described by Zafra et al. [33] in which a mixture of 20 : 5 : 25 (v/v/v) BSTFA-pyridine-ethyl acetate (containing phenolic sample) is allowed to stand for 2 minutes at room temperature. The procedure is enough to get adequate derivatization.

A 0.1 g of crude sample was dissolved in ethyl acetate under sonication and the volume was completed to the mark in 100 mL volumetric flask with the same solvent. 12 μL of

this prepared solution was mixed with 488 μL ethyl acetate using digital micropipette. 50 μL of the previous solution was mixed in 10 μL pyridine and 40 μL BSTFA. The mixture was shaken well for two minutes in order to derivatize the phenolic compounds. 50 μL of the last solution was mixed with 50 μL of 1 mg/L IS solution to give a solution mixture of crude sample and 0.5 mg/L of IS. Finally, a volume of 1 μL of the prepared solution was injected into GC-MS instrument.

2.5. Preparation of Calibration Standards and Curves. The isolated pure phenolic compounds as shown in Section 2.3 above were used as reference materials. Standard mixture solutions of 0.05, 0.1, 0.5, 1, 2, and 4 mg/L of the derivatized compounds (*trans*-Cinnamic acid, Ferulic acid, *p*-Coumaric acid, Caffeic acid, Vanillic acid, Tyrosol, and Hydroxytyrosol) with 0.5 mg/L IS were prepared in order to define the linear working range. Internal standard method was preferred in order to correct any loss of phenolic compounds during sample preparation.

2.6. Determination the Limit of Detection and Limit of Quantification. The limit of detection (LOD) for each standard phenolic compound was calculated from a chromatogram of a diluted standard mixture solution, on the basis of signal-to-noise (S/N) ratio of 3.

The limit of quantification (LOQ) for each standard phenolic compound was calculated from a chromatogram of a diluted standard mixture solution, on the basis of signal-to-noise (S/N) ratio of 10.

2.7. Instrumentation

2.7.1. UV Detection. Constituents were detected as spots in each fraction after separation from the column using short UV radiation lamp at wavelength of 254 nm.

2.7.2. NMR Analysis. ^{1}H and ^{13}C-NMR spectra were recorded at 300 and 75.5 MHz on a BRUKER DPX spectrometer, using TMS as an internal standard in order to elucidate the structure of the isolated compounds.

2.7.3. HRMS Analysis. High-resolution mass spectrometry (BRUKER APEX IV) was used in order to identify the correct molecular ion for each compound. The ionization method used was electrospray ionization (ESI) with a resolution of 180000 (at *m/z* 400).

2.7.4. GC-MS Analysis. The gas chromatographic analysis was performed using an Agilent 6890 Series II. A gas chromatograph fitted with an autosampler injector. A capillary column HP-5 fused silica column (30 m $\times$ 0.25 mm, film thickness 0.25 μm, (5%)-biphenyl-(95%)-dimethylsiloxane copolymer) was used. A silanized injector liner split/splitless (2 mm I.D.) was used. Detection was carried out with a 7683 mass-selective single quadrupole detector (Agilent Technologies). The injector temperature was 250°C. The oven temperature was held at 80°C for 3 minutes, and then increased to 240°C at a heating rate of 13°C min^{-1}, and the temperature was held for 20 min. The total run time was 38.5 min. The detector temperature was 280°C. The carrier gas used was helium (purity 99.999%) at a flow rate of 1.0 mL min^{-1}. The samples were injected in the splitless mode and the splitter was opened after 7 min (delay time). The sample volume in the direct injection mode was 2 μL. The ion energy used for the electron impact ionization (EI) mode was 70 eV. The mass range scanned was 150–550 *m/z*. Single ion monitoring (SIM) acquisition mode was used.

3. Results and Discussion

3.1. Isolation and Identification of Constituents. The chemical structures of the seven pure isolated, characterized, and studied phenolic compounds are reported in Figure 2. For the mass spectrometric identifications of the seven phenoilc compounds, the following exact values (as *m/z*) were used: *trans*-Cinnamic acid (147.04572), Tyrosol (137.06082), Vanillic acid (167.03479), Hydroxytyrosol (153.05556), p-Coumaric acid (163.03961), Ferulic acid: 1(93.05043), and Caffeic acid (179.03473).

Trans-Cinnamic Acid (1). This compound belongs to group A (I) which was isolated from silica gel column of the crude sample as a white powder using the solvent (100% chloroform).

