

## TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY EVALUATION OF OLIVE MILL POMACE EXTRACT

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### Abstract

Olive oil production generates different by-products such as olive mill wastewater and olive pomace OP, considered low-cost sources of bioactive compounds including polyphenols that show remarkable antioxidant properties. The objective of this work study was to conduct an assessment of the total polyphenol content TPC and antioxidant activity of different OP extracts recovered through liquid-liquid solvent extraction. OP samples were obtained from both two and three-phase extraction processes of olive oil production. Several solvents (water, methanol, ethanol, and n-hexane) have been used to extract phenolic compounds with ultrasound-assisted techniques (UAE). Folin-Ciocalteu assay was used to determine the TPC in olive oil pomace samples. The total antioxidant activity of phenolic extracts was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and phosphomolybdate assay. The highest extraction efficiency of phenolic compounds was obtained using a combination of ethanol:water (80:20). Three-phase decanter system showed higher values of TPC (0.7-0.8 g gallic acid equivalent/L extract), antioxidant activity (80-105% antiradical activity), and total antioxidant capacity (90-109% TAC) compared with the two-phase. Based on the results, OP has antioxidant qualities and may be useful in food formulation.

**Key words:** total phenolic content, antioxidant activity, olive pomace, food formulation.

### INTRODUCTION

Olive oil production is an essential global agro-industry, especially in Mediterranean countries, and is considered a key element associated with several health-beneficial effects (Takaç 2009; Alkhalidi 2023).

One of the most iconic and representative ingredients in the Mediterranean diet are the oils and fats of *Olea europaea* L. as a good source of unsaturated fatty acids (around 72% monounsaturated fatty acids primarily oleic acids, and 14% polyunsaturated fatty acids (Barjol 2013; Wani et al., 2018)).

Various studies have reported that olive oil consumption is associated with several health benefits, including reduction of risk factors of coronary heart disease and prevention of several chronic diseases (such as atherosclerosis), cancer, chronic inflammation, strokes, and other degenerative diseases (Skaltsounis et al., 2015; Pavez et al., 2019).

These beneficial health effects have been attributed to the bioactive and phenolic

compounds which are considered major contributors to their antioxidant and health-promoting effects by analytical and technical approaches of modern medicine as well as food science and chemistry (Zhao et al., 2022).

Olive oil production involves several mechanical extraction processes, such as i) press olive oil extraction, ii) three-phase centrifugal olive oil extraction, and; iii) two-phase centrifugal olive oil extraction (Ochando-Pulidon et al., 2020).

The olive oil industry generates a large amount of by-product, solid wastes, and liquid effluents such as olive pomace (OP), olive mill waste (OMW), and olive mill wastewater (OMWW) depending on the extraction techniques used for olive oil manufacture (Del Mar Contreras et al. 2020; Abbattista et al., 2021). Industrial olive processing byproducts and wastes have been recognized as one of the most critical problems in terms of environmental pollution due to their high content of organic matter and phenolic compounds (Roig et al., 2006). Discharges of processing biomass, particularly liquid effluent,

cause toxicity, contamination, pollution, and economic damage to the respective manufacturers (Safarzadeh Markhali, 2021). They cause diverse environmental impacts in terms of resource depletion, land degradation, air emissions, and waste generation (Pampuri et al., 2021). However, they also contain valuable nutritional substances (Quero et al., 2022). Nowadays these discharges are valorized by several pharmaceutical and food industries, mainly due to their high phenolic content (Rathi et al., 2018).

Olive pomace (OP) produced from a two-phase centrifugation system contains around 65% up to 70% moisture while the pomace generated from a three-phase decanter contains a lower amount of moisture quantity (45%). (Skaltsounis et al., 2015).

This by-product contains mainly water, seed, and pulp and is considered harmful to the environment, due to the phytotoxic and antimicrobial properties, low pH, relatively high salinity and organic load, and the phenolic and lipid constituents (Piotrowska et al., 2011).

Olive pomace as the main residue derived from olive oil extraction, presents a typical composition of lignocellulosic biomass which contains lignin (30.0–41.6%), cell wall polysaccharides (35.3–49.0%) as cellulose, pectic polymers and hemicellulose (xylans, glucoroxylans, xyloglucans and, manans), oil (7.5–14%) and minerals (4.4–6%) (Rodrigues et al., 2015; Miranda et al., 2019;).

