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Profiling and evaluation of phenolic compounds in olive mill wastewater in Jordan

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ABSTRACT

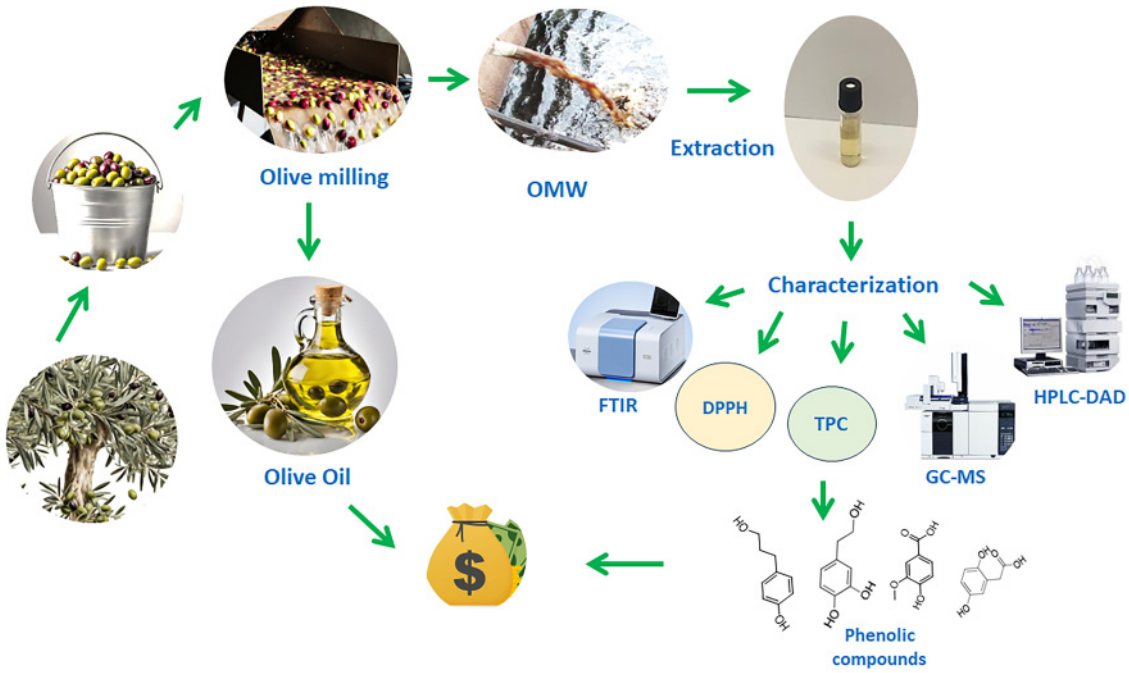
Phenolic compounds (PCs) are prevalent in olive mill wastewater (OMW), offering numerous health benefits but concurrently posing environmental challenges. This study aims to comprehensively characterize PCs in OMW, both qualitatively and quantitatively. An accurate, sensitive, and cost-effective method was successfully developed for extracting and quantifying seven PCs in OMW. The extraction procedure was optimized to achieve the maximum recovery using a liquid-liquid extraction method. Concentrations of the identified PCs were determined using a high-performance liquid chromatography-diode array detector (HPLC-DAD). The seven PCs peaks were successfully separated on an RP-C8 column within a 23-minute runtime using a gradient mobile phase. The method underwent thorough validation, producing satisfactory results. Subsequently, the developed method was applied to analyze OMW from four olive mills in Jordan, revealing PC concentrations ranging from 139 to 430 mg/L. Tyrosol and hydroxytyrosol were identified as the most abundant compounds. Additionally, gas chromatography-mass spectrometry (GC-MS) separated and identified forty PCs. The total phenolic content was quantified, reaching a value of 1839 mg/L. Moreover, the antioxidant activity was assessed, yielding a maximum value of 95.8%. These results underscore the substantial levels of PCs in OMW, highlighting the importance of economically utilizing this water.

Key words: GC–MS, HPLC-DAD, Jordan, olive mill wastewater (OMW), phenolic compounds

HIGHLIGHTS

- A novel HPLC-DAD method has been developed to determine PCs in OMW.
- This method was employed for the analysis of PCs in OMW samples from Jordan, revealing that tyrosol and hydroxytyrosol were the most abundant compounds.
- Forty PCs were identified in OMW utilizing GC/MS.
- The results underscore the richness of PCs in OMW and the potential for economically harnessing this resource.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Olive oil is a key component of the Mediterranean diet due to its many uses, health benefits, and long shelf life. The International Olive Council estimated worldwide production at 2,444 tons in 2015 (Kiritsakis *et al.* 2017). Approximately 10 million hectares of land worldwide are home to a staggering 900 million olive trees, with the Mediterranean Basin containing 98% of them (Zghari *et al.* 2017). The olive oil production system generates olive oil, solid residue, and liquid phase. The liquid phase is called the olive mill wastewater (OMW).

Global OMW production exceeds 30 million cubic meters annually (De Marco *et al.* 2007).

Jordan ranks 8th in the world in the production of olive and olive oil. The olive milling industry is a major contributor to the Jordanian economy, with olive oil generating approximately 100 million Jordanian dinars in annual revenue (Abdel & Hiary 2015). In Jordan, there are 25 million olive trees that produce approximately 220,000 tons of olives, 35,000 tons of oil, 60,000 tons of olive pomace, and about 200,000 m^3 of OMW annually. These products come from around 130 olive mills scattered throughout the Kingdom of Jordan (Khdair *et al.* 2019).

Olive oil can be obtained through two main processes, a two-phase process and a three-phase process (Frascari *et al.* 2016). The three-phase process typically generates more OMW, producing 1,200 L/ton compared to 200 L/ton for the two-phase process (Khdair & Abu-Rumman 2020).

OMW consists mainly of vegetation water (from the olive fruit), washing water, and water added during malaxation and during pressing (Khdair *et al.* 2019).

OMW is acidic, and varies in color from dark red to black, with a mixture of water (83–96%), minerals (2%), and organic matter (4–16%) (Zghari *et al.* 2017). The organic content of OMW includes sugars, tannins, nitrogen-containing compounds, pectins, lipids, and phenolic compounds (PCs) (Belaid *et al.* 2013). The chemical composition of OMW is subject to various factors, such as weather conditions, olive variety, fruit ripeness, and the method used for olive oil extraction (Khdair *et al.* 2019).

PCs are a significant group of naturally occurring compounds in plants. The health benefits of PCs and their use in the food, cosmetics, and pharmaceutical industries can justify the necessary work required to extract these compounds (Jerman Klen & Mozetič Vodopivec 2011). The beneficial health properties of PCs can be attributed to their antioxidant, anti-inflammatory, antimicrobial, and anticarcinogenic activities (Chanioti & Tzia 2018).

The PCs in olive fruits and their pulp are more soluble in water than in oil. Therefore, PC concentrations in the aqueous phase tend to be higher than in the oil phase, with concentrations in the OMW ranging from 0.5 to 25 g/L (Kallel *et al.* 2009). Thus, recently, OMW has been widely viewed as a natural source of PCs.

On the other hand, the presence of PCs in wastewater is one of the biggest environmental challenges facing the olive milling industry. The toxic effect of PCs on plants, microorganisms, and aquatic life limits the possibility of the direct reuse of OMW in irrigation. Therefore, OMW is usually dumped in landfills or waterways. This, in turn, generates another problem due to the unpleasant odors that arise from the interactions of PCs with other materials (Solomakou & Goula 2021). Moreover, there is an additional cost associated with transporting the OMW away from agricultural land for dumping.

The development of analytical methods for determining PCs in OMW serves a dual purpose, addressing environmental concerns and regulatory compliance, as well as ensuring efficient wastewater treatment and the overall sustainability of olive oil production processes. In addition, it allows for tapping into the economic potential of these compounds.

Several studies focused on developing new extraction methods for PCs in order to utilize them as natural antioxidants in OMW (Venturi *et al.* 2017). Some researchers employed liquid–liquid extraction, with ethyl acetate as the major solvent, while other articles reported the use of solid-phase extraction (SPE) (Jerman Klen & Mozetič Vodopivec 2011).

