MULTISTAGE RECOVERY PROCESS OF BIOPHENOLIC ANTIOXIDANTS WITH FOCUS ON HYDROXYTYROSOL FROM OLIVE MILL WASTEWATER CONCENTRATES

by

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To my mum and dad..

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ABSTRACT

MULTISTAGE RECOVERY PROCESS OF BIOPHENOLIC ANTIOXIDANTS WITH FOCUS ON HYDROXYTYROSOL FROM OLIVE MILL WASTEWATER CONCENTRATES

A number of phenolic compounds (PCs) and their environmental contamination problems as consequences of olive oil production process in the countries surrounding the Mediterranean Sea propel the need for extraction of high-added value phenolic antioxidants from olive mill wastewater (OMWW) to reduce the toxicity of the effluent. Since existing treatment methods for OMWW are not efficient for recovery approaches of phenolic antioxidants due to the consumption of high amount of solvent, new methods of isolation that require low solvent will still be on-demand in foreseeable future. In this regard, this research utilizes concentrated decanter and lagoon OMWW treated with mechanical vapor recompression evaporator to recover natural antioxidants mainly hydroxytyrosol (HTyr). A multistage recovery process including pretreatment by acidification, extraction with organic solvent and/or solid phase extraction (SPE) using nonionic polymeric adsorbent was applied to both types of concentrates. While solvent/solid ratio, extraction time and stage were the parameters used to optimize solid-liquid extraction (SLE) process, for aqueous two-phase extraction (ATPE) ethanol concentration, temperature and extraction time were optimized by 2^3 full factorial experimental design. The results clearly revealed the requirement of ultrasonic assistance for the extraction of PCs from decanter concentrate. The acidification of OMWW concentrates enhanced the overall recovery of HTyr and the highest recoveries were 6.6 g/kg and 4.6 g/kg achieved by ATPE from lagoon and decanter concentrates, respectively. The results also showed that HTyr selectivity of the SPE process using XAD16N is pretty high with approximately 81 % HTyr recovery from the extracts of both concentrates.

ÖZET

ZEYTİNYAĞI KARASU KONSANTRELERİNDEN BİYOFENOLİK ANTİOKSİDANLARIN, BAŞLICA HİDROKTİROSOLÜN, ÇOK BASAMAKLI GERİ KAZANIM İŞLEMİ

Akdeniz'i çevreleyen ülkelerdeki zeytinyağı üretim süreci sonucunda oluşan zeytin karasuyunun içinde bulunan fenolik bileşiklerin neden olduğu çevresel kirlilik problemleri, zeytin karasuyundan yüksek değerli fenolik antioksidanları geri kazanarak bu atık suyun toksisitesinin düşürülmesi ihtiyacını doğurmuştur. Zeytin karasuyunun arıtılmasında kullanılan mevcut yöntemler fenolik antioksidanların geri kazanım yaklaşımları için yüksek miktarda çözücü tüketiminden dolayı verimli olmadığından, az çözücü tüketimine olanak tanıyan yeni geri kazanım metotları geliştirilmesi önem taşımaktadır. Bu bağlamda, bu araştırmada fenolik antioksidanları, özellikle hidroksitirosolü geri kazanmak için mekanik buhar sıkıştırmalı evaporatörde işlemden geçirilmiş dekantör ve lagünden alınan zeytin karasuyu konsantreleri kullanılmaktadır. Asitleme ile ön işlemden geçirme, organik çözücü ile ektraksiyon ve/veya iyonik olmayan polimerik adsorban kullanılarak katı faz ekstraksiyonunu kapsayan çok-aşamalı geri kazanım işlemi her iki konsantre çeşidine de uygulanmıştır. Çözücü/katı oranı, ekstraksiyon süresi ve basamak sayısı katı-sıvı ekstraksiyon işlemini optimize etmek için kullanılan parametrelerdir. Sulu iki fazlı ekstraksiyon için ise, etanol konsantrasyonu, sıcaklık ve ekstraksiyon süresi 2^3 tam faktöriyel deney dizaynı ile optimize edilmiştir. Sonuçlar dekantör konsantresinden fenolik bileşiklerin ekstraksiyonu için ultrasonik yöntem kullanma gereksinimini açıkça ortaya koymuştur. Zeytin karasuyu konsantrelerinin asidifikasyonu toplam geri kazanılan hidroksitirosolün miktarını artırmış ve en yüksek geri kazanımı sulu iki-fazlı ekstraksiyon ile sırasıyla lagün ve dekantör konsantrelerinden elde edilen 6.6 g/kg ve 4.6 g/kg olmuştur. Sonuçlar ayrıca XAD16N reçinesinin yaklaşık 81 % geri kazanım oranı ile hidroksitirosol seçiciliğinin oldukça yüksek olduğunu ve bu antioksidanın saflaştırılmasında kullanılabilecek bir adsorpsiyon reçinesi olduğu sonucunu ortaya koymuştur.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Unit
A	Adsorption Ratio	%
AA	Antioxidant Activity	%
AAE	Ascorbic Acid Equivalent	
AF	Amorphous Fluoroplastic	
AS	Ammonium Sulfate	
ATPE	Aqueous Two-Phase Extraction	
ATPS	Aqueous Two-Phase System	
BOD	Biological Oxygen Demand	g/L
BSA	Bovine Serum Albumin	
CAE	Caffeic Acid Equivalent	
CE	Collusion Energy	V
CXP	Collusion Cell Exit Potential	V
CO_2	Carbon Dioxide	
COD	Chemical Oxygen Demand	g/L
CPE	Cloud Point Extraction	
D	Desorption Efficiency	%
DHP	Dipotassium Hydrogen Phosphate	
DP	Declustering Potential	V
DPPH	2,2-diphenyl-1-picrylhydrazyl	
DW	Deionized Water	
EC	Electrical Conductivity	ms/cm
EE	Extraction Efficiency	%
EP	Entrance Potential	V
EtOAc	Ethyl Acetate	
EtOH	Ethanol	
EU	European Union	
FA	Formic Acid	
FAS	Ferrous Ammonium Sulfate	
GAC	Granular Activated Carbon	

GAE	Gallic Acid Equivalent	
H_2O_2	Hydrogen Peroxide	
H_2SO_4	Sulfuric Acid %	
HCl	Hydrochloric Acid	
HPLC	High Performance Liquid Chromatography	
HTyr	Hydroxytyrosol	
K	Partition Coefficient	%
K_2HPO_4	Dipotassium Hydrogen Phosphate	
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectroscopy	
LLE	Liquid-Liquid Extraction	
m	Mass	g
MeOH	Methanol	
MIP	Molecularly Imprinted Polymers	
MQ	Milli-Q	
MVR	Mechanical Vapor Recompression	
MW	Molecular Weight	g/mole
$(NH_4)_2SO_4$	Ammonium Sulfate	
OC	Organic Carbon	
OMWC	Olive Mill Wastewater Concentrate	
OMWW	Olive Mill Wastewater	
PC	Phenolic Compound	
Q_1	Parent Ion	m/z
Q_3	Daughter Ion	m/z
SLE	Solid-Liquid Extraction	
SFE	Supercritical Fluid Extraction	
SPE	Solid Phase Extraction	
TKN	Total Kjeldahl Nitrogen	ppm
TP	Total Phenols	mg GAE/g
TPC	Total Phenolic Content	mg GAE/g
TSS	Total Suspended Solids	g/L
Tyr	Tyrosol	
UAE	Ultrasound Assisted Extraction	
V_r	Volume Ratio	
Z	Charge	

1. INTRODUCTION

Nowadays, the growing attention of replacing synthetic with natural antioxidant additives has oriented researchers to scan plants, fruits, vegetables, and other agricultural sources for recovering phytochemical substances with health-beneficial characteristics (Galanakis et al., 2010; Szabo et al., 2010). Phenols are today among the most important group of natural antioxidants which contain at least one hydroxyl group (polar fraction) directly bonded to an aromatic ring (nonpolar fraction) and frequently exist in plants as esters or glycosides, rather than as free molecules (Queimada et al., 2009). Considering the investigations in this field, it is demonstrated that olive mill effluents as consequences of olive oil production process create a natural and inexpensive source of biophenolic compounds with strong antioxidant activity.

Olive oil production is one of the most important industrial sectors in Mediterranean countries since early times. During the last few decades, olive oil has an increasing demand through worldwide as a primary component of the Mediterranean diet. Throughout the world, annual consumption of olive oil scaled up 1.8-fold between 1990/91 to 2017/18 by reaching 3 million tons in the last years. Mediterranean countries contribute up to 97 % of the total olive oil production while Spain is the utmost olive oil-producing country (1,090,500 t in 2017/18), then Italy (300,000 t), Greece (320,000 t), Turkey (287,000 t) and Tunisia (220,000 t), followed by Morocco, Algeria and Portugal (International Olive Oil Council, 2017).

Batch and continuous olive oil processing are the two main olive oil production pathways where the latter applied with three-phase centrifugal system or two-phase centrifugal system depending on the separation methods used (Azbar et al., 2004). Although each extraction route produces both liquid and solid effluents, significantly large volume of liquid waste, which is called as olive mill wastewater (OMWW), is generated by both three-phase centrifugal and press systems compared to two-phase centrifugal system. However, considerable amount of semi-solid or slurry effluent, which is so-called pomace, is also discharged by two-phase centrifugation pathway (Takac and Karakaya, 2010). Despite from the fact that characteristics of the effluents vary based on the production pathway, climate, soil composition, agronomic conditions of the region, as well as the type, maturity and quality of cultivated olives, high organic pollution load of OMWW creates severe environmental problems due to the presence of phenolic compounds (PCs) with ecotoxicological effects and low

biodegradability (Obied et al., 2005; Paraskeva and Diamadopoulos, 2006; Leouifoufi et al., 2014; Ochando-Pulido et al., 2016).

Although the application of untreated olive mill effluents to cereal crops and olive trees is suggested to supply benefits for soil and plant (Paraskeva and Diamadopoulos, 2006), the direct discharge of olive mill effluents obviously creates serious environmental problems such as soil and water contamination, plant growth inhibition, leakage to the underground, sharp odor nuisance, impacts on aquatic fauna and ecological balance, as well as phenol and sulfur dioxide emissions through air (Paredes et al., 1999; Rinaldi et al., 2003; Sellami et al., 2016). Therefore, legal limitations prohibit the direct discharge of OMWW to the environment or municipal sewage collection system. In the light of these facts, advanced treatment strategies used to reduce pollution load of OMWW are inevitable. Recently, separation systems mainly with membrane technologies are investigated treatment processes for OMWW. However, the most important handicap of the operation of these applications is the excessive fouling potential of the system primarily caused by microorganisms, soluble organics, and colloidal compounds (Ochando-Pulido, 2016). On the other hand, Mechanical Vapor Recompression (MVR) Evaporator can be an efficient and economical alternative to reduce the volume of wastewater.

MVR evaporator can have potential to provide several benefits for OMWW treatment compared to the conventional evaporation achieved in lagoons. First, only small space is required for the system operation. Furthermore, quick and economic evaporation/condensation process in a closed system results in high COD reduction in distillate that may be discharged to sewage collection systems or may be further treated to lower its COD content. More importantly, after having evaporation, the resulting olive mill wastewater concentrate (OMWC) can be a source for high-added value organic compounds such as phenolic antioxidants, pectin, recalcitrant and important enzymes (Rahmanian et al., 2014). Considering the critical advantages of the removal of PCs from OMWW which are mainly responsible from its high COD content and phytotoxic characteristics, the development of industrial scale recovery applications of PCs from this natural waste source not only creates a profitable, socio-economic, and environmentally safe production of valuable phenolic antioxidants but also minimize environmentally hazardous characteristics of OMWC.

Many researches have been investigated for years to recovery PCs from OMWW (Katsoyannos et al., 2006; Agalias et al., 2007; De Marco et al., 2007; Scoma et al., 2012; Bedouhene et al., 2014; Leouifoufi et al., 2014; Xynos et al., 2015; Zagklis et al., 2015; Víctor-Ortega et al., 2016; Yangui et

al., 2017). However, application of the current methods is not still promising to build an energy efficient industrial production of PCs, because poor yield problems, requirement of complicated plants and long-time periods, use of toxic and large volume of solvent.

Over recent years, PCs such as phenyl alcohols, phenyl acids, flavonoids, secoiridoids and their antioxidant activity isolated from OMWW have been investigated (Dagdelen et al., 2013; Leouifoufi et al., 2014; Stamatopoulos et al., 2014; Kelebek et al., 2015; Kaleh and Geißen, 2016; Cabrera-Bañegil et al., 2017). Among more than 30 PCs found in OMWW, hydroxytyrosol (HTyr), tyrosol (Try) and oleuropein are the most abundant and bioactive phenols since they exert an in vitro protective effect against low-density lipoprotein oxidation as well as being effective at low concentrations to protect human erythrocytes and DNA against oxidative damages (Obied et al., 2005; Rahmanian et al., 2014; Bedouhene et al., 2014). Olive polyphenols are also effective compounds to prevent chronic human diseases including cardiovascular and inflammatory disorders, as well as pathological diseases such as atherosclerosis, cancer and neurodegenerative diseases (Bedouhene et al., 2014). Particularly HTyr is the most valuable and expensive PC with its solid antioxidant, antibacterial, anti-viral, anti-inflammatory, anti-hypertensive and anti-angiogenic characteristics, which also has a high demand in food, cosmetic and pharmaceutical industries (Bedouhene et al., 2014 ; Frascari et al., 2016; Putnik et al., 2017). However, current market price of HTyr is very high because of its slow and costly chemical production processes (De Marco et al., 2007).

These facts clearly show that the high pollution of effluents from olive oil production processes and associated environmental and socio-economical issues are some of the most important problems of the worldwide olive oil industry. Development of an efficient recovery process for high-added value phenolic antioxidants from olive mill effluents enables new market and industrial sectors by turning this serious pollution problem into an advantage.

Due to the aforementioned concerns, this research aims to apply a multistage process on OMWW for the recovery of biophenolic antioxidants with focus on HTyr. OMWCs achieved from decanter and lagoon OMWW treated by pilot scale MVR evaporator were subjected to different solvent extraction processes at different experimental conditions to enhance the efficiency and selectivity of antioxidant recovery. The evaluation of solid phase extraction applied on the extracts was investigated for further purification of the product.

2. LITERATURE REVIEW

2.1. Potential Production of OMWW by Olive Oil Industry

Olive oil extraction process from olive fruits can be accomplished by traditional discontinuous press process or in modern units by continuous extraction systems (three-phase and two-phase) (Azbar et al., 2004; Dermeche et al., 2013). Regardless of the type of system, water is used for washing of olive fruits and malaxing of crushed olives, to favor coalescence and agglomeration of small oil droplets. Further addition of water depends upon the type of system. While in continuous two-phase system water is not consumed in the following stages of extraction process, in batch and continuous three-phase centrifugal extraction system water is added during the decantation and separation stages for simplifying the separation of oil and water phase (Dermeche et al., 2013). Also, olive fruit includes 40-60 % vegetation water, in addition to 10-30 % oil and 30 % solid matter, the COD content of OMWW is 200 to 400-fold higher than domestic wastewater because of its high organic content (Hocaoglu et al., 2018).

In continuous two-phase centrifugal systems, the small amount of added water and vegetation water remain in solid residue which is called as pomace whereas in batch and continuous three-phase centrifugal systems, both solid and liquid effluents are generated (Sayadi et al., 2000). Although only small amount of wastewater is produced during the process, olive pomace is the main effluent of the two-phase extraction system (Hocaoğlu et al., 2015). A comparison for the volume of wastewater generated in different stages of three- and two-phase centrifugation systems is illustrated in Figure 2.1. The water addition in malaxation stage was rarely applied in two-phase systems according to the water content of olive while water is not added in decanters. On the other hand, the considerable volume of the wastewater generated by three-phase centrifugal system comes from the decantation stage. In some other operations high volume of water (200 L/tonne) can also be added in the last stage of the process (Borja et al., 2006). All these indicated that the volume of OMWW generated from the olive oil production processes may change depending on various factors such as air conditions, origin of the olives and season of harvesting (Hocaoğlu et al., 2015).

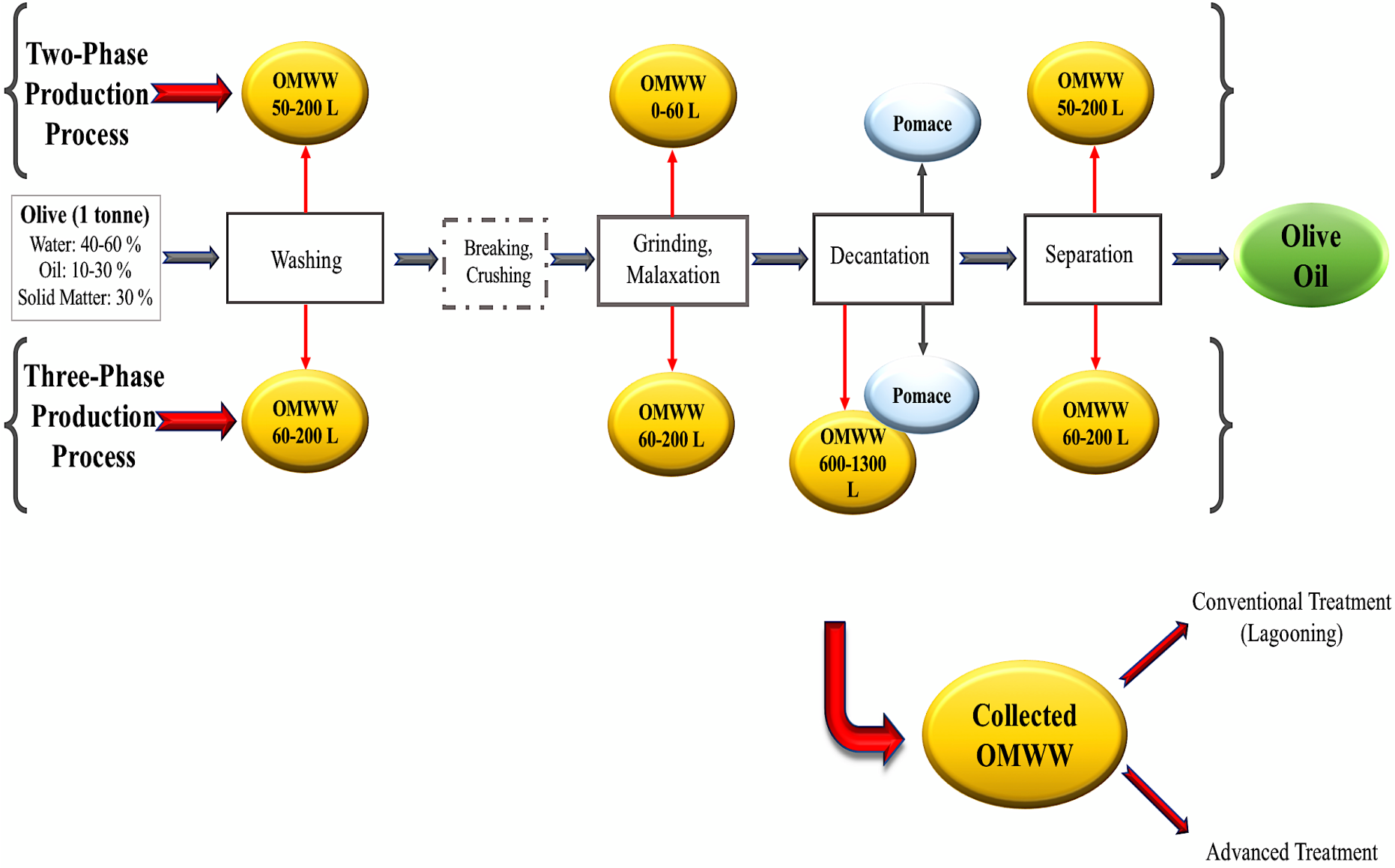


Figure 2.1. The volume of OMWW produced by two-phase and three-phase centrifugation systems.

2.1.1. Composition of OMWW

In general, OMWW has a high pollution load, although its physicochemical characteristics vary depending on the olive cultivation conditions as well as extraction processes (Table 2.1) (Ochando-Pulido et al., 2017). In addition to 83-92 % water content, other components including PCs, sugars, organic acids, polyalcohols, several mineral nutrients (e.g. potassium), lipids, pectins, proteins are the main constituents of OMWW (De Marco et al., 2007; Rahmanian et al., 2014; Leouifoufi et al., 2014). OMWW is characterized by a brownish black color (52,270- 180,000 mg/L Pt-Co units), slightly acidic nature (pH 3-6), strong specific olive oil odor, high electrical conductivity, and high content of polyphenols (0.4-5.45 %), reducing sugar (1.5-19.3 %), potassium as a major inorganic element (4 g/L), organic load (0.8-220 g/L COD; 0.3-110 g/L BOD; 25-45 g/L TOC) and suspended solids (1-9 g/L) (Mouncif et al., 1993; Paredes et al., 1999; García-Gómez et al., 2003; Dermeche et al., 2013; Leouifoufi et al., 2014; Sellami et al., 2016; Ochando-Pulido et al., 2017; Majbar et al., 2017). The variations of physicochemical parameters are listed in Table 2.1 with respect to type of process in which OMWW is produced.

Table 2.1. Characteristics of OMWW from batch and continuous olive oil extraction processes (Ochando-Pulido, 2016).

Process	**COD (g/L)**	**BOD_5 (g/L)**	**TSS (g/L)**	**pH**	**EC (mS/cm)**	**TP (g/L)**
Olives cleaning	0.8-2.2	0.3-1.5	8-18	5,5-6.6	2.5-3.0	0-0.1
Batch press	30-130	90-100	10-12	4.5-5.0	2.0-5.0	1.0-2.4
Three-phase	30-200	5-45	5-35	3.5-5.5	2.0-7.9	0.3-7.5
Two-phase	4-16	0.8-6.0	2-7	3.5-6.0	1.5-2.5	0.1-1.0

COD: Chemical oxygen demand, BOD: Biological oxygen demand, TSS: Total suspended solid, EC: Electrical conductivity, TP: Total phenol

In addition to the contamination parameters mentioned above, the characterization of organic molecules in OMWW was previously studied for the use of OMWW in agricultural purposes and for the development of OMWW treatment approaches. Literature values for the characteristic parameters of OMWW and its solid fraction are compiled in Table 2.2.

Table 2.2. Characterization of OMWW and its solid fraction.

Source	pH	TP	TKN	OC	Protein	Carbohydrate	Lipid	Reference
OMWW	4.50	-	0.46 g/L	21.83 g/L	-	-	9.0 g/L	(Mouncif *et al.*,
OMWW	4.73	64.78 g/L[a]	0.75 g/L	103.12 g/L	-	-	6.5 g/L	1993)
OMWW	5.17	2.21 %	0.88 %[b]	47.52 %	-	12.22 %	4.27 %	(Paredes *et al.*,
OMWW Sludge	5.41	-	1.74 %[b]	47.90 %	-	-	-	1999)
OMWW Solids	5.63	-	11.6 g/kg[b]	626.3 g/kg	-	-	13.8 %	(García-Gómez *et*
OMWW Solids	5.80	-	15.1 g/kg[b]	551.5 g/kg	-	-	10.2 %	*al.*, 2003)
OMWW	2.24-5.9	0.63-5.45 %	-	20.19-39.8 g/L	-	1.5-12.22 %	0.03-4.25 %	(Dermeche *et al.*,
OMWW	1.42-4.0	0.4-2.43 %	-	-	2.87-7.2 %	0.83-19.3 %	3.76-18.0 %	2013)
OMWW	5.03	3.69 g/L	-	-	4.55 %	10.56 %	10.83 g/L	(Sellami *et al.*,
OMWW	4.75	0.98 g/L	-	-	3.74 %	6.23 %	10.71 g/L	2016)
OMWW	5.42	-	0.86 %	43.36 %	-	-	-	(Majbar *et al.*,
								2017)

[a] Tanic Acid Equivalents, [b] Total Nitrogen, TP: Total Phenol, TKN: Total Kjeldahl Nitrogen, OC: Organic Carbon

The key risk potential of disposal of OMWW to the environment is not only due to its high organic pollution load but also the presence of considerable amount of PCs (Ramos-Cormenzana et al., 1996) that form a lignin-like structure and establish the highest recalcitrant portion of this effluent (Sayadi et al., 2000). OMWW includes almost 100-fold concentrated polyphenols than in olive oil (Lesage-Meessen et al., 2001) and the concentration of PCs in OMWW can reach 5-10 g per liter depending on the harvest season, cultivation and extraction processes (D'Annibale et al., 2004). PCs are responsible from the brownish black color of OMWW as well as its phytotoxic and antibacterial characteristics (Aggelis et al., 2003).

2.1.2. Regulations for the Management of OMWW

There is not specific provision for the management of olive oil manufacturing waste in European Union (EU) (Inglezakis et al., 2012). Individual EU countries develop national legislation, which should be in line with Water Framework Directive. In Turkey technical issues to be followed in the management of wastewater produced in olive oil facilities are determined by the Republic of Turkey Ministry of Environment and Urbanization in 2015 (Çevre ve Şehircilik Bakanlığı, 2015). As in the case of EU countries, the direct discharge into environment is strictly forbidden in Turkey and the discharge standards of this sectors's wastewater are given in Table 2.3 (Çevre ve Orman Bakanlığı, 2004).

Table 2.3. The discharge standards of wastewater produced from olive oil industry defined by the Regulation of Water Pollution Control.

Parameter	**Composite Sample (2 hours)**	**Composite Sample (24 hours)**
COD (mg/L)	250	230
Oil and Grease (mg/L)	60	40
pH	6-9	6-9
Color (Pt-Co)	280	260

Turkey like other olive oil producing countries is facing severe environmental pollution problems due to the environmentally hazardous characteristics of OMWW (Takac and Karakaya, 2010). Considering the high pollution load of OMWW (Table 2.1 and 2.2), the use of chemical, physical, biological and combine treatment techniques must be necessary before its disposal into the environment.

2.1.3. Current OMWW Treatment Technologies

The common OMWW treatment in Mediterranean countries is to use evaporation ponds (lagooning) (Kavvadias et al., 2017). However, due to problems (e.g. nuisance due to odors, requirement of large area) of this physical treatment, the development of more efficient and environmentally safe treatment applications is needed. In order to decrease the pollution load of OMWW, several treatment technologies have been developed so far, including physical, chemical, biological and thermal treatment as well as combinations thereof (Paraskeva and Diamadopoulos, 2006; Takac and Karakaya, 2010; Rahmanian et al., 2014) and these current treatment technologies of OMWW with their detailed methodology are listed in Table 2.4.

While conventional physicochemical treatments are not effective for the abatement of pollution load of OMWW, the advanced techniques with success have limited application due to high overall treatment cost. Therefore, the investigations have been focused on the removal of PCs, which are responsible for the requirement of advanced treatment, but there is still need for efficient and economic methods.

Table 2.4. Treatment technologies of OMWW.

Category	Methodology	Results	References
Physical	Sedimentation, Filtration, Flotation and Centrifugation	70 % COD removal, 30 % oil recovery	(Velioglou et al., 1987; Georgacakis and Dalis, 1993)
	Micro-, Ultra-, Nano-Filtration and Reverse Osmosis	99 % COD removal, but membrane fouling resulting in high cost	(Canepa et al., 1988; Turano et al., 2002; Stoller and Chianese, 2006; Russo, 2007; Paraskeva et al., 2007)
	Dilution		(Paraskeva and Diamadopoulos, 2006)
	Evaporation		(Paraskeva and Diamadopoulos, 2006)
	Sedimentation (Settling)		(De Martino et al., 2011)
	Filtration		(Akdemir and Ozer, 2009; Galanakis et al., 2010)
	Centrifugation		(De Leonardis et al., 2007)
	Adsorption/Desorption		(Ena et al., 2012)
	Solar Distillation		(Paraskeva and Diamadopoulos, 2006)
	Combined		(Paraskeva and Diamadopoulos, 2006)
Thermal	Evaporation, Distillation	20–80 % COD removal, requirement for further treatment, high energy demand	(Rozzi and Malpei, 1996; Tsagaraki et al., 2007)
	Combustion, Pyrolysis	Toxic gases generation and expensive operation with excessive energy demand	(Rozzi and Malpei, 1996; Caputo et al., 2003)

Table 2.4. Treatment technologies of OMWW (cont.).

