



## Quantitative analysis of phenolic compounds in Syrian olive mill wastewater by spectrophotometry and HPLC

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### ABSTRACT

Olive mill wastewater (OMW), generated by the oil extraction industry, represents a severe environmental problem due to its highly polluting organic load arising from phenols with low biodegradability. However, with its more than 30 phenols, OMW is regarded as a potential source of natural antioxidants. This study aims to identify phenolic acids from Syrian OMW. The phenols were extracted using different solvents: methanol, chloroform and ethylacetate. High performance liquid chromatography (HPLC) and spectrophotometric approaches were used to determine the phenols of OMW samples. Ethylacetate is the best solvent with phenolic compounds (493 mg/100 g). The isolated phenolic acids were ellagic acid, chlorogenic acid, caffeic acid, p-coumaric acid, cinamic acid.

### Introduction

The growing consumer demand for olive oil, as a consequence of its proved benefits for human health<sup>[1]</sup>, has become a positive factor for higher productions of this natural fat. Olive oil extraction industries are mainly located in Mediterranean countries: Spain, Italy, Greece, Portugal, Tunisia, Syria (4.8%), Turkey, Morocco and Algeria<sup>[2]</sup>.

Olive oil production involves one of the following extraction processes: i) press olive oil extraction, ii) three-phase centrifugal olive oil extraction, and; iii) two-phase centrifugal olive oil extraction. Each process generates waste products of different characteristics. Pressure and three-phase centrifugation systems produce considerably more liquid effluent which is so-called olive mill wastewater (OMW), vegetation water, or alpechin than the two-phase centrifugation process<sup>[3,4]</sup>. The properties of OMW vary enormously with type of the process, climatic conditions and region.

The production process of olive oil usually yields next to 20% olive oil, a 30% semi-solid waste and 50% aqueous liquor<sup>[3]</sup>. 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<sup>3</sup> per year of OMW<sup>[5]</sup>.

OMW is a dark red- to black- colored, acidic (pH 3 - 6) liquid of high conductivity. This black wastewater liquid

is highly pollutant, since it presents high Biological oxygen demand (BOD) values (50 – 100 g O<sub>2</sub>/L) as well as chemical oxygen demand (COD) values (80 – 200 g O<sub>2</sub>/L)<sup>[6]</sup>. These are 200 – 400 times higher than those of a typical municipal sewage<sup>[7]</sup>.

The low pH and the high amounts of organic matter include sugars, tannins, phenols, polyalcohols, pectins, and lipids<sup>[8]</sup> make it very difficult to be purified. Deterioration of natural water bodies due to olive mill wastes is a serious problem as indicated by coloring, appearance of an oily shine, and increased oxygen demand.

The powerful pollutants prohibit OMW to be directly discharged into water or onto land. Several treatment procedures including physical, chemical, biological or combined technologies have been tested to reduce undesirable properties of OMW prior to disposal.

The major factor of the environmental problem is because of the high phenols of OMW streams, that is to say it may contain up to 10 g/L of phenols<sup>[9]</sup>. These compounds are difficult to decompose<sup>[10]</sup> and present phytotoxicity<sup>[11,12]</sup>, toxicity against aquatic organisms<sup>[13]</sup>, or suppression of soil microorganisms<sup>[14]</sup>. The olive fruit is very rich in phenols, but only 2% of the total phenols of the olive fruit pass in the oil phase, while the remaining amount is lost in the OMW (approx. 53%) and in the pomace (approx. 45%)<sup>[15]</sup>.

In general, phenols are thought to deliver health benefits by several mechanisms, including: (1) direct free radical quenching, (2) protection and regeneration of other

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dietary antioxidants, (3) chelation of metal ions. So they act as antibiotic/antiviral, anti-inflammatory, and protective agents from diseases<sup>[16,17]</sup>. Notably, OMW can provide a cheap source of phenols with strong antioxidant properties.

Olive phenols are biologically active compounds and contain numerous simple and complex substances that are characterized by multifunctional moieties. The simple phenols such as vanillic acid, gallic acid, cumaric acid, caffeic acid, tyrosol and hydroxytyrosol possess alkene, alcoholic, and carboxylic groups whereas more complex phenols such as secoiridoids (oleuropein and ligstroside) possess glycosidic and monoterpenoid units<sup>[18]</sup>.

Recovery of phenols from OMW by means of appropriate technologies will not only avoid waste disposal problem, but also will allow obtaining added-value phenols as by-products. These phenols may be used as natural ingredients in variety of food products to enhance their quality.