For the quantitation of PCs, chromatographic methods were mainly applied; for example, Zghari *et al.* (2017) applied gas chromatography with a mass spectrometry detector (GC–MS) for the determination of tyrosol, catechol benzoic acid, and *p*-hydroxyl coumaric acid in OMW, and Azaizeh *et al.* (2012) used high-performance liquid chromatography with an ultraviolet detector (HPLC-UV) for the determination of hydroxytyrosol (HTy), tyrosol, vanillic acid, ferulic acid, and *p*-coumaric acid in OMW.

However, up to our literature survey, no HPLC coupled with a diode array detector (HPLC-DAD) method is available for the simultaneous determination of HTy, protocatechuic acid, tyrosol, homovanillyl alcohol, 4-hydroxy benzoic acid, caffeic acid, and vanillic acid in OMW.

The main objective of this study was to provide a comprehensive understanding of the quantitative and qualitative aspects of phenolic content in OMW in Jordan. This goal was achieved by (1) developing a cost-effective and efficient technique for extracting PCs from OMW, (2) fine-tuning an HPLC-DAD method for precisely quantifying seven PCs in OMW, (3) profiling the PCs present in OMW using GC–MS, (4) quantifying the total phenolic content of OMW, (5) evaluating the antioxidant activity of OMW, and (6) analyzing the Fourier transform infrared (FTIR) spectra of OMW samples.

2. MATERIALS AND EQUIPMENT

2.1. Standards

4-hydroxy benzoic acid (HBA) (99%), homovanillyl alcohol [4-hydroxy-3-methoxyphenethyl alcohol] (HVA) (99%), tyrosol [2-(4-hydroxyphenyl) ethanol] (Tyr) (98%), caffeic acid (CA) (98%), vanillic acid (VA) (97%), protocatechuic acid (PCA) (99.71%), hydroxytyrosol [3-hydroxytyrosol] (HTy) (98%), homogentisic acid (HGA) (98%) were used as internal standard (IS) for HPLC-DAD. These compounds along with N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) and 1 mg/L of α-naftol as IS for GC–MS were procured from Sigma Aldrich. Folin-Ciocalteu reagent (2 M) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (98%) were bought from Sigma Aldrich (Germany). All the solvents employed were of HPLC or HPLC/GC grade.

2.2. Sampling

Samples of OMW were obtained from four olive mills across different regions of Jordan: Al-Salt, Irbid, Karak, and Amman, as depicted in Figure 1. These mills employ a three-phase extraction method for olive oil production. Sampling was done in October and November 2021 during the middle–late milling period. Each sample was taken from the discharge of the olive mills and had a volume of 20 l. The collected samples exhibited an opaque, dark brown-red color and were turbid. Subsequently, all samples were stored in sealed plastic containers at a temperature of 4 °C to prevent the auto-oxidation of PCs.

2.3. Chromatographic instruments and conditions for the quantitative analysis of PCs

The optimized chromatographic analysis was performed utilizing a Shimadzu HPLC system (LC-20A) featuring a DAD (SPD-M20A). The HPLC-DAD method involves the separation of PCs on a C8 column (4.6 × 150 mm, 5 μm) that was maintained at a constant temperature of 40 °C. The mobile phase was comprised of solvent A, which was 0.5% (v/v) acetic acid deionized water, and solvent B, which was methanol. The system was run with the following gradient elution program 0–10 min, 5% B;

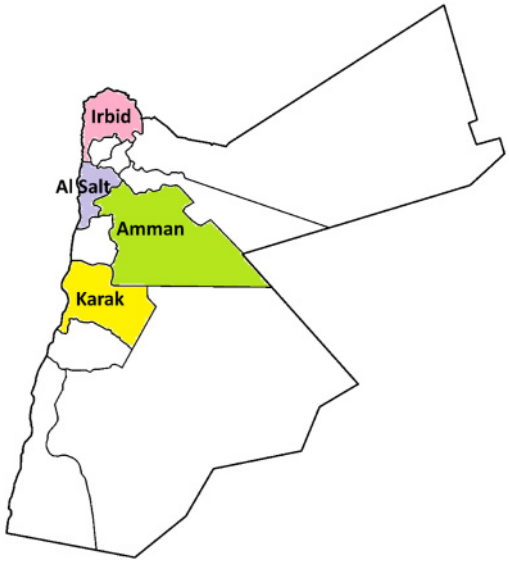


Figure 1 | Jordan map showing the selected sampling locations of OMW.

10–12 min, 30% B; 12–17 min, 33% B; 17–18 min, 38% B; 18–20 min, 50%; 20–23 min, 70%; 23–27 min, 95%; 27–35 min, 5%. The flow rate was 0.7 mL/min, the injection volume was 10.0 μL, and the data were collected at 279 nm.

2.4. The extraction method of PCs for HPLC-DAD analysis

2.4.1. Liquid–liquid extraction

The optimized extraction procedure was started by adding 1 mL of 0.1 M NaOH to 10 mL of filtered OMW in a 50 mL centrifuge tube. The solution was vortexed (VELP Scientifica) and centrifuged (Hermle, Germany). Then, the solution was extracted two times with 15 mL of *n*-hexane (3,000 rpm for 5 min) to remove lipid content. Next, the hexane layer was discarded, and the pH was adjusted for the aqueous layer to 2 with 2 M HCl. Then, the aqueous layer was extracted two times with 10 mL of ethyl acetate (3,000 rpm for 5 min). After that, the ethyl acetate layer was collected and evaporated using a nitrogen evaporator (Biobase, China) at 40 °C. Finally, the extract was reconstituted with 1.5 mL of methanol containing 100 mg/L of HGA (IS), and it was then transferred into a screw cap vial, and 10 μL was injected into the HPLC-DAD system. Figure 2 shows the summary of the optimized liquid–liquid extraction (LLE) procedure.

2.4.2. Solid-phase extraction

A SPE C18 cartridge was conditioned with 5 mL of *n*-hexane. Then, a mixture of 5 mL of the OMW sample and 5 mL of *n*-hexane was uploaded on the cartridge and eluted under vacuum. After that, PCs were eluted by 10 mL of methanol. Finally, 10 μL of this eluent was injected into the HPLC-DAD.

2.5. Development and validation of the HPLC-DAD method

A blank solution of OMW was prepared by extracting and cleaning the OMW sample 10 times. Then, a series of standard mixture solutions (5, 20, 40, 50, 100, 150, and 200 mg/L) containing all the investigated PCs (HTy, PCA, Tyr, HVA, HBA, CA, and VA) and the IS (100 mg/L) were prepared. The peak area of each compound relative to the peak of IS was calculated and plotted against the concentration to construct the calibration curves.

2.5.1. Specificity

To account for the specificity of the method, three injections were performed: (1) blank solution, (2) blank spiked with IS, and (3) the standard mixture of 20 mg/L of PC.

2.5.2. Limit of detection, limit of quantification, and precision

The blank OMW was spiked with IS and injected six times into HPLC-DAD to find the limit of detection and limit of quantification (LOD and LOQ).