Category	Methodology	Results	References
Physicochemical	Neutralization, Precipitation and Adsorption	30–50 % and 80–95 % COD removal by individual and combined practices, respectively.	(Lolos et al., 1994; Adhoum and Monser, 2004; Inan et al., 2004; Kestioğlu et al., 2005; Sarika et al., 2005)
	Oxidation and Advanced Oxidation	40–60 % and 85 % COD removal by simple oxidation and combined processes, respectively.	(Mantzavinos et al., 1997; Benitez et al., 1999; Gernjak et al., 2003; Rizzo et al., 2008; Chatzisymeon et al.,2009)
	Wet H_2O_2 Photocatalytic Oxidation		(Azabou et al., 2007)
	Electro-Fenton		(Khoufi et al., 2009)
	Ozonation		(Chedeville et al., 2009)
	Lime Treatment		(Aktas et al., 2001)
	Electrocoagulation		(Adhoum and Monser, 2004; Inan et al., 2004; Khoufi et al., 2008)
	Cloud Point Extraction (CPE)		(Gortzi et al., 2008)
	Combined		(De Marco et al., 2007)

Table 2.4. Treatment technologies of OMWW (cont.).

Category	Methodology	Results	References
Biological	Anaerobic Processes	60-80 % COD removal in 3-5 days; while 90 % COD removal in 25 days	Dalis et al., 1996; Sabbah et al., 2005; Ammary, 2005; Azbar et al., 2009a; Azbar et al., 2009b
	Aerobic Processes	55-75 % COD removal in a few days; while 80 % COD removal for longer time	Velioĝlu et al., 1992; Benitez et al., 1997; Paraskeva and Diamadopoulos, 2006; El Hajjouji et al., 2007
	Mixing and Digestion	75-90 % COD removal	Marques et al., 1998; Marques, 2001; Jaouani et al., 2003; Paraskeva and Diamadopoulos, 2006; Azbar et al., 2008
	Enzymatic		Jaouani et al., 2003; Dias et al., 2004
Combined	Oxidation and Biological Processes	75 % TP and 80-99 % COD removal; but expensive operation due to combined processes	Bressan et al., 2004; Drouiche et al., 2004; Beccari et al., 2009; Justino et al., 2010

2.1.4. An Alternative OMWW Treatment Technology: Mechanical Vapor Recompression Evaporator

Commonly used evaporation process in lagoons for the treatment of OMWW is very slow and evaporation ponds require very large land area. Therefore, an alternative application to reduce the volume of wastewater can be the use of Mechanical Vapor Recompression (MVR) evaporator. Although this evaporation technique has been used for the treatment of limited number of wastewater (McCabe and Vivona, 1999; Li and Watkinson, 2011; Sivakumar et al., 2013) and for the salinity reduction of wastewater and sea water (Karameldin and Mekhemar, 2003; Bahar et al., 2004; Liang and Han, 2011; Liang et al., 2013: Han et al., 2017), there is not any research for its application to the OMWW. The working principle of the MVR process is based on removal of 90-95 % water content of wastewater by thermal process in which temperature reaches more than 100 °C in a closed reactor. Hence, quick and economic water removal within small area of the reactor (5-10 m^2) can be obtained. The configuration and principle parts of the MVR evaporator are illustrated on Figure 2.2.

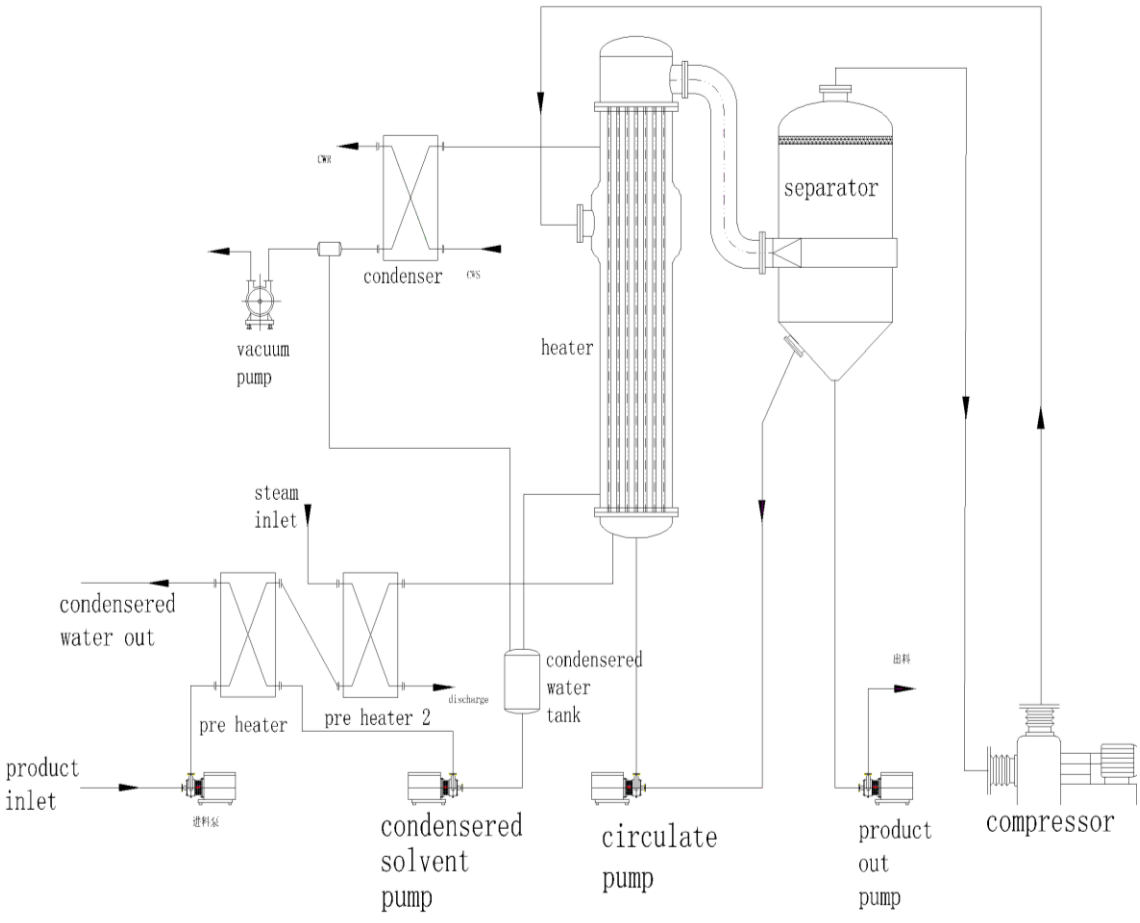


Figure 2.2. Basic components of a typical MVR evaporator.

2.2. Biophenolic Antioxidants in OMWW

Over recent years, PCs such as phenyl alcohols (hydroxytyrosol (HTyr), tyrosol (Tyr)), phenyl acids (vanillic acid, caffeic acid), flavonoids (luteolin, luteolin-7-glucoside), secoiridoids (oleuropein, oleuropein aglycon, ligstroside, verbascoside) and lignans ((+)-Pinoresinol, (+)-1-Acetoxypinoresinol, (+)-1-Hydroxypinoresino) have been detected in OMWW with various studies (Table 2.5) (Dagdelen et al., 2013; Leouifoufi et al., 2014; Stamatopoulos et al., 2014; Kelebek et al., 2015; Kaleh and Geißen, 2016; Cabrera-Bañegil et al., 2017).

Among more than 30 PCs found in OMWW, HTyr, Try, and oleuropein are the most abundant and bioactive phenols. These compounds exert an in vitro protective effect against low-density lipoprotein oxidation as well as being effective at low concentrations to protect human erythrocytes and DNA against oxidative damages (Obied et al., 2005; Takac and Karakaya, 2010; Rahmanian et al., 2014; Bedouhene et al., 2014). Besides, over the last decade, the patented applications in this field have generally focused on the recovery of HTyr, Tyr, oleuropein and several phenolic acids that possess stronger antioxidant characteristics compared to other PCs (Cuomo and Rabovskiy, 2002; Crea, 2004; Guzman et al., 2004; Fasiello et al., 2005; López et al., 2008). These olive polyphenols are also effective compounds to prevent chronic human diseases including cardiovascular and inflammatory disorders, as well as pathological diseases such as atherosclerosis, cancer, neurodegenerative diseases (Bedouhene et al., 2014).

Since current chemical and enzymatic processes for the synthesis of phenolic antioxidants such as HTyr are very slow and expensive, the recovery of these compounds from OMWW creates an environmentally safe and sustainable approach as well as a promising field for the market introduction of phenolic antioxidants from recovered compounds (De Marco et al., 2007; Galanakis et al., 2015).

Particularly HTyr is one of the most valuable and expensive PC with its strong antioxidant, antibacterial, anti-inflammatory and anti-angiogenic properties, that exerts a high demand in cosmetic, food and pharmaceutical industries (Bedouhene et al., 2014; Frascari et al., 2016; Putnik et al., 2017).

Table 2.5. Structural information of the major PCs in OMWW.

Phenolic Compounds	Substituents	MW (g/mole)	Chemical Structure
Benzoic Acid and Derivatives			
3-Hydroxybenzoic acid	3-OH	138	
p-Hydroxybenzoic acid	4-OH	138	
3,4-Dihydroxybenzoic acid	3,4-OH	154	
Gentisic acid	2,5-OH	154	
Vanillic acid	3-OCH_3, 4-OH	168	
Gallic acid	3,4,5-OH	170	
Syringic acid	3,5-OCH_3, 4-OH	198	
Cinnamic acids and derivatives			
o-Coumaric acid	2-OH	164	
p-Coumaric acid	4-OH	164	
Caffeic Acid	3,4-OH	180	
Ferulic Acid	3-OCH_3, 4-OH	194	
Sinapinic Acid	3,5-OCH_3, 4-OH	224	
Phenyl ethyl alcohols			
Tyrosol [(*p*-hydroxyphenyl)ethanol] or *p*-HPEA	4-OH	138	
Hydroxytyrosol [(*3,4*-dihydroxyphenyl)ethanol] or 3,4-DHPEA	3,4-OH	154	

Table 2.5. Structural information of the major PCs in OMWW (cont.).

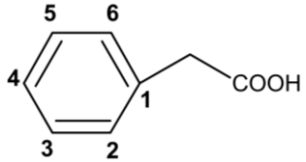
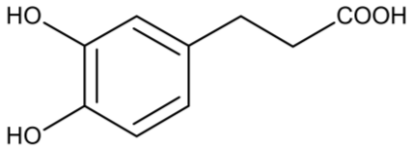
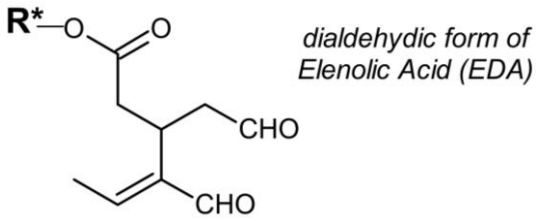
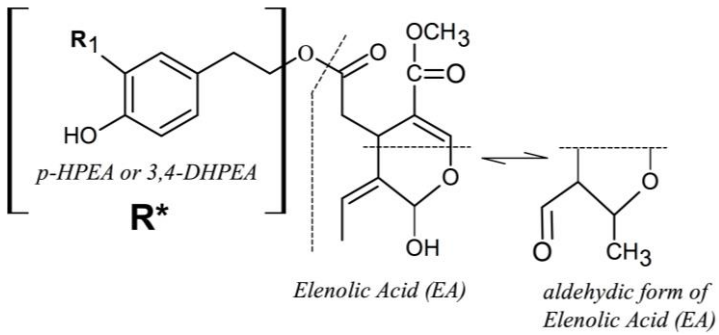
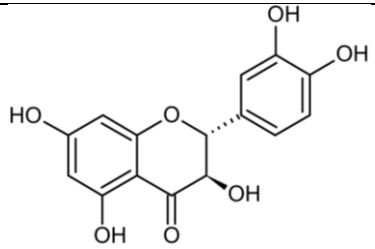
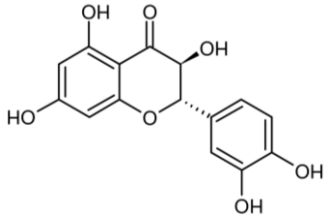
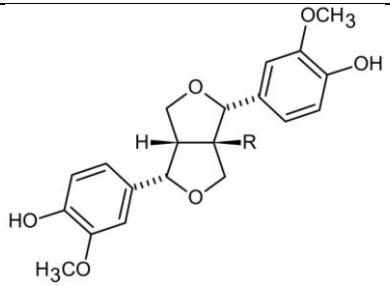
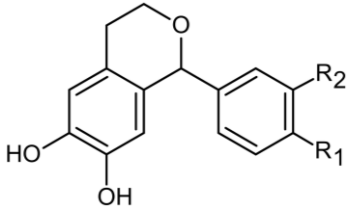
Phenolic Compounds	Substituents	MW (g/mole)	Chemical Structure
Other phenolic acids and derivatives			
p-Hydroxyphenylacetic acid	4-OH	152	
3,4-Dihydroxyphenylacetic acid	3,4-OH	168	
4-Hydroxy-3-methoxyphenylacetic acid	3-OCH_3, 4-OH	182	
3-(3,4-Dihydroxyphenyl)propanoic acid		182	
Dialdehydic forms of secoiridoids			
Decarboxymethyloleuropein aglycon (3,4-DHPEA-EDA)	R_1-OH	304	
Decarboxymethyl ligstroside aglycon (*p*-HPEA-EDA)	R_1-H	320	
Secoiridoid Aglycons			
Oleuropein aglycon or 3,4-DHPEA-EA	R_1-OH	378	
Ligstroside aglycon or *p*-HPEA-EA	R_1-H	362	
Aldehydic form of oleuropein aglycon	R_1-OH	378	
Aldehydic form ligstroside aglycon	R_1-H	362	

Table 2.5. Structural information of the major PCs in OMWW (cont.).

Phenolic Compounds	Substituents	MW (g/mole)	Chemical Structure
Flavonols			
(+)-Taxifolin		304	
Flavones			
Apigenin	R_1-OH, R_2-H	270	
Luteolin	R_1-OH, R_2-OH	286	
Lignans			
(+)-Pinoresinol	R-H	358	
(+)-1-Acetoxypinoresinol	R-$OCOCH_3$	416	
(+)-1-Hydroxypinoresino	R-OH	374	
Hydroxyisochromans			
1-Phenyl-6,7-dihydroxyisochroman	R_1, R_2-H	242	
1-(3-Methoxy-4-hydroxy)phenyl-6,7-dihydroxyisochroman	R_1-OH, R_2-OCH_3	288	

2.3. Strategies for the Recovery of Phenolic Antioxidants

In the past, great efforts have been spent for dephenolization of OMWW to reduce its toxicity. After realizing the beneficial effects of biophenols in OMWW, recent studies have been concentrated on characterization (De Marco et al., 2007; Leouifoufi et al., 2014; Castro-López et al., 2017), quantification (Kelebek et al., 2015), and recovery of PCs from wastewater (Ferri et al., 2011; Bertin et al., 2011; Kaleh and Geißen, 2016). Recovery of PCs from OMWW requires the pretreatment of samples by acidification and residual lipid removal processes, the extraction of PCs from pretreated samples and then the purification of phenolic antioxidants.

2.3.1. Pretreatment

Both acidification and lipid removal are the most common pretreatment methods prior to recovery of biophenols from olive mill effluents.

2.3.1.1. Acidification. Acidification of olive mill effluents favors the hydrolysis of oleuropein to HTyr by the reaction scheme on Figure 2.3 (Allouche et al., 2004; De Marco et al., 2007).

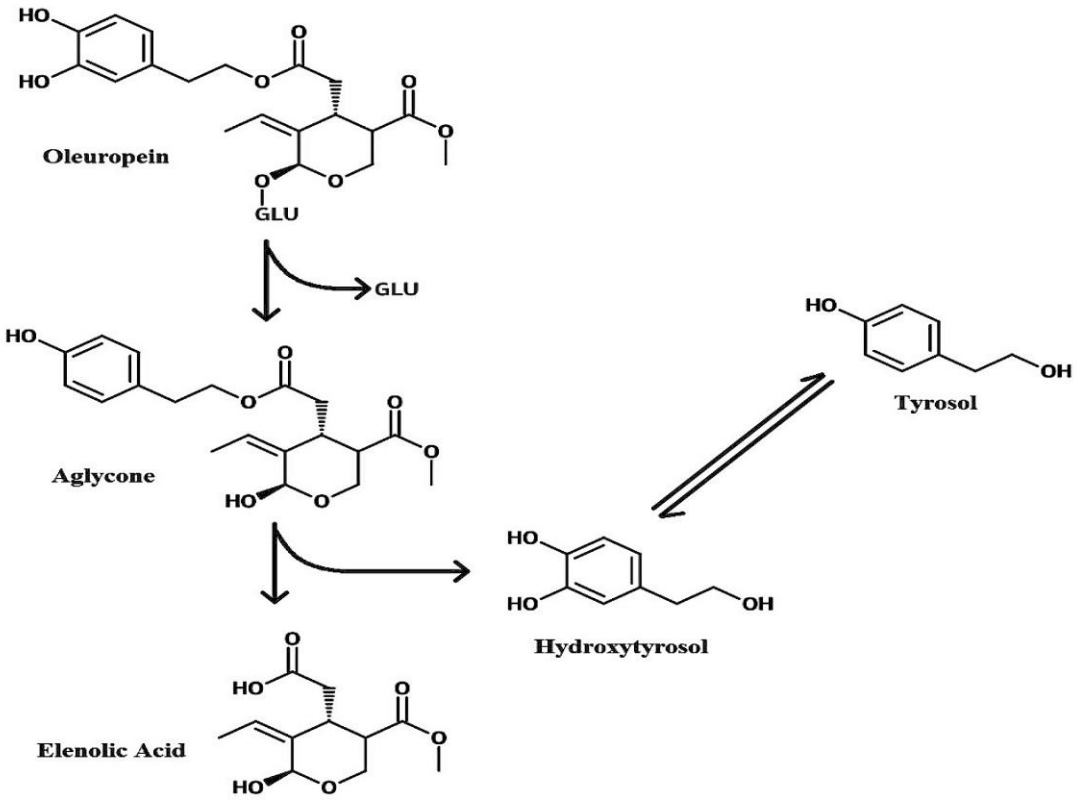


Figure 2.3. Acidic hydrolysis of oleuropein to HTyr and elenoic acid.

The release of biophenolic compounds, such as HTyr and Tyr, bonded to polymeric phenolic fraction can also require acidification (Rubio-Senent et al., 2013). The results of the research investigated by Sellami et al. (2016) supported this information by enhancing the release of HTyr in OMWW under acidic conditions. However, in the same study it was shown that the amount of Tyr and caffeic acid exhibited a decreasing trend by the acidification. In another study, the instability of caffeic acid and gallic acid was detected under acidic conditions (Friedman and Jürgens, 2000). The reduction in the content of HTyr or other PCs found in olive mill effluents was allocated to auto-oxidation arising under acidic environment or to the instability of these natural substances (Sellami et al., 2016).

Hydrochloric acid (HCl) is most commonly used for the acidification of olive mill effluents by lowering the pH around 2 (De Marco et al., 2007; Lafka et al., 2011; Leouifoufi et al., 2014; Bedouhene et al., 2014; Sellami et al., 2016; Rubio-Senent et al., 2013), while a patented study involved the addition of citric acid to increase the quantity of oleuropein aglycon in OMWW (Emmons and Guttersen, 2005). In another patented study, besides citric acid, other organic acids such as tartaric, maleic, malic, malonic, adipic, and fumaric acids were tested to extract PCs and dietary fibers, such as pectin, from OMWW (Tornberg and Galanakis, 2008). The acidification using these organic acids resulted in the precipitation of insoluble fibers in organic acid/EtOH system, while PCs and soluble fibers remained soluble in solvent.

Besides the benefits mentioned above, acidification also favors the stabilization of the vegetation water which is rich in low molecular weight biophenols, and avoids its fermentation (Crea, 2004). In the patented research of Crea, the use of different type of acids including mineral acids, such as sulfuric, hydrochloric, nitric and phosphoric acids, as well as organic acids belonging to the carboxylic acid, sulfonic acid and phenolic groups, especially citric acid was suggested for the acid treatment of olive vegetation water.

2.3.1.2. Oil Removal. Although advanced skimming and centrifugation systems are applied during oil extraction, residue of oil can remain in OMWW that should be removed prior to additional treatment and recovery studies (Visioli et al., 1999; Ceccon et al., 2001; De Marco et al., 2007; Lafka et al., 2011; Leouifoufi et al., 2014; Bedouhene et al., 2014; Rubio-Senent et al., 2013; Sellami et al., 2016).

2.3.2. Solvent Extraction

Liquid-liquid extraction (LLE) under acidic conditions is one of the most desirable extraction techniques due to its simplicity, effectivity and convenience for PCs found in OMWW (Leouifoufi et al., 2014). Solvent extraction processes are performed either individually or in combination with further purification activities such as membrane and chromatographic separations.

LLE has been investigated by using various organic solvents including ethyl acetate, ethanol, methanol, acetone, diethyl ether, n-butanol, isopropanol, and a mixture of ethanol/diethyl ether (De Marco et al., 2007; Takac and Karakaya, 2010; Venturi et al., 2017). Although the results of most of studies published in relevant literature are not comparable because of the different expressions of the extraction performance and recovery results, solvent extractions with ethanol, methanol, acetone, and ethyl acetate generally achieves high recovery of PCs. A recent publication by Galanakis et al. (2013) also supported these experimental findings with predicted activity coefficients of fifteen PCs in seven different solvent systems (water, ethanol, methanol, ethyl acetate, acetone, dichloromethane, diethyl ether) at different temperatures (298.15, 313.15 and 333.15 ºK). Diethyl ether (the less polar solvent, log P = 0.76) and water (the most polar solvent, log P = -0.29) are weak solvents for polyphenols. PCs with longer aliphatic fragments like HTyr and Tyr are more solubilized in polar protic mediums such as ethanol and methanol, followed by acetone and ethyl acetate, while their solubility is pretty low in diethyl ether, water and dichloromethane. Additionally, the solubility of oleuropein is highest in ethanol, followed by acetone, methanol, ethyl acetate, dichloromethane, diethyl ether and water (Galanakis et al., 2013).

Many researches have been investigated the extraction of PCs with ethyl acetate, while the patented invention of Visioli et al. (1999) reported that LLE with ethyl acetate is selective towards low and medium molecular weight PCs and heavier PCs are not extracted which remain in the water phase. De Marco et al. (2007) quantified PCs in the combined extracts of hexane and ethyl acetate. The amount of total phenolic content (TPC) was found 2.5 g Tyr/L of acidified OMWW whereas it was 1.1 g Tyr/L in crude OMWW.

In the following studies, the same methodology described by De Marco et al. (2007) was applied by Leouifoudi et al. (Leouifoufi et al., 2014) and Bedouhene et al**.** (2014). While TPC was found in the range of 5.27-10.1 g gallic acid equivalent (GAE)/L in one of the studies (Leouifoudi et al., 2014), the achieved phenolic extract included 0.148 mg GAE/mg in the other study (Bedouhene et al., 2014).

In another recent study, TPC of the ethyl acetate extracts of OMWW taken from two different source was reported as caffeic acid equivalents (CAE) which varies between 0.56 and 2.48 g CAE/L (Sellami et al., 2016). The study of Sellami et al. (2016) also revealed that the acidification of OMWW increased TPC from 0.58-0.98 g CAE/L to 2.48-3.69 g CAE/L of OMWW.

Ethanol generally regarded as safe solvent was also used for the extraction of PCs. Tercan and Seker achieved 2.19 g/L and 2.62 g/L polyphenols as a result of ethanol extraction from both liquid and solid fraction of OMWW, respectively (Tercan and Seker, 2012). In a patented work performed by Tornberg and Galanakis, up to 70 % (v/v) ethanol and an organic acid, preferably citric acid, in the range of 0.5 % to 3 % by weight of the extraction solution were used to extract PCs from OMWW and the maximum amount of TP was achieved as 1,254 ppm (Tornberg and Galanakis, 2008). In a recent study, the extraction performances of ethanol and ethanol/diethyl ether solvents were compared and resulted with the recovery of 0.43 and 0.29 g GAE/L of solvent extracts of OMWW, respectively (Venturi et al., 2017).

Lafka et al. (2011) compared the extraction performances of methanol, ethanol, a mixture of ethanol and water (1:1, v:v), ethyl acetate, n-propanol and isopropanol under different extraction conditions to recover PCs from olive mill wastes. In addition to the solvent extraction methods, the extracts obtained from supercritical fluid extraction (SFE) using carbon dioxide (CO_2) were tested in their study. TPC of the extracts estimated as CAE varied from 0.43 % to 1.29 % (w/w) depending on the type of solvent used. It was also reported that SFE yielded low recovery results compared to the extracts achieved by solvent extraction processes except ethyl acetate. At the end of the 24 h extraction process, methanol showed the highest extraction efficiency, then ethanol, ethanol/water mixture (1:1), propanol, isopropanol, SFE/CO_2, and ethyl acetate. However, the ethanol extracts demonstrated the highest (59.8 % inhibition) antiradical activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method, followed by the extracts of methanol (58.7 %), isopropanol (54.5 %), ethanol/water (49.3 %), n-propanol (45.8 %), SFE/CO_2 (41.7 %) and ethyl acetate (34.2 %), respectively. Apparently, higher antioxidant activity depended upon the higher TPC, and the extracts with low TPC recovery (SFE/CO_2 and ethyl acetate) showed lower antioxidant activity.

The highly variable results of biophenols recovery cannot be attributed to the extraction performance since TPC of OMWW is highly dependent upon various factors as mentioned above. However, in any solvent extraction processes, the extract includes several components of OMWW such as organic acids, carbohydrates, secoiridoid derivatives, and flavonoids besides the PCs (Ceccon

et al., 2001), and supplementary purification stages are necessary for the isolation of PCs (Oreopoulou and Tzia, 2007). Galanakis et al. (2013) suggested the use of sequential extraction stages in order to eliminate other extractable matters such as sugars and organic acids. According to this suggestion, the polar mixtures such as ethanol/water or methanol/water could be used in the first extraction stage prior to fractionation of PCs with other solvents such as ethyl acetate or acetone in the second stage. This theoretical approach has successfully been applied for the recovery of HTyr, Tyr and oleuropein from olive leaves (Papoti and Tsimidou, 2009; Lee et al., 2015).

2.3.3. Solid Phase Extraction

Solid phase extraction (SPE) is another promising alternative technology for the recovery of phenols from OMWW. The process is an attractive application used especially in separation and purification activities thanks to its ease of operation, design and scale up, minimized consumption of toxic elution solvents, as well as its insensitivity to toxic molecules, simplicity of reactivation and cost-effectiveness of the process and minimized degradation possibility of target molecules (Soto et al., 2011). Activated carbon (granular activated carbon), non-ionic polymeric adsorption resins (XAD4, XAD7HP, XAD16, XAD16N), non-polar resins (ENV+), weak-base anion exchange resin (IRA96, Amberlyst IRA76), strong-base anionic resin (Amberlite A26), and several other resin types (AFs, MIPs, TRI-SBA-15, TRI-P-10) were used for the recovery of PCs from OMWW, which are discussed in the following paragraphs.

Although, activated carbon is used for the treatment of OMWW to reduce its phenolic content, it is not convenient for the recovery of PCs due to lower desorption performance (Anbia and Amirmahmoodi, 2011; Bertin et al., 2011). However, Ena et al. (2012) employed Azolla (a freshwater aquatic fern, strain Azolla caroliniana used as biofilter) and granular activated carbon (GAC) originating vegetable matrices for the recovery of biophenols from OMWW. Overall adsorption/desorption potential of GAC was found higher by reaching 3.5-fold higher HTyr recovery (3.31 mg/g) than that of Azolla (0.97 mg/g) resulting that GAC has higher affinity toward HTyr. In this study, different amount of adsorbent concentrations defined in the range of 12.5 to 200 g/L were employed for adsorption experiment, indicating that adsorbed phenolic content increased with increasing resin concentration by reaching almost 2.7 g/L PCs adsorption using maximum amount of adsorbent (200 g/L).

Instead of activated carbon, various types of polymeric adsorbents have been used for the recovery of PCs from OMWW in numerous studies (Agalias et al., 2007; Azzam et al., 2010; Bertin et al., 2011; Ferri et al., 2011; Scoma et al., 2012; Ena et al., 2012; Rahmanian et al., 2014; Xynos et al., 2015; Zagklis et al., 2015; Frascari et al., 2016; Kaleh and Geißen, 2016; Víctor-Ortega et al., 2016). Agalias et al. (2007) tested non-ionic XAD7HP and XAD16 polymeric adsorbents for both the isolation of polyphenols and the reduction of environmental contamination problem of OMWW. The application of the process resulted in an odorless wastewater with 99.99 % reduced content of PCs and 98 % reduced COD.