The mass spectrum showed the molecular ion at *m/z* 147 (M-H), which corresponds to the correct molecular formula $C_9H_8O_2$.

The ^{1}H-NMR ($CDCl_3$) spectrum exhibited signals for a monosubstituted benzene ring as a multiplet at δ 7.36 which corresponds to C2-H and C6-H, another multiplet signal at δ 7.15 corresponds to C3-H, C4-H, and C5-H. The *trans*-olefinic proton C7-H appeared downfield as doublet at δ 7.48 (J = 16.1 Hz), while the C8-H resonated at δ 6.27 (J = 16.1 Hz) as doublet.

The ^{13}C-NMR ($CDCl_3$) spectrum showed signals for eight carbon atoms. The signal at δ 167.3 corresponds to the carbonyl carbon. The four signals for monosubstituted aromatic atoms at δ 132.7, 128.3, 126.9, and 126.1 correspond to C1, (C2 and C6), C4, and (C3 and C5), respectively. The olefinic carbon atoms appeared at δ 143.3 and 116.3 corresponding to C7 and C8, respectively.

The DEPT (135) ($CDCl_3$) spectrum showed only five signals in the positive direction at δ 128.3, 126.9, 126.1, 143.3, and 116.3 ppm which correspond to C1, (C2 and C6), C4, (C3 and C5), C7 and C8, respectively.

Tyrosol (2). This compound belongs to group A (II) and group B (I) who was isolated from silica gel column of the crude sample as a white crystalline powder using the solvent ethyl acetate/benzene (20 : 80).

The mass spectrum showed the molecular ion at *m/z* 137 (M-H), which corresponds to the correct molecular formula $C_8H_{10}O_2$.

The ^{1}H-NMR (CD_3OD) spectrum showed the presence of two doublets at δ 6.68 and 6.96 each with coupling

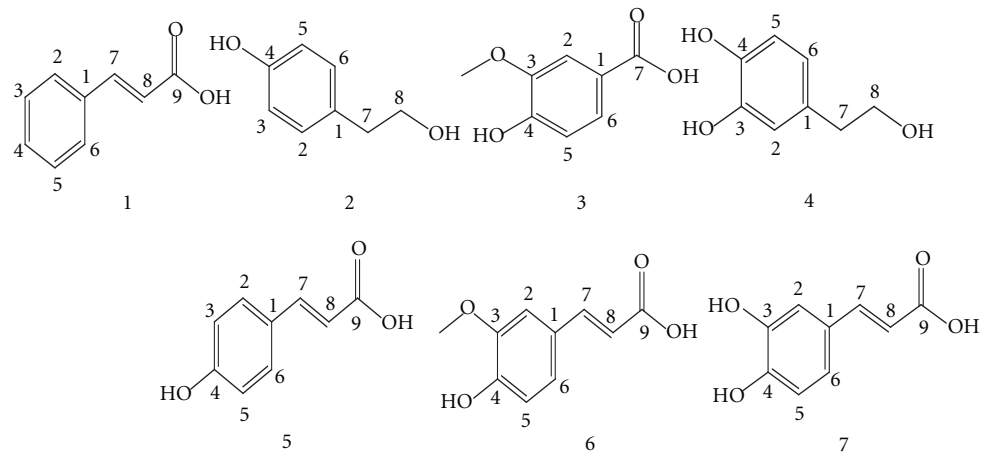

Figure 2: Structures of the isolated phenolic compounds 1–7.

constant of 8.4 Hz assigned to the protons at (C-3 and C-5) and (C-2 and C-6), respectively. The triplet at δ 3.68 integrating to two protons corresponds to the two geminal protons linked to the hydroxyl aliphatic group present in the molecule. Another triplet appearing at δ 2.65 and integrating for two protons corresponds to the methylene linked to the aromatic ring.

The ^{13}C-NMR (CD_3OD) spectrum showed the presence of six signals at (δ 155.3, 129.7, 129.6, 114.8, 63.3, and 38.0). The first two signals are assigned to the two quaternary carbons of the aromatic ring and for its displacement; the first one corresponds to C-4 and the second one to C-1. Because the molecule is symmetric, the signals corresponding to (C-2 and C-6) and (C-3 and C-5), appear as two signals at δ 129.6, 114.8, respectively. Finally, the upfield signals were assigned to the two side chain carbons: the carbon linked to the hydroxyl group (δ 63.3) and to the carbon linked to the aromatic ring (δ 38.0), respectively.