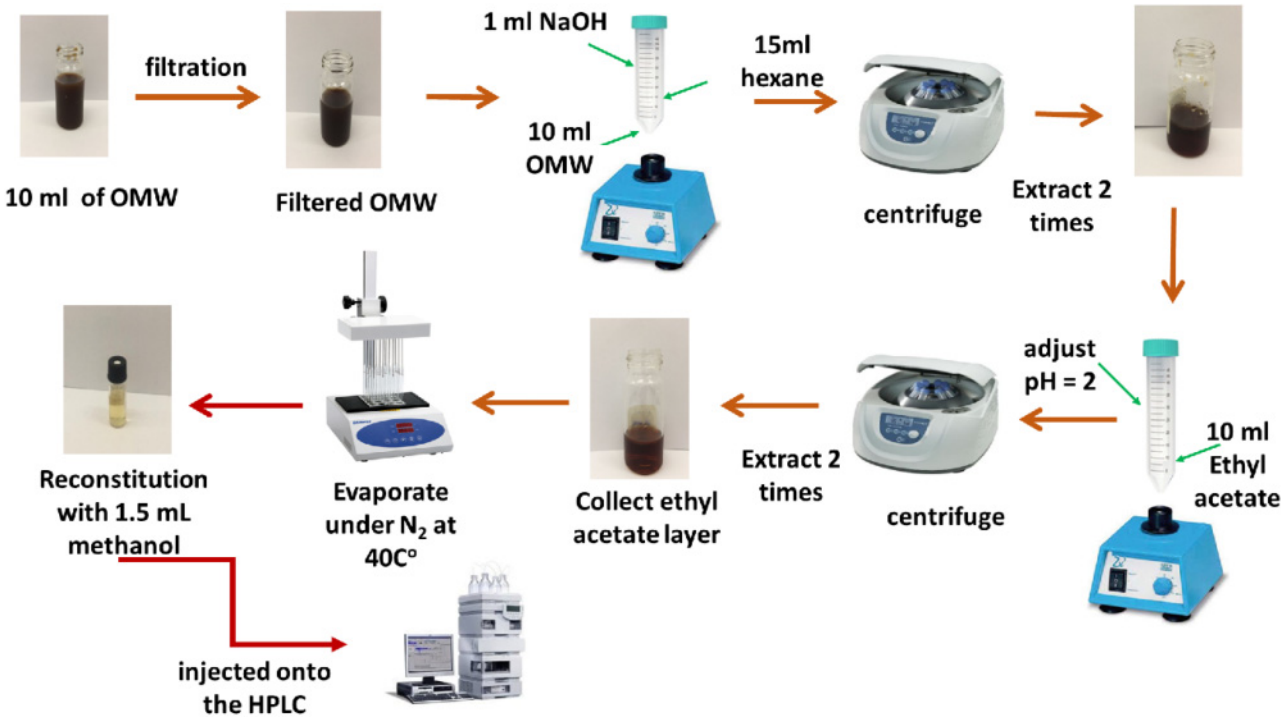


Figure 2 | Summary of the extraction procedure of PCs from OMW.

The precision of the instrument was evaluated through the injection of standard mixtures of PCs six times at three levels (50, 80, and 100 mg/L).

2.5.3. Recovery studies

Three solutions were prepared by fortifying 10.0 mL of a blank OMW sample with a standard mixture of PCs, resulting in concentrations of 50, 100, and 150 mg/L. Subsequently, these solutions underwent the extraction, purification, and analysis steps, as outlined in Figure 2. The recovery tests were conducted in triplicate.

2.5.4. Robustness test

The robustness of the method was evaluated by running the analysis of a standard mixture of PCs of 100 mg/L at different conditions of flow rate (0.6, 0.7, 0.8, 0.9, and 1 mL/min), column oven temperature (30, 35, 40, and 45 °C), and detector wavelength (279, 280, and 281 nm).

2.6. Estimation of the concentration of PCs in OMW using the developed HPLC-DAD method

Every OMW sample was meticulously prepared following the steps outlined in Figure 2, and it was subsequently injected into the HPLC-DAD column three times ($n = 3$). The relative peak area for each compound present in the chromatogram was determined, and the concentration of each compound was then computed by employing the previously established calibration curves.

2.7. Sample preparation for GC/MS analysis

For GC–MS analysis, the ethyl acetate extract was subjected to derivatization to enhance the volatility and thermal stability of PCs. BSTFA is mostly used as a derivatizing agent due to its high reactivity. Derivatization of PCs was performed according to the procedure described by Zafra *et al.* (2006). A mixture of 20 : 5 : 25 (v/v/v) BSTFA–pyridine–ethyl acetate (containing PCs) was prepared. Then, the mixture was shaken well for 2 min to derivatize the PCs. After that, 50 μL of the last solution was mixed with 50 μL of 1 mg/L of α-naftol as IS. Finally, a volume of 1 μL of the prepared solution was injected into the GC–MS instrument. To obtain a methanol extract, the same method was used but with methanol as the solvent instead of ethyl acetate.

The GC analysis was performed using a gas chromatograph fitted with an autosampler injector (Shimadzu QP2010 Ultra, Japan). A capillary column HP-5-fused silica column (30 m × 0.25 mm, film thickness 0.25 μm, (5%)-biphenyl–(95%)-dimethylsiloxane copolymer) was used. A silanized injector liner split/splitless (2 mm ID) was employed. The detection was carried out with a mass-selective single quadrupole detector. The injector temperature was 250 °C. The oven temperature was held at 80 °C for 3 min 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 mode was 70 eV. The mass range scanned was 150–550 m/z.

2.8. Total phenolic content

The total phenolic content (TPC) in each extract was evaluated spectrophotometrically using the Folin-Ciocalteu method described by Waterhouse (2002). Firstly, a series of gallic acid standard solutions were prepared (50, 100, 250, 500, 250, 500, and 750 mg/L). Then, 0.5 mL of each standard/sample was treated with Folin-Ciocalteu reagent. After that, 7.5 mL of a 20% sodium carbonate solution was added to the mixture, the volume was completed to 50 mL with deionized water, and the reaction was incubated for 2 h. In conclusion, the absorbance of the prepared solutions was measured at 765 nm.

2.9. Antioxidant activity

The DPPH radical scavenging assay described by Chanioti & Tzia (2018) was used to assess the antioxidant activity of OMW samples. First, 0.1 mL of the sample extract, which was constituted in methanol, was added to 3.9 mL of a 25 mg/L DPPH solution. Then, the solution was incubated in the dark at room temperature for 20 min. After that, the absorbance was measured at 515 nm. A blank solution was prepared by mixing 0.1 mL of methanol with 3.9 mL of 25 mg/L DPPH solution.

2.10. FTIR analysis

FTIR spectra were acquired utilizing a Bruker Vector 22 spectrometer from Germany, equipped with an integrated Michelson interferometer, and operated with Opus 5.5 software. The spectra were obtained by applying crude samples onto an attenuated reflection cell that featured a diamond crystal.

2.11. Statistical analysis

The experimental outcomes were replicated three times, and the results were expressed as means ± standard deviation. An analysis of variance test was conducted to estimate significant differences among samples. Differences among samples were considered to be statistically significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Optimization of the HPLC-DAD method

The identification of PCs in the OMW extracts was performed by HPLC-DAD by comparing the relative retention times with those of standard solutions. In the optimization of the HPLC method, the most suitable was selected for the following parameters: stationary phase, mobile phase, flow rate, wavelength, and column oven temperature. The criteria were to achieve the highest response and the best resolution at a suitable run time.

3.1.1. Selection of a stationary phase

In this study, different stationary phases (C8, C18, and diphenyl) were tested. Figure 3(a) shows chromatograms of the seven targeted PCs separated at these stationary phases. It can be observed that the best resolution, highest response, and suitable run time were achieved upon using the C8 column. This could be attributed to the higher polarity of the C8 column compared with the other columns. Therefore, it enables better separation and resolution of the polar PCs.

3.1.2. Optimization of the mobile phase

The second important step in the HPLC method development is the choice of a suitable mobile phase. Methanol and acetonitrile were tested as organic solvents in the HPLC system. Methanol gave better resolution for the seven PCs, as shown in Figure 3(b). The pH value for the aqueous mobile phase plays an important role in the separation of the target compounds. The most suitable pH was 3.1, which gave the best resolution. Several factors can affect the elution of the examined target