Bertin et al. (2011) and Ferri et al. (2011) performed two complementary researches with several adsorbents, namely XAD4, XAD7, XAD16, IRA96 and Isolute ENV+, by testing the desorption efficiency through water, methanol, ethanol and acidified ethanol elution. The nonpolar ENV+ resin exhibited the highest overall process productivity, while IRA96 gave the best adsorption performance of PCs. The highest desorption rate of TP was achieved by the employment of acidified ethanol as desorbing agent while the highest HTyr recovery was obtained with ethanol elution.

In a recent study, Zagklis et al. (2015) implemented XAD4, XAD16 and XAD7HP resins for both the recovery of PCs and their isolation from the carbohydrate fraction of OMWW treated by membrane process. After batch scale adsorption experiments, XAD4 and XAD16N showed better performance compared to XAD7HP which gave poor results for phenols adsorption. The desorption performance of triple distilled water, ethanol (EtOH) and acetone were tested. Distilled water and EtOH were found the most selective solvents towards the desorption of carbohydrate and phenolic fractions, respectively. On the other hand, acetone removed the fractions of both carbohydrate (100 %) and phenols (80 %) at high percentages. The recovered and concentrated phenols under vacuum evaporation yielded a final concentration of 378 g GAE/L TP including 845 g/L HTyr. In the studies examined by Bertin et al. (2011) and Ferri et al. (2011), the similar performance of the XAD resins was observed indicating that XAD7 resin gave the poorest potential for the adsorption of PCs, while the adsorption potential of XAD16 and XAD4 were close to each other.

Scoma et al. (2012) and Frascari et al. (2016) studied a research with XAD16 resin for the selective recovery of PCs from OMWW by acidified ethanol elution. Scoma et al. reported approximately 81 % and 53 % adsorption and desorption ratios, respectively. Although the XAD16 resin has a high selectivity towards PCs, in the study of Frascari et al., the purity of final product was found lower than 30 % due to the very low abundance of PCs in tested OMWW.

To increase the purity of recovered biophenols, Xynos et al. (2015) used a combined system including an extraction of polyphenol rich extract from XAD4 resin followed by the isolation of HTyr with centrifugal partition chromatography, and at the end of this process 54.3 % HTyr recovery with 95 % purity was achieved. Kaleh and Geißen (2016) tested AFs (carbonaceous resins derived from a synthetic polymer; AF5, AF6 and AF7) in addition to commonly used thirteen polymeric resins for the isolation of PCs from crude OMWW. AF5 resin demonstrated the highest isolation of HTyr and Tyr, while their desorption efficiency with water elution was low. Additionally, MIPs (molecularly imprinted polymers) attained a promising alternative with 79 % and 95 % selectivity towards HTyr and Tyr, respectively. In addition to nonionic resins used for the recovery of PCs, Victor-Ortega et al. (2016) suggested the utilization of strong-base (Amberlite A26) and weak-base (Amberlyst IRA76) anion exchange resins for the isolation of polyphenols from wastewater. The strong-base anion exchange resin exhibited faster adsorption kinetics (6.1 g/mg min) and consequently better adsorption capacity (22 mg/g) compared to the weak-base anion exchanger (2.3 g/mg min and 7.5 g/mg, respectively) using the model solutions prepared by dissolving reagent-grade phenol in water.

In addition to polymeric resins, different types of adsorbents were used to recover the biophenols from OMWW. Yangui et al. (2017) synthesized amine-modified mesoporous silica adsorbents (TRI-SBA-15, TRI-P-10) to recover PCs, and the overall process efficiency for HTyr with these adsorbents was found remarkably higher than that achieved with activated carbon. Although among polymeric adsorbents, non-ionic styrene-divinylbenzene resin, XAD16 seems one of the most commonly used and promising adsorbents, the results of different studies exhibited considerable variations for the efficiency of different resins that can be attributed to the OMWW characteristics as well as the effects of pretreatment activities. While the similar performances of XAD7, IRA96 and Isolute ENV+ were shown for the recovery of PCs from OMWW in the study of Bertin et al., 2011, XAD16 and IRA96 resins were found as the most effective adsorbents with 90 % and 76 % the recovery of PCs by Agalias et al. (2007) and Ferri et al., (2011), respectively. On the other hand, Frascari et al., (2016) achieved a final product with <30 % PC purity by using XAD16 resin.

In the aforementioned studies, water, methanol, ethyl acetate, cyclohexane, ethanol and mixture of these have been used as desorption solvent. Zagklis et al. (2015) and Agalias et al.(2007) used different solvents systems to separate carbohydrates and purify specific PCs retained on XAD4 and XAD16 adsorption resins, respectively. It was also shown that the desorption efficiency of PCs was higher at low pH value (Bertin et al., 2011; Rahmanian et al., 2014; Kaleh and Geißen, 2016). All the studies in the literature on the treatment of OMWW with SPE are summarized in Table 2.6.

Table 2.6. Solid phase extraction processes performed for the recovery of PCs from OMWW.

Source	Type of Adsorbent	Type of Elution Solvent	Polyphenol Recovery %	Purity of Phenols	References
Filtered OMWW	XAD4, XAD7HP, XAD16	Cyclohexane/ EtOAc/MeOH/H_2O	0.58 kg HTyr/1 m^3 OMWW by XAD16	90 % pure HTyr by XAD16	Agalias et al. (2007)
OMWW	XAD4, XAD7, XAD16, IRA96, Isolute ENV+ (High performance: ENV+)	Acidified Ethanol (0.5 % 0.1 M HCl, v/v)	Process productivity by ENV+ 59-84 %		Bertin et al. (2011)
OMWW	XAD7, XAD16, IRA96 and Isolute ENV+ (High performance: ENV+)	Ethanol	Max. adsorption by IRA96: 76 % Max. TP recovery by ENV+: 60 %		Ferri et al. (2011)
OMWW	XAD16	Acidified Ethanol (0.5 % 0.1 M HCl, v/v)	Adsorption: 81.4 % Desorption: 52.7 %		(Scoma et al., 2012)
OMWW	XAD16	Acidified Ethanol (0.5 % 0.1 M HCl, v/v)	Desorption: 65 -74 %	<30 % purity	Frascari et al. (2016)
OMWW	XAD4, XAD16, XAD7HP (High performance: XAD4,)	Among water, EtOH and acetone High performance	378 g GAE/L TP including 845 g/L HTyr.		Zagklis et al. (2015)

2.3.4. Cloud Point Extraction

In recent studies, a new method, namely cloud point extraction (CPE) which is suggested as a simple, fast, low-cost, sensitive, clean and selective method, has been used for the recovery and pre-concentration of PCs from OMWW (Katsoyannos et al., 2006; El-Abbassi et al., 2014; Giovanoudis et al., 2017). This extraction method requires the addition of 4-12 % surfactant. El-Abbassi et al. recovered up to 66.5 % phenols from OMWW using one step CPE with 10 % Triton X-100 surfactant at 90 °C (El-Abbassi et al., 2014). Katsoyannas et al. used 4-6 % Triton X-114 for the recovery of more than 96 % TPs from OMWW with one or more CPE steps (Katsoyannos et al., 2006). In another recent study, food-grade surfactants were tested for the separation of PCs from OMWW and after the three steps CPE process, the highest recovery was achieved 100 % by PEG 8000 and Tween 80, 98 % by Lecithin and Genapol X-080 (Giovanoudis et al., 2017).

2.3.5. Ultrasound-Assisted Extraction

Ultrasound assisted extraction (UAE) of PCs is generally investigated for the solid waste of olive oil extraction process namely olive pomace as well as olive fruits, olive leaves used as a source of PCs (Jerman et al., 2010; Achat et al., 2012; Şahin and Şamli, 2013; Xynos et al., 2015; Icyer et al., 2016; Shirzad et al., 2017; Mojerlou and Elhamirad, 2018). Both methanol and ethanol are the two most common solvents using in UAE as seen in Table 2.7.

Considering the studies mentioned in Table 2.7., temperature, sonication time, solvent concentration and extraction stage are the most important parameters which must be optimized to increase the phenolic recovery from different olive-based sources. According to results achieved at optimum conditions, the TP recovery increases with decreasing ethanol concentration at around 50-56 % (v/v) by water (Şahin and Şamli, 2013 ; Xynos et al., 2014; Shirzad et al., 2017). Also, the positive effect of the increasing temperature was generally observed on TPC recovery (Jerman et al., 2010; Xynos et al., 2014; Icyer et al., 2016; Shirzad et al., 2017; Mojerlou and Elhamirad, 2018). On the other hand, the effect of sonication time differs depending on sample type and characteristics.

Table 2.7. Ultrasound assisted extraction of PCs from olive-based sources.

Sample Source	Solvents	Tested Range of Experimental Parameters and Optimum Conditions	Total Phenolic Content and Antioxidant Activity	References
Olive Fruit	MeOH	Solvent (80-100%) Sonication Time (4-30 min) Temperature (25-45 °C) Extraction Stage (1-5) *Optimum: 100 % MeOH, 20 min, 45 °C, 3 stages	TPC: 94.1 to 98.7 %	(Jerman et al., 2010)
Olive Oil and Olive Leaves	MeOH	Sonication Time (15-45 min) Temperature (25-51 °C) Ultrasonic Power (27-52 W) *Optimum: 60 W, 45 min, 16 °C	TPC: 414.3 mg oleuropein/g of oil AA: 25.5%	(Achat et al., 2012)
Olive Leaf	EtOH	Solvent (0-100%) Solid:Solvent Ratio (25-50 mg/mL) Sonication Time (20-60 min) *Optimum: 50 % EtOH, 50 mg/mL, 60 min	TPC: 25.06 mg GAE/g of dried olive leaf AA: 95.6%	(Şahin and Şamli, 2013)
Olive Leaves	EtOH	Solvent (0-100%) Sonication Time (5-25 min) Temperature (40-190 °C) Extraction Stage (1-3) *Optimum: 56% EtOH, 190 °C, 3 stages	AA: 118.8 μg/mL Oleuropein: 26.1 % Max. yield: 46.64 %	(Xynos et al., 2014)

TPC: Total Phenol Content, AA: Antioxidant Activity

Table 2.7. Ultrasound assisted extraction of PCs from olive-based sources (cont.).

Sample Source	Solvents	Tested Range of Experimental Parameters and Optimum Conditions	Total Phenolic Content and Antioxidant Activity	References
Virgin Olive Oil Waste	MeOH	Solvent (0-100 %) Sonication Time (10-30 min) Temperature (30-60 °C) Amplitude (20-100 %) *Optimum: 51.1 % MeOH, 21 min 60 °C, 13.72 % amplitude	TPC: 1.6 to 45 mg GAE/g	(Icyer et al., 2016)
Olive Leaves	EtOH	Solvent (25-75 %) Sonication Time (5-15 min) Temperature (35-65 °C) *Optimum: 51%, 65 °C, 15 min	TPC: 183.4 mg GAE/g AA: 78.98 %	(Shirzad et al., 2017)
Olive Cake	MeOH	Solid/Solvent Ratio (2-10 %) Sonication Time (2-10 min) Temperature (35-60 °C) Duty Cycle (on time/off time) (0.35-0.8 s) *Optimum: 3.6%, 3 min, 56 °C, 0.6 s	TPC: 4.34 mg GAE/g AA: 73.5 %	(Mojerlou and Elhamirad, 2018)

TPC: Total Phenol Content, AA: Antioxidant Activity

3. MATERIALS AND METHODS

3.1. Materials

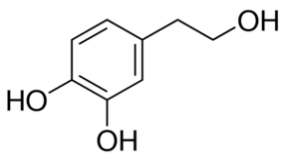
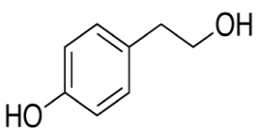
3.1.1. Olive Mill Wastewater Concentrates

Two different oil mill wastes were selected in this research as sources of phenolic antioxidants to compare their physicochemical characteristics, antioxidant capacity, and antioxidant recovery efficiency. These samples were the concentrated OMWW taken from a decanter and an open-air lagoon between December 2017-January 2018. While obtained residue of lagoon wastewater has sticky solid nature, the concentrated decanter wastewater was semisolid due to its higher moisture content. The gathered OMWC were stored at + 4 °C to guarantee stability until analysis of the samples.

3.1.2. Antioxidants

HTyr and Tyr were two antioxidants investigated in the study. The stock solutions of these antioxidants were prepared by dissolving the standards (≥98 % and 98 % purity from Sigma Aldrich, respectively) in MeOH at concentration levels of 5 and 1 mg/mL, respectively and stored at -20 °C prior to analysis. The physicochemical properties and structures of HTyr and Tyr are given in Table 3.1.

Table 3.1. Physicochemical characteristics of HTyr and Tyr.

Antioxidant	Hydroxytyrosol (HTyr)	Tyrosol (Tyr)
Structure		
Molecular Weight (g/mol)	154.170	138.164
Water Solubility (g/L)	17.4	25.3
pK_a	9.45	10.20
K_{ow}	6.44	4.90

3.1.3. Adsorbent

Amberlite XAD16N polymeric adsorbent, a nonionic, hydrophobic, and cross-linked polymer, was used for SPE operations. Its macroreticular structure (including both a continuous polymer and a continuous pore phases), large surface area and the aromatic nature of its surface are responsible for strong adsorptive characteristics of the resin. To minimize the growth of microorganisms within the resin, that is stored as a water wet product soaked with sodium chloride (NaCl) and sodium carbonate (Na_2CO_3). Therefore, it was washed several times as mentioned in section 3.2.1.5 before its use. The specifications of Amberlite XAD16N (Dow Chemical Company, n.d.) are listed in Table 3.2.

Table 3.2. Technical properties of Amberlite XAD16N polymeric adsorption resin.

Matrix	Macroreticular aliphatic cross-linked polymer
Physical form	White beads
Surface area	≥ 800 m^2/g
Porosity	≥ 0.55 mL/mL
Fines content (0.212 mm)	< 0.350 mm : 2.0 % max
Coarse beads	> 1.18 mm : 2.0 % max
Uniformity coefficient	≤ 2.0
Specific gravity	1.015 to 1.025
Moisture holding capacity	62 to 70 %
pH range	0-14
Harmonic mean size	0.56 – 0.71 mm
Maximum temperature limit	150 °C

3.1.4. Other Chemicals

All chemicals that were employed throughout the research are listed in Table 3.3.

Table 3.3. List of chemical substances used in the experiments.

Chemical Name	Molecular Formula	CAS Number	Experimental Use	Supplier
Hydrochloric acid	HCl	7647-01-0	pH Adjustment	Sigma-Aldrich
Hexane	C_6H_{14}	110-54-3	Oil Removal	Emboy
Ethyl acetate	$C_4H_8O_2$	141-78-6	Extraction	Sigma-Aldrich
Ethanol	C_2H_5OH	64-17-5	Extraction	Riedel de Haën
Ammonium Sulfate	$(NH_4)_2SO_4$	7783-20-2	ATPE	Sigma-Aldrich
Dipotassium hydrogen phosphate	K_2HPO_4	7758-11-4	ATPE	Merck
Potassium dichromate	$K_2Cr_2O_7$	7778-50-9	OC/COD	Merck
Ferrous ammonium sulfate hexahydrate	$Fe(NH_4)_2(SO_4)_2.6H_2O$	7783-85-9	OC	Merck
1.10-phenanthroline monohydrate	$C_{12}H_8N_2.H_2O$	5144-89-8	OC	Sigma-Aldrich
Iron (II) sulfate heptahydrate	$FeSO_4.7H_2O$	7782-63-0	OC	Sigma-Aldrich
Sulfuric acid	H_2SO_4	7664-93-9	OC/TKN/Carbohydrate/COD	Sigma-Aldrich
TKN Indicator			TKN	Hach
Polyvinyl alcohol	$(CH_2CHOH)_n$	9002-89-5	TKN	Hach
Nessler reagent			TKN	Hach
Mineral stabilizer			TKN	Hach
Hydrogen peroxide	H_2O_2	7722-84-1	TKN	Sigma-Aldrich
Potassium hydroxide	KOH	1310-58-3	TKN	Merck

Table 3.3. List of chemical substances used in the experiments (cont.).

Chemical Name	Molecular Formula	CAS Number	Experimental Use	Supplier
Folin-Ciocalteu Reagent			TP	Merck
Sodium Carbonate	Na_2CO_3	497-10-8	TP	Merck
Gallic acid	$C_7H_6O_5$	149-91-7	TP	Merck
Mercury (II) Sulfate	$HgSO_4$	7783-35-9	COD	Sigma-Aldrich
Silver Sulfate	Ag_2SO_4	10294-26-5	COD	Sigma-Aldrich
Alpha-D-glucose	$C_6H_{12}O_6$	492-62-6	Carbohydrate Analysis	Sigma-Aldrich
Anthrone	$C_{14}H_{10}O$	90-44-8	Carbohydrate Analysis	Sigma-Aldrich
Bradford Reagent			Protein Analysis	Sigma-Aldrich
2,2-diphenyl-1-picrylhydrazyl	$C_{18}H_{12}N_5O_6$	1898-66-4	Antioxidant Activity	Sigma-Aldrich
L-Ascorbic Acid	$C_6H_8O_6$	50-81-7	Antioxidant Activity	Sigma-Aldrich
Methanol	CH_3OH	67-56-1	Antioxidant Activity	Sigma-Aldrich
Formic Acid	HCOOH	64-18-6	HPLC and LC-MS/MS	Sigma-Aldrich
Milli-Q Water	H_2O		HPLC	Millipore
Acetonitrile	C_2H_3N	75-05-8	HPLC	Sigma-Aldrich
Water	H_2O	7732-18-5	LC-MS/MS	Merck
Ammonium Formate	HCO_2NH_4	540-69-2	LC-MS/MS	Sigma-Aldrich
Sodium Lauroyl Sarcosine	$C_{15}H_{28}NNaO_3$	137-16-6	LC-MS/MS	Amresco
Methanol	CH_3OH	67-56-1	LC-MS/MS	Merck

3.2. Methods

3.2.1. Multistage Recovery Process

A multistage recovery process of biophenolic antioxidants was initiated by reducing the water content of OMWW and assembling OMWC in a pilot scale MVR Evaporator operated in Ayvalık district of Balıkesir. Then, the concentrated wastewaters were pretreated prior to extraction stage by the employment of oil removal and acidification. The pretreated OMWCs were subsequently extracted with solid-liquid extraction (SLE) or aqueous two-phase extraction (ATPE) for the recovery of PCs using organic solvents, namely ethyl acetate (EtOAc) for only SLE and EtOH for both SLE and ATPE. Afterwards, the final stage was performed by the SPE for further purification of PCs. The systematic representation of multistage recovery process is shown in Figure 3.1.

Due to very viscous and sticky nature, the lagoon OMWC was heated at 30-40 °C to be taken for the application of the processes and analysis whereas there was no need for sample preparation for the decanter OMWC prior to analysis. After a certain amount of OMWC has been weighed, it was pretreated for oil removal and acidification as described in the following paragraphs for process details and potential merits.

3.2.1.1. Pretreatment of OMWC for Lipid Removal. Lipid removal was performed to improve SLE performance. Considering the physicochemical characteristics of samples used in this research, oil removal was applied only to the concentrated decanter wastewater and it was accomplished by hexane extraction. After dissolving 0.1 g dry weight of the sample in 5 mL deionized water (DW), the extraction was carried out with 10 mL hexane at 2000 rpm for 10 min. The separation of the lipid containing hexane phase was carried out by centrifugation at 3000 rpm for 3 min.

3.2.1.2. Pretreatment of OMWC by Acidification. The lagoon and decanter OMWCs were acidified at pH 2 with 0.1 M HCl prior to extraction of the PCs in order to hydrolyze oleuropein to HTyr and to liberate PCs bonded to polymeric phenolic fraction. Acid-free lagoon and decanter samples were also used as controls.

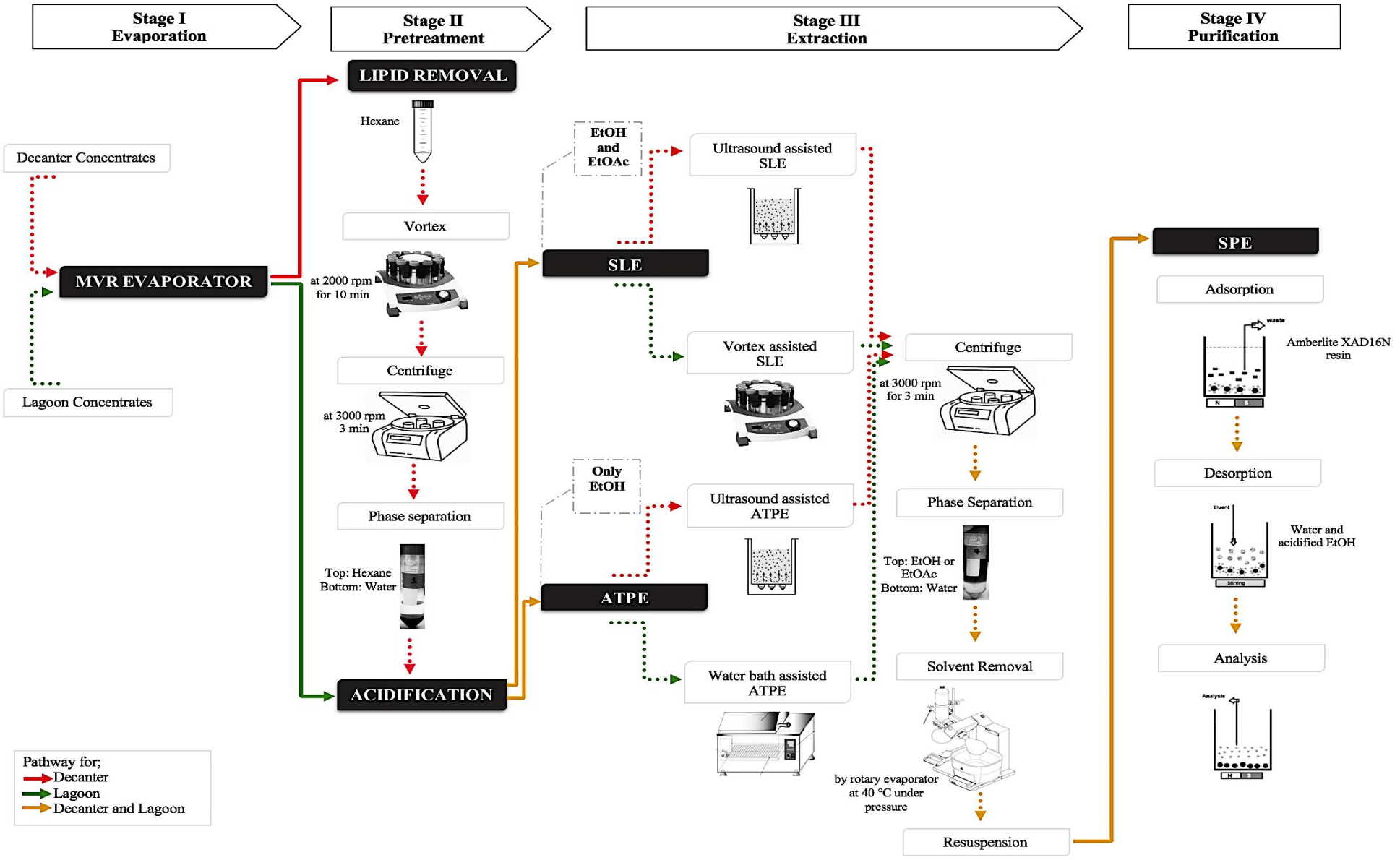


Figure 3.1. Multistage recovery process of phenolic antioxidants from OMWW.

3.2.1.3. Solid-Liquid Extraction of OMWC. In the first step of the extraction process, 0.1 g of the lagoon and decanter OMWCs placed in falcon tubes were dissolved in 5 mL DW. Then, specified volumes of nonaqueous solvents, EtOAc or EtOH, were added to separate sample tubes to test the effect of the solvent/sample ratio on the extraction efficiency. The mixtures were extracted at 2000 rpm for 10 min and then were centrifuged at 5000 rpm for 5 min.

Since the decanter OMWC has water insoluble fraction, as opposed to the lagoon sample the effect of ultrasound on the extraction performance was searched only for this sample to facilitate the release of PCs bonded to complex molecules. In experimental method, the acidified suspension of decanter OMWC was extracted at different sonication times and extraction stages using predefined volumes of EtOAc and EtOH solvents. After the centrifugation of samples, solvents were removed with rotary evaporator (Heidolph Laborata 4000) under negative pressure at 40 °C. Followed by the removal of the solvent, the concentrated extract was resuspended with 40 mL of DW and filtered through 0.2 μm syringe filters (Sartoriuos). The filtered fraction was used for the following analysis (except for COD analysis).

3.2.1.4. Aqueous Two-Phase Extraction of OMWC. The systems selected for the estimation of partition behavior of PCs found in OMWC were determined according to the study examined by Xavier et al. (2017) who previously investigated the extraction of phenols from eucalyptus wood wastes by ATPE. Two different types of salt, which are dipotassium hydrogen phosphate, K_2HPO_4, and ammonium sulfate, $(NH_4)_2SO_4$, were tested to induce salting out effect.

During the preparation of the system, the salt was first dissolved in DW in a falcon tube and then EtOH was added dropwise by mixing the solution with a magnetic stirrer. After cloud point determination, excess amount of EtOH was added at the given concentration and a ternary diagram was drawn to show the final concentration of each component in the systems. Finally, 0.1 g of acidified samples, which were previously prepared in separate falcon tubes, were mixed with the system. The volume of 0.1 M HCl used for acidification of the samples was considered in the preparation of the systems to keep system dynamics constant. Assessment of the selected salt performance for the recovery of PCs from OMWC samples was performed by using vortex-assisted ATPE at room temperature and at 2000 rpm for 10 min.

According to the results of preliminary experiments performed by vortex assisted ATPE, ammonium sulfate was selected for further experiments. Because of the difference in physicochemical

nature of OMWCs, mechanical shaker and ultrasound assisted ATPEs were applied for lagoon and decanter OMWCs placed in 250 mL Erlenmeyer flasks, respectively to achieve successful mixing and to increase the interaction of system components.

Different extraction conditions have been tested in order to find the best compromise between efficiency and EtOH concentration, temperature, time of extraction. After centrifugation of the mixtures achieved at each extraction condition at 3000 rpm for 3 min, EtOH phase was separated and the solvent was removed with a rotary evaporator at 40 °C. The residue remained was resuspended with 40 mL of DW and used for analysis. The experimental process of ATPE is illustrated in Figure 3.2.