The present study deals with the identification of phenolic acids by spectrophotometry and HPLC from OMW.

## Materials and Methods

**Chemicals and reagents:** All chemicals and reagents used were either analytical or HPLC grade. The Folin Ciocalteu phenol reagent, gallic acid and phosphoric acid were purchased from Sigma-Aldrich (St. Louis, USA). Analytical grade of hydrochloric acid, sodium hydroxide, sodium carbonate, methanol, chloroform and ethylacetate were purchased from Merck (Merck, Darmstadt, Germany).

**OMW samples preparation:** OMW was collected from olive mill press located in Sweida - Syria. The OMW was stored in closed plastic containers in the dark at 4°C.

**Extraction of phenolic compounds:** A modification of procedure for extraction of phenols published by Winkelhausen *et al.*,<sup>[19]</sup> was employed. In the first step, the samples were extracted, three times successively, with hexane in the ratio 1:4 (v/v) for OMW to remove the residual oil and pigments. In the second step, the phenols were extracted from the samples using methanol adjusted to pH 9 with NaOH. The ratio of OMW and methanol was 1:6 (v/v). After two successive extractions, the total methanol extract was filtered (0.45 µm) and then concentrated by a rotary-evaporator model IKA RV 10 digital with oil free air compressor model SL400G 220v 50Hz with integrated heating bath HB 10 digital (3 liters) at 30°C and 45 rpm until we get the dry residue and then dissolved in 25 ml methanol. Also, phenols were extracted in OMW according to Winkelhausen *et al.*,<sup>[19]</sup>. The samples were extracted, three times successively, with hexane in the ratio 1:4 (v/v) for OMW to remove the residual oil and pigments. In the next step, phenols were extracted from the samples using chloroform. The ratio of OMW and chloroform

was 1:6 (v/v). After two successive extractions, the total chloroform extract was filtered (0.45 µm) and then concentrated by a rotary-evaporator model IKA RV 10 digital with oil free air compressor model SL400G 220v 50Hz with integrated heating bath HB 10 digital (3 liters) at 30°C and 45 rpm until we get the dry residue and then dissolved in 25 ml methanol. Finally, phenols were extracted in OMW using ethylacetate according to the method of Elena De Marco *et al.*,<sup>[20]</sup>. Ten ml of OMW were mixed with 15 ml of hexane; the mixture was vigorously shaken and centrifuged for 5 min at 3000 rpm. The phases were separated and washing was repeated two times successively. Extraction of phenols was then carried out with 10 ml of ethylacetate after acidification by HCl to pH 2. The phases were separated and the extraction was repeated four times successively. The ethylacetate was evaporated under vacuum and the dry residue was dissolved in 3 ml of methanol and this solution was used for determination of phenols.

**Determination of total phenols in olive extracts:** Phenol concentrations were determined using the Folin-Ciocalteu assay<sup>[21]</sup>. The Folin-Ciocalteu method (FCM), based on the reduction of a phosphowolframate-phosphomolybdate complex by phenols to blue reaction products, was used to determine phenols. Briefly, 3 ml of Nanopure water, 2 ml of sample, and 0.2 ml of Folin-Ciocalteu reagent were added to a 10 ml volumetric flask. The contents were mixed and allowed to stand for 5-8 min at room temperature. Next, 4 ml of a 7% sodium carbonate solution was added, followed by the addition of Nanopure water filled to volume. Solutions were mixed and allowed to stand at room temperature for 2 h. Sample aliquots were filtered through a Whatman 0.45 µm (polytetrafluoroethylene) filter prior to the determination of phenols' concentration using a Beckman DU 7400 spectrophotometer monitoring at 750 nm against blank (100 µl water). The absorbance was measured twice for each sample. The absorbance of the samples was compared with calibration curve to obtain the phenols' content. The calibration curve was prepared using gallic acid as the standard phenol at concentrations of 0.2, 0.4, 0.6, 0.8 and 1 mM. The absorbance was determined at 306 nm. The linearity range for this assay is shown in Fig.1 with ( $R^2 = 0.9990$ ).

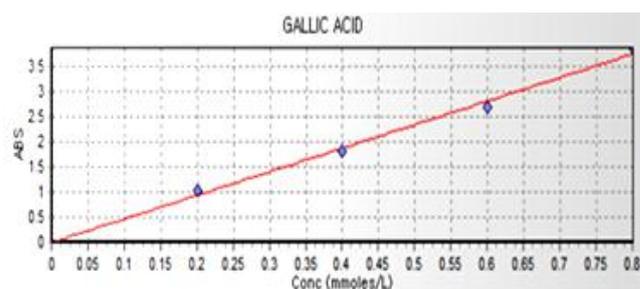


Fig. 1: The calibration curve using gallic acid.