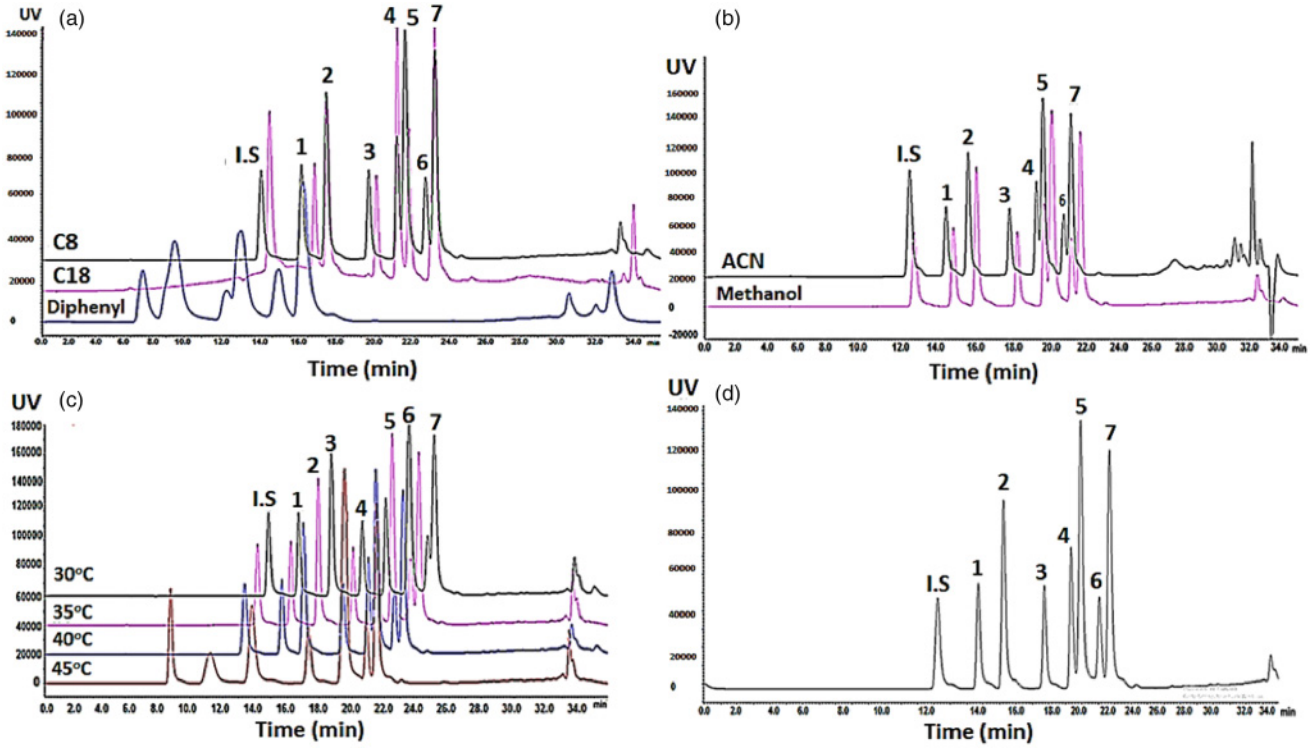


Figure 3 | Chromatograms of PCs standard mixture of 100 mg/L (a) at different stationary phases; (b) at different mobile phase solvents; (c) at different temperatures; and (d) representative chromatogram at the optimized conditions. (Peaks numbering are according to Table 1).

compounds. Firstly, the polarity of the individual PCs is reflected by the log *p*-value, as indicated in Table 1 where the lower log *p*-value refers to a less polar compound (Agach *et al.* 2014). The second factor is the molecular weight since PCs with low molecular weight are more polar and strongly interact with the relatively polar C8 stationary phase. Thirdly, the pKa values of each compound (Table 1) are important because when the pH value of the mobile phase is within the range of pKa ± 1, the compound will be in its unionized form. Increasing the pH above the range leads to the ionization of the compound. The combined effect of these factors, including the type of stationary phase, causes the compounds to elute in the following order: HTy, PCA, Tyr, HVA, HBA, CA, and VA.

Table 1 | Log *p*, pKa, retention times (t_R) of phenolic compounds, and system suitability results

No.	Target compound	Log *p*	pKa	t_R (min)	Number of theoretical plates (*N*)	Tailing factor	Capacity factor (k')	Selectivity factor	Resolution
	Acceptance criteria	–	–	–	>2,000	<2	>1	>1	>1.5
	HGA (IS)	–	–	12.2	–	–	–	–	–
1	HTy	0.89	9.45	14.6	22,742	1.6	1.9	1.3	4.9
2	PCA	0.86	4.48	16.0	26,687	1.6	2.2	1.6	3.4
3	Tyr	0.03	10.17	18.4	52,284	1.5	2.7	1.3	3.6
4	HVA	0.47	10.23	20.0	42,746	1.1	3.0	1.0	3.8
5	HBA	1.58	4.54	20.4	50,662	1.3	3.1	1.1	1.4
6	CA	1.15	4.58	21.6	42,213	1.1	3.3	1.0	2.5
7	VA	1.43	4.45	22.1	48,944	1.4	3.4	1.1	1.4

3.1.3. Selection of a flow rate

Different flow rates (0.6, 0.7, 0.8, 0.9, and 1 mL/min) were examined, and the best resolution was achieved at a flow rate of 0.6 mL/min. However, flow rate (0.7 mL/min) gave a slightly lower resolution at a shorter run time; hence, it was selected.

3.1.4 Selection for wavelength

In the optimization of wavelength values 279, 280, and 281 nm wavelengths for detector reading were tested, and the highest average response for the seven target compounds was at 279 nm.

3.1.5. Selection for column oven temperature

The chromatograms of the target PCs were obtained at four different oven temperatures, 30, 35, 40, and 45 °C, as shown in Figure 3(c). It can be noted that the best resolution was observed at a temperature of 40 °C. Meanwhile, at 45 °C, some peaks of the target compounds were absent, since it seems that HBA co-eluted with HVA at this temperature.

Therefore, the optimized conditions include using C8 as a stationary phase, methanol in the mobile phase, a flow rate of 0.7 mL/min, temperature adjusted to 40.0 °C, and a 279 nm detector wavelength. To the best of our knowledge, this is the first method involving the separation of the seven PCs: HTy, PCA, Tyr, HVA, HBA, CA, and VA and their determination using HPLC-DAD. Figure 3(d) represents a typical chromatogram of a standard mixture of the seven PCs under optimal conditions.

3.2. System suitability

System suitability is a test that is based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated (Branch 2005). System suitability parameters were evaluated for the newly developed HPLC-DAD method according to the Food and Drug Administration (FDA) guidelines (FDA & CDER 2018).

The evaluated system suitability parameters were the number of theoretical plates/efficiency (N), capacity factor (k), resolution (R_s), tailing factor or asymmetry factor (A_s), and separation factor (α).

Table 1 shows the calculated system suitability parameters and the acceptance criteria for each one. It can be observed that all system suitability parameters for all target compounds met the accepted criteria (FDA & CDER 2018). However, the resolution of HBA and VA was less than the required criteria.

3.3. Optimization of the extraction procedure

The optimization of the extraction procedure involves selecting the extraction method, extraction solvent, solvent ratio, and adding sodium hydroxide. The criteria were to achieve the highest recovery, analytical signal, and the simplest procedure. The chosen extraction procedures were evaluated and applied for the extraction of the analytes using several replicates of the same sample.

3.3.1. Extraction method

The first step in the optimization of the extraction method is to decide whether to apply LLE or SPE. The LLE was preferred for its simplicity and convenience. Figure 4(a) shows chromatograms of PCs extracted using LLE and SPE, and it can be noticed that LLE gives a higher recovery than SPE.

3.3.2. Adding sodium hydroxide

Figure 4(b) shows that adding NaOH before the *n*-hexane step gives a higher response compared to the response with no NaOH added. This can be attributed to the fact that sodium hydroxide ionizes the PCs, thus increasing their solubility in the aqueous layer. Therefore, the addition of NaOH enables the hexane layer to be discarded without worrying about the presence of residual PCs in it. It should be clarified also that *n*-hexane was added in order to remove most of the lipids and nonpolar compounds from the sample.

3.3.3. Acidification

Acidification is expected to increase the solubility of PCs in organic solvents (de Marco *et al.* 2007). Therefore, the pH was adjusted to 2 after the removal of the *n*-hexane layer to ensure that all PCs were in their unionized form.