The DEPT (135) (CD_3OD) spectrum showed only four signals, two of them appeared in the positive direction at δ 129.6 and 114.8 ppm which correspond to (C-2 and C-6) and (C-3 and C-5), respectively, while the other signals appeared in the negative direction at δ 38.0 and 63.3 ppm corresponding to C-7 and C-8, respectively.

Vanillic Acid (3). This compound belongs to group A (II) and group B (II) which was isolated from the crude sample by TLC on silica gel plates as a pale yellow powder using the solvent methanol/dichloromethane/ammonia (30 : 70 : vapor). The lowest band ($R_f = 0.29$) was desorbed from silica using a mixture of distilled methanol and chloroform. The solvent was evaporated to give this compound.

The mass spectrum showed the molecular ion at *m/z* 167 (M-H), which corresponds to the correct molecular formula $C_8H_8O_4$.

The ^{1}H-NMR (CD_3OD) spectrum revealed the presence of two doublets at δ 6.71 and 7.43 each with coupling constant of 8.2 Hz assigned to the two protons at C-5 and C-6, respectively. The aromatic proton at C-2 occurred as a sharp singlet at δ 7.42. The three methoxyl protons in position 3 appeared as sharp singlet at δ 3.71.

The ^{13}C-NMR (CD_3OD) spectrum showed the presence of eight carbons in the spectrum in which three were methine carbons at 114.8, 112.4, and 124.0 ppm assigned to C-2, C-5 and C-6, respectively. The four quaternary carbons at 121.7, 147.3, 151.3, and 168.8 ppm were assigned to C-1, C-3, C-4 and C-7, respectively. The methoxyl carbon atom showed a signal at δ 55.0 ppm.

The DEPT (135) (CD_3OD) spectrum showed only four signals in the positive direction at δ 114.8, 112.4, 124.0, and 55.0 ppm corresponding to C-2, C-5, C-6 and methoxyl carbon atom, respectively.

Hydroxytyrosol (4). This compound belongs to group A (II) and group B (III) which was isolated from silica gel column of the crude sample as a yellowish-brown powder using the solvent ethyl acetate/benzene (40 : 60).

The mass spectrum showed the molecular ion at *m/z* 153 (M-H), which corresponds to the correct molecular formula $C_8H_{10}O_3$.

The ^{1}H-NMR (CD_3OD) spectrum revealed the presence of two doublets at δ 6.68 and 6.53 each with coupling constant of 8.0 Hz assigned to the two protons at C-5 and C-6, respectively. Another aromatic proton on position C-2 occurred as sharp singlet at δ 6.71. A triplet appearing at δ 3.68 and integrating for two protons corresponds to the two geminal protons linked to the hydroxyl aliphatic group present in the molecule, while the two protons appearing triplet at δ 2.67 were assigned to the methylene linked to the aromatic ring.

The ^{13}C-NMR (CD_3OD) spectrum showed the presence of eight signals in which three were methine carbons at 115.2, 115.9, and 120.2 ppm assigned to C-2, C-5 and C-6, respectively. The three quaternary carbons at 130.6, 144.7, and 143.2 ppm were assigned to carbon signals at positions C-1, C-3, and C-4, respectively. Finally, the chemical shift signals at δ 38.2 and 63.3 were assigned to the two side chain carbons C-7 and C-8, respectively.

The DEPT (135) (CD_3OD) spectrum showed only five signals, three of which appeared in the positive direction at δ 115.2, 115.9, and 120.2 ppm corresponding to C-2, C-5, and C-6, respectively, while the other signals appeared in the negative direction at δ 63.3 and 38.0 ppm corresponding to C-7 and C-8, respectively.

p-Coumaric Acid (5). This compound belongs to group A (II) and group B (II) which was isolated from the crude sample by TLC on silica gel plates as a white powder using the solvent methanol/dichloromethane/ammonia (30 : 70 : vapor). The middle band ($R_f = 0.4$) was desorbed from silica using a mixture of distilled methanol and chloroform. The solvent was evaporated to give this compound.

The mass spectrum showed the molecular ion at *m/z* 163 (M-H), which corresponds to the correct molecular formula $C_9H_8O_3$.

The ^{1}H-NMR (CD_3OD) spectrum showed the presence of two aromatic protons signals resonating as two doublets at δ 6.45 and 7.13 ppm ($J = 8.4$ Hz) which were assigned to (C3-H and C5-H) and (C2-H and C6-H), respectively. Also, the spectrum showed the presence of two olefinic protons resonating as two doublets, one of them attached to the aromatic ring and has a chemical shift at δ 7.31 ppm ($J = 16.0$ Hz) while the other attached to the carboxylic acid group and has a chemical shift at δ 5.93 ($J = 16.0$ Hz).