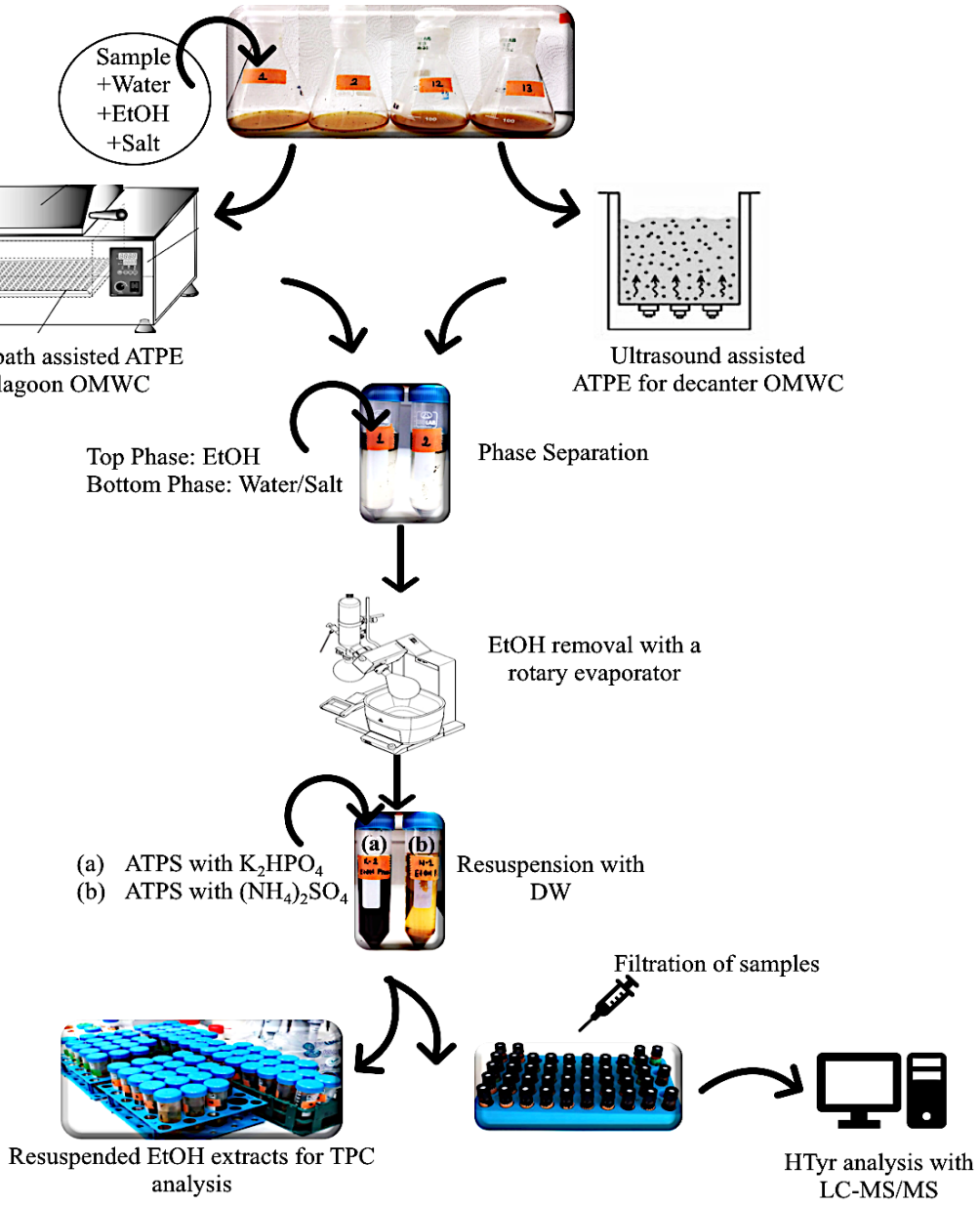


Figure 3.2. Experimental procedure of ATPE.

3.2.1.5. Purification of Extracts with Solid Phase Extraction. A nonionic polymeric resin Amberlite XAD16N was used for further purification of PCs. Prior to use, it was washed with acidified EtOH (0.5 % 0.1 M HCl, v/v), which was then removed by rinsing the resin several times with DW and finally the resin was dried at 105 °C in a laboratory oven. The purification capacity of the resin was determined at different dosages with 10 mL OMWC extract placed in 50 mL Erlenmeyer flasks. Batch equilibrium experiments were performed with an orbital shaker at 140 rpm for 3 hours by samplings every 15 min. After the equilibration, the resin was washed first with 20 mL DW three times to remove carbohydrate fraction, then with 25 mL acidified EtOH (0.5 % 0.1 M HCl, v/v) twice to desorbed phenolic fraction.

3.2.2. Analytical Methods

Analytical methods used in this research for the quantification of PCs and their relative antioxidant activity, as well as for the characterization of OMWC are described in the following sections.

3.2.2.1. Total Phenol Content. TPC of the samples was determined colorimetrically according to Folin-Ciocalteu method (Singleton and Rossi, 1965). Aliquots of 0.5 mL sample were mixed with 2.5 mL of 0.2 N Folin-Ciocalteu reagent. Afterwards, 2 mL sodium carbonate solution (7.5 %, w/v in DW) was added to create a basic condition (pH of ~10) for the redox reaction between PCs and Folin-Ciocalteu reagent, and the assay tubes were incubated in a water bath at 50 °C for 5 min before cooling at room temperature. After the incubation period, the greenish yellow color of the sample matrix turned to light to dark blue depending on the concentration of PCs. Phenolic protons dissociate under the basic conditions resulting in the formation of phenolate ions, which favor the reduction of Folin-Ciocalteu reagent. Among the components of Folin-Ciocalteu reagent, molybdates are the most reducible components suggesting that most of the electron transfer reactions in the matrix are between the reductants (phenolate ions) and the molybdates (Sánchez-Rangel et al., 2013) as represented in the following reactions.

Redox Reaction at pH ~10

$$\text{Mo (VI)} + e^- \text{ (from PCs and other reducing compounds)} \longrightarrow \text{Mo (V)} \qquad (3.1)$$

$$\text{Sample in Folin reagent (pH ~3)} + \text{Sodium Carbonate} \longrightarrow \text{Phenolate ions (pH ~10)} \qquad (3.2)$$

The absorbance of the assays was measured at 765 nm by using a double beam spectrophotometer (Shimadzu UV-1208) which was used for all further spectrophotometric measurements against reagent blank. In Folin-Ciocalteu method, blank sample was prepared by the addition of 0.5 mL DW instead of sample solution. The calibration curve was constructed using gallic acid standards prepared in DW in the range of 10 mg/L to 80 mg/L. The development of the color change after the salt addition is shown in Figure 3.3, and the results were expressed as gallic acid equivalents (GAE). Sample dilution was performed when the recorded absorbance value exceeded linear range of the calibration curve. The calibration graph of gallic acid for TP analysis is given in Appendix C.

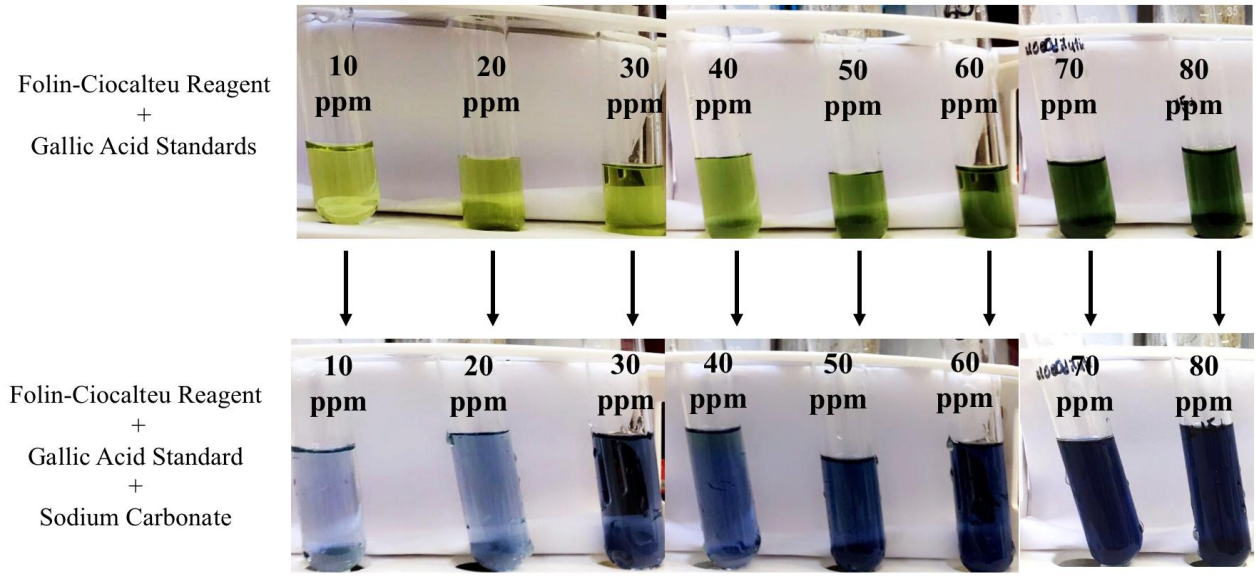


Figure 3.3. The color development in TP analysis by Folin-Ciocalteu method.

3.2.2.2. HPLC Analysis. The analysis of HTyr and Tyr was performed by a HPLC instrument (Agilient 1100 series) equipped with a binary pump, diode array detector (DAD) and an autosampler. Chemstation software was adapted to run the instrument. The chromatographic separations of target antioxidants in extracts were performed using Phenomenex Gemini-NX C18 column (150 x 4.6 mm with 3 μm particle size) at 40 °C. A binary mobile phase consisting of 0.1 % formic acid (FA) in milliQ (MQ) water (Mobile Phase A) and 0.1 % FA in acetonitrile (Mobile Phase B) were used with a gradient elution program (Table 3.4). The injection volume of 10 μL was run at a flow rate of 0.8 mL/min. After the sample injection, the system was washed with mobile phases.

Table 3.4. The gradient flow of mobile phases used for HPLC analysis.

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
10	90	10
25	70	30
45	50	50
50	30	70
60	90	10

Detection of phenolic antioxidants was carried out at 280 nm. Standard solutions for the calibration curve were prepared in mobile phase and MQ water (1:1, v/v). The calibration curves of HTyr and Tyr were drawn with fresh standards at five different concentrations (1, 2.5, 5, 7.5 and 10 ppm) prepared just before the analysis. The area under the peaks of the analytes in the samples was determined to quantify their concentration with respect to the calibration curves. The calibration curves of HTyr and Tyr are given in Appendix A.

3.2.2.3. LC-MS/MS Analysis of Hydroxytyrosol. As a confirmation of HTyr concentrations, the samples were analyzed by a triple quadrupole mass spectrometer, AB Sciex QTRAP 4500 LC-MS/MS system combined with Eksigent ekspert ultraLC system 110. The measurements were performed with electro spray ionization (ESI) probe in the negative ion mode at -4500 V ion spray voltage (IS) and a temperature of 550 °C (TEM) using turbo spray ion source. Data were collected at 152.9, 122.8 and 122.6 m/z.

As a preliminary step, MS/MS conditions for targeted antioxidant (100 ppb in mobile phase A) were generated by manual infusion with a syringe pump and the parameters of the ESI source were optimized. After the parent ion (Q1) of HTyr was determined, 2 daughter ions (Q3s), which are the ions with the highest intensities among the other ions generated after the fragmentation of the parent ion in the collision cell, as well as optimum declustering potential (DP), entrance potential (EP), collusion energy (CE) and collision cell exit potential (CXP) to generate those Q3s were determined by “Compound Optimization” mode. The optimum system conditions are given in Table 3.5.

Table 3.5. Optimum MS/MS conditions for HTyr analysis.

Compound	Q1 (m/z)	Q3 (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
HTyr	152.9	122.8	-65	-10	-20	-3
HTyr	152.9	122.6	-65	-10	-20	-9

In LC-MS/MS system chromatographic conditions were optimized for the standard of HTyr with gradient elution program through a reversed phase column to achieve optimum separation conditions for the analytes. During 14 min analysis period, elution was performed through Phenomenex Kinetex C18 column (50 x 3.0 mm with 2.6 μm particle size) at 40 °C with a flow rate of 0.5 mL/min, using 0.1 % FA and 10 mM ammonium formate in MeOH (Mobile Phase A), and 0.1 % FA and 10 mM ammonium formate in water (Mobile Phase B). The gradient flow of LC-MS/MS analysis is given in Table 3.6.

Table 3.6. The gradient flow of mobile phases used for LC-MS/MS analysis.

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
1.5	0	100
2	50	50
4	50	50
5	100	0
14	100	0

The calibration curve of HTyr was constructed with fresh standards at eight different concentrations (0.01, 0.05, 0.1, 0.5, 1, 2, 5 and 10 ppb) prepared from HTyr stock solution. The dilution of samples and standards was performed using mobile phase mixture (1:1, v/v) and an internal standard, sodium lauroyl sarcosine with the same concentration of the standard which was added and with the concentration of 0.05 ppb for sample dilutions, prepared in MeOH:Water (1:1, v/v) prior to analysis. The calibration curve of HTyr and its mass spectrum are given in Appendix B and Figure 3.4, respectively.

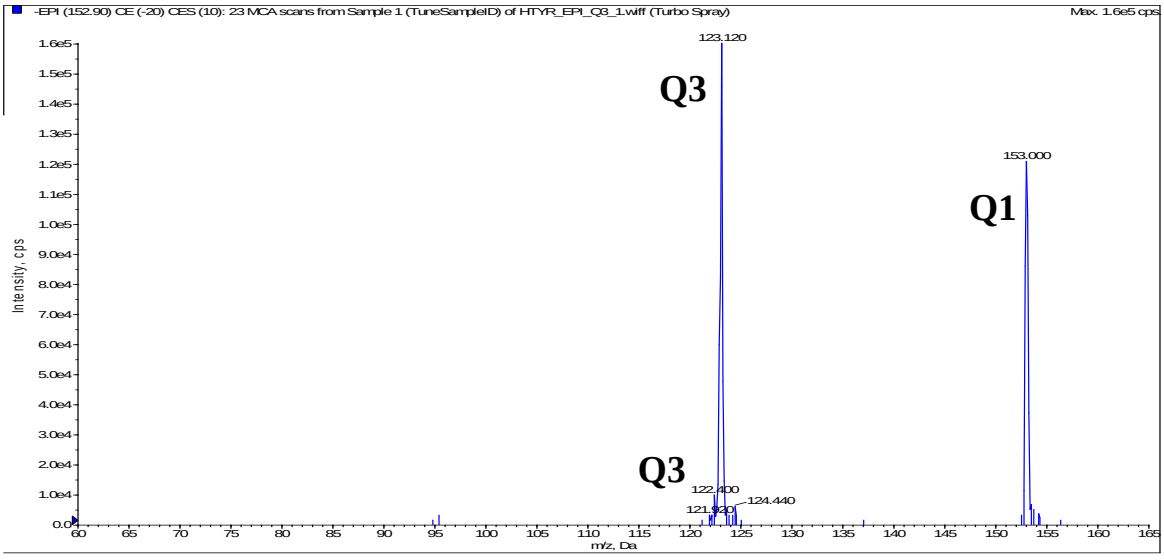
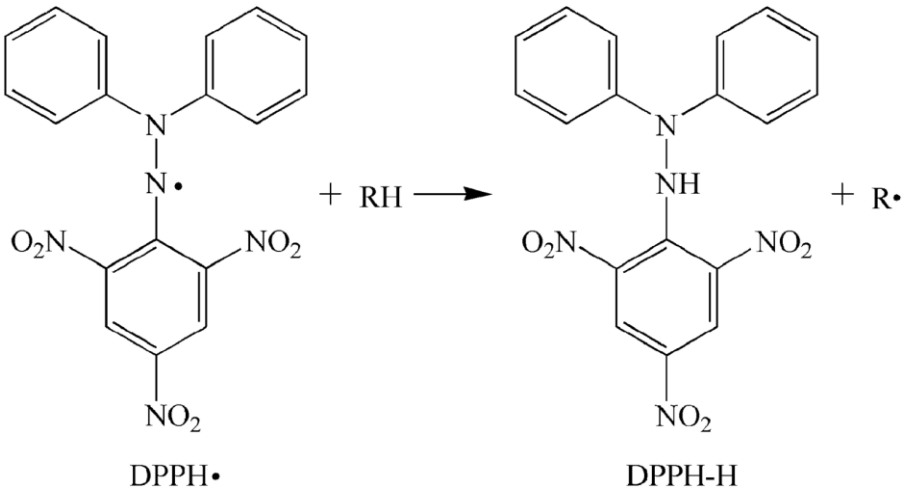


Figure 3.4. Mass spectrum and fragmentations of HTyr standard by LC-MS/MS analysis.

3.2.2.4. Antioxidant Activity. The antioxidant activity of the extracts was estimated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay which was originally developed by Blois (Blois, 1958). DPPH is a stable radical depending on the paramagnetism given by the lone electron; in other words, delocalization of the spare electron on the molecule as a whole. The DPPH solution prepared in methanol has dark violet color and represents a strong absorption at 517 nm. In the presence of an electron or hydrogen radical provided by an antioxidant, DPPH radical is reduced to become a stable diamagnetic molecule as indicated in the reaction below (Brand-Williams et al., 1995). As a result, its color alters from deep to pale violet by reducing its absorption, indicating that this extract has antioxidant effect by mechanism of free radical scavenging activity.



(3.3)

The method described by Hendel et. al. (Hendel et al., 2016) was adapted to estimate antioxidant activity of achieved phenolic extracts of OMWC. The assays were prepared by adding 50 μL of sample to 5 mL of 0.004 % (w/v) DPPH solution in methanol. After 30 min incubation in the dark at room temperature, absorbance values were recorded at 517 nm.

The DPPH free radical scavenging activity was calculated as follow:

$$\% \text{ Antioxidant Activity} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100 \quad (3.4)$$

$A_{control}$ = The absorbance of control solution containing only MeOH and DPPH

A_{sample} = The absorbance of the extract after 30 min incubation

The results were also expressed as ascorbic acid equivalents (AAE) by using L-ascorbic acid (5 to 30 μg/mL) standard calibration curve (Appendix C).

3.2.2.5. pH. The pH values of the samples and extracts were measured with WTW pH 330 pH-meter or pH-indicator strips by dissolving 0.1 g of solid and semisolid concentrates in 40 mL DW and diluting the extracts to 40 mL with DW.

3.2.2.6. Moisture Content. The moisture content of samples was determined by Kern DBS Version 1.0 Electronic Moisture Analyser setting the program condition at 120 °C. Drying was automatically completed when the preset weight loss remained constant for 30 seconds.

3.2.2.7. Organic Carbon Content. The OC content of samples was determined by the oxidation reaction as described in TS 8336 Method of Turkish Standards Institute (TSE), which has been developed according to Walkley-Black Method.

Lagoon and decanter OMWC samples (5 to 35 mg) were digested in the presence of 3 mL of 1 N potassium dichromate ($K_2Cr_2O_7$) and 3 mL of concentrated sulfuric acid (H_2SO_4) in closed glass tubes at 150 °C for 2 h. After digestion, excess dichromate in the test tube was back titrated with 0.5 N of ferrous ammonium sulfate (FAS) solution by adding 3-4 drops of ferroin indicator. The ferroin indicator was prepared by dissolving 1.5 g of 1.10-phenanthroline monohydrate and 0.1 g of iron (II) sulfate heptahydrate in 100 mL DW.

The OC content of OMWC was estimated using the following equation.

$$\text{OC \%} = \frac{((B-S) \times N_k \times 0.389)}{T} \qquad (3.5)$$

B = Volume of FAS used for the blank sample (mL)
S = Volume of FAS used for the sample (mL)
N_k = Normality of FAS solution (N)
T = Weight of OMWC (g)

3.2.2.8. Total Kjeldahl Nitrogen Content. The TKN content of the OMWC was examined by the standard digestion procedure published by Hach company using H_2SO_4 and hydrogen peroxide (H_2O_2) (Hach Company, 2014). In the digestion process, lagoon and decanter OMWCs (0.25 g to 0.5

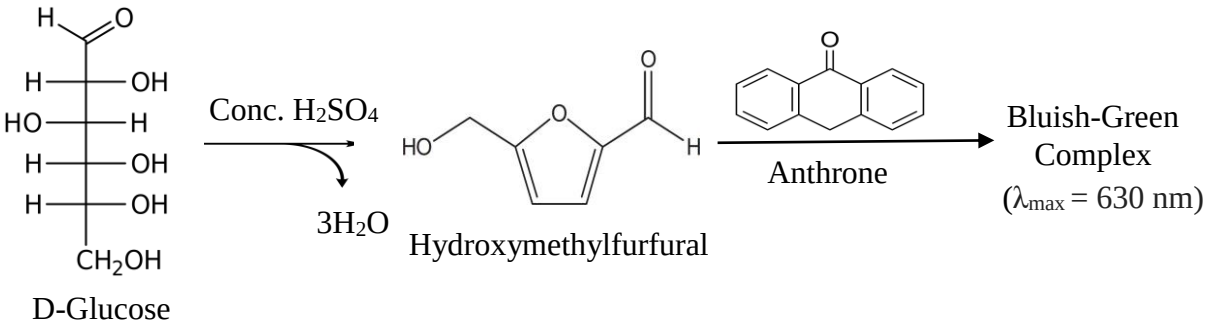
g) were digested with 4 mL H_2SO_4 using the Digesdahl digestion apparatus until reflux started at around 440 °C and for further 4 min. After the digestion period, 10 mL H_2O_2 was added to the flask and excess H_2O_2 was boiled off by heating for one more min. Finally, the digested sample was diluted to 100 mL with DW and stored at 4 °C until analysis.

The TKN content of the digested samples was determined according to the spectrophotometric Nessler method (Hach Company, 2014). The method involves the sequential addition of 5 mL DW, 3 mL digested sample and 1 drop TKN indicator in a volumetric flask. This solution was then titrated by the dropwise addition of 8 N potassium hydroxide (KOH) until the permanent blue color was observed. Afterwards, the solution was diluted to 20 mL with DW, followed by the addition of 3 drops of mineral stabilizer and 3 drops of polyvinyl alcohol. After diluting the solution to 25 mL with DW, 1 mL of Nessler reagent was added to this solution. The TKN content (mg/L) was determined by measuring the absorbance at 460 nm immediately after the 2 min incubation period (Eq 3.6). Blank sample was prepared by using 25 mL of DW for digestion step while the following procedure for blank was same as the method described above.

$$\text{TKN (ppm)} = \frac{75 \text{ x } A}{B \text{ x } C} \tag{3.6}$$

A: Value read from the display of spectrophotometer (mg/L)
B: Mass of sample taken for digestion (g)
C: Analysis volume of digested sample (mL)

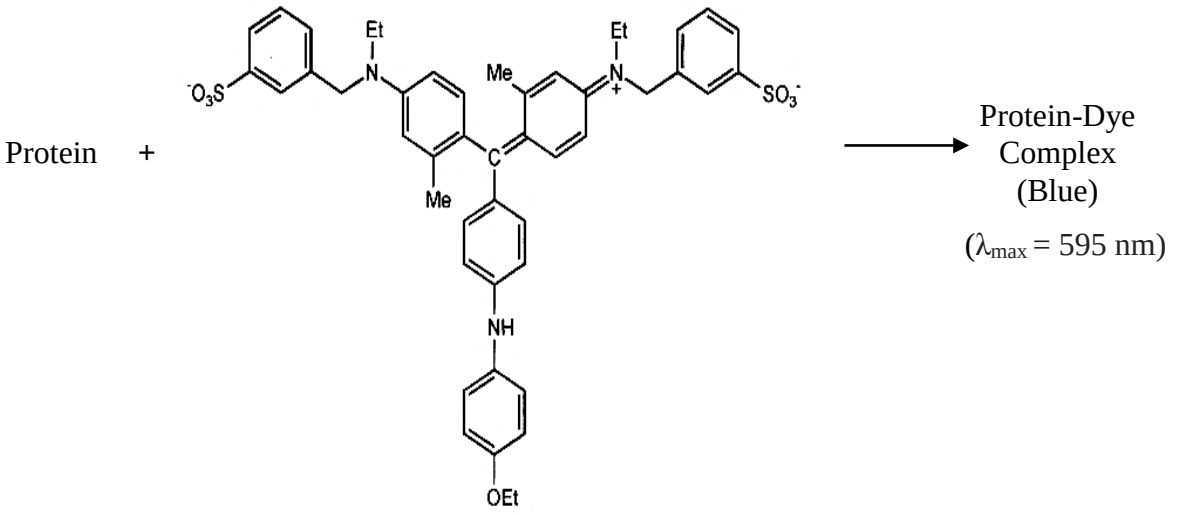
3.2.2.9. Carbohydrate Content. The carbohydrate content of lagoon and decanter OMWC samples and the extracts was estimated according to the Anthrone method (Dreywood, 1946; Jagannathan et al., 2010). The filtered sample was mixed with anthrone reagent, which was prepared by dissolving 200 mg anthrone in 100 mL concentrated sulfuric acid (2 g/L), at 1:4 (mL/mL) volume ratio and incubated in boiling water bath for 10 min. In the presence of sulfuric acid, carbohydrates are first hydrolyzed to simple sugars; which are then dehydrated to hydroxymethylfurfural in hot acidic medium. In the medium, the anthrone reagent was used as a coloring agent reacts with the furfural derivatives, forming a blue-green complex as shown in the following reaction.



(3.7)

After the completion of reaction, the assay tubes were cooled at room temperature and the absorbance values were measured at 630 nm. The calibration curve was constructed using alpha-D-glucose (20-100 mg/L) standards and the results were described as glucose equivalents (mg glucose/g of extract). The calibration curve of the method is given in Appendix C.

3.2.2.10. Protein Content. The Bradford method (Bradford, 1976) was used to estimate the protein content of OMWC and extracts. Briefly, 40 μL of the filtered sample was added to 2 mL of conventionally ready Bradford reagent, which contains the dye Coomassie Brilliant Blue G-250 and mixed thoroughly. During the reaction between the Bradford reagent and the protein fraction of the sample, red color of the dye is converted to blue upon binding to protein (Eq 3.8).



(3.8)

Coomassie Brilliant Blue G-250

This binding is very fast process (nearly 2 min) and the protein-dye complex remains persistent in the solution for approximately 1 h. For this reason, assay tubes were stored at room temperature for 5 min and the absorbance values were measured at 595 nm. A calibration curve was created with bovine serum albumin (BSA) protein standards prepared at concentrations ranging from 0.01 g/L to 2 g/L. The calibration curve of BSA is given in Appendix C.

3.2.2.11. Lipid Content. The partition-gravimetric method was used to quantify lipid content of the samples. A representative portion of lagoon and decanter samples was dissolved in 15 mL DW and extracted with 15 mL of n-hexane in 2 stages. After the collection of hexane extracts, the solvent was evaporated at 70 °C. The residual lipid content was quantified according to the following equation after sufficient cooling in the desiccator.

$$\text{Lipid (mg/g of sample)} = \frac{W_T - W_B}{W_S} \quad (3.9)$$

W_T = weight of flask + oil content of sample (g)
W_B = weight of flask + oil content of blank (g)
W_T = weight of sample used (g)

The experimental procedure of lipid removal is shown in Figure 3.5.

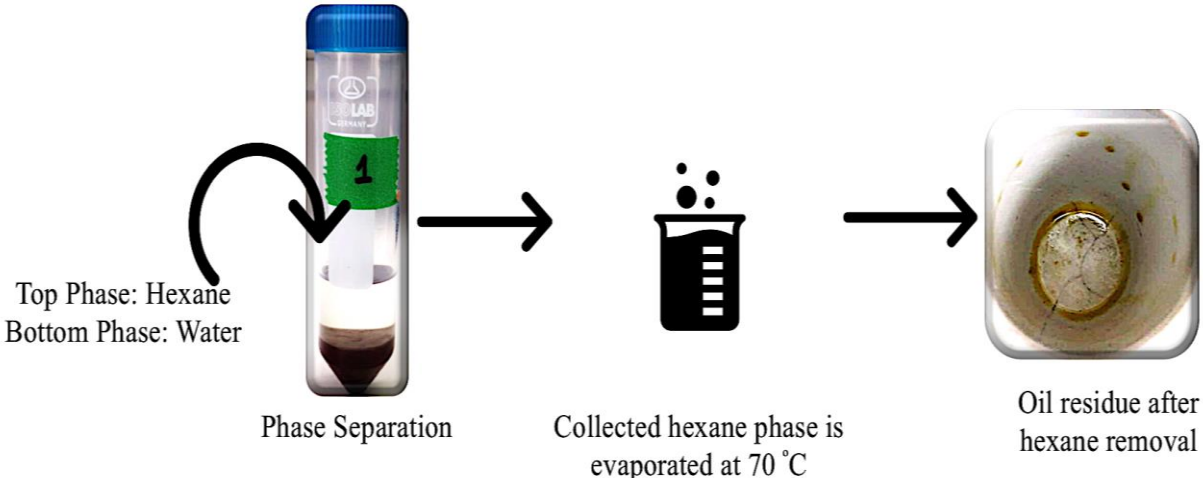


Figure 3.5. Experimental process of lipid removal.

3.2.3. Experimental Design and Statistical Evaluation

Optimum ATPE conditions for the highest recovery of PCs and HTyr was investigated using 2^3 full factorial experimental design by Minitab 18 (Minitab Inc.) software.

4. RESULTS AND DISCUSSION

4.1. Characteristics of Olive Mill Waste Samples

Prior to recovery studies, the concentrates of lagoon and decanter wastewater were characterized by physicochemical parameters presented in Table 4.1. These data were later used to establish a relationship between recovery efficiencies of TP and HTyr, and sample properties, as well as to decide how to handle the stepwise operation of multistage recovery process.

Table 4.1. Physicochemical characterization of lagoon and decanter OMWCs (n=4).

	Lagoon	**Decanter**
Moisture (%)	0.32 ± 0.04	45.97 ± 0.21
pH	6.5 ± 0.09	5.2 ± 0.05
TKN (g/L)	4.99 ± 0.15	10.97 ± 0.24
OC (%)	47.7 ± 1.63	48.8 ± 1.58
Protein (mg BSA/g)	153.12 ± 3.33	87.23 ± 2.93
Carbohydrate (mg glucose/g)	57.38 ± 2.89	129.82 ± 1.57
Lipid (mg lipid/g)	0.78 ± 0.02	87.91 ± 1.32

(All analyses were performed considering dry weights of the samples.)