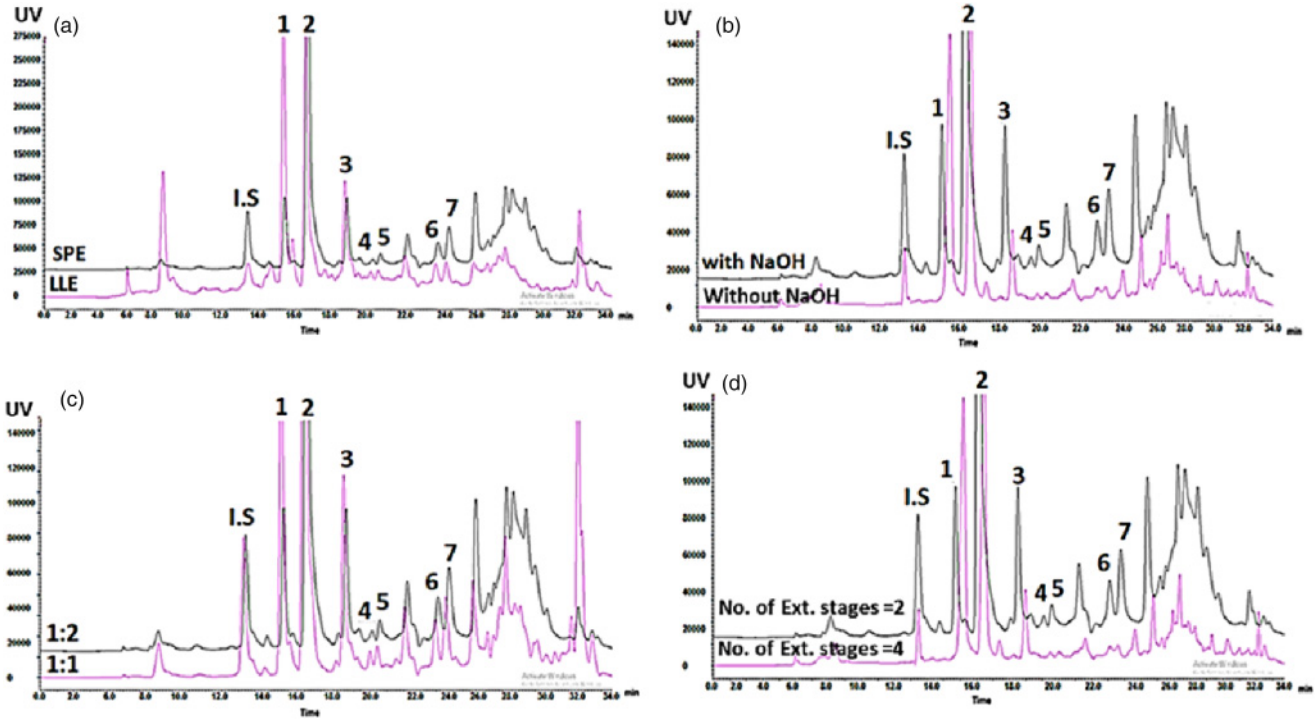


Figure 4 | Chromatogram of PCs from the Karak OMW sample extracted (a) using LLE and SPE; (b) with NaOH and without NaOH; (c) at different ratios of ethyl acetate to OMW; and (d) at a different number of extraction stages.

3.3.4. Extraction solvent

Ethyl acetate is the most used solvent to extract PCs from aqueous matrices such as OMW. Ethyl acetate was more effective at extracting PCs than other solvents, such as methyl ethyl ketone and diethyl ether (Zghari *et al.* 2017). Therefore, it was selected in our study as the extraction solvent.

3.3.5. Extraction solvent ratio

Two ratios of solvent to the sample were tested: 1:1 and 1:2. As shown in Figure 4(c), the 1:1 ratio gave the best recovery for the seven compounds.

3.3.6. Number of extraction stages

The number of replicates for the extraction step with ethyl acetate was adjusted. Figure 4(d) shows chromatograms of PCs after extraction 2 and 4 times, and as can be noticed, the highest recovery was achieved. This may be attributed to the loss of some PCs upon employing more extraction steps.

Considering all the tested optimizations, the optimal extraction conditions for the PCs from OMW include LLE with the preliminary treatment of the sample using NaOH and hexane. This is followed by extraction with ethyl acetate in a ratio of 1:1 with the sample, and the pH is adjusted to 2. These conditions, along with a full description of the extraction process, are shown in Figure 2.

3.4. Validation of the developed HPLC-DAD method

The developed HPLC-DAD method was validated in terms of linearity, precision, accuracy, recovery, robustness, the limit of detection, and limit of quantitation. The results of the validation process are discussed in the following sections.

3.4.1. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present (ICH 2005). To account for the specificity of the method, three injections into the HPLC-DAD system were performed.

Figure 5 shows chromatograms for the blank solution, the blank spiked with IS, and the standard mixture of PCs at 20 mg/L. Thus, no interference peaks between the blank and the compounds peaks.

3.4.2. Linearity

Calibration curves were constructed for each targeted PC by plotting the area ratio versus concentration. The area ratio of each target compound is calculated using Equation (1):

$$\text{Area ratio} = \text{Area of target compound}/\text{Area of I.S} \quad (1)$$

Table 2 shows the slope, intercept, and the correlation of determination R^2 for the seven target PCs. R^2 values ranged between 0.95 and 0.99 for all compounds. This indicates a good linear relationship between the area ratio and the concentration.

3.4.3. LOD and LOQ

The LOD and LOQ values were calculated according to Equations (2) and (3):

$$\text{LOD} = 3S_b/m \quad (2)$$

$$\text{LOQ} = 10S_b/m \quad (3)$$

where S_b is the standard deviation of blank and m is the slope of the calibration curve for each target analyte

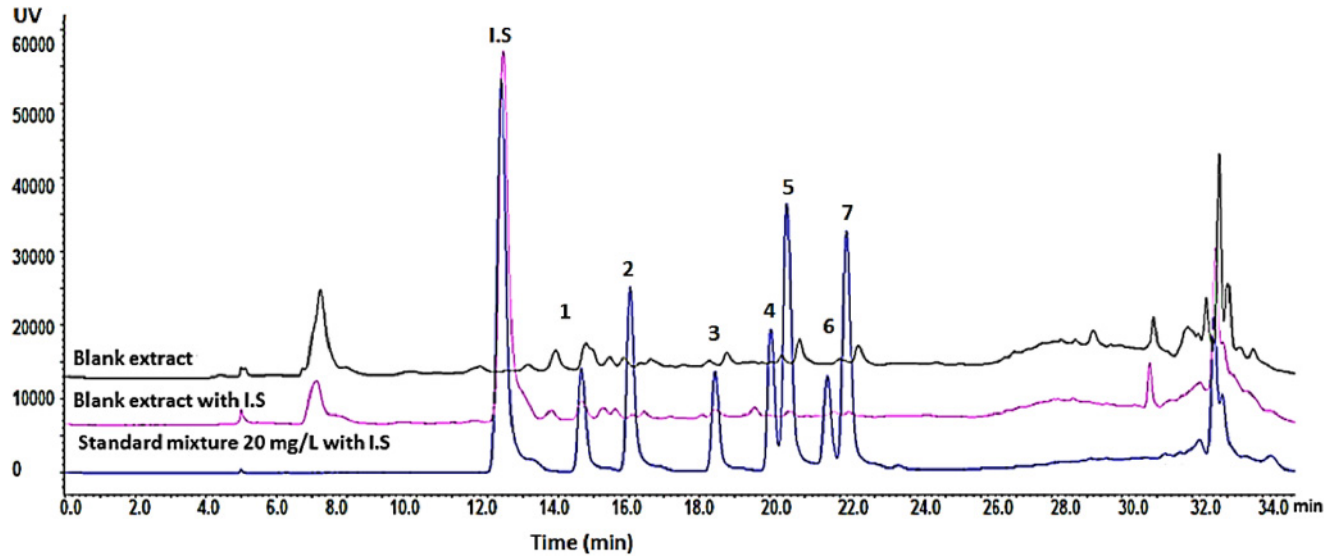


Figure 5 | Chromatograms of blank, blank with IS, and a standard mixture of PCs (C8, methanol, pH = 3.1).

Table 2 | Peak number, phenolic compound name, slope, intercept, LOD, LOQ, and recovery

Order of elution (peak number)	Compound name	Slope	Intercept	R^2	LOD (mg/L)	LOQ (mg/L)	Average %recovery
1	HTy	0.035	0.032	0.998	0.044	0.146	81.6
2	PCA	0.088	0.398	0.951	0.006	0.019	90.2
3	Tyr	0.015	0.025	0.997	0.064	0.213	94.7
4	HVA	0.011	0.015	0.986	0.042	0.140	88.1
5	HBA	0.023	0.091	0.995	0.006	0.021	95.6
6	CA	0.111	0.444	0.985	0.069	0.233	93.6
7	VA	0.019	0.136	0.996	0.006	0.021	97.6

Table 2 represents the LOD and LOQ values of each PC with their retention times. For example, the LOD value of HVA was 0.042 mg/L, which is lower than the value obtained by Shmeis *et al.* (2021) (1.57 mg/L) using HPLC-UV.