The ^{13}C-NMR (CD_3OD) spectrum showed seven signals for nine carbon atoms. The signal at δ 168.0 corresponds to the carbonyl carbon, the four aromatic signals at δ 124.1, 128.0, 113.7, and 158.0 correspond to C1, (C2 and C6), (C3 and C5), and C4, respectively. Finally, the olefinic carbon atoms appeared at δ 143.7 and 112.4 corresponding to C7 and C8, respectively.

The DEPT (135) (CD_3OD) spectrum showed only four signals in the positive direction at δ 128.0, 113.7, 143.7, and 112.4 ppm corresponding to (C2 and C6), (C3 and C5), C7, and C8, respectively.

Ferulic Acid (6). This compound belongs to group A (II) and group B (II) which was isolated from the crude sample by TLC on silica gel plates as a white powder using the solvent methanol/dichloromethane/ammonia (30 : 70 : vapor). The third highest band ($R_f = 0.5$) was desorbed from silica using a mixture of distilled methanol and chloroform. The solvent was evaporated to give this compound.

The mass spectrum showed the molecular ion at *m/z* 193 (M-H), which corresponds to the correct molecular formula $C_{10}H_{10}O_4$.

The ^{1}H-NMR (CD_3OD) spectrum showed the presence of one methoxyl group signal at δ 3.68 ppm and three aromatic protons signals resonating as two doublets at δ 6.62 and 6.89 ppm ($J = 8.12$ Hz) corresponding to C5-H and C6-H and a singlet at δ 6.98 ppm corresponding to C2-H. Also, the spectrum showed the presence of two olefinic protons resonating as two doublets, one of them was assigned for the proton attached to the aromatic ring (δ 7.43 ppm, $J = 15.9$ Hz) while the other assigned to the proton attached to the carboxylic acid group (δ 6.13, $J = 15.9$ Hz).

The ^{13}C-NMR (CD_3OD) spectrum showed signals for ten carbon atoms. The signal at δ 168.0 was assigned to the carbonyl carbon. The six signals for aromatic carbon at δ 124.8, 113.4, 147.4, 146.3, 112.8, and 120.9 were assigned to C1, C2, C3, C4, C5, and C6, respectively. The methoxyl carbon resonated a signal at δ 53.4. Finally, the olefinic carbon atoms appeared at δ 143.9 and 108.6 corresponding to C7 and C8, respectively.

The DEPT (135) (CD_3OD) spectrum showed only six signals in the positive direction at δ 113.4, 112.8, 120.9, 143.9, 108.6, and 53.4 ppm which correspond to C2, C5, C6, C7, C8, and methoxyl carbon, respectively.

Caffeic Acid (7). This compound belongs to group A (III) which was isolated from silica gel column of the crude sample as a yellow powder using the solvent methanol/chloroform (10 : 90).

The mass spectrum showed the molecular ion at *m/z* 179 (M-H), which corresponds to the correct molecular formula $C_9H_8O_4$.

The ^{1}H-NMR (CD_3OD) spectrum revealed the presence of two doublets at δ 6.68 and 6.83 each with coupling constant of 8.1 Hz assigned to the two protons at C-5 and C-6, respectively. The sharp singlet at δ 6.93 was assigned to the aromatic proton at C-2. The two methine protons at positions C-7 and C-8 appeared as two doublets at δ 7.45 ($J = 15.8$ Hz) and 6.11 ($J = 15.8$ Hz), respectively.

The ^{13}C-NMR (CD_3OD) spectrum showed the presence of nine carbons in the spectrum in which five were methine carbons (three aromatic carbons and two aliphatic carbons) at 113.5, 112.1, 119.9, 144.1, and 112.5 ppm assigned to C-2, C-5, C-6, C-7, and C-8, respectively. The four quaternary carbons at 124.8, 143.8, 146.5, and 168.1 ppm were assigned to C-1, C-3, C-4, and C-9, respectively.

The DEPT (135) (CD_3OD) spectrum showed only five signals in the positive direction at δ 113.5, 112.1, 119.9, 144.1, and 112.5 ppm which correspond to C-2, C-5, C-6, C-7, and C-8, respectively.