**HPLC analysis:** The OMW phenols' analysis was performed in liquid chromatography equipped with Varian Pump model 9010, Rheodyne injector, Dyode-Array detector Varian ProStar model 330, column Heater Jones Chromatography model 7981 and using LC WorkStation Software. The analyses were carried out with the internal standard calibration method. The sample was injected in the HPLC system with a C18 column (Merck LichroCart 125-3), thermostated at 35.2°C. The mobile phase consisted of phosphoric acid at pH 2.5 (eluent A) and methanol (eluent B). The injection volume for all samples was 10 µl. The elution was done with flow rate 0.5 ml/min and the gradient program was as follows: 95–83% A (10 min), 83–74% A (10 min), 74–42% A (20 min), 42–5% A (10 min) and 5–95% A (10 min). The phenols' analyses were done at 280 nm and identification was carried out using the peak retention time and spectrum compared with those from commercial standards [22].

### Results and discussion

Using the system setup and methods described, it was possible to extract phenols in the samples. The dry weight and the percentage are shown in Table 1.

According to the dry weight for the extracts, chloroform extract had the highest phenols (8% w/v) while the other extracts presents in lesser amount.

#### Total phenol contents of OMW

Comparing with the results in dry weight, the larger

amount of phenols must be in chloroform, but chloroform can extract other components in OMW, so we cannot take the dry weight as a standard to determine the amount of phenols. The total phenol contents must be determined in the samples by Folin method. The calibration curve using gallic acid (Fig.1) gives the formula ( $AbS = 4.699 * C$ ). The total phenols of different extracts are shown in Table 2.

Ethylacetate extract had the highest Phenols (493 mg/100g). On the other hand, methanol and chloroform were present in lesser extent than ethylacetate (Table 2).

Solvents with relatively lower polarity like ethylacetate were more efficient in general for extracting phenols [23,24] in OMW. On the other hand, pure solvents with higher polarity extracted significantly higher amounts of phenols than non-polar solvent. Chloroform is considered as a non-polar solvent while ethylacetate is considered as an aprotic solvent and methanol is considered as a protic solvent. The solvent most suitable for the extraction of phenols appeared to be ethylacetate in OMW [24], and that what can explain the ability of chloroform to extract larger amount of phenol than methanol in this study.

The presented results conform to those of another study, that ethylacetate exhibits a higher extraction power compared to other solvents such as methyl isobutyl ketone, methyl ethyl ketone, and diethyl ether, which was shown by Allouche [25].

Based on the abovementioned results, HPLC analysis was employed to define the qualitative content of phenolic acids in the ethylacetate extract in OMW.

**Table 1:** Percentage and dry weight for phenols extracts using ethylacetate, chloroform and methanol.

Sample	Solvent	Volume (ml)	Dry weight (g/ml)	% extract (w/v)
OMW	Ethylacetate	50	1.3	2.6
	Chloroform	50	4	8
	Methanol	50	2.8	5.6

**Table 2:** Total phenol contents (C) in mg/100g of dry weight at absorbent on 750nm at concentration 10 mM for each solvent.

Solvent	Concentration mM	AbS (750 nm)	C Total phenol content (mg/100g dry weight)
Ethylacetate	10	1.395	493
Chloroform	10	0.950	340
Methanol	10	0.919	333

### HPLC analysis for OMW extract

HPLC chromatogram are presented in Fig. 2, ellagic acid, chlorogenic, caffeic, p-coumaric, and cinnamic acids were identified in the investigated extracts by comparing their retention times (RT) and on-line ultraviolet (UV) spectra with those of standards Fig. 3.