3.4.4. Instrument precision

The instrument precision was evaluated by injecting a standard mixture of PCs six times at three concentration levels and calculating the RSD. The RSD values for all the target compounds ranged from 0.24 to 1.07%. This indicated good instrument precision where RSD values meet the accepted criteria (15%) for trace analyses related to the HPLC-DAD instrument (Gustavo González & Ángeles Herrador 2007).

3.4.5. Recoveries of PCs

Recovery was tested at three levels 50, 100, and 150 mg/L, and the average recoveries were calculated using Equation (4).

$$\text{recovery}\% = \frac{\text{spiked sample concentration} - \text{non} - \text{spiked sample concentration}}{\text{spiked concentration}} \quad (4)$$

The recovery outcomes are detailed in Table 2. It is notable that all recovery percentages fell within the range of 81.6–97.6%, which aligns with the acceptable bounds for trace analyses (70–120%) as suggested by Gustavo González & Ángeles Herrador (2007). This range surpasses the limits described by Rahmanian *et al.* (2014).

3.4.6. Robustness test

The results suggest that the method demonstrated robustness in the face of variations in both mobile phase flow rate and wavelength. This is evident from the fact that the relative standard deviation (RSD%) values remained within the acceptable range (Gustavo González & Ángeles Herrador 2007). In contrast, the method exhibited susceptibility to changes in the column temperature, as evidenced by a relatively high RSD% value. This highlights the importance of exercising caution when adjusting the column temperature during analysis.

The significance of this method lies in its applicability to any study focused on the removal of the seven PCs from OMW. Moreover, the method has the potential to evaluate the economic feasibility of extracting these PCs from OMW by leveraging information about their initial concentrations.

3.5. Application of the method in the determination of PCs in OMW

The established HPLC-DAD method was utilized to assess the concentrations of PCs in OMW samples gathered from four olive mills in Jordan. The concentration for each specific compound was computed by employing the predefined multi-point calibration curve. The concentration of each compound in each sample as well as the summation of the seven PCs (Σ7PCs) are displayed in Table 3. Figure 6 illustrates chromatograms of sample extracts from each OMW sample, displaying the detected peaks of the investigated compounds.

Figure 7 depicts the distribution of PCs in OMW samples. A significant difference was observed in the total concentration of the seven PCs (Σ7PC) among the samples ($p < 0.05$). Notably, the Amman sample exhibited the highest levels of the seven PCs, reaching 430 mg/L. The observed variation can be attributed to climate differences, considering that the four olive mills are located in distinct regions in Jordan with average annual temperatures of 17.4, 17.6, 18.7, and 26.3 °C in Irbid, Amman, Al-Salt, and Karak, respectively. This implies that colder weather might promote higher levels of PCs, as increased temperatures are recognized for their role in accelerating the degradation and oxidation of PCs.

The olive cultivar types could also contribute to variations in the levels of PCs. However, it is important to note that the specific olive types collected from each region are unknown, and there is a possibility that the samples consist of a mixture of different olive varieties (Khdair *et al.* 2019).

Additionally, it was noted that HTy emerged as the most prevalent compound, with an average concentration of 99.5 mg/L in both Amman and Irbid samples, followed by tyrosol with an average value of 65.3 mg/L. This result is consistent with findings reported by Di Mauro *et al.* (2017), where HTy was identified as the most abundant compound. However, it is worth noting that the reported concentration in their study (545 mg/L) is higher than our findings. Meanwhile, in the Karak sample, the PCA was identified as the dominant compound.

Table 3 | Concentrations of target PCs compounds in the investigated samples

	Concertation (mg/L) ($\bar{X} \pm s$)				
Compound	**Karak**	**Irbid**	**Amman**	**Al-Salt**	**Average**
HTy	26.7 ± 0.4	157 ± 0.6	198 ± 0.5	16.2 ± 0.4	99.5 ± 0.96
PCA	77.3 ± 0.5	5.00 ± 0.2	37.7 ± 0.2	5.66 ± 0.09	31.4 ± 0.58
Tyr	52.2 ± 0.6	96.1 ± 0.1	67.6 ± 0.5	45.2 ± 0.8	65.3 ± 1.1
HVA	15.9 ± 0.05	10.0 ± 0.5	23.1 ± 0.7	11.4 ± 0.07	15.1 ± 0.86
HBA	11.2 ± 0.1	6.75 ± 0.4	16.9 ± 0.1	7.11 ± 0.06	10.5 ± 0.42
CA	34.3 ± 0.4	50.8 ± 0.8	39.7 ± 0.4	29.4 ± 0.3	38.6 ± 0.10
VA	10.0 ± 0.08	15.3 ± 0.05	46.3 ± 0.05	23.9 ± 0.5	23.9 ± 0.51
Total of 7 PCs (Σ7PCs)	228 ± 0.9	341 ± 1.2	429 ± 1.1	139 ± 1.1	284 ± 1.9
TPC (mg/L)	748	1,839	1,239	485	1,078
% Scavenging	95.0	95.3	93.8	95.8	95.0

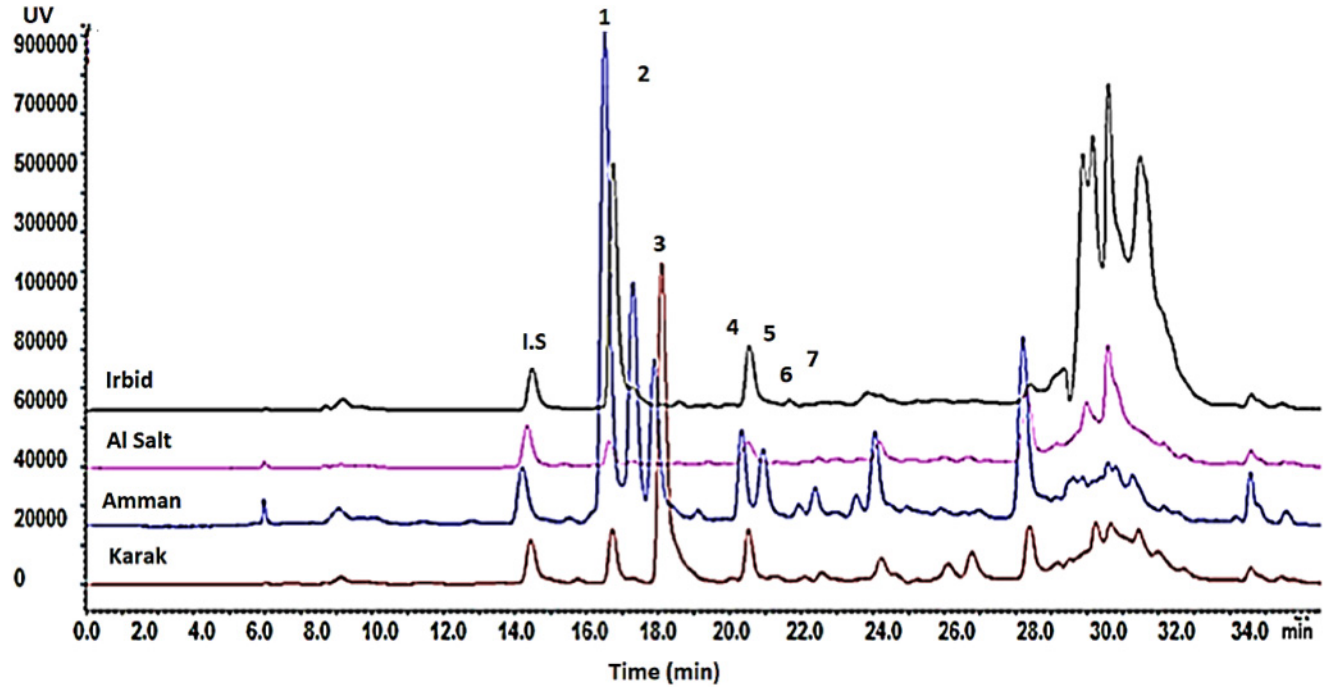


Figure 6 | Chromatograms of PCs extracted from the four OMW samples in Jordan.