Slightly acidic nature is a typical characteristic of OMWW whose pH value was found in the range of 4 to 6 in various studies (Mouncif et al., 1993; Paredes et al., 1999; García-Gómez et al., 2003; Dermeche et al., 2013; Bouknana et al., 2014; Sellami et al., 2016; Majbar et al., 2017). Similarly, lagoon and decanter OMWCs demonstrated a slightly acidic nature. However, the pH value of lagoon samples was found higher in comparison to decanter samples (Table 4.1). This is well expected result since wastewater stored in lagoon could be exposed to various fate processes; consequently, all the characteristics varied by the storage time.

The OC contents of both lagoon and decanter OMWCs were very similar while other organic constituents such as carbohydrate and protein were quite different. Since the application of MVR evaporation resulted in the removal volatile organics from OMWW, the OC content of the decanter OMWC was not higher than that of lagoon sample although the carbohydrate content was about 2.3-fold higher.

As can be seen from the Table 4.1 there is not a positive correlation between the TKN and the protein content of lagoon and decanter samples. Actually, the Bradford assay used for the estimation of protein content can be interfered by certain chemical/protein and chemical/dye interactions (Bio-Rad Laboratories Inc., n.d.), and the interferences can also be originated from non-protein species, such as flavonoids (Compton and Jones, 1985; Fanger, 1987).

4.2. Effect of Acidification on the Extraction of Phenolic Antioxidants in Water

Considering high water solubility of PCs in water, preliminary experiments with acidified lagoon and decanter OMWC samples were carried out to evaluate the effects of water/solid ratio, extraction time, and the number of extraction stage on the recovery of TPC. It should be noted that the acidification of samples resulted in the formation of insoluble substances that could be attributed to the precipitation of the derivatives of dietary fibers (Tornberg and Galanakis, 2008). The obtained results at pH 2 by changing one of the parameters and holding the others constant are presented in Figure 4.1 (a-c).

As shown in Figure 4.1 (a) in which TPC of the samples is shown as a function of water/solid ratio. The recovered TPC from both lagoon and decanter OMWCs considerably increased with an increase in water to solid ratio and the maximum amounts of TP for lagoon and decanter samples were about 41 mg GAE/g and 25 mg GAE/g, respectively. It should be noted that decanter sample contains particulate matter without acidification and the insoluble fraction was increased by acidification. However, the Figure 4.1 (a) also indicates that increasing water/solid ratio above the certain value did not provide further dissolution of TPC from decanter sample within 10 min of extraction time.

The experiments performed by extending the extraction time from 5 to 30 min revealed that the recovery of TPC was obtained within short extraction time period (Figure 4.1 (b)). While about 20 % increase in TPC of decanter OMWC was achieved by the extension of extraction time from 5 to 10 min, this ratio was 11 % for lagoon OMWC and further prolonging the extraction time did not have a remarkable effect on the recovery of TPC in both samples. In addition, the effect of second stage extraction of the precipitates collected by the centrifugation of the samples was evaluated using the same amount of water at the first stage. However, the contribution of this second stage extraction on the TPC was limited for both of the samples (Figure 4.1 (c)).

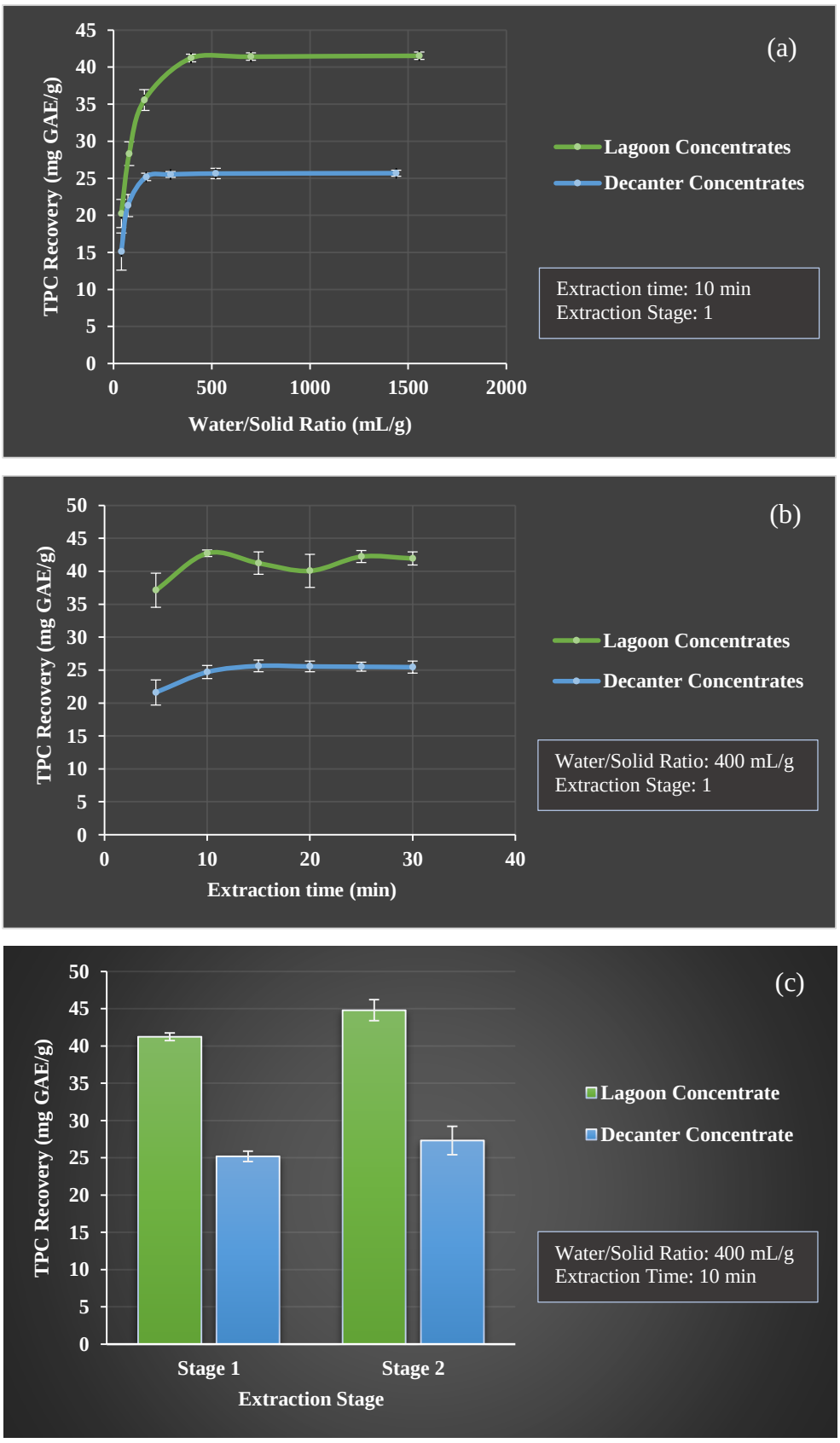


Figure 4.1. The effect of water extraction conditions on TPC recovery from lagoon and decanter OMWCs; (a) water/solid ratio, (b) extraction time, (c) extraction stage (n=2).

According to Sanchez-Rangel et. al. (2013) and Everette et al. (2010), although Folin-Ciocalteu assay is the most commonly used method for the estimation of TPC, various nonphenolic components of sample matrix that can possess a reactivity towards the Folin-Ciocalteu reagent, and these reactions can interfere the assay. The possible interfering compounds include vitamins, amino acids, proteins, unsaturated fatty acids, carbohydrates, organic acids, inorganic ions, nucleotide bases, aldehydes, ketones, thiols and metal complexes. Therefore, many researchers suggest that the Folin-Ciocalteu assay cannot be seen as a measure of TPC, but rather a measure of overall antioxidant activity of the sample (Prior et al., 2005; Dudonné et al., 2009; Everette et al., 2010).

Considering the aim of the study, HTyr and Tyr concentrations were determined in water extracts at five different pH values with additional experiments in which water/sample ratio were 400 mL/g and 165 mL/g for lagoon and decanter OMWCs, respectively. In these studies, selected antioxidants were quantified by the HPLC analysis. For the acidification of the OMWCs, 0.1 M HCl was used as in many studies (De Marco et al., 2007; Lafka et al., 2011; Leouifoufi et al., 2014; Bedouhene, 2014; Sellami et al., 2016; Rubio-Senent et al., 2013). The lagoon and decanter OMWCs were also analyzed without acidic treatment as a control sample and all the results are presented in Figure 4.2.

The abundancy of HTyr in control sample (without acidification) was higher in the decanter sample with initial mildly acidic nature than the lagoon sample. On the other hand, HTyr and Tyr were found under the detection limit in lagoon concentrates without acidified (pH 6.5). Acidification of the OMWC can be an important factor to increase the solubility of PCs and to hydrolyze oleuropein to HTyr (Allouche et al., 2004; De Marco et al., 2007) according to the reaction scheme represented in Figure 2.3. In addition, several biophenolic compounds including HTyr and Tyr bounded to polymeric organics can be liberated under acidic conditions (Rubio-Senent et al., 2013). Accordingly, the recovery of both HTyr and Tyr from lagoon and decanter samples were enhanced by reducing pH as clearly seen in Figure 4.2. Actually, the results achieved for HTyr and Tyr in lagoon and decanter OMWC samples were comparable with the results of TPC and confirmed the higher antioxidant content of lagoon OMWC. The acidification can provide a further contribution to the recovery of the phenolic alcohols since precipitation of dietary fibers can eliminate some organic fraction of OMWC. The carbohydrate analysis performed in the water extracts revealed 20.1 % and 21.5 % reduction by the acidification of lagoon and decanter OMWCs, respectively.

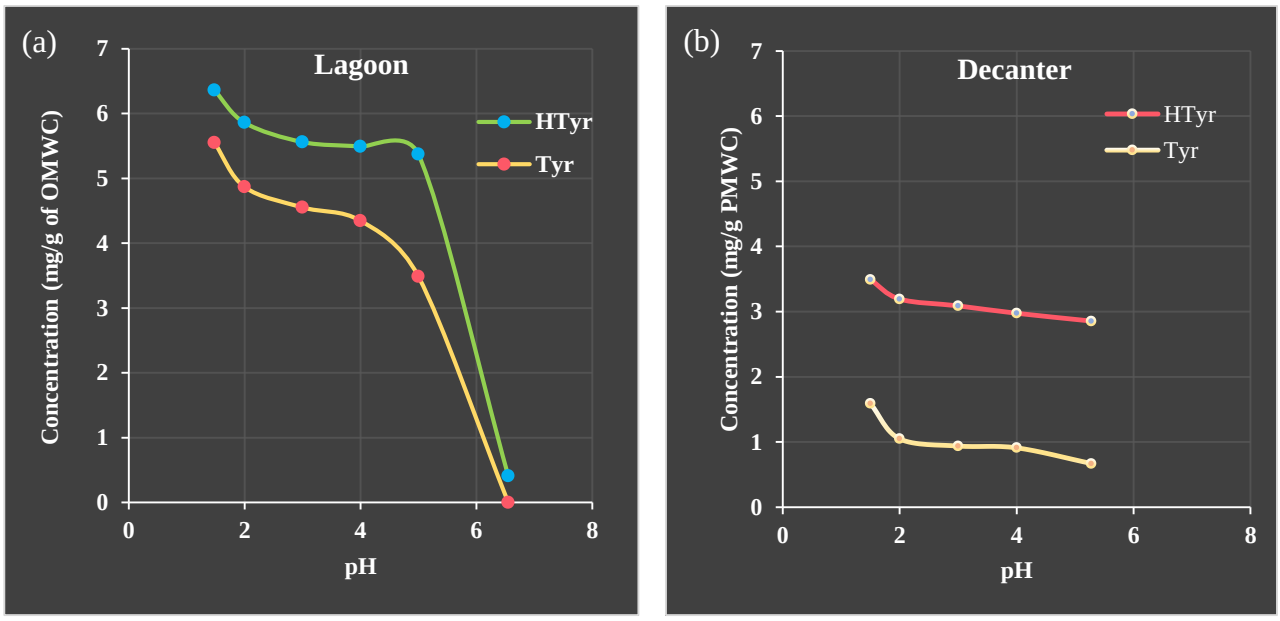


Figure 4.2. Effect of pH on the water extractable HTyr and Tyr from (a) lagoon and (b) decanter OMWC.

In the light of these findings, further investigations were performed at pH 2. Because of the concern about the instability of HTyr and Tyr with the addition of strong acid (Friedman and Jürgens, 2000; Sellami et al., 2016), the acidification was also carried out using acetic acid in separate experiments. However, pH of sample matrix could not be lowered below 2.53 with this weak acid, and the resulting HTyr and Tyr concentrations in lagoon OMWC sample were limited with 4.64 and 3.14 mg/g, respectively. It is apparently seen that not only pH value of the sample matrix but also the type of acid can influence the solubility of these phenolic alcohols.

4.3. Solid-Liquid Extraction of Phenolic Compounds

4.3.1. Recovery of TPC from OMWCs

SLE with EtOAc or EtOH was applied to lagoon and decanter OMWC samples at pH 2 considering the results mentioned in Section 4.2. SLE was assisted with vortex and ultrasound for lagoon and decanter OMWCs, respectively. The extraction efficiency in terms TPC recovery was determined under different experimental conditions applied in section 4.2 and the results achieved for both lagoon and decanter OMWCs are comparably presented in Figure 4.3 (a-f).

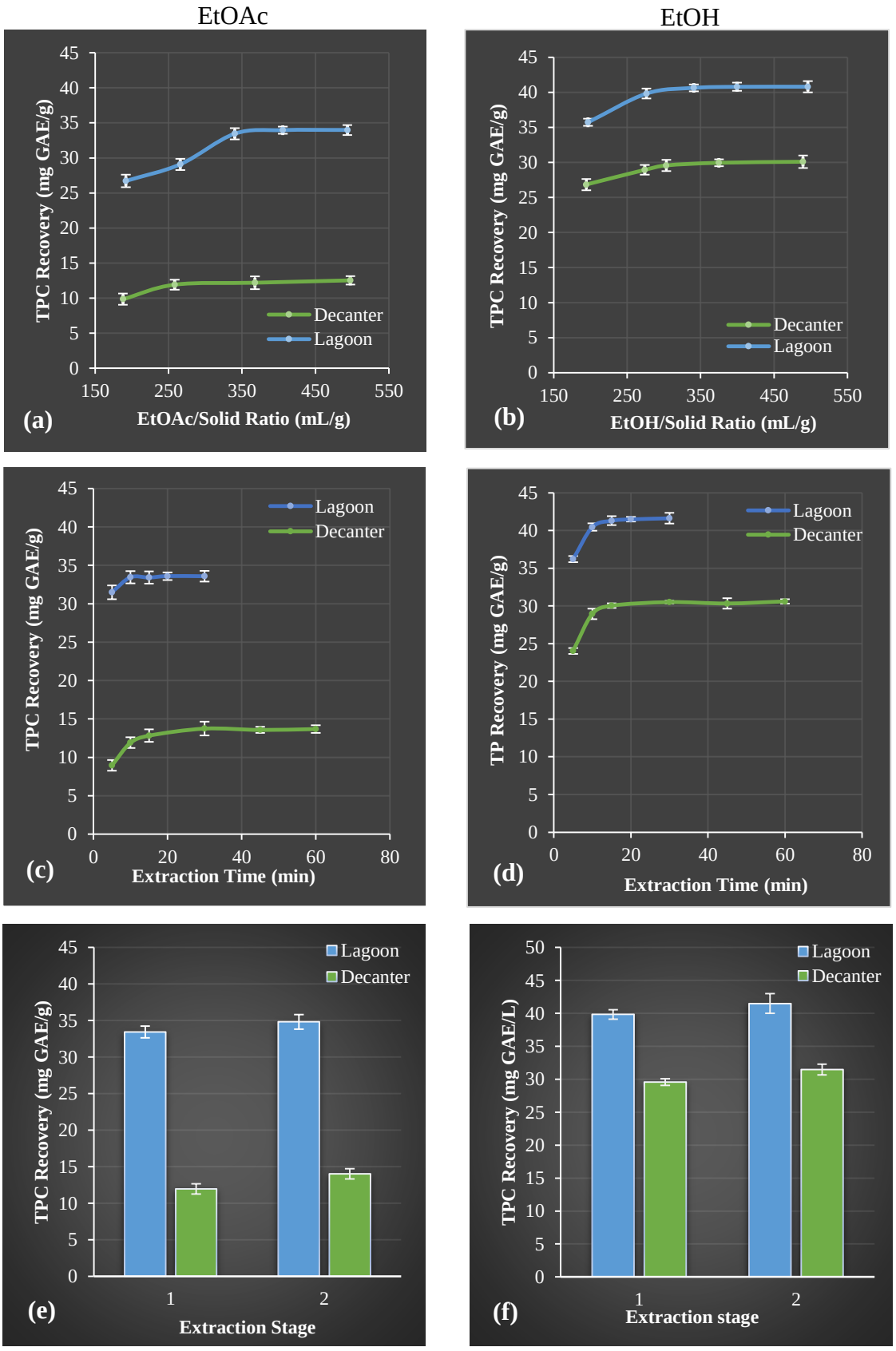


Figure 4.3. The effect of solvent extraction conditions on TPC recovery from lagoon and decanter OMWCs; (a-b) solvent/solid ratio, (c-d) extraction time, (e-f) extraction stage using EtOAc and EtOH, respectively (n=2).

As in case of water extracts, TPC in organic solvent extract achieved from lagoon OMWC was remarkably higher than that of decanter sample. The recovery patterns of TPC in extracts from both lagoon and decanter samples were similar with the increasing solvent/solid ratio, while they possessed limited enhancement with the extension of extraction time and the increasing of extraction stage (Figure 4.3 (a-f)). However, PCs recovered by EtOH extraction was higher than those achieved by EtOAc at each individual point tested (Figure 4.3). This is well expected since the polar protic nature of EtOH and H_2O with high dielectric constants (25 and 80, respectively) and high dipole moments (1.69 and 1.85, respectively) resulting from the presence of hydroxyl group allows superior solubility of phenols in these mediums compared to polar aprotic solvents such as EtOAc. Consequently, the partitioning of TPs between water immiscible EtOAc and water resulted in lower recovery and the highest TPC achieved from EtOAc extracts of lagoon and decanter OMWCs were 35 mg GAE/g and 14 mg GAE/g, respectively. On the other hand, at the optimum conditions of EtOH extraction, TPCs were 41 mg GAE/g and 30 mg GAE/g for the lagoon and decanter OMWCs, respectively.

As can be deduced from Figure 4.1 (a) and 4.3 (a-b) smaller volume of extraction solvent either EtOAc or EtOH, was required to achieve highest recovery of TPC from the lagoon OMWC compare to the results achieved with water extraction. However, this was not valid for the decanter OMWC and obviously, water insoluble PCs necessitated higher amount of organic solvent.

The results for the amount of recovered TPs from OMWCs revealed that wastewater can be a potential resource for PCs while the results achieved in the literature exhibited lower recovery rates for TPC. For instance, the extraction of olive leaves by ultrasound assisted SLE using ethanol yielded 25 mg GAE/g (Şahin and Şamli, 2013). Mojerlou and Elhamirad (2018) recovered only 4.34 mg GAE/g TPC from olive cake by using ultrasound assisted methanol extraction while Icyer et. al. (2016) recovered 1.6 to 45 mg GAE/g phenols from virgin olive oil waste with EtOH that was markedly favorable for a successful phenol recovery from olive sources.

Although the extraction of TPC into EtOAc phase was not efficient due to high solubility of PCs in water, some of the individual PCs (e.g HTyr and Tyr) can possess higher affinity to EtOAc according to the study performed by Galanakis et al. (2013) who denoted the estimation of activity coefficients of different natural phenols in different solvents. Moreover, Visioli et al. (1999) reported the selectivity of EtOH for the extraction of low and medium molecular weight phenols by leaving heavier phenols in the water phase. Therefore, it can be concluded that the use of EtOAc is not adequate to extract all PCs; on the other hand, the recovery of HTyr and Tyr can be higher in EtOAc

than those achieved in water. Furthermore, the elimination of other chemical constituents of sample matrix by EtOAc extraction, which are more soluble in water, can increase the purity of recovered HTyr and Tyr. To evaluate these assumptions, the extracts were subjected to HPLC for both HTyr and Tyr. In addition, the quantification of HTyr was confirmed by LC-MS/MS analyses.

4.3.2. The Recovery of HTyr from OMWCs

The results of HTyr analysis in the extracts of lagoon and decanter OMWCs are presented in Table 4.2.

Table 4.2. Concentration of HTyr determined by LC-MS/MS analysis.

		LC-MS/MS
Sample	**Extraction with**	**HTyr (g/kg)**
Lagoon	Water	0.434
Lagoon	Acidified water at pH 2	3.145
Lagoon	EtOAc	3.208
Lagoon	EtOH	3.813
Decanter	Water	3.086
Decanter	Acidified water at pH 2	3.192
Decanter	EtOAc	3.284
Decanter	EtOH	3.567

The pre-acidification of both lagoon and decanter OMWCs caused an increase in concentrations of HTyr and Tyr similar to the results achieved for TPC. These results supported the assumption on the hydrolysis of oleuropein to HTyr as well as liberation of bounded polyphenols from polymeric phenolic fraction under acidic condition. Comparing the results achieved with water and EtOH extraction it seems that the use of EtOH did not provide an advantage over the acidified water in terms of the recovery of TPC. However, the extraction with EtOH enhanced 20.4 and 17.5 percentage the recoveries of HTyr from the lagoon and decanter OMWCs, respectively. Although the highest recovery of HTyr was achieved by EtOH extraction from OMWCs, EtOAc extraction was more selective for HTyr as suggested in section 4.3.1 and as shown in the HPLC chromatograms of aqueous and nonaqueous solvent extracts (Figure 4.4 a-d and 4.5 a-d).

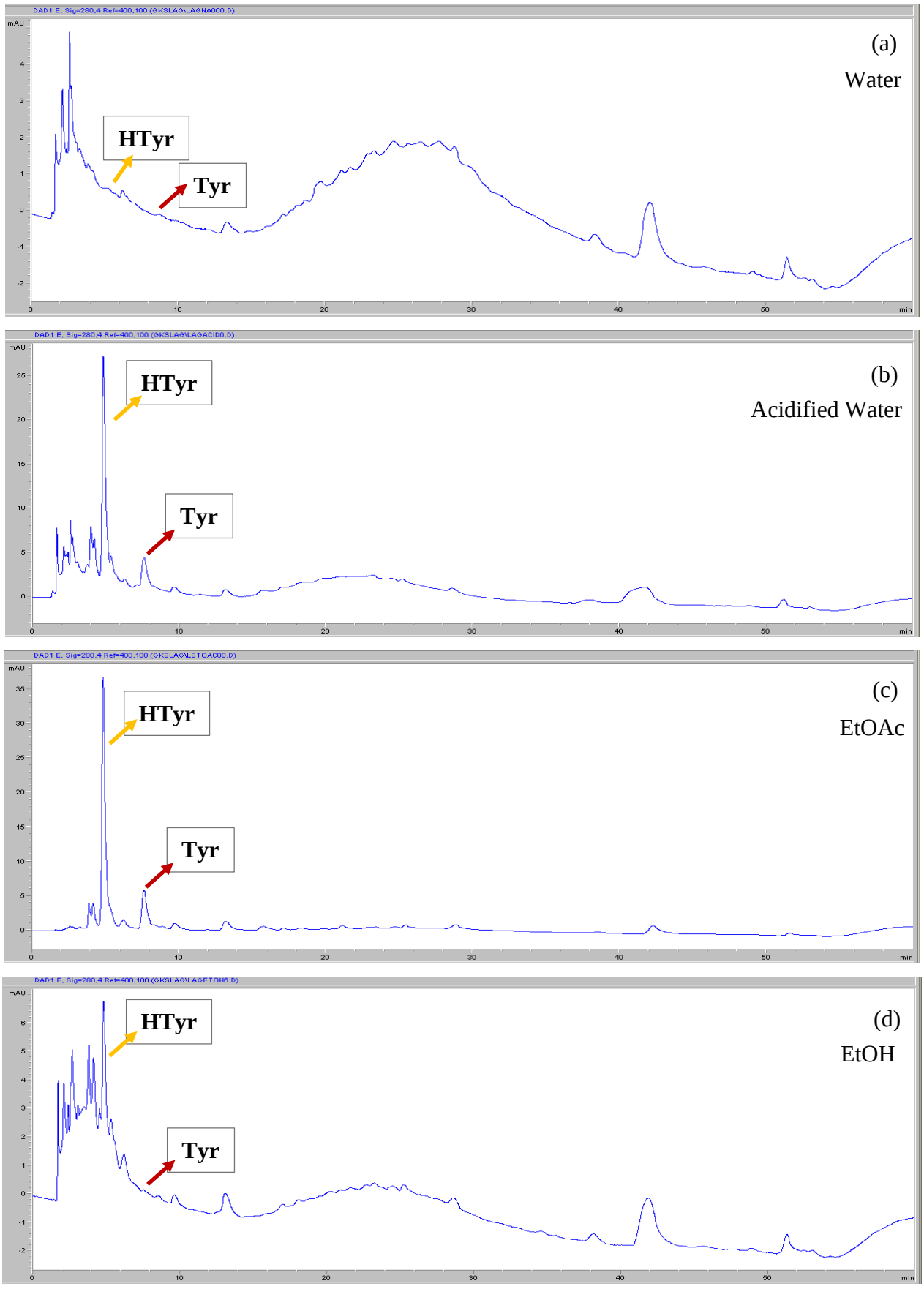


Figure 4.4. HPLC Chromatograms of lagoon OMWC extracts achieved by (a) water, (b) acidified water, (c) EtOAc, and (d) EtOH.

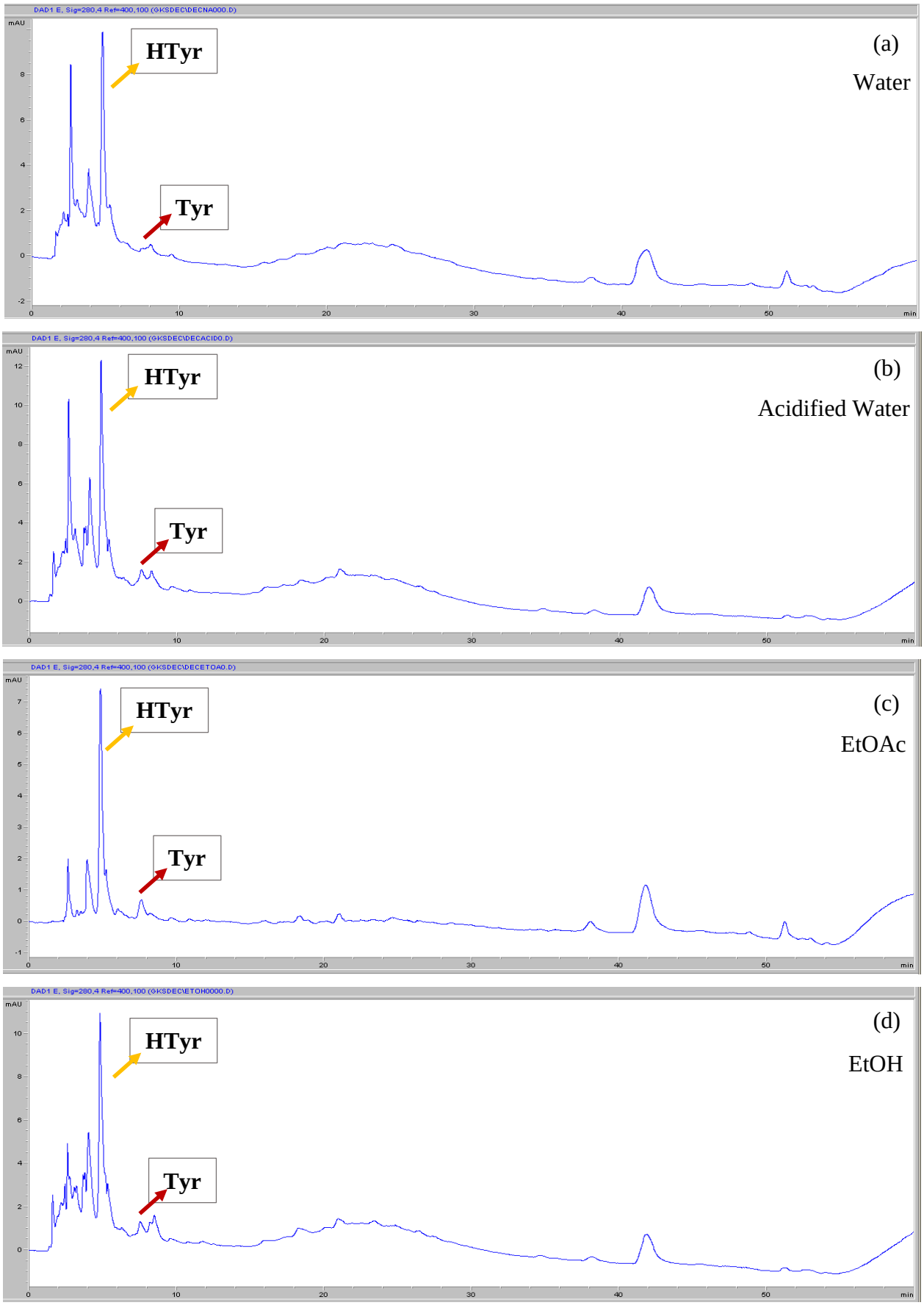


Figure 4.5. HPLC Chromatograms of decanter OMWC extracts achieved by (a) water, (b) acidified water, (c) EtOAc and (d) EtOH.