Olive pomace is also a valuable food by-product containing natural phenols and polyphenols with health benefits related to their antioxidant activities (Zhao et al., 2022).

It contains large amounts of bioactive compounds with a wide range of physiological properties, such as anti-atherogenic, anti-allergenic, anti-cancer, anti-inflammatory, antioxidant, anti-microbial, anti-thrombotic,

## MATERIALS AND METHODS

### *Sample collection*

The olive pomace was obtained directly from three olive oil industries located in the southern and central parts of Albania. Olive pomace samples labeled with a specific code (OP1, OP2, and OP3) were collected during the crop year 2022-2023.

cardioprotective, and vasodilatory effects (Lozano-Sánchez et al., 2014).

Olive pomace is an excellent source of high-added value compounds such phenolic compounds, carbohydrates, and proteins (Bermúdez-Oria et al., 2019). It is characterized by containing squalene and has been reported as the source of several phytochemicals such as tocopherols, peptides, and quercetin (Rodrigues et al., 2015).

OP and OMWW could be considered also a low-cost and renewable natural source of antioxidants due to its high content of phenolic compounds with potential influence on human health, such as hydroxytyrosol, tyrosol, and luteolin (Caporaso et al., 2018; Speroni, 2020). Since there are many benefits that phenols of OP bring, several authors have reported its use as a raw material for obtaining multiple valuable compounds with health-promoting properties (Niknam et al., 2021). Thus, thanks to its low cost and large availability, several extraction techniques for recovery of olive pomace antioxidant components have been developed to re-valorize the olive oil by-product and minimize the environmental impact associated with its disposal (Cepo et al., 2018; Gulon et al., 2020;).

In Albania, olive oil production is one of the major agricultural sector, with 15.500 tons produced in the 2022 crop year according to the International Olive Council (IOC) (Dawson et al., 2022).

The main goal of the current study is to evaluate the total phenolic content (TPC) and antioxidant activity of different OP extracts recovered through liquid-liquid solvent extraction. To this aim, it is necessary to consider the extraction techniques and solvents used to recover antioxidant compounds of OP samples obtained from both two and three-phase decanter of olive oil production systems.

Samples of OP1 and OP3 were obtained by a 3-phase centrifugal extraction process, while the samples of OP2 were obtained from the processing of olives using a Perialisi Leopard Decanter with multi-phase decanter technology. Samples of OP1 were obtained by the production of extra virgin olive oil from the processing of organic Kalinjoti cultivar using a three-phase extraction system. According to genetic studies, Kalinjoti is recognized as the

most common native cultivar growing mostly in the Vlora region of Southern Albania (Thomaj and Panajoti, 2003).

Samples of OP2 and OP3 were collected from the processing of various olive varieties after the centrifugation step of two and three-phase extraction systems, respectively.

Samples of OP were dried at 45°C to 50°C for 48h in a tray dryer to prevent the degradation of phenolic compounds (Pikuli et al., 2023). To obtain smaller particle sizes (diameter 1 mm), dried OP samples were milled for 7 s, with a flour mill.

The moisture content of olive pomace samples was determined gravimetrically by drying 10 g of olive pomace for 48h till constant weight in a force air drying oven LBX OVF series, at 105±10°C (Moya et al., 2020).

#### *Extraction of phenolic compounds*

The recovery of phenolic compounds from olive pomace has been performed by solid-liquid extraction methods using different solvents, including water, methanol, ethanol, and hexane (Gullon 2020).

Pikuli et al. (2023) optimized the extraction of phenolic compounds from olive pomace using ethanol:water (80:20) at temperature of 30-40°C and an extraction times of 60 to 180 min. Ultrasound-assisted solid-liquid extraction of bioactive compounds from OP was performed at 30°C with ultrasound pulses every 5 s using an ultrasonic bath, model Cole-Parmer 8893 (47 kHz, 230 W). UAE was carried out using 10 g of OP and 50 mL of each extraction solvent (water, methanol:water (80:20), ethanol:water (50:50), ethanol:water (80:20), and n-hexane) (Pampuri et al., 2021).

The supernatants of phenolic compounds were filtered using 0.45 µm Millipore syringe filters and stored at - 20°C for further analysis.