3.6. PCs identified in OMW using GC/MS

Figure 8 depicts gas chromatograms illustrating the ethyl acetate and methanol extracts obtained from the OMW sample collected at the Irbid olive mill. Over 40 distinct PCs were identified within this specific sample. To tentatively identify these compounds, their mass spectra were compared with entries in the Nist 14 mass spectral library. The results of this identification are summarized in Table S1. These findings underscore the substantial phenolic content present in OMW. The number of compounds identified in this study exceeds the number obtained by Deeb *et al.* (2012), who only identified seven compounds.

3.7. Determination of TPC

Gallic acid was employed as a representative PC, and the TPC was quantified and expressed in mg/L of gallic acid equivalent. The TPC was determined utilizing the linear equation: $y = 0.0018x - 0.0774$, where y represents the absorbance measured at

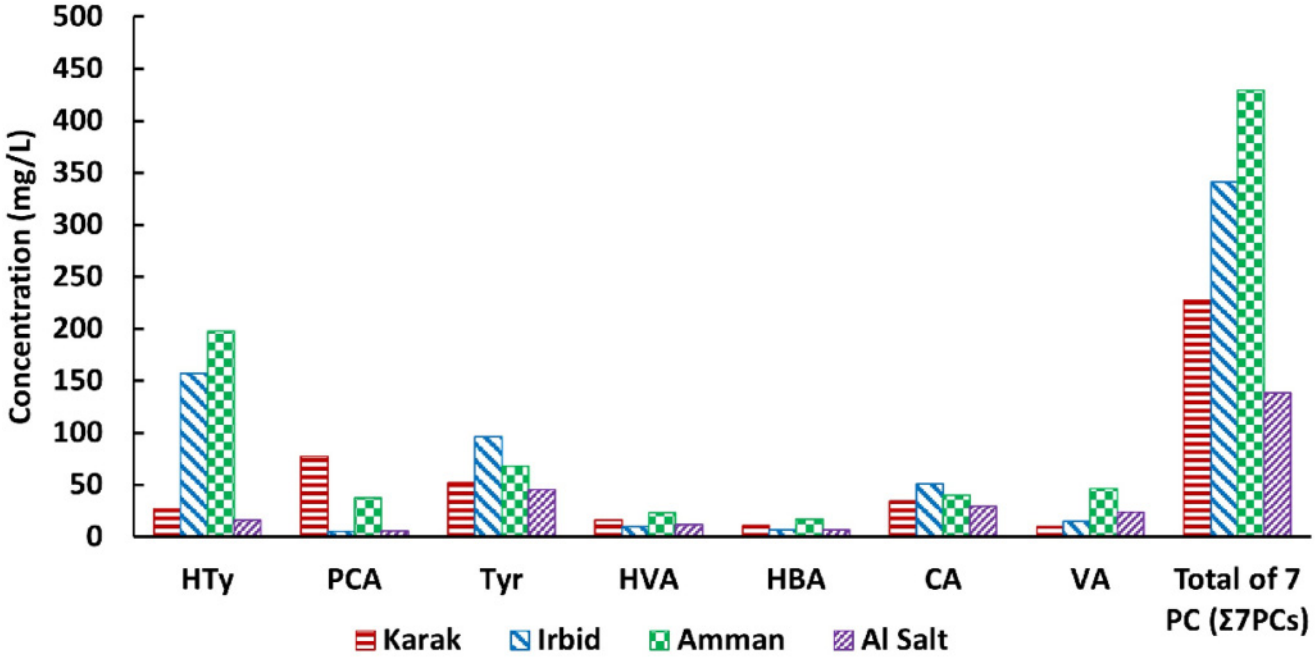


Figure 7 | Concentration of each phenolic compound in extracted samples.

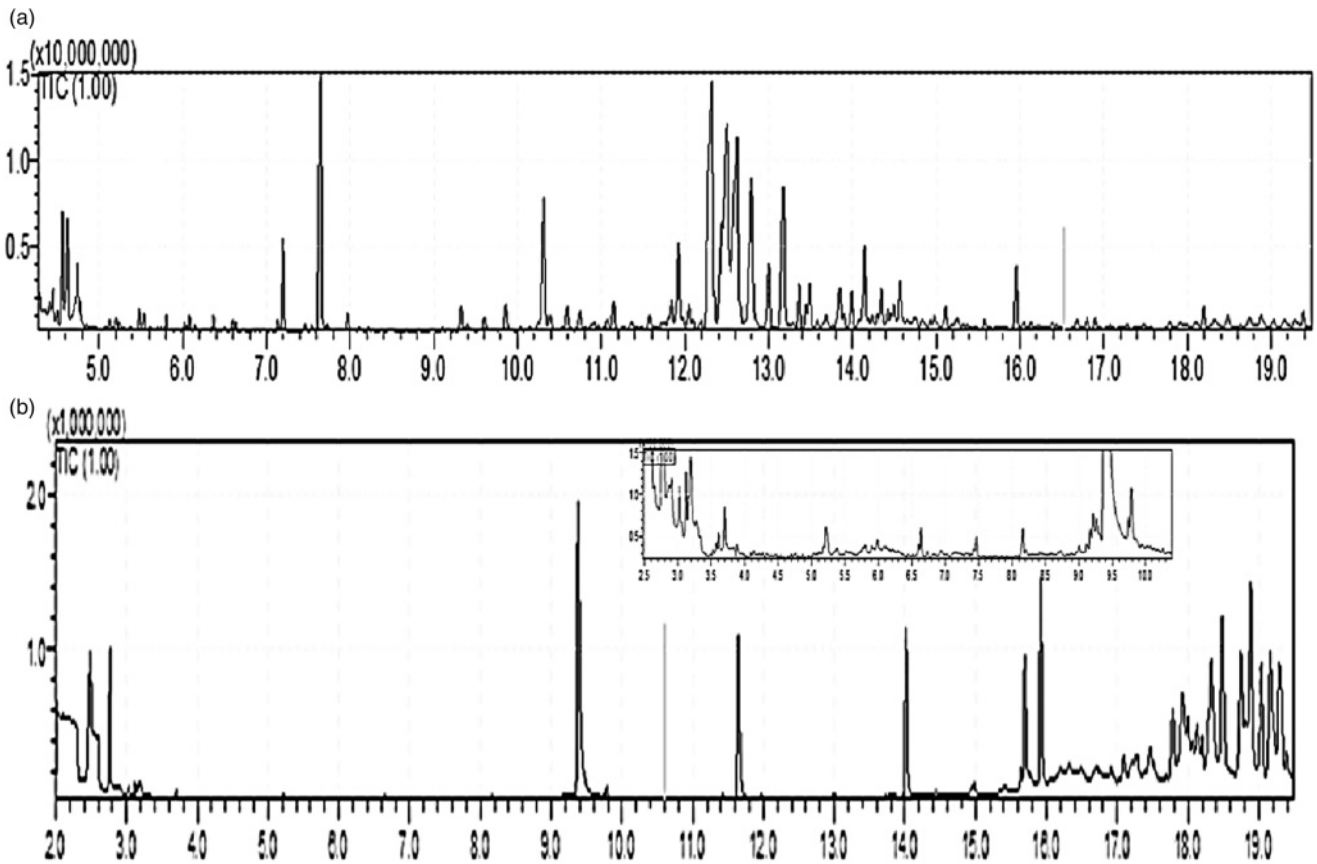


Figure 8 | Gas chromatograms of (a) ethyl acetate extract of OMW and (b) methanol extract of OMW.

760 nm, and x corresponds to the total phenolic content derived from the calibration curve, exhibiting a coefficient of determination (R^2) of 0.997.

The results of TPC in each extract are tabulated in Table 3. A significant difference ($p < 0.05$) was observed in TPC amounts among the different samples. Among the examined OMW, Irbid OMW was found to be the highest TPC with an average value of 1,839 mg/L, followed by Amman with 1,239.1 mg/L, Karak with 748.1 mg/L, and least TPC in Al-Salt with an average value of 485.2 mg/L. However, these results were higher than the Σ7 PCs, as the tested samples are expected to contain PCs other than the target seven PCs such as Gentisic acid, 4-Coumaric acid, and Acetovanillone (Shmeis *et al.* 2021). Also, the richness of the samples in PCs has been proven by the results of GC – MS analysis and is indicated in Figure 8 and Table S1.