3.2. Quantitative Determination of Phenols in Crude Sample. The concentrations of the phenolic compounds identified in the crude sample of OMWW were determined quantitatively by preparing a solution of crude the sample with 0.5 mg/L IS and then the compounds were quantified by comparing the relative peak area (RPA) for each compound in the chromatogram of the diluted crude sample with the RPA of the same compound in the chromatogram of the standard solution.

The GC-MS chromatogram of the prepared diluted sample is shown in Figure 3.

Table 1 reports the retention times, characteristic ions (*m/z*), as well as the LOD and LOQ extracted from the GC-MS spectra of the seven isolated phenolic compounds.

Calibration curves were constructed by a linear regression of the peak area ratio of the individual phenolic standard to the IS, versus the concentration of each phenolic standard in the solution mixture. As a result, the calibration curves

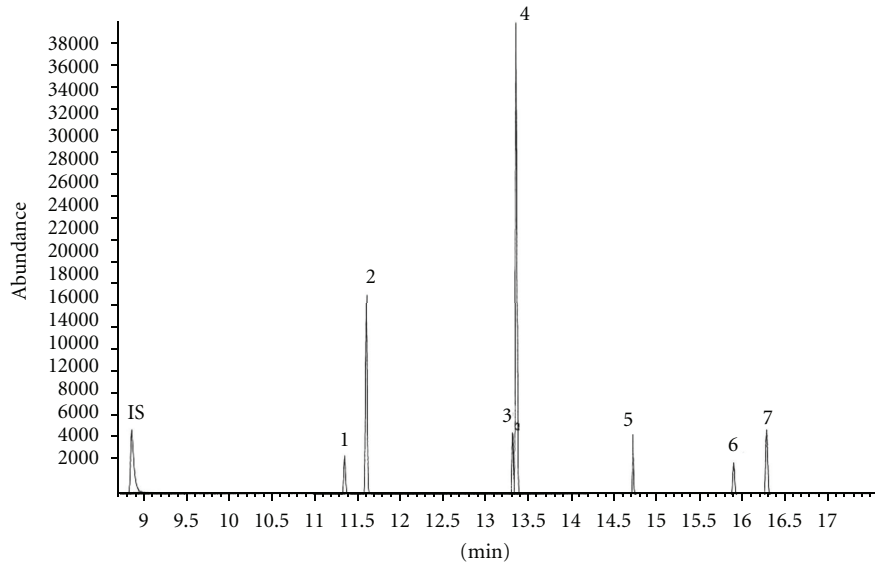

Figure 3: GC-MS chromatogram of the prepared crude sample with 0.5 mg/L IS.

Table 1: GC-MS parameters of silylated derivatives of the phenolic compounds 1–7.

Compound	Retention time (min)	Identified ions (*m/z*)	LOD (ppb)	LOQ (ppb)
1	11.37	220, 205, 131, 103	2.93	9.78
2	11.62	282, 267, 179, 73	1.23	4.09
3	13.34	312, 297, 282, 267, 253, 223	1.54	5.15
4	13.38	370, 267, 179, 73	0.33	1.09
5	14.74	308, 293, 219, 73	3.74	12.45
6	15.92	338, 323, 308, 249, 73	1.19	3.98
7	16.30	396, 381, 219, 191, 73	2.82	9.40

were linear for all phenolic compounds with regression coefficient of $r^2 \geq 0.9913$ in the working range.

The results of the analysis of the extract from OMWW are reported in Table 2. The concentrations are expressed as the amount (mg) of each phenolic compounds detected in the extract obtained from 1 L wastewater.

4. Conclusions

In conclusion, separation and analysis of the phenolic compounds extracted from Jordanian OMWW was achieved using a new procedure. Hydroxytyrosol and Tyrosol are the predominant products in Jordanian OMWW with relatively high concentrations of 315.9 mg/L and 210.6 mg/L, respectively. GC-MS has appeared to be a simple and sensitive analytical tool for the determination of phenolic compounds in olive mill wastewater.

Table 2: Concentration of identified phenolic compounds in OMWW.

Compound	Concentration of OMWW (mg/L)
trans-Cinnamic acid	105.3
Tyrosol	210.6
Vanillic acid	128.7
Hydroxytyrosol	315.9
p-Coumaric acid	117.0
Ferulic acid	93.6
Caffeic acid	140.4

We recommend studying the following parameters and their effect on the constituents obtained and their respective concentrations: (a) effect of method of olive oil pressing, (b) type of olive trees, (c) age of olive trees, (d) area where olive trees are grown, and (e) storage of wastewater.

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