#### *Evaluation of the Total Phenolic Content (TPC)*

The total phenolic content was carried out using the Folin-Ciocalteu method with some modifications described previously by Spinelli et al. (2015). Total phenolic compounds were quantified by a calibration curve previously built (0-5 mg GA/mL;  $R^2 = 0.9997$ ;  $y = 2.6276x - 0.0168$ ) using a standard solution of gallic acid (Sigma-Aldrich), and the total phenolic content was expressed as gram gallic acid equivalents

per liter (g GAE/L extract) (Quero et al., 2022). All analytical tests were carried out in triplicate. The experimental procedure is described below. Initially, 20µl of phenolic extract (diluted in water), 2500µl of water, and 400µl of Folin-Ciocalteu phenol were added to each test tube.

The test tubes were vigorously mixed using a vortex, and placed in the dark for 8 minutes. 500 µl of sodium carbonate Na<sub>2</sub>CO<sub>3</sub> (7%) was added to each tube and mixed again. All test tubes were placed in a water bath previously preheated to 40°C to react for 30 minutes. After that, the test tubes were removed from the water bath and allowed to reach room temperature. The absorbance of each solution was measured in a UV-vis spectrophotometer at a wavelength of λ=750nm.

#### *Total Antioxidant Activity (TAA) of Phenolic Extract*

The total antioxidant activity of phenolic extracts was carried out using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay according to Ballesteros et al. (2014). A calibration curve was previously built using 3mg DPPH dissolved in 100 ml of methanol to prepare the 1, 1-diphenyl-2-picrylhydrazyl solution. The experiment was conducted on microtiter plates. The following information is a brief description of the experimental procedure. The phenolic extract was obtained from the extraction of olive pomace. A quantity of 10mg of phenolic extract was dissolved in 1 ml of water. After that 20 µl of dissolved phenolic extract (dissolved in water), 980 µl of methanol, and 1000 µl of DPPH solution were added to each test tube. The test tubes were mixed in a vortex and were incubated in the dark for 30 minutes. DPPH is a stable radical that appears in purple and absorbs at a wavelength of 517 nm. The absorbance of each test tube's material was measured using a UV-vis spectrophotometer at the same wavelength. Total antioxidant activity was expressed as % RAC (anti-radical activity). To calculate the DPPH scavenging activity of the different fractions, the following formula was used:

Percentage inhibition (%) = [(Abs of control - Abs of the sample) / (Abs of control)] x 100.

### Total Antioxidant Capacity (TAC) of Phenolic Extract

Total antioxidant capacity of the extracts was evaluated by the phosphomolybdenum method according to the procedure described by Jan et al. (2013), with some modifications.

Briefly, 20  $\mu$ l aliquot of OP extracts was added into test tubes. A pre-prepared buffer solution (phosphomolybdate reagents) of 3 ml was added. The tubes were well-mixed and incubated in a pre-heated water bath at 95°C for 90 minutes. After cooling down the samples to room temperature, the absorbance of each mixture was measured in a UV-vis spectrophotometer at a wavelength of  $\lambda=695$  nm. Ascorbic acid was used as a standard to calculate the total antioxidant capacity expressed as a percentage (%TAC). Total antioxidant capacity was calculated using ascorbic acid as a standard according to the following formula:

$$\%TCA = \frac{[(\text{Abs. of control} - \text{Abs. of sample}) / (\text{Abs. of control})] \times 100}{}$$

All reagents utilized were of analytical grade and purchased from Sigma Aldrich.

### Statistical analysis

All analyses were performed in triplicate, and the results were expressed as mean values  $\pm$  standard deviation. The statistical analyses were evaluated by one-way ANOVA Tukey's test with a significance level  $\alpha$  set at 0.05, using SPSS ver. 22 software.

## RESULTS AND DISCUSSIONS

The olive pomace (OP) chemical composition is influenced by the olive cultivar, growing conditions, and the extraction process used (Poti et al., 2022). Samples of OP had a brown dark color and pH values ranged from 4.6 to 5.4. The moisture content of olive pomace samples is displayed in Figure 1.

The moisture content of sample OP2, collected from the two-phase centrifugation process, ranged from 38% to 49%. On the other hand, the olive pomace samples obtained by the three-phase extraction systems (OP1 and OP3) contained a larger amount of water, with moisture levels ranging from 60% to 72%.

The recovery of phenolic compounds from OP has been carried out using various solvents in solid-liquid ultrasound-assisted extraction,

including water, methanol:water (80:20), ethanol:water (50:50), ethanol:water (80:20), and n-hexane. The extraction yield was determined by using gravimetric analysis. Briefly 1 mL of each extract was weighed before and after being placed at  $100 \pm 1^\circ\text{C}$  overnight. Figure 2 displays the amount of extract (in grams) obtained from 10 grams of dry OP (%w/w).