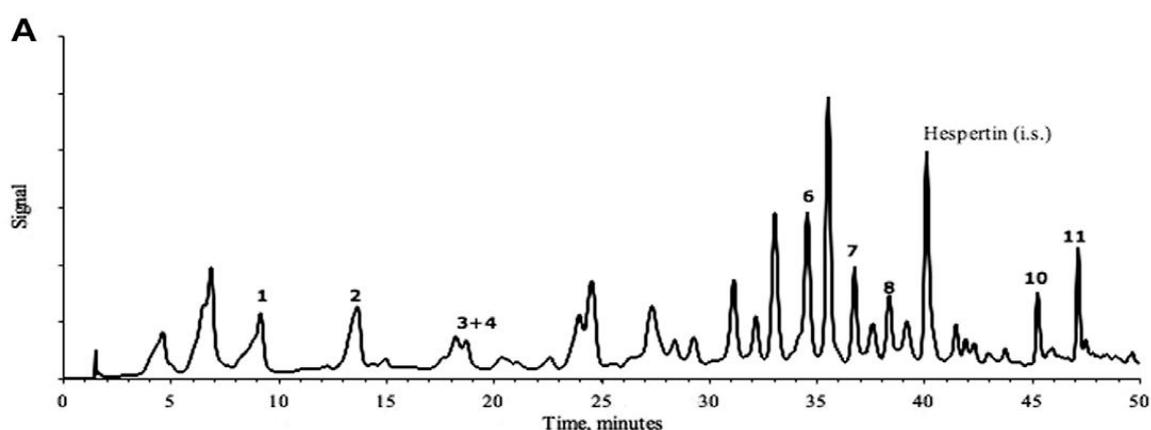
The benzoic group appeared before the cinnamic acid group because the first has low molecular weight and higher polarity coming from large number of OH radical in the benzoic group.

These identified phenols were already reported in OMW except for ellagic and chlorogenic acid. (Table 3). Ellagic and chlorogenic acid were first reported in this study. Retention times for the identified phenolic acids are shown in Table 4.

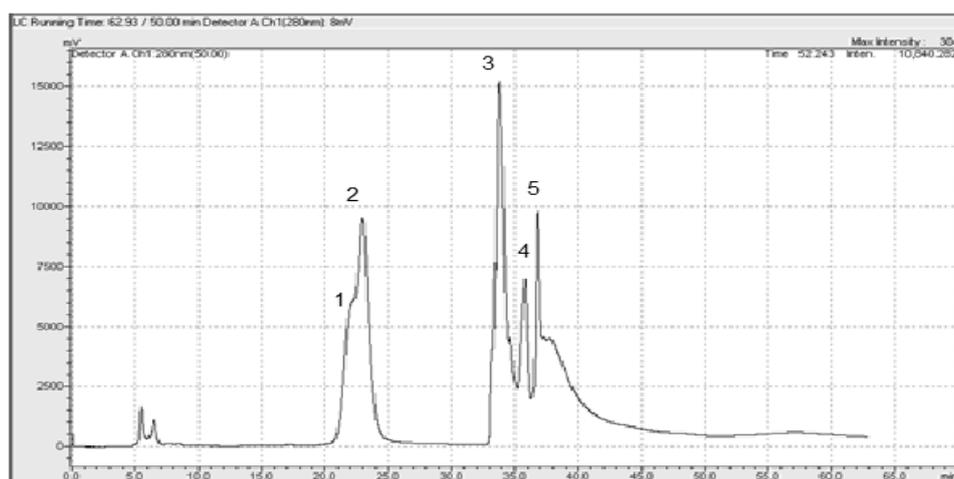
### Conclusions

Extraction for phenols and determination for phenolic acids from Syrian OMW was achieved using three solvents. Ethylacetate was the solvent in extracting phenols with concentration of (493 mg/100g). HPLC has appeared to be a simple and sensitive tool for the determination of phenolic acids since it showed unreported phenolic acids (ellagic and chlorogenic acids) in Syrian OMW.

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.



**Fig.2:** Typical HPLC chromatogram of phenols from Montesinho Natural Park. Phenolic acids are: (1) protocatechuic acid, (2) phydroxibenzoic acid, (3) caffeic acid, (4) chlorogenic acid, (5) p-coumaric acid, (6) ellagic acid and (7) cinnamic acid. Flavonoids are: (8) naringenin, (9) kaempferol, (10) pinocembrin and (11) chrysin. [22].



**Fig.3:** (1) Caffeic acid, (2) Chlorogenic acid, (3) p-coumaric acid, (4) Ellagic acid, (5) Cinnamic acid.

**Table 3:** References of phenolic acids.

Phenolic acid compounds	References
Caffeic acid	[26-32]
p-Coumaric acid	[27, 29-32]
(Cinnamic acid) ferulic and sinapic acid	[29, 31-34]

**Table 4:** RT for the identified phenolic acids.

Phenolic acid	Retention time, RT
Caffeic acid	18
Chlorogenic acid	19
p-Coumaric acid	34
Ellagic acid	35
Cinnamic acid	36

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