4.4. Aqueous Two-Phase Extraction of Phenolic Compounds

The recovery yields of both TPC and HTyr were higher with SLE using EtOH as mentioned in the previous section. Besides, EtOH is a favorable solvent compared to EtOAc when recovered antioxidants are considered for pharmaceutical, medicine, cosmetic and food industries. ATPE was preferred to separate the EtOH phase from water phase. Two different salts with acidic and basic nature were selected, ammonium sulfate ($(NH_4)_2SO_4$) and dipotassium hydrogen phosphate (K_2HPO_4), to induce phase separation for the preparation of the aqueous two-phase systems (ATPS). The selected concentrations of system components depending upon the results of cloud point titration and their ternary diagram are shown in Table 4.3 and Figure 4.6, respectively.

Table 4.3. Experimental data of ATPS.

EtOH/K_2HPO_4/Water System			
System	Ethanol (%, w/w)	K_2HPO_4 (%, w/w)	Water (%, w/w)
1	10	31.3	58.7
2	20	22.5	57.5
3	35	9.3	55.7
EtOH/$(NH_4)_2SO_4$/Water System			
System	Ethanol (%, w/w)	$(NH_4)_2SO_4$ (%, w/w)	Water (%, w/w)
1	15	28	57
2	25	19.5	55.5
3	40.6	12	47.4

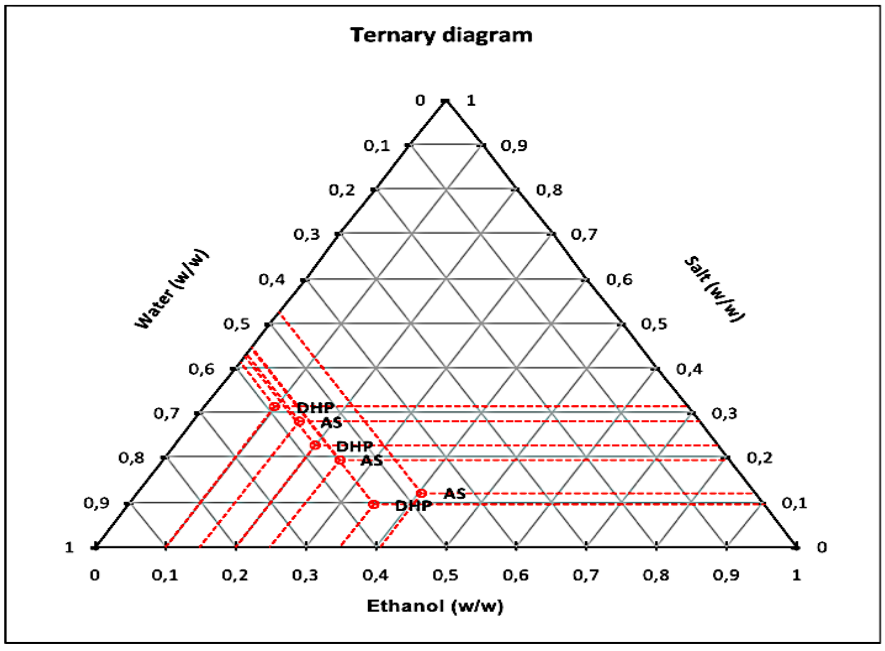


Figure 4.6. Phase diagram of ATPS composed of EtOH, dipotassium hydrogen phosphate (DHP) or ammonium sulfate (AS) salts and water.

In ATPS the recovery yield of target antioxidants can be enhanced by selective separation of PCs. Depending on the system constituents and salt performances, a partitioning of PCs can be expected between water and EtOH phases. Each tested system was evaluated with the volume ratio (Vr) of top (T; EtOH) and bottom (B; water) phases, the partition coefficient (K) of TPC, and the extraction efficiency (EE) calculated with following equations and the results are listed in Table 4.4.

$$V_r = \frac{V_T}{V_B} \quad (4.1)$$

where V_T and V_B are the volume of top and bottom phases, respectively.

$$K = \frac{C_T}{C_B} \quad (4.2)$$

where C_T and C_B are the TP concentrations (mg GAE/g) in the top and bottom phases, respectively.

$$\%\ EE_j = \frac{C_j x\ V_j}{C_B\ x\ V_B + C_T\ x\ V_T}\ x\ 100 \quad (4.3)$$

where EE_J is the percentage ratio of the TPC in the top or bottom phase to the sum in both phases.

Table 4.4. ATPS performance for TPC recovery.

EtOH/K_2HPO_4/Water System								
System	V_r	K	TPC (mg GAE/g)		EE (%)		pH	
			Phase		Phase		Phase	
			Top	Bottom	Top	Bottom	Top	Bottom
1	0.30	3.158	26.233	8.306	48.65	51.35	10.32	10.23
2	1.11	6.018	23.703	3.939	87.02	12.98	10.31	10.27
3	7.20	18.931	25.462	1.345	99.27	0.73	10.21	10.18
EtOH/$(NH_4)_2SO_4$/Water System								
System	V_r	K	TPC (mg GAE/g)		EE, %		pH	
			Phase		Phase		Phase	
			Top	Bottom	Top	Top	Bottom	Top
1	0.29	2.201	20.851	9.474	39.21	60.79	7.25	6.45
2	1	6.383	27.866	4.366	86.45	13.55	6.27	5.88
3	4.25	44.952	30.163	0.671	99.48	0.52	6.27	5.90

Considering the salt performances for the separation of phases and the recovery of PCs, ammonium sulfate showed more promising result most probably due to its acidic characteristic. The systems including dipotassium hydrogen phosphate cause the collection of insoluble components of matrix at the interface of two phases. In addition, clear extracts were not achieved after the solvent evaporation and resuspension of residue. Therefore, ammonium sulfate was selected to optimize the ATPE selected as the salting out chemical for enhancing the recoveries of TP and HTyr. The formation of precipitates during the ATPE containing different salts is shown in Figure 4.7.

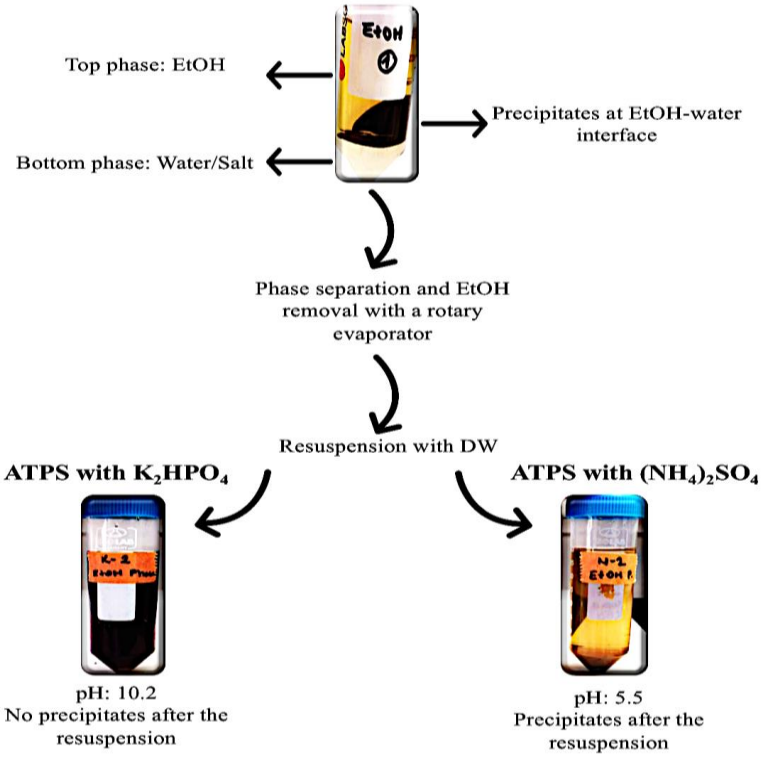


Figure 4.7. The formation of precipitates during the ATPE containing different salts.

As seen in Table 4.4, the partition coefficients of the systems depended upon the system components. Ammonium sulfate showed higher partitioning coefficient with efficient phase separation at the high ethanol concentration. Similarly, 12 % (w/w) ammonium sulfate with 40.6 % (w/w) EtOH in ATPE provided highest recovery of TPs from eucalyptus wood in a recent study (Xavier et al. 2017).

4.4.1. 2^3 Full Factorial Experimental Design for the Optimization of ATPE

2^3 full factorial experimental design was used for the evaluation of selected factors on the performance of ATPE process. The investigated range of coded design factors, which are EtOH

concentration, temperature and extraction time are given in Table 4.5 and Table 4.7 for lagoon and decanter OMWCs, respectively. In these investigations efficient contact of samples with the components of ATPS was achieved with mechanical shaker and ultrasound for lagoon and decanter OMWC, respectively as in the case of SLE. The system responses were the recoveries of TPC and HTyr.

The results of system performances of each 19 experiments for lagoon and decanter OMWCs are shown in Table 4.6 and 4.8, respectively.

Table 4.5. The range of extraction parameters tested in 2^3 full factorial design for lagoon concentrate.

Codes	Factor Names-Lagoon	Low	High
A	Ethanol Conc. (%, w/w)	15	40
B	Temperature (°C)	25	65
C	Extraction Time (min)	15	60

Table 4.6. 2^3 full factorial design table for the coded factors and the responses obtained from ATPE of lagoon concentrate.

Experiment Number	Process Parameter Levels			Responses	
	A	B	C	TPC (mg GAE/g of OMW)	HTyr (mg/g)
1	-	-	-	19.709	6.177
2	-	-	-	19.568	6.016
3	-	-	+	18.264	4.760
4	-	-	+	18.450	4.994
5	-	+	-	17.983	5.359
6	-	+	-	17.818	5.279
7	-	+	+	19.340	5.422
8	-	+	+	18.972	5.309
9	0	0	0	23.769	6.413
10	0	0	0	23.957	6.546
11	0	0	0	24.031	6.570
12	+	-	-	24.903	6.647
13	+	-	-	24.712	6.450
14	+	-	+	27.829	6.883
15	+	-	+	27.506	6.574
16	+	+	-	26.463	6.443
17	+	+	-	26.277	6.123
18	+	+	+	29.740	6.603
19	+	+	+	27.772	6.374

Table 4.7. The range of extraction parameters tested in 2^3 full factorial design for decanter concentrate.

Codes	Factor Names-Lagoon	Low	High
A	Ethanol Conc. (%, w/w)	15	40
B	Temperature (°C)	25	65
C	Sonication Time (min)	5	30

Table 4.8. 2^3 full factorial design table for the coded factors and the responses obtained from ATPE of decanter concentrate.

	Process Parameter Levels			Responses	
Experiment Number	A	B	C	Total Phenols (mg GAE/g of OMW)	HTyr (mg/g)
1	-	-	-	20.553	3.879
2	-	-	-	20.758	3.993
3	-	-	+	21.738	3.902
4	-	-	+	21.794	3.990
5	-	+	-	21.516	4.255
6	-	+	-	21.609	4.353
7	-	+	+	21.139	3.904
8	-	+	+	20.703	3.960
9	0	0	0	27.471	4.606
10	0	0	0	27.814	4.792
11	0	0	0	27.424	4.685
12	+	-	-	27.234	4.475
13	+	-	-	27.483	4.497
14	+	-	+	30.718	4.809
15	+	-	+	30.905	4.901
16	+	+	-	44.056	4.546
17	+	+	-	44.098	4.597
18	+	+	+	30.291	4.609
19	+	+	+	30.250	4.574

The statistical significances of effects of the selected independent variables on the performance of the ATPE process were evaluated and the significant factors ($p<0.05$) are shown by Pareto charts in Figure 4.8 and Figure 4.9. According to these figures, it is clearly seen that EtOH concentration is the most significant factor for the TP and HTyr recoveries. Although all factors and their interactions were significant for the TP recovery from lagoon OMWC, the use of maximum levels of the interaction of three factors negatively affected the TP recovery as for HTyr recovery. In addition, the individual increase of temperature and extraction time negatively affected the HTyr recovery from lagoon samples.

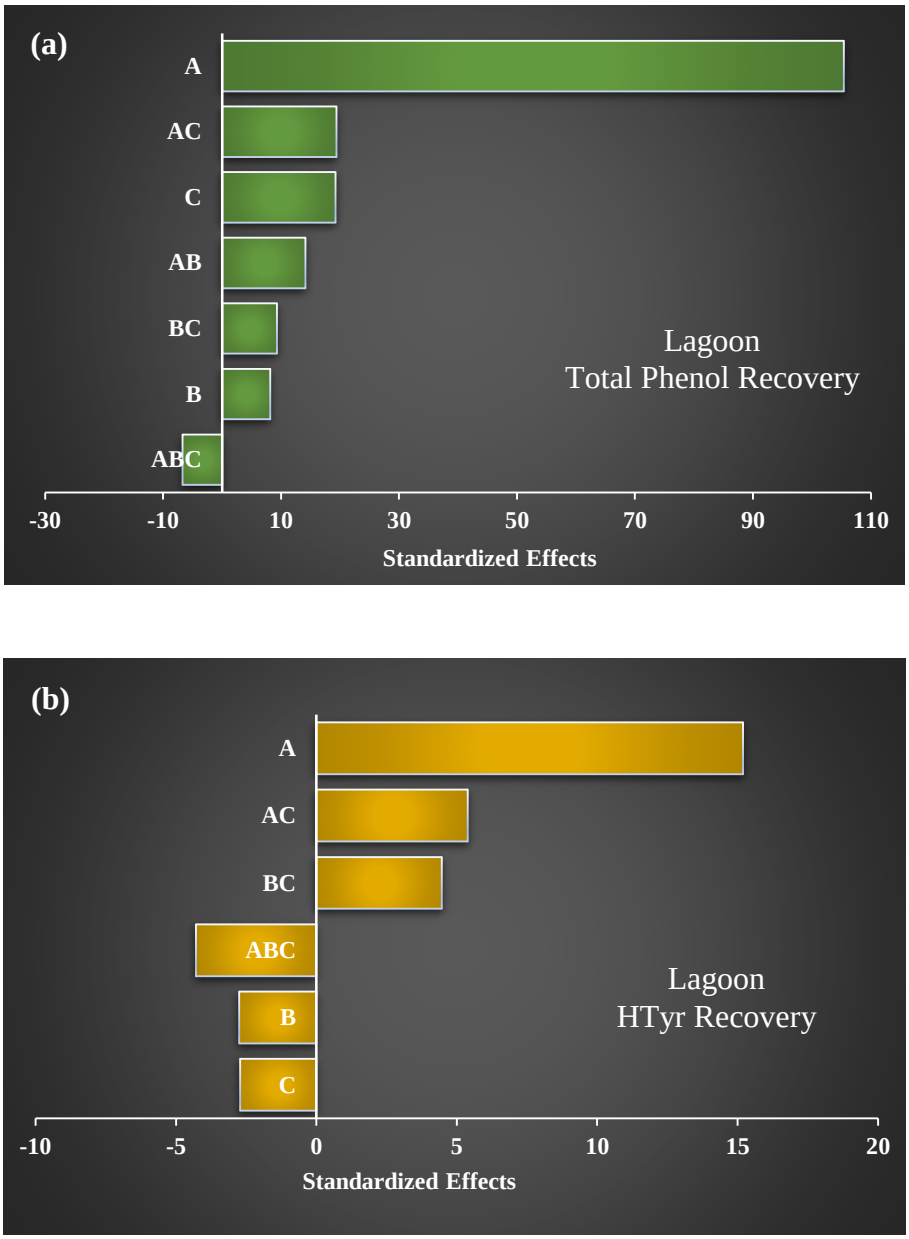


Figure 4.8. Pareto charts of the standardized effects in lagoon OMWC (a) TP recovery, (b) HTyr recovery; (A: EtOH concentration; B: temperature; C: extraction time).

For decanter samples, the individual and combined effects of solvent concentration and temperature have positive influence on the recovery of TPC while the interaction of three factors exert a negative influence on the TP recovery. On the other hand, increasing sonication time negatively affected the TP recovery particularly when it increases parallel to temperature. For the HTyr recovery, only the increasing EtOH concentration and the combined effect of increasing sonication time indicated a positive significant effect. Longer extraction time with ultrasound has also a negative effect on TP recovery but this factor did not have significant effect on HTyr.

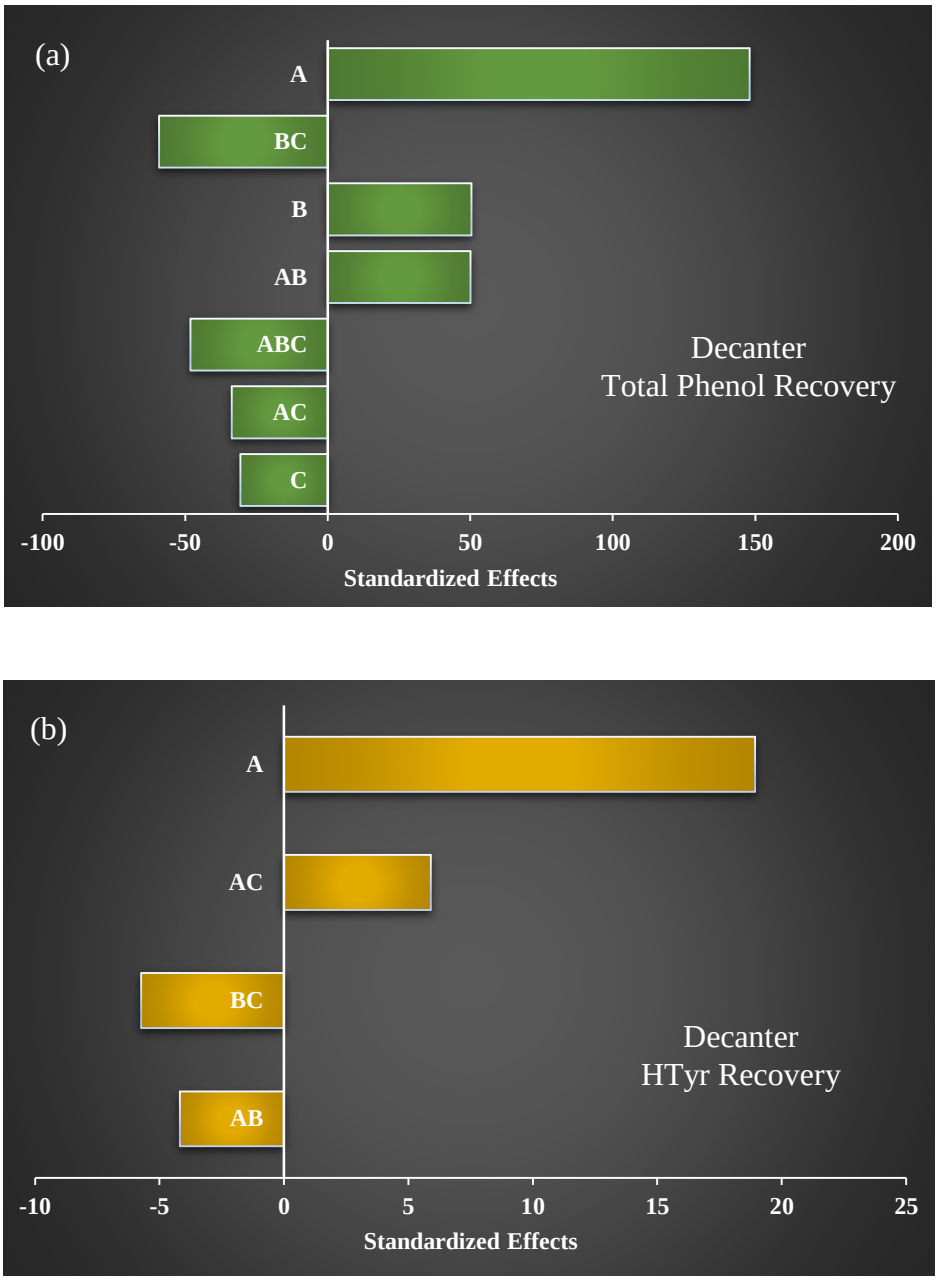


Figure 4.9. Pareto charts of the standardized effects in decanter OMWC (a) TP recovery, (b) HTyr recovery; (A: EtOH concentration; B: temperature; C: extraction time).

4.5. Overall Performance Evaluation of the Extraction Processes

The overall performance of the SLE and ATPE processes were evaluated by the recovery of HTyr, and elimination of proteins and carbohydrates. The most promising results were achieved by ATPE using ethanol. The highest HTyr recoveries were 6.6 g/kg and 4.6 g/kg from lagoon and decanter concentrates by ATPE, respectively. Also, carbohydrate contents of the extracts were only 24 and 13 mg glucose/g while their protein contents were 15 and 4 mg BSA/g of lagoon and decanter OMWCs, respectively after the ATPE process.

Considering the achieved concentration of HTyr detected by LC-MS/MS analysis, it is obviously said that the recovery processes followed in this research gave remarkably higher yield of HTyr compared to HTyr concentrations detected by previous studies from different olive-based sources. Kanakis et al. (2013) determined HTyr concentrations as 1.37 g/kg from olive paste, 0.12 g/kg from olive drupes, only 5.8 mg/kg and 2.4 mg/kg from the first (taken after decanter treatment) and the final olive oil (taken at the end of the olive oil extraction process), respectively. The compositions of the extracts achieved after each optimum extraction process including optimum solvent/sample ratios (s/s) were illustrated in Figure 4.10 and Figure 4.11 for lagoon and decanter concentrates, respectively.

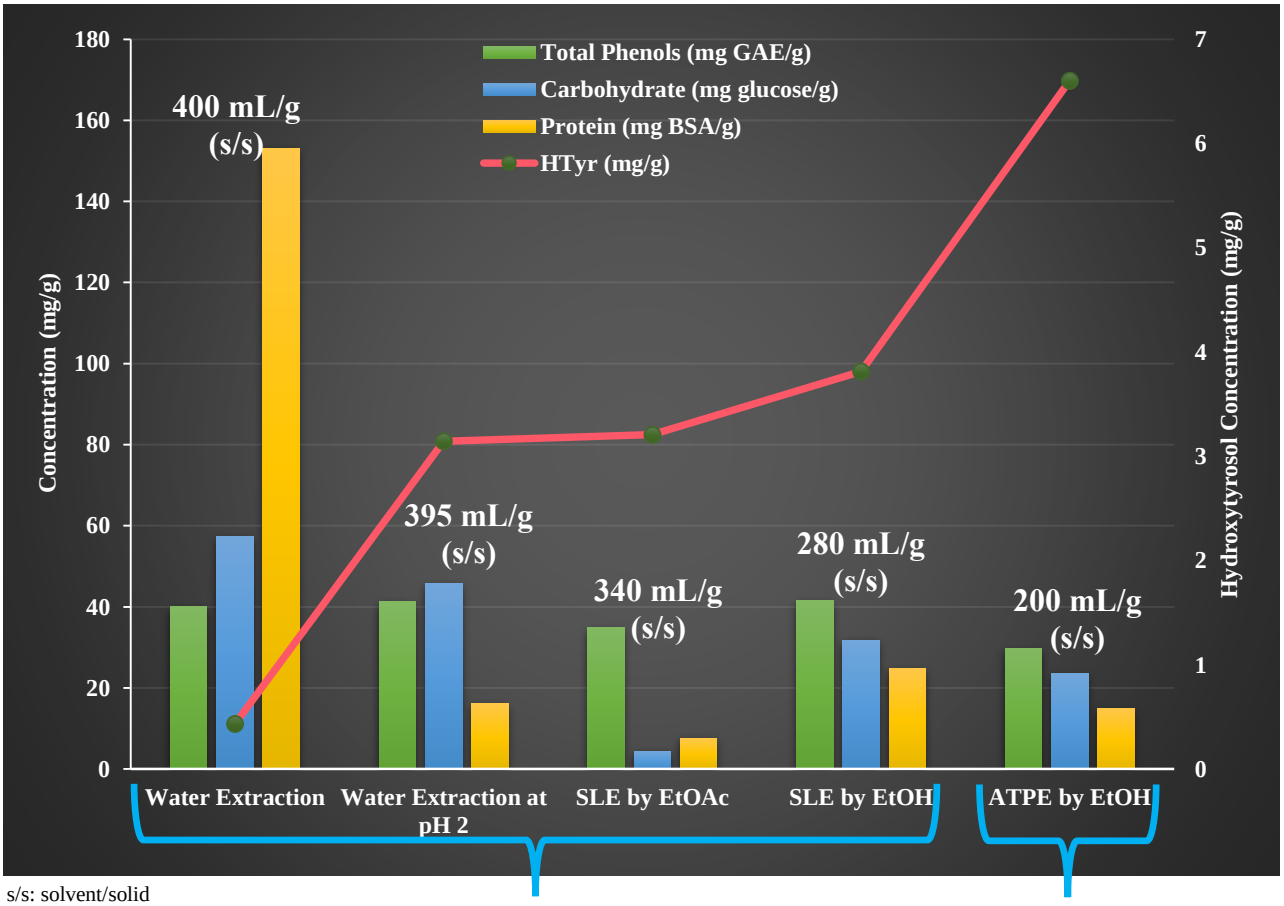


Figure 4.10. Composition of lagoon samples achieved at optimum condition after water extraction with and without acidification; SLE by EtOAc and EtOH; and ATPE by EtOH.

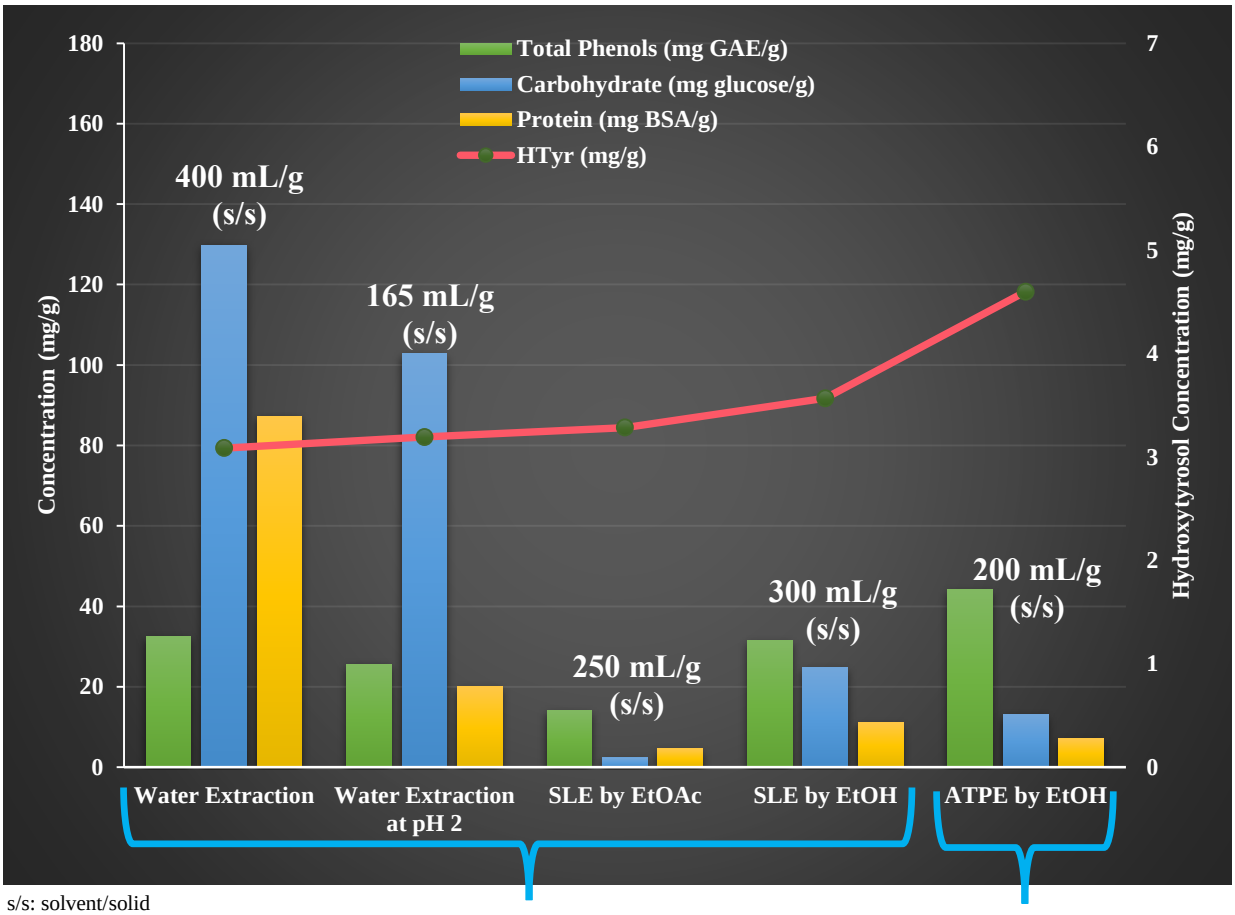


Figure 4.11. Composition of decanter samples achieved at optimum condition after water extraction with and without acidification; SLE by EtOAc and EtOH; and ATPE by EtOH.