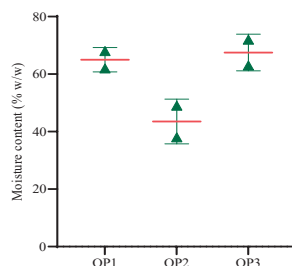


Figure 1. Moisture content of olive pomace samples

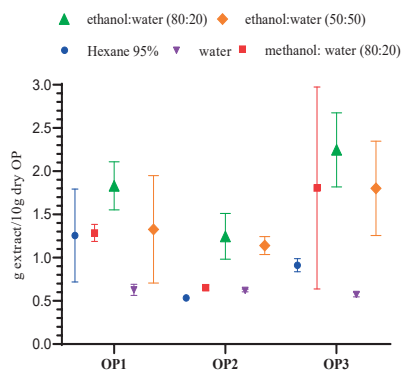


Figure 2. Yield extract of olive pomace provided by ultrasound assisted extraction (UAE)

Data regarding total phenolic content, total antioxidant activity, and capacity of olive pomace samples are presented in Table 1. TPC of OP samples was quantified by Folin-Ciocalteu assay which depends on the reduction of Folin-Ciocalteu reagent by phenolic compounds under alkaline condition.

The highest amount of TPC, TAA, and TAC compounds was recorded in the samples of olive pomace OP1 and OP3, obtained from three-phase production systems. The highest TPC values in olive pomace samples were obtained by using methanol:water (80:20) and

ethanol:water (80:20) as extraction solvents, 0.7505±0.0105 mg GAE/L extract and 0.7832±0.0460 mg GAE/L extract (Tukey's test with  $\alpha = 0.05$ ), respectively.

Studies conducted in Spain (Gómez et al., 2020) and Italy (Aliakbarian et al., 2018) have reported similar range of TPC in olive pomace, obtained through various combinations of water-solvent extraction. Generally, solvents have been found to extract a higher TPC than water and acidified water (Zhao et al., 2022). However, this is the first report in Albania on the extractable TPC range (0.7505-0.7832 mg GAE/L extract) from olive pomace. In this study, the combination of ethanol:water (80:20) was the most common solvent used for phenolic compounds extraction, considered a green, nontoxic, and environmentally friendly medium compared to other organic solvents (Rodriguez-Rojo et al., 2012).

Table 1. Total phenolic content (TPC), total antioxidant activity (TAA), and total antioxidant capacity (TAC) of olive pomace samples

TPC (g GAE/L extract)			
Solvents-UAE	OP1	OP2	OP3
water	0.5521 ±0.0145	0.2446 ±0.0013	0.5245 ±0.0788
methanol:water (80:20)	0.7505 ±0.0105	0.3341 ±0.0066	0.5113 ±0.0657
ethanol:water (80:20)	<b>0.7832</b> <b>±0.0460</b>	0.3841 ±0.0250	0.5457 ±0.0039
ethanol:water (50:50)	0.7652 ±0.0512	0.3681 ±0.0560	0.5842 ±0.01707
TAA (%RAC)			
	OP1	OP2	OP3
water	67.2911 ±5.2556	60.2112 ±1.8586	70.5365 ±2.1262
methanol:water (80:20)	79.1557 ±8.2821	85.6641 ±0.8125	101.6614 ±0.5534
ethanol:water (80:20)	<b>104.242</b> <b>±5.5341</b>	84.6522 ±4.9167	91.1491 ±7.8173
ethanol:water (50:50)	90.3727 ±2.2457	83.2298 ±1.8750	88.8198 ±2.2998
%TAC			
	OP1	OP2	OP3
water	67.165 ±3.3524	41.6651 ±5.6148	70.9350 ±1.2689
methanol:water (80:20)	79.3032 ±6.3572	43.7053 ±1.6522	47.6354 ±8.7512
ethanol:water (80:20)	<b>109.604</b> <b>±0.9864</b>	52.2223 ±2.6422	89.9117 ±4.8113
ethanol:water (50:50)	99.7032 ±3.4680	37.942 ±5.6443	73.7440 ±6.1254

According to the data presented in Table 1, olive pomace samples obtained from the Kalinjoti cultivar (OP1) showed higher levels of TPC, TAA, and TTAC compared with OP samples obtained from other cultivars. Hence, it can be concluded that the OP obtained from the Kalinjoti cultivar is a good source of bioactive

compounds and may be useful in food formulation.