Comparisons with TPC in OMW from other countries revealed significant variations in results. In Greece, Sygouni *et al.* (2019) reported a maximum TPC value of 1,020 mg/L. Additionally, Baazaoui *et al.* (2023) obtained a TPC value of 8,300 mg/L, which is considerably higher than the findings in this study. This variability can be ascribed to distinctions in weather conditions, olive cultivar varieties, fruit ripeness, and the methodology employed for olive oil extraction (Khdair *et al.* 2019).

3.8. Antioxidant activity

The antioxidant activity was performed using the DPPH radical scavenging assay. The percentage of the scavenging activity was calculated using Equation (5).

$$\%\text{Scavenging} = (A_o - A_s/A_o) * 100 \tag{5}$$

where A_o is the absorbance of blank (0.1 mL of methanol + 3.9 mL of 25 mg/L DPPH solution); A_s is the absorbance of the sample (0.1 mL of OMW extract + 3.9 mL of 25 mg/L DPPH solution).

The computed percentages of scavenging activities are detailed in Table 3. The Al-Salt OMW sample exhibited the highest scavenging activity percentage. It is worth noting that although a portion of the % scavenging activity can be attributed to the presence of PCs (as reflected by the TPC values), the Al-Salt sample does not possess the highest TPC. This could be attributed to substances with antioxidant properties other than PC, such as β-carotene (Murillo-Cruz *et al.* 2021). The percentage of scavenging activity ranged from 93.8 to 95.8% in the samples. These results reveal relatively high levels of scavenging activity in comparison to findings in Germany and Greece, where the range was 56–66 and 34.2–59.8%, respectively (Lafka *et al.* 2011; Azaizeh *et al.* 2012).

3.9. Characterization of OMW FTIR spectroscopic analysis

FTIR spectra of OMW extracts are shown in Figure 9. The spectra revealed the presence of the O–H hydroxyl group indicated by the broad peak at 3,306.3 cm^{-1}. The peak at 1,605.6 cm^{-1} is attributed to the C=C bond (1,650–1,600) cm^{-1}, the C–H bond is observed at 2,979.1 cm^{-1} (around 3,000), and aromatic CH is confirmed by the peaks in the range 1,600–1,500 cm^{-1}, while the carbonyl group C=O of the carboxylic acid at 1,707.5 cm^{-1}. Also, C–C is noticed from the peak at 1,381.4 cm^{-1}. It can be observed that the four OMW extracts have similar functional groups. FTIR results of this study are comparable to the results obtained by Zghari *et al.* (2017) who found similar FTIR spectra in OMW samples from Morocco.

Although the work presented in this study shares a common purpose with other reports, it distinguishes itself through several novel aspects. Firstly, it signifies the inaugural analysis of PCs in Jordan, emphasizing a unique exploration of this crucial aspect. The methodology employed demonstrates innovation by substituting acetonitrile with the more abundant methanol and incorporating NaOH in the extraction process. A distinctive feature of this study lies in the simultaneous application of liquid and gaseous chromatography, offering a comprehensive analytical approach. Notably, our results surpass previous studies, attesting the efficacy of our methodology. Furthermore, our research breaks new ground by providing, for the first time, data on total phenolic contents and antioxidant activity in treated wastewater from Jordan. Lastly, the study addresses a gap in the existing literature by conducting an HPLC-DAD analysis in OMW, contributing uniquely to this specific field of research.

3.9.1. Future prospects

The study's outcomes demonstrate the abundance of PCs in OMW, positioning it as a sustainable source with significant health and nutritional benefits. Consequently, future research should prioritize an economic feasibility study to explore the potential of upscaling the presented extraction method for generating commercial quantities of PCs. Given the established

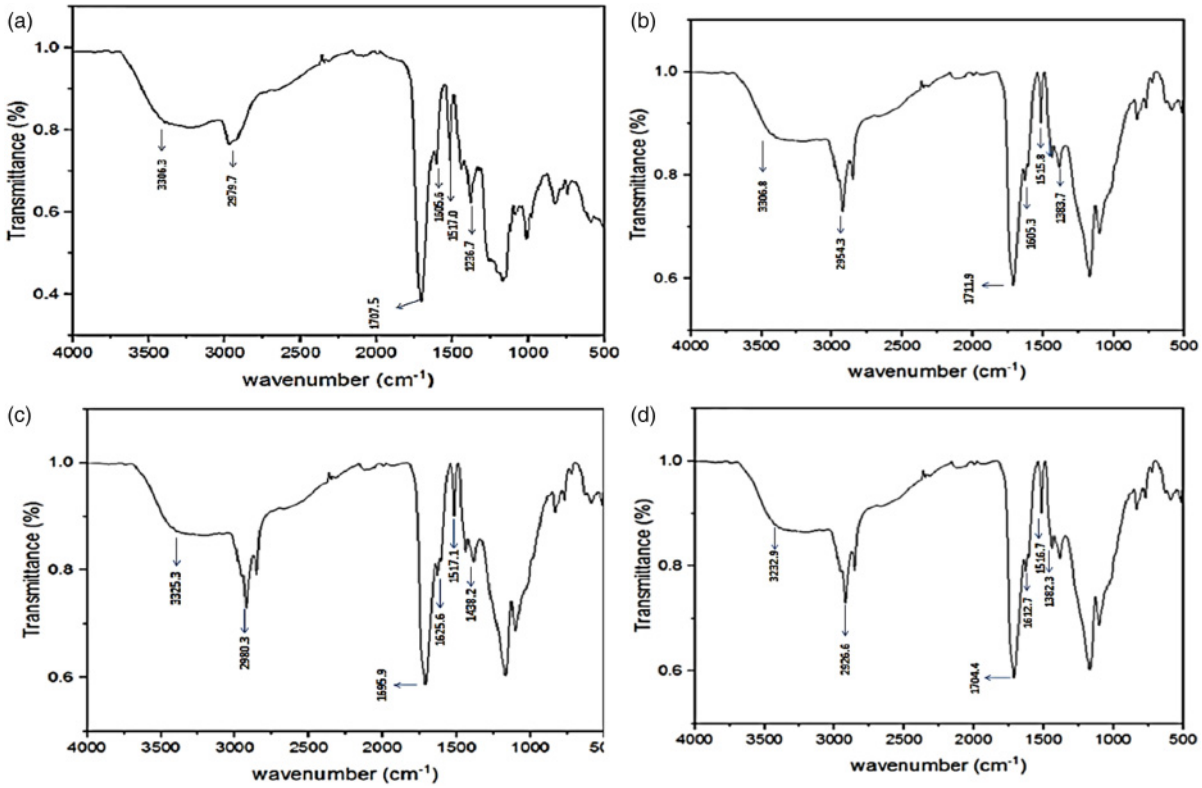


Figure 9 | IR spectrum for (a) Amman, (b) Al-Salt, (c) Irbid, and (d) Karak OMWs extracted samples.

value of HTy, the primary phenolic component in our OMW samples (valued at $1,000–2,000 per gram for scientific/experimental purposes), we anticipate the feasibility of scaling up the extraction process. This transformation of what was once an environmental concern into a financially viable resource exemplifies the achievement of sustainability.

Moreover, the knowledge gained about PC concentrations in OMW holds paramount importance for developing future methods to treat OMW, enabling its safe reuse in irrigation or proper disposal. Subsequent studies could further expand on this research by exploring additional PCs through preparative HPLC and incorporating a larger sample size of OMW for a more comprehensive understanding.

4. CONCLUSION

In this study, we developed a novel, efficient, and cost-effective method for extracting and quantifying seven key PCs present in OMW. The method was modified to establish optimal conditions for extraction, separation, and analysis. Through a comprehensive validation process and adherence to international guidelines for method analysis, efficacy and success were demonstrated by the method. The developed method was then employed to assess PC levels in OMW in Jordan. Significant levels of PCs were observed, with HTy identified as the most abundant compound. All target compounds were found in all the samples, albeit at varying concentrations and distributions that could be attributed to differences among the samples in soil quality, climate, surrounding environment, type of olive fruit, and fruit ripeness. More careful sampling should be considered in the future to account for these variations. The total phenolic content and antioxidant activity were also evaluated and revealed consistently high values across all samples, reinforcing the substantial value of OMW. The economic potential of OMW as a source of PCs, boasting both health and economic benefits, is highlighted by this study. Additionally, a database for selecting the proper treatment method for OMW is provided.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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