4.5.1. Antioxidant Activity of OMWC Extracts

The DPPH assay is the most commonly used method for the determination of antioxidant activity of the OMWW and other olive components, therefore, this method was selected in this research for the determination of antioxidant activity of phenol containing extracts of lagoon and decanter OMWCs.

The antioxidant activity of the extracts estimated by DPPH assay is given in Table 4.9.

Table 4.9. Antioxidant activity of the phenolic extracts.

	% Antioxidant Activity		mg AAE*/g	
	Lagoon	Decanter	Lagoon	Decanter
Water (SLE)	26.2	15.0	36.8	22
Acidified Water (SLE)	27.7	9.0	38.8	14.1
EtOAc (SLE)	22.0	7.8	31.3	12.5
EtOH (SLE)	22.6	16.2	32.1	23.6
EtOH (ATPE)	30.8	23.4	43	33.1

*AAE: Ascorbic Acid Equivalent

In accordance to the results of TP and HTyr recoveries, the highest antioxidant activity percentages were achieved by ATPE of lagoon and decanter OMWCs and the antioxidant activity of lagoon extracts was higher than that of decanter. These achieved results for antioxidant activity could not be compared with those found in literature since DPPH method was modified in various composition of DPPH solution and incubation time.

4.6. Purification by Solid Phase Extraction

4.6.1. Adsorption Potential of Amberlite XAD16N Resin

For the further purification of the extracts achieved from OMWCs batch SPE experiments were carried out with Amberlite XAD16N polymeric nonionic resin since this resin has been investigated for PCs and OMWW in various studies. The adsorption capacity was determined in term of the adsorption ratio (A, %) of TPC in each extract by the following equation:

$$A\ \% = \frac{C_0 - C_{eq}}{C_0} \times 100 \qquad (4.4)$$

C_0 = Initial TP concentration (mg GAE/L)
C_{eq} = Equilibrium TP concentration (mg GAE/L)

To define the amount of resin for the adsorption of PCs in acidified water and ethyl acetate extracts of lagoon OMWC, experiments were carried out with three different dosages of the resin (0.5 g, 1 g and 2 g/10 mL extract), and the results were presented in Figure 4.12.

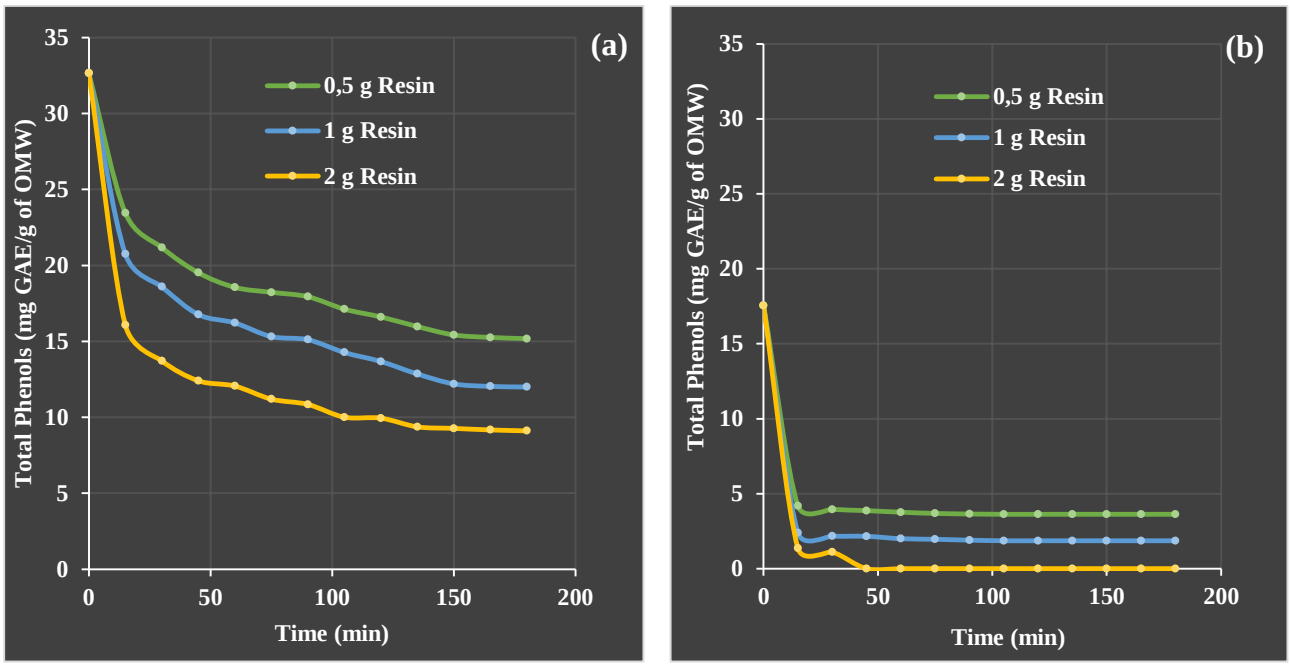


Figure 4.12. SPE with Amberlite XAD16N adsorption resin (a) acidified water and (b) EtOAc extracts of lagoon samples.

In Table 4.10, the adsorption ratios of TP in two different extract at equilibrium are listed.

Table 4.10. The adsorption ratio (A, %) of the acidified water and ethyl acetate extracts of lagoon OMWC with XAD16N.

	A, %		
Extracts	**0.5 g Resin**	**1 g Resin**	**2 g Resin**
Acidified Water/SLE	63.2	70.9	78.1
Ethyl Acetate/SLE	89.2	94.4	100

As can be deduced from the results, the adsorption ratio of TPs was high in the ethyl acetate extract of lagoon OMWC due to the selective extraction of PCs. However, the various components of water extract caused a competition for adsorption of TPs on the resin. The extracts of ethyl acetate were much more clear than acidified water extract by eliminating other chemical constituents through the extraction process as seen in HPLC chromatograms (Figure 4.4 (a-c)).

For the lagoon and decanter OMWC extracts achieved by ATPE, SPE was performed with a higher dose range of the resin (2-4 g/10 mL of extract) to ensure the complete adsorption of TPs. While the TP concentrations are shown in terms of adsorption time at three different dosages of the resin in Figure 4.13 for lagoon and decanter OMWC extracts, respectively.

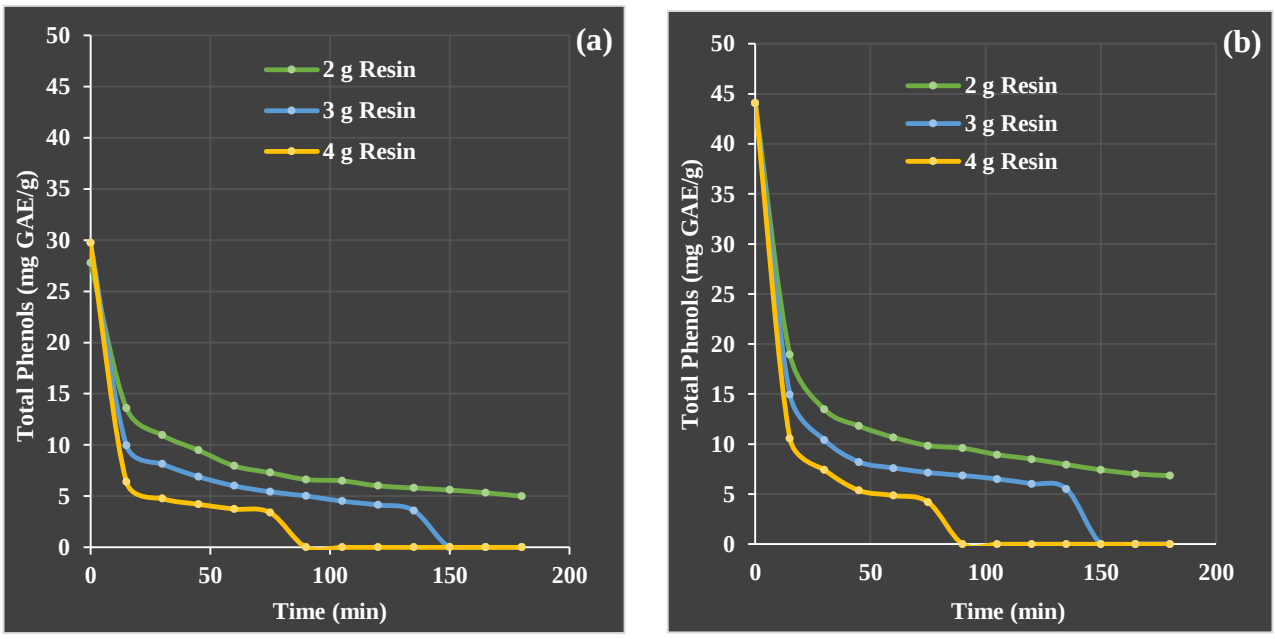


Figure 4.13. SPE with Amberlite XAD16N adsorption resin (a) lagoon extracts and (b) decanter extracts achieved by ATPE.

In Table 4.11, the adsorption ratios of TP in the extracts of OMWCs at equilibrium are given.

Table 4.11. The adsorption ratio (A, %) of the aqueous two-phase extracts of lagoon and decanter OMWC with XAD16N.

	A, %					
Extracts	**2 g Resin**		**3 g Resin**		**4 g Resin**	
	Lagoon	Decanter	Lagoon	Decanter	Lagoon	Decanter
Ethanol/ATPE	82	84.5	100	100	100	100

4.6.2. Desorption Efficiency and Process Productivity of SPE for the Recovery of HTyr

After having equilibration of PCs on the resin, elution was performed with acidified ethanol. For the elution of organic compounds other than phenolics, the resin was washed with water prior to solvent elution as performed in the study of Zagklis et al. (2015) in which 92.8 % reduction in the carbohydrate content was achieved during the recovery of phenols. The desorption efficiency or process productivity (D, %) was calculated using the following equation:

$$D\ \% = \frac{C_{ad} - C_d}{C_{ad}} \times 100 \qquad (4.5)$$

where C_{ad} is the adsorbed TPC (mg GAE/g) and C_d is the desorbed TPC (mg GAE/g).

The equation above was engaged in the overall evaluation of the whole recovery process of PCs and HTyr as well as the removal process of carbohydrate and protein from the extracts of OMWC. Throughout the SPE, the experiment productivity for TPC was 60 % and 61.4 % for lagoon and decanter extracts, respectively. Compared to Zagklis et al., the TPC recovery in this study is slightly smaller. The difference between the TPC recovery efficiencies may be related with the type of resin. Since Zagklis et al. was initially evaluated the phenol adsorption performances of XAD16N and XAD4 resins resulting that XAD4 has better performance than XAD16N. However, HTyr selectivity of the process is pretty higher with 80-82 % HTyr recovery from the OMWC extracts concluding that XAD16N is a promising adsorption resin which can be used for further purification of HTyr.

The desorption efficiency of carbohydrate and protein as well as the TP and HTyr selectivity of the process are given in Table 4.12 for lagoon and decanter OMWC extracts achieved by ATPE.

Table 4.12. Performance evaluation of SPE considering TP and HTyr recovery, and carbohydrate and protein removal efficiency.

		Lagoon ATPE/EtOH	**Decanter ATPE/EtOH**
HTyr	Adsorbed C_{HTyr} (mg/g)	6.603	4.546
	C_{HTyr} desorbed by water (mg/g)	1.063	0.752
	C_{HTyr} desorbed by EtOH (mg/g)	5.310	3.708
	% HTyr Selectivity	80.4 %	81.6 %
TP	Adsorbed $C_{Total\ Phenols}$ (mg glucose/g)	30	44
	$C_{Total\ Phenols}$ desorbed by water (mg glucose/g)	9	14
	$C_{Total\ Phenols}$ desorbed by EtOH (mg glucose/g)	18	27
	% $D_{Total\ Phenol\ Recovery}$	60 %	61.4 %
Protein	Adsorbed $C_{Protein}$ (mg BSA/g)	15	7
	$C_{Protein}$ desorbed by water (mg BSA/g)	7	3
	$C_{Protein}$ desorbed by EtOH (mg BSA/g)	5	-
	% $D_{Protein\ Removal}$	53 %	100 %
Carbohydrate	Adsorbed $C_{Carbohydrate}$ (mg glucose/g)	24	13
	$C_{Carbohydrate}$ desorbed by water (mg glucose/g)	15	8
	$C_{Carbohydrate}$ desorbed by EtOH (mg glucose/g)	7	4
	% $D_{Carbohydrate\ Removal}$	70.8 %	69.2 %

5. CONCLUSIONS

In scope of this research, a multistage recovery process of biophenolic antioxidants with focus on HTyr from the lagoon and decanter OMWCs was investigated. The following findings underline the major results of the research.

- Some of the physicochemical characteristics of lagoon and decanter OMWCs achieved from the MVR evaporation of OMWW were different. While decanter OMWC has a slightly acidic nature, lagoon sample exhibited a higher pH value together with differences in the organic components of OMWC that could be attributed to possible fate processes facing by the storage time. Also, almost 8.8 % lipid content was remained in the decanter OMWC which needs to be removed by pretreatment process.

- The pre-acidification of both lagoon and decanter OMWCs caused an increase in concentrations of HTyr and Tyr similar to the results achieved for TPC. The assumption on the hydrolysis of oleuropein to HTyr as well as liberation of bounded polyphenols from polymeric phenolic fraction under acidic condition could explain the obtained results. In addition, the acidification of lagoon and decanter OMWCs, led to 20.1 % and 21.5 % carbohydrate reduction in water extracts, respectively. It can be suggested that pre-acidification can provide a further contribution to the recovery of the phenolic alcohols due to the precipitation of dietary fibers.

- In overall SLE process supplied higher TPC recovery from lagoon OMWC compare to decanter sample. On the other hand, the recovery patterns of TPC in extracts from both lagoon and decanter samples at room temperature were similar with the increasing solvent/solid ratio, while they possessed limited enhancement with the extension of extraction time and the increasing of extraction stage. The SLE process performed with EtOH at about 280 mL/g solvent to solid ratio within 10 min provided 41 mg/g and 30 mg/g TPC recovery from lagoon and decanter OMWC, respectively. However, at these extraction conditions the recoveries of HTyr were 3.813 mg/g and 3.567 mg/g, respectively from lagoon and decanter OMWC.

- Although the highest recovery of HTyr were achieved with EtOH extraction from OMWCs, EtOAc extraction was more selective for HTyr. The partitioning of TPs between water

immiscible EtOAc and water resulted in lower recovery and the highest TPC achieved from EtOAc extracts of lagoon and decanter OMWCs were 35 mg GAE/g and 14 mg GAE/g, respectively.

- In order to increase the selectivity of EtOH extraction, salt induced phase separation was investigated using ATPS. Ammonium sulfate as salting out chemical showed more promising potential for the recovery of both TPC and HTyr due to its acidic characteristic. Among the tested parameters namely ethanol concentration, temperature, extraction time, for the optimization of ATPE process, EtOH concentration is the most significant factor for the TP and HTyr recoveries from both OMWCs.

- For the ultrasound assisted ATPE of decanter samples, the individual effects of temperature and time were not significant for HTyr recovery, but their interactions exhibited negative influence on the HTyr recovery.

- During the olive oil production process, 900 tonnes of OMWW are produced from the treatment of 1000 tonnes of olive. After the 70-90 % concentration of OMWW, 41 mg GAE/g TPC by SLE and 6.6 mg/g HTyr by ATPE were achieved from lagoon OMWC, while 44 mg GAE/g TPC and 4.6 mg/g HTyr by ATPE were achieved from decanter OMWC.

- The highest antioxidant activities of lagoon and decanter OMWC were 43 mg AAE/g and 33 mg AAE/g, respectively detected by DPPH assay were achieved with the extracts from ATPE of the concentrates, and the results of antioxidant test and HTyr recovery were consistent. The lagoon extracts showed higher antioxidant activity at each extraction strategy than those achieved by decanter concentrates.

- SPE was applied for further purification HTyr achieved from ATPE, which recovered the highest amount of HTyr. The experiment productivity for TPC was 60 % and 61.4 % for lagoon and decanter extracts, respectively. However, HTyr selectivity of the process is pretty higher with 80-82 % HTyr recovery from the OMWC extracts, concluding that XAD16N is a promising adsorption resin which can be used for further purification of HTyr.

REFERENCES

Achat, S., Tomao, V., Madani, K., Chibane, M., Elmaataoui, M., Dangles, O., Chemat, F., 2012. Direct enrichment of olive oil in oleuropein by ultrasound-assisted maceration at laboratory and pilot plant scale. Ultrasonics Sonochemistry, 19, 777–786.

Adhoum, N., Monser, L., 2004. Decolourization and removal of phenolic compounds from olive mill wastewater by electrocoagulation. Chemical Engineering and Processing: Process Intensification, 43, 1281–1287.

Agalias, A., Magiatis, P., Skaltsounis, A. L., Mikros, E., Tsarbopoulos, A., Gikas, E., Spanos, I., Manios, T., 2007. A new process for the management of olive oil mill waste water and recovery of natural antioxidants. Journal of Agricultural and Food Chemistry, 55, 2671–2676.

Aggelis, G., Iconomou, D., Christou, M., Bokas, D., Kotzailias, S., Christou, G., Tsagou, V., Papanikolaou, S., 2003. Phenolic removal in a model olive oil mill wastewater using *Pleurotus ostreatus* in bioreactor cultures and biological evaluation of the process. Water Research, 37, 3897–3904.

Akdemir, E.O., Ozer, A., 2009. Investigation of two ultrafiltration membranes for treatment of olive oil mill wastewater, Desalination, 249, 660–666.

Aktas, E.S., Imre, S., Ersoy, L., 2001. Characterization and lime treatment of olive mill wastewater, Water Research, 35, 2336–2340.

Allouche, N., Fki, I., Sayadi, S., 2004. Toward a high yield recovery of antioxidants and purified hydroxytyrosol from olive mill wastewaters, Journal of Agricultural and Food Chemistry, 52, 267–273.

Ammary, B.Y., 2005. Treatment of olive mill wastewater using an anaerobic sequencing batch reactor, Desalination, 177, 157–165.

Anbia, M., Amirmahmoodi, S., 2011. Adsorption of phenolic compounds from aqueous solutions using functionalized SBA-15 as a nano-sorbent, Scientia Iranica, 18, 446–452.

Azabou, S., Najjar, W., Gargoubi, A., Ghorbel, A., Sayadi, S., 2007. Catalytic wet peroxide photo-oxidation of phenolic olive oil mill wastewater contaminants. Part II. Degradation and detoxification of low-molecular mass phenolic compounds in model and real effluent, Applied Catalysis B: Environmental, 77, 166–174.

Azbar, N., Bayram, A., Filibeli, A., Muezzinoglu, A., Sengul, F., Ozer, A., 2004. A review of waste management options in olive oil production, Critical Reviews in Environmental Science and Technology, 34, 209–247.

Azbar, N., Keskin, T., Yuruyen, A., 2008. Enhancement of biogas production from olive mill effluent (OME) by co-digestion, Biomass and Bioenergy, 32, 1195–1201.

Azbar, N., Tutuk, F., Keskin, T., 2009a. Biodegradation performance of an anaerobic hybrid reactor treating olive mill effluent under various organic loading rates, International Biodeterioration and Biodegradation, 63, 690–698.

Azbar, N., Tutuk, F., Keskin, T., 2009b. Effect of organic loading rate on the performance of an up-flow anaerobic sludge blanket reactor treating olive mill effluent, Biotechnology and Bioprocess Engineering, 14, 99–104.

Azzam, M.O.J., Al-Malah, K., Al-Gazzawi, Z. and Al-Omari, S.A., 2010. Dynamic treatment response of olive mills wastewater using series of adsorption steps, Clean - Soil, Air, Water, 38, 822–830.

Bahar, R., Hawlader, M.N.A., Woei, L.S., 2004. Performance evaluation of a mechanical vapor compression desalination system, Desalination, 166, 123–127.

Beccari, M., Bertin, L., Dionisi, D., Fava, F., Lampis, S., Majone, M., Valentino, F., Vallini, G., Villano, M., 2009. Exploiting olive oil mill effluents as a renewable resource for production of biodegradable polymers through a combined anaerobic-aerobic process, Journal of Chemical Technology and Biotechnology, 84, 901–908.

Bedouhene, S., Hurtado-Nedelec, M., Sennani, N., Marie, J. C., El-Benna, J., Moulti-Mati, F., 2014. Polyphenols extracted from olive mill wastewater exert a strong antioxidant effect in human neutrophils, International Journal of Waste Resources, 4, 161.

Benitez, F.J., Beltran-Heredia, J., Torregrosa, J., Acero, J.L., 1999. Treatment of olive mill wastewaters by ozonation, aerobic degradation and the combination of both treatments, Journal of Chemical Technology and Biotechnology, 74, 639–646.

Bertin, L., Ferri, F., Scoma, A., Fava, F., 2011. Recovery of high added value natural polyphenols from actual olive mill wastewater through solid phase extraction, Chemical Engineering Journal, 171, 1287–1293.

Bio-Rad Laboratories Inc., no date. Quick start Bradford protein assay. California, U.S.A.

Blois, M. S., 1958. Antioxidant determination by the use of a stable free radical, Nature, 181, 1199–1200.

Borja, R., Raposo, F., Rincón, B., 2006. Treatment technologies of liquid and solid wastes from two-phase olive oil mills, Grasas y aceites, 57, 32–46.

Bouknana, D., Hammouti, B., Salghi, R., Jodeh, S., Zarrouk, A., Warad, I., Aouniti, A., Sbaa, M., 2014. Physicochemical characterization of olive oil mill wastewaters in the eastern region of Morocco, Journal of Materials and Environmental Science, 5, 1039–1058.

Bradford, M.M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of dye-binding, Analytical Biochemistry, 72, 248–254.

Brand-Williams, W., Cuvelier, M.E., Berset, C., 1995. Use of a free radical method to evaluate antioxidant activity, LWT - Food Science and Technology, 28, 25–30.

Bressan, M., Liberatore, L., D'Alessandro, N., Tonucci, L., Belli, C., Ranalli, G., 2004. Improved combined chemical and biological treatments of olive oil mill wastewaters, Journal of Agricultural and Food Chemistry, 52, 1228–1233.

Cabrera-Bañegil, M., Schaide, T., Manzano, R., Delgado-Adámez, J., Durán-Merás, I., Martín-Vertedor, D., 2017. Optimization and validation of a rapid liquid chromatography method for determination of the main polyphenolic compounds in table olives and in olive paste, Food Chemistry, 233, 164–173.

Canepa, P., Marignetti, N., Rognoni, U., Calgari, S., 1988. Olive mills wastewater treatment by combined membrane processes, Water Research, 22, 1491–1494.

Caputo, A. C., Scacchia, F., Pelagagge, P. M., 2003. Disposal of by-products in olive oil industry: Waste-to-energy solutions, Applied Thermal Engineering, 23, 197–214.

Castro-López, C., Ventura-Sobrevilla, J.M., González-Hernández, M.D., Rojas, R., Ascacio-Valdés, J.A., Aguilar, C.N., Martínez-Ávila, G.C.G., 2017. Impact of extraction techniques on antioxidant capacities and phytochemical composition of polyphenol-rich extracts, Food Chemistry, 237, 1139–1148.

Ceccon, L., Saccu, D., Procida, G., Cardinali, S., Saccù, D., 2001. Liquid chromatographic determination of simple phenolic compounds in waste waters from olive oil production plants, Journal of AOAC International, 84, 1739– 1744.

Çevre ve Orman Bakanlığı, 2004. Su kirliliği kontrolü yönetmeliği, Official Newspaper, 25687. Ankara, Türkiye.

Çevre ve Şehircilik Bakanlığı, 2015. Zeytinyağı tesislerinde oluşan atıksularon yönetiminde uyulması gereken teknik hususlar. Ankara, Türkiye.

Chatzisymeon, E., Diamadopoulos, E., Mantzavinos, D., 2009. Effect of key operating parameters on the non-catalytic wet oxidation of olive mill wastewaters, Water Science and Technology, 59, 2509–2518.

Chedeville, O., Debacq, M., Porte, C., 2009. Removal of phenolic compounds present in olive mill wastewaters by ozonation, Desalination, 249, 865–869.

Clesceri, L.S., Greenberg, A.E., Eaton, A.D. (Eds), 1998. Standard methods for examination of water and wastewater. Twentieth Ed., Washington, D.C, U.S.A.

Compton, S.J., Jones, C.G., 1985. Mechanism of dye response and interference in the Bradford protein assay, Analytical Biochemistry, 151, 369–374.

Crea, R., 2004. An hydroxytyrosol-rich composition from olive vegetation water and method of use thereof. WO 2004005228 A1, U.S.A.

Cuomo, J., Rabovskiy, A.B., 2002. Antioxidant compositions extracted from a wastewater from olive oil production. US 6361803 B1, U.S.A.

D'Annibale, A., Ricci, M., Quaratino, D., Federici, F., Fenice, M., 2004. Panus tigrinus efficiently removes phenols, color and organic load from olive-mill wastewater, Research in Microbiology, 155, 596–603.

Dagdelen, A., Tümen, G., Özcan, M.M., Dündar, E., 2013. Phenolics profiles of olive fruits (*Olea europaea* L.) and oils from Ayvalik, Domat and Gemlik varieties at different ripening stages, Food Chemistry, 136, 41–45.

Dalis, D., Anagnostidis, K., Lopez, A., Letsiou, I., Hartmann, L., 1996. Anaerobic digestion of total raw olive-oil wastewater in a two-stage pilot- plant (up-flow and fixed-bed bioreactors), Bioresource Technology, 57, 237–243.

De Leonardis, A., Macciola, V., Lembo, G., Aretini, A., Nag, A., 2007. Studies on oxidative stabilisation of lard by natural antioxidants recovered from olive-oil mill wastewater, Food Chemistry, 100, 998–1004.

De Marco, E., Savarese, M., Paduano, A., Sacchi, R., 2007. Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters, Food Chemistry, 104, 858–867.

De Martino, A., Arienzo, M., Iorio, M., Vinale, F., Lorito, M., Prenzler, P. D., Ryan, D., Obied, H.K., 2011. Detoxification of olive mill wastewaters by zinc-aluminium layered double hydroxides, Applied Clay Science, 53, 737–744.

Dermeche, S., Nadour, M., Larroche, C., Moulti-Mati, F., Michaud, P., 2013. Olive mill wastes: Biochemical characterizations and valorization strategies, Process Biochemistry, 48, 1532–1552.

Dias, A.A., Bezerra, R.M., Pereira, A.N., 2004. Activity and elution profile of laccase during biological decolorization and dephenolization of olive mill wastewater, Bioresource Technology, 92, 7–13.

Dow Chemical Company, no date. Amberlite XAD16N Product Data Sheet No. 177-03120-0313, Michigan, U.S.A.

Dreywood, R., 1946. Qualitative test for carbohydrate material, Industrial and Engineering Chemistry - Analytical Edition, 18, 499.

Drouiche, M., Le Mignot, V., Lounici, H., Belhocine, D., Grib, H., Pauss, A., Mameri, N., 2004. A compact process for the treatment of olive mill wastewater by combining OF and UV/H2O2 techniques, Desalination, 169, 81–88.

Dudonné, S., Vitrac, Z., Coutiere, P., Woillez, M., Merillon, J.M., 2009. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays, Journal of Agricultural and Food Chemistry, 57, 1768–1774.

El-Abbassi, A., Kiai, H., Jihane, R., Abdellatif, H., 2014. Cloud point extraction of phenolic compounds from pretreated olive mill wastewater, Journal of Environmental Chemical Engineering, 2, 1480–1486.

Emmons, W., Guttersen, C., 2005. Isolation of oleuropein aglycon from olive vegetation water. US 2005/010371 A1, U.S.A.

Ena, A., Pintucci, C., Carlozzi, P., 2012. The recovery of polyphenols from olive mill waste using two adsorbing vegetable matrices, Journal of Biotechnology, 157, 573– 577.

Everette, J.D., Bryant, Q.M., Green, A.M., Abbey, Y.A., Wangila, G.W., Walker, R.B., 2010. Thorough study of reactivity of various compound classes toward the Folin-Ciocalteu reagent, Journal of Agricultural and Food Chemistry, 58, 8139–8144.

Fanger, B.O., 1987. Adaptation of the Bradford protein assay to membrane-bound proteins by solubilizing in glucopyranoside detergents, Analytical Biochemistry, 162, 11–17.

Ferri, F., Bertin, L., Scoma, A., Marchetti, L., Fava, F., 2011. Recovery of low molecular weight phenols through solid-phase extraction, Chemical Engineering Journal, 166, 994–1001.

Frascari, D., Bacca, A.E.M., Zama, F., Bertin, L., Fava, F., Pinelli, D., 2016. Olive mill wastewater valorisation through phenolic compounds adsorption in a continuous flow column, Chemical Engineering Journal, 283, 293–303.

Friedman, M., Jürgens, H. S., 2000. Effect of pH on the stability of plant phenolic compounds, Journal of Agricultural and Food Chemistry, 48, 2101–2110.

Galanakis, C.M., Goulas, V., Tsakona, S., Manganaris, G.A., Gekas, V., 2013. A knowledge base for the recovery of natural phenols with different solvents, International Journal of Food Properties, 16, 382–396.

Galanakis, C.M., Martinez-Saez, N., Dolores, M., Barba, F.J., Mitropoulou, V.S., 2015. Patented and Commercialized Applications. In Galanakis, C.M. (Eds), Food Waste Recovery: Processing Technologies and Industrial Techniques, 337–360, Academic Press, U.S.A.

Galanakis, C.M., Tornberg, E., Gekas, V., 2010. Clarification of high-added value products from olive mill wastewater, Journal of Food Engineering, 99, 190–197.

García-Gómez, A., Roig, A., Bernal, M.P., 2003. Composting of the solid fraction of olive mill wastewater with olive leaves: organic matter degradation and biological activity, Bioresources Technology, 86, 59–64.

Georgacakis, D., Dalis, D., 1993. Controlled anaerobic digestion of settled olive-oil wastewater, Bioresource Technology, 46, 221–226.

Gernjak, W., Krutzler, T., Glaser, A., Malato, S., Caceres, J., Bauer, R., Fernández-Alba, A.R., 2003. Photo-fenton treatment of water containing natural phenolic pollutants, Chemosphere, 50, 71–78.

Giovanoudis, I., Athanasiadis, V., Nanos, G., Gortzi, O., Lalas, S., 2017. Phenols separation from olive mill wastewater by cloud point extraction with food grade surfactants. Greece.

Gortzi, O., Lalas, S., Chatzilazarou, A., Katsoyannos, E., Papaconstandinou, S., Dourtoglou, E., 2008. Recovery of natural antioxidants from olive mill wastewater using Genapol- X080, JAOCS, Journal of the American Oil Chemists Society, 85, 133–140.

Guzman, J. F.-B., Moreno, A., Gutierrez, G., Arcos, R., Araujo, A., Bejarano, R., 2004. Method for obtaining purified hydroxytyrosol from products and by-products derived from the olive tree. US 6849770B2, U.S.A.

El Hajjouji, H., Fakharedine, N., Ait Baddi, G., Winterton, P., Bailly, J.R., Revel, J.C., Hafidi, M., 2007. Treatment of olive mill waste-water by aerobic biodegradation: An analytical study using gel permeation chromatography, ultraviolet-visible and Fourier transform infrared spectroscopy, Bioresource Technology, 98, 3513–3520.

Hach Company. 2014. Total Kjeldahl Nitrogen, Method 8075, DOC316.53.01091. U.S.A.

Hendel, N., Larous, L., Belbey, L., 2016. Antioxidant activity of rosemary (*Rosmarinus officinalis* L.) and its in vitro inhibitory effect on *Penicillium digitatum*, International Food Research, 23, 1725–1732.

Hocaoglu, S.M., Hande, B., Haksevenler, G., Basturk, I., Talazan, P., Aydoner, C., 2018. Assessment of technology modification for olive oil sector through mass balance: A case study for Turkey, Journal of Cleaner Production, 188, 786– 795.

Hocaoğlu, S.M., Haksevenler, H.G., Baştürk, İ., Aydöner, C., 2015. Zeytin sektörü atıklarının yönetimi projesi nihai rapor. Project No. 5148602. The Scientific and Technical Research Council of Turkey.

Icyer, N.C., Toker, O.S., Karasu, S., Tornuk, F., Bozkurt, F., Arici, M., Sagdic, O., 2016. Combined design as a useful statistical approach to extract maximum amount of phenolic compounds from virgin olive oil waste, LWT - Food Science and Technology, 70, 24–32.

Inan, H., Dimoglo, A., Şimşek, H., Karpuzcu, M., 2004. Olive oil mill wastewater treatment by means of electro-coagulation, Separation and Purification Technology, 36, 23–31.

Inglezakis, V.J., Moreno, J.L., Doula, M., 2012. olive oil waste management: current situation and policy recommendations, International Journal of Chemical and Environmental Engineering Systems, 3, 65–77.

International Olive Oil Council, 2017. World olive oil consumption figures.

Jagannathan, S., Vijayakumar, R., RahulGandhi, P., Ananthi, M., Charles, C., Premkumar, A., Venkataramana, K.N., 2010. Analysis of carbohydrates in newly developed liquid state rabies vaccine, Journal of Microbial and Biochemical Technology, 2, 147–151.

Jaouani, A., Sayadi, S., Vanthournhout, M., Penninckx, M. J., 2003. Potent fungi for decolourisation of olive oil mill wastewaters, Enzyme and Microbial Technology, 33, 802–809.

Jerman, T., Trebše, P., Mozetič Vodopivec, B., 2010. Ultrasound-assisted solid liquid extraction (USLE) of olive fruit (*Olea europaea*) phenolic compounds, Food Chemistry, 123, 175–182.

Justino, C., Marques, A. G., Duarte, K. R., Duarte, A. C., Pereira, R., Rocha-Santos, T., Freitas, A. C., 2010. Degradation of phenols in olive oil mill wastewater by biological, enzymatic, and photo-Fenton oxidation, Environmental Science and Pollution Research, 17, 650–656.

Kaleh, Z., Geißen, S.U., 2016. Selective isolation of valuable biophenols from olive mill wastewater, Journal of Environmental Chemical Engineering, 4, 373–384.

Kanakis, P., Aikaterini, T., Michel, T., Gikas, E., Halabalaki, M., Skaltsounis, A.-L., 2013. From olive drupes to olive oil: An HPLC-orbitrap-based qualitative and quantitative exploration of olive key metabolites, Planta Medica, 79, 1576–1587.

Karameldin, A., Mekhemar, A.L.S., 2003. The Red Sea area wind-driven mechanical vapor compression desalination system, Desalination, 153, 47–53.

Katsoyannos, E., Chatzilazarou, A., Gortzi, O., Lalas, S., Konteles, S., Tataridis, P., 2006. Application of cloud point extraction using surfactants in the isolation of physical antioxidants (phenols) from olive mill wastewater. Fresenius Environmental Bulletin, 15, 1122–1125.

Kavvadias, V., Elaiopoulos, K., Theocharopoulos, S., Soupios, P., 2017. Fate of potential contaminants due to disposal of olive mill wastewaters in unprotected evaporation ponds, Bulletin of Environmental Contamination and Toxicology, 98, 323–330.

Kelebek, H., Kesen, S., Selli, S., 2015. Comparative study of bioactive constituents in Turkish olive oils by LC-ESI/MS/MS, International Journal of Food Properties, 18, 2231–2245.

Kestioğlu, K., Yonar, T., Azbar, N., 2005. Feasibility of physico-chemical treatment and Advanced Oxidation Processes (AOPs) as a means of pretreatment of olive mill effluent (OME), Process Biochemistry, 40, 2409–2416.

Khoufi, S., Aloui, F., Sayadi, S., 2008. Extraction of antioxidants from olive mill wastewater and electro-coagulation of exhausted fraction to reduce its toxicity on anaerobic digestion, Journal of Hazardous Materials, 151, 531–539.

Khoufi, S., Aloui, F., Sayadi, S., 2009. Pilot scale hybrid process for olive mill wastewater treatment and reuse, Chemical Engineering and Processing: Process Intensification, 48, 643– 650.

Lafka, T.-I., Lazou, A.E., Sinanoglou, V.J., Lazos, E.S., 2011. Phenolic and antioxidant potential of olive oil mill wastes, Food Chemistry, 125, 92–98.

Lee, O.H., Lee, B.Y., Lee, J., Lee, H.B., Son, J.Y., Park, C.S., Shetty, K., Kim, Y.C., 2009. Assessment of phenolics-enriched extract and fractions of olive leaves and their antioxidant activities, Bioresource Technology, 100, 6107–6113.

Leouifoufi, I., Zyad, A., Amechrouq, A., Oukerrou, M.A., Mouse, H.A. and Mbarki, M., 2014. Identification and characterisation of phenolic compounds extracted from Moroccan olive mill wastewater, Food Science and Technology, 34, 249–257.

Lesage-Meessen, L., Navarro, D., Maunier, S., Sigoillot, J.C., Lorquin, J., Delattre, M., Simon, J.L., Asther, M., Labat, M., 2001. Simple phenolic content in olive oil residues as a function of extraction systems, Food Chemistry, 75, 501–507.

Li, Y., Watkinson, A.P., 2011. Deposit formation in evaporation of a pulp mill effluent, Heat Transfer Engineering, 32, 258–263.

Liang, L., Han, D., Ma, R., Peng, T., 2013. Treatment of high-concentration wastewater using double-effect mechanical vapor recompression, Desalination, 314, 139–146.

Liang, L., Han, D., 2011. Energy-saving study of a system for ammonium sulfate recovery from wastewater with Mechanical Vapor Compression (MVC), Research Journal of Applied Sciences, Engineering and Technology, 3, 1227–1232.

Lolos, G., Skordilis, A., Parissakis, G., 1994. Polluting characteristics and lime precipitation of olive mill wastewater, Journal of Environmental Science and Health. Part A: Environmental Science and Engineering and Toxicology, 29, 1349–1356.

López, M.J.A., Streitenberger, S.A., Peñalver, M.M. and Martinez, O.P., 2008. Process and apparatus for the production of hydroxytyrosol containing extract from olives and solids containing residues of olive oil extraction. US 8236993B2, U.S.A.

Majbar, Z., Rais, Z., Haji, M.E., Abbou, M.B., Bouka, H., Nawdali, M., 2017. Olive mill wastewater and wine by-products valorization by co-composting', Journal of Materials and Environmental Science, 8, 3162–3167.

Mantzavinos, D., Hellenbrand, R., Livingston, A.G., Metcalfe, I.S., 1997. Wet oxidation as a pretreatment method for wastewaters contaminated by bioresistant organics, Water Science and Technology, 36, 109–116.

Marques, I.P., Teixeira, A., Rodrigues, L., Dias, S.M., Novais, J.M., 1998. Anaerobic treatment of olive mill wastewater with digested piggery effluent, Water Environment Research, 70, 1056–1061.

Marques, I.P., 2001. Anaerobic digestion treatment of olive mill wastewater for effluent re-use in irrigation, Desalination, 137, 233–239.

McCabe, D.L., Vivona, M.A., 1999. Treating process wastewater employing vacuum distillation using mechanical vapor recompression, Environmental Progress, 18, 30–33.

Mojerlou, Z., Elhamirad, A., 2018. Optimization of ultrasound-assisted extraction (UAE) of phenolic compounds from olive cake, Journal of Food Science and Technology, 55, 977–984.

Mouncif, M., Tamoh, S., Faid, M., Achkari-Begdouri, A., 1993. A study of chemical and microbiological characteristics of olive mill waste water in Morocco, Grasas y Aceites, 44, 335–338.

Obied, H.K., Allen, M.S., Bedgood, D.R., Prenzler, P.D., Robards, K., Stockmann, R., 2005. Bioactivity and analysis of biophenols recovered from olive mill waste, Journal of Agricultural and Food Chemistry, 53, 823–837.

Ochando-Pulido, J.M., Fragoso, R., Macedo, A., Duarte, E., Ferez, A. M., 2016. A brief review on recent processes for the treatment of olive mill effluents. In: Boskou D., Clodoveo M.L. (Eds), Products from Olive Tree, 283–300, InTech, Rijeka, Crotia.

Ochando-Pulido, J.M., 2016. A review on the use of membrane technology and fouling control for olive mill wastewater treatment, Science of the Total Environment, 563–564, 664– 675.

Ochando-Pulido, J.M., Pimentel-Moral, S., Verardo, V., Martinez-Ferez, A., 2017. A focus on advanced physico-chemical processes for olive mill wastewater treatment, Separation and Purification Technology., 179, 161–174.

Oreopoulou, V., Tzia, C., 2007. Utilization of plant by-products for the recovery of proteins, dietary fibers, antioxidants, and colorants, In: Oreopoulou, V., Russ, W. (Eds), Utilization of By-Products and Treatment of Waste in the Food Industry, 209–232, Springer, New York, E.U.

Papoti, V.T., Tsimidou, M.Z., 2009. Looking through the qualities of a fluorimetric assay for the total phenol content estimation in virgin olive oil, olive fruit or leaf polar extract, Food Chemistry, 112, 246–252.

Paraskeva, P., Diamadopoulos, E., 2006. Technologies for olive mill wastewater (OMW) treatment: a review, Journal of Chemical Technology and Biotechnology, 81, 1475–1485.

Paraskeva, C.A., Papadakis, V.G., Tsarouchi, E., Kanellopoulou, D.G., Koutsoukos, P.G., 2007. Membrane processing for olive mill wastewater fractionation, Desalination, 213, 218–229.

Paredes, C., Cegarra, J., Roig, A., Sánchez-Monedero, M.A., Bernal, M.P., 1999. Characterization of olive mill wastewater (alpechin) and its sludge for agricultural purposes, Bioresource Technology, 67, 111–115.

Prior, R.L., Wu, X., Schaich, K., 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements, Journal of Agricultural and Food Chemistry, 53, 4290–4302.

Putnik, P., Barba, F.J., Španić, I., Zorić, Z., Dragović-Uzelac, V., Bursać Kovačević, D., 2017. Green extraction approach for the recovery of polyphenols from Croatian olive leaves (*Olea europea*), Food and Bioproducts Processing, 106, 19–28.

Queimada, A.J., Mota, F.L., Pinho, S.P., Macedo, E.A., 2009. Solubilities of biologically active phenolic compounds: Measurements and modeling, Journal of Physical Chemistry B, 113, 3469–3476.

Rahmanian, N., Jafari, S.M., Galanakis, C.M., 2014. Recovery and removal of phenolic compounds from olive mill wastewater, JAOCS, Journal of the American Oil Chemists' Society, 91, 1–18.

Ramos-Cormenzana, A., Juárez-Jiménez, B., Garcia-Pareja, M.P., 1996. Antimicrobial activity of olive mill wastewaters (alpechin) and biotransformed olive oil mill wastewater, International Biodeterioration & Biodegradation, 38, 283–290.

Rinaldi, M., Rana, G., Introna, M., 2003. Olive-mill wastewater spreading in southern Italy: Effects on a durum wheat crop, Field Crops Research, 84, 319–326.

Rizzo, L., Lofrano, G., Grassi, M., Belgiorno, V., 2008. Pre-treatment of olive mill wastewater by chitosan coagulation and advanced oxidation processes, Separation and Purification Technology, 63, 648–653.

Rozzi, A., Malpei, F., 1996. Treatment and disposal of olive mill effluents, International Biodeterioration & Biodegradation, 38, 135–144.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Fernández-Bolaños, J., 2013. Chemical characterization and properties of a polymeric phenolic fraction obtained from olive oil waste, Food Research International, 54, 2122–2129.

Russo, C., 2007. A new membrane process for the selective fractionation and total recovery of polyphenols, water and organic substances from vegetation waters (VW), Journal of Membrane Science, 288, 239–246.

Sabbah, I., Yazbak, A., Haj, J., Saliba, A., Basheer, S., 2005. Biomass selection for optimal anaerobic treatment of olive mill wastewater, Environmental Technology, 26, 47–54.

Şahin, S., Şamli, R., 2013. Optimization of olive leaf extract obtained by ultrasound-assisted extraction with response surface methodology, Ultrasonics Sonochemistry, 20, 595–602.

Sánchez-Rangel, J.C., Benavides, J., Heredia, J.B., Cisneros-Zevallos, L., Jacobo-Velázquez, D.A., 2013. The Folin–Ciocalteu assay revisited: improvement of its specificity for total phenolic content determination, Analytical Methods, 5, 5990.

Sarika, R., Kalogerakis, N., Mantzavinos, D., 2005. Treatment of olive mill effluents Part II. Complete removal of solids by direct flocculation with poly-electrolytes, Environment International, 31, 297–304.

Sayadi, S., Allouche, N., Jaoua, M., Aloui, F., 2000. Detrimental effects of high molecular-mass polyphenols on olive mill wastewater biotreatment, Process Biochemistry, 35, 725–735.

Scoma, A., Pintucci, C., Bertin, L., Carlozzi, P., Fava, F., 2012. Increasing the large scale feasibility of a solid phase extraction procedure for the recovery of natural antioxidants from olive mill wastewaters, Chemical Engineering Journal, 198–199, 103–109.

Sellami, M. K., Khemakhem, I., Mkadmini, K., Bouzouita, N., 2016. Chemical composition, antioxidant potential and phenolic profile of oil mill waste water from Tunisian olive varieties (Chetoui and Chemlali), 5, 605-614.

Shirzad, H., Niknam, V., Taheri, M., Ebrahimzadeh, H., 2017. Ultrasound-assisted extraction process of phenolic antioxidants from Olive leaves: a nutraceutical study using RSM and LC–ESI–DAD–MS, Journal of Food Science and Technology, 54, 2361–2371.

Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents, American Journal of Enology and Viticulture, 16, 115–144.

Sivakumar, M., Ramezanianpour, M., O'Halloran, G., 2013. Mine water treatment using a vacuum membrane distillation system, APCBEE Procedia, 5, 157–162.

Soto, M.L., Moure, A., Domínguez, H., Parajó, J.C., 2011. Recovery, concentration and purification of phenolic compounds by adsorption: A review, Journal of Food Engineering, 105, 1–27.

Stamatopoulos, K., Katsoyannos, E., Chatzilazarou, A., 2014. Antioxidant activity and thermal stability of oleuropein and related phenolic compounds of olive leaf extract after separation and concentration by salting-out-assisted cloud point extraction, Antioxidants, 3, 229–244.

Stoller, M., Chianese, A., 2006. Technical optimization of a batch olive wash wastewater treatment membrane plant, Desalination, 200, 734–736.

Szabo, M.R., Radu, D., Gavrilas, S., Chambre, D., Iditoiu, C., 2010. Antioxidant and antimicrobial properties of selected spice extracts, International Journal of Food Properties, 13, 535–545.

Takac, S., Karakaya, A., 2010. Recovery of phenolic antioxidants from olive mill wastewater, Recent Patents on Chemical Engineering, 2, 230–237.

Tercan, S., Seker, M., 2012. Comparison of polyphenol extractions from olive pomace and solid fraction of olive mill waste water, Natural Product Research, 26, 1837–1841.

Tornberg, E., Galanakis, C., 2008. Olive waste recovery. WO 2008082343 A1.

Tsagaraki, E., Lazarides, H.N., Petrotos, K.B., 2007. Olive mill wastewater treatment, In: Oreopoulou, V., Russ, W. (Eds), Utilization of By-Products and Treatment of Waste in the Food Industry, 133–157, Springer, New York, E.U.

Turano, E., Curcio, S., De Paola, M.G., Calabrò, V., Iorio, G., 2002. An integrated centrifugation-ultrafiltration system in the treatment of olive mill wastewater, Journal of Membrane Science, 209, 519–531.

Velioglou, S., Curi, K., Camillar, S., 1987. Laboratory experiments on the physical treatment of olive oil wastewater, International Journal for Development Technology, 5, 49–57.

Venturi, F., Sanmartin, C., Taglieri, I., Nari, A., Andrich, G., Terzuoli, E., Donnini, S., Nicolella, C., Zinnai, A., 2017. Development of phenol-enriched olive oil with phenolic compounds extracted from wastewater produced by physical refining, Nutrients, 9, 916.

Víctor-Ortega, M. D., Ochando-Pulido, J. M., Martínez-Férez, A., 2016. Phenols removal from industrial effluents through novel polymeric resins: Kinetics and equilibrium studies, Separation and Purification Technology, 160, 136–144.

Visioli, F., Romani, A., Mulinacci, N., Zarini, S., Conte, D., Vincieri, F. F., Galli, C., 1999. Antioxidant and other biological activities of olive mill waste waters, Journal of Agricultural and Food Chemistry, 47, 3397–3401.

Xavier, L., Freire, M. S., Vidal-Tato, I., 2017. Recovery of phenolic compounds from Eucalyptus wood wastes using ethanol-salt-based aqueous two-phase systems, Maderas, Ciencia y tecnología, 19, 3-14.

Xynos, N., Papaefstathiou, G., Gikas, E., Argyropoulou, A., Aligiannis, N., Skaltsounis, A.L., 2014. Design optimization study of the extraction of olive leaves performed with pressurized liquid

extraction using response surface methodology, Separation and Purification Technology, 122, 323–330.

Xynos, N., Abatis, D., Argyropoulou, A., Polychronopoulos, P., Aligiannis, N., Skaltsounis, A.L., 2015. Development of a sustainable procedure for the recovery of hydroxytyrosol from table olive processing wastewater using adsorption resin technology and centrifugal partition chromatography, Planta Medica, 81, 1621–1627.

Yangui, A., Abderrabba, M., Sayari, A., 2017. Amine-modified mesoporous silica for quantitative adsorption and release of hydroxytyrosol and other phenolic compounds from olive mill wastewater, Journal of the Taiwan Institute of Chemical Engineers, 70, 111–118.

Zagklis, D.P., Vavouraki, A.I., Kornaros, M.E., Paraskeva, C.A., 2015. Purification of olive mill wastewater phenols through membrane filtration and resin adsorption/desorption, Journal of Hazardous Materials, 285, 69–76.

APPENDIX A: HPLC CALIBRATION CURVES OF HTYR AND TYR

Figure A.1. Calibration curve of HTyr by HPLC.

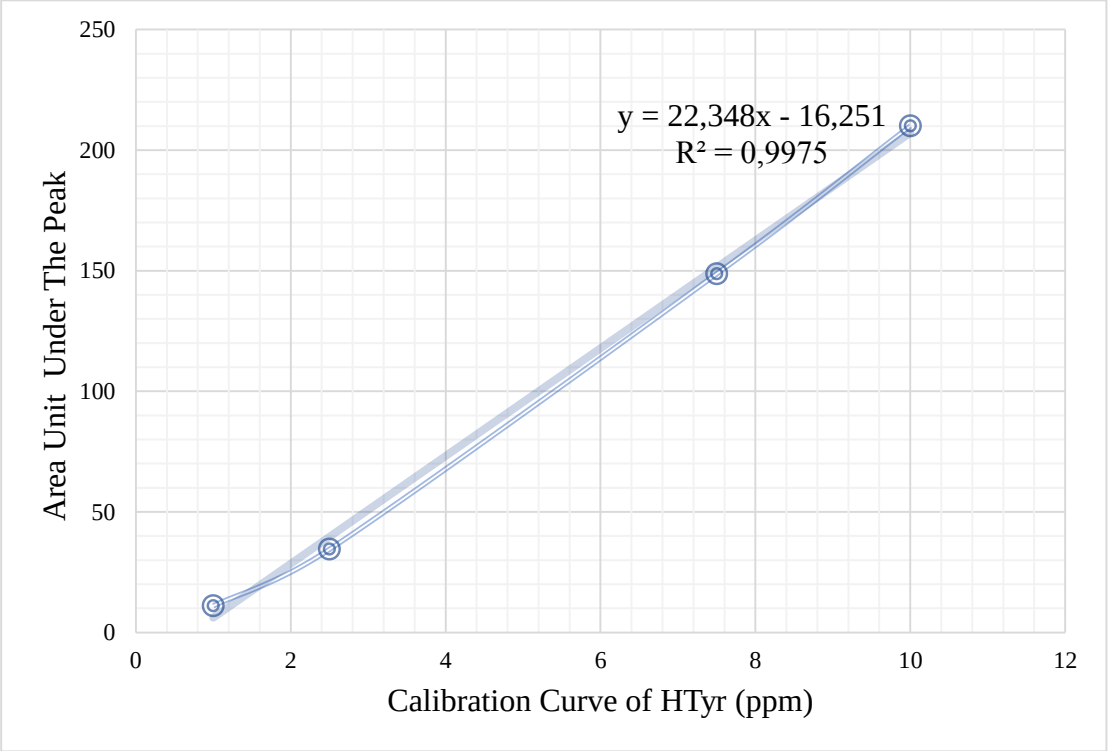
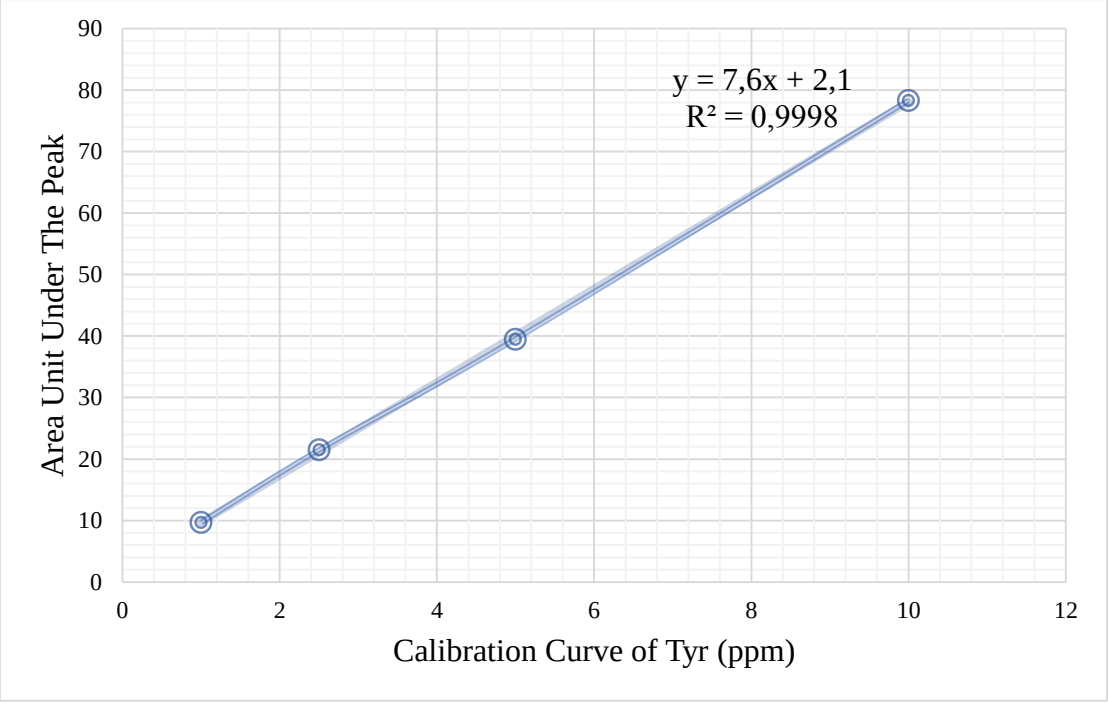