The content of bioactive polyphenols in olive pomace is variable depending on numerous factors: olive cultivar, the olive oil extraction process, and the type of pomace that remains as the by-product (traditional extraction, two-phase process or three-phase process, etc.) (Dermeche, 2013).

The influence of the extraction solvent (water, methanol, or ethanol) and the olive oil production processes (2 or 3-phase centrifugation systems) on the yield extract of phenolic compounds, Total Phenolic Content, Total Antioxidant Activity by DPPH (% Anti-Radical Activity), and Total Antioxidant Capacity by phosphomolybdate test (%TAC) of olive pomace samples are shown in Figure 3.

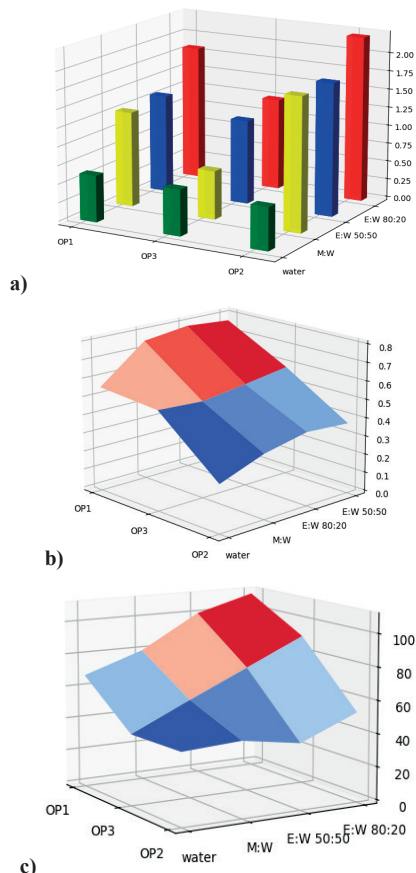


Figure 3. Three-dimensional plots showing the effect of extraction solvent and olive oil production processes on extraction yield (a); on TPC (b); and on TAC (c) of olive pomace

The optimum extraction efficiency of antioxidant compounds was obtained using a combination ethanol:water (80:20) as extraction solvent (Figure 3a). The highest levels of TPC and TAA were obtained from olive pomace samples of 3-phase centrifugation systems using ethanol:water (80:20) (OP1 and OP3) as extraction solvent (Figure 3b,c). The influence of different olive oil production processes (2 and 3-phase extraction systems) on the recovery rate of TPC, TAA, and TAC is presented in Figure 4.

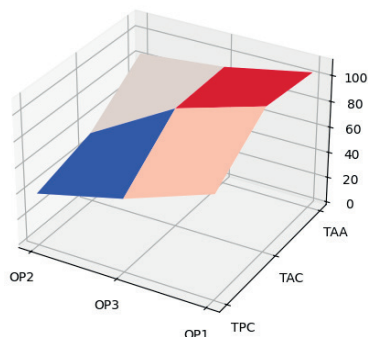


Figure 4. Three-dimensional plots showing the influence of olive oil production processes on the recovery rate of TPC, TAA, and TAC from olive pomace using ethanol:water (80:20) as extraction solvent

The results showed that significantly higher levels of TPC, TAA, and TAC in an aqueous solution of ethanol (80:20) were obtained from olive pomace generated by 3-phase centrifugation processes (OP1 and OP3) in comparison to 2-phase centrifugation systems (OP2).

## CONCLUSIONS

In the present study, we focused mainly on the evaluation of the total phenolic content and antioxidant activity of olive pomace samples generated from the manufacturing of olive oil using two and three-phase centrifugation systems. Among different food-grade solvents, ethanol:water (80:20) was selected as the most appropriate solvent for phenolic compounds recovery from OP. The obtained results showed that olive pomace extracts contained a significant amount of phenolic compounds and antioxidant activity. However, they significantly depended on some factors such as olive cultivars, olive oil production processes, and the polarity of the extraction solvent. Satisfactory

phenolic and antioxidant yields proved that OP was a low-cost, renewable, and abundant source of bioactive compounds. Simple solvent extraction using food-grade solvents could be successfully applied. The study highlights the usage of olive pomace generated from the olive oil industry as a valuable source of bioactive compounds in food formulations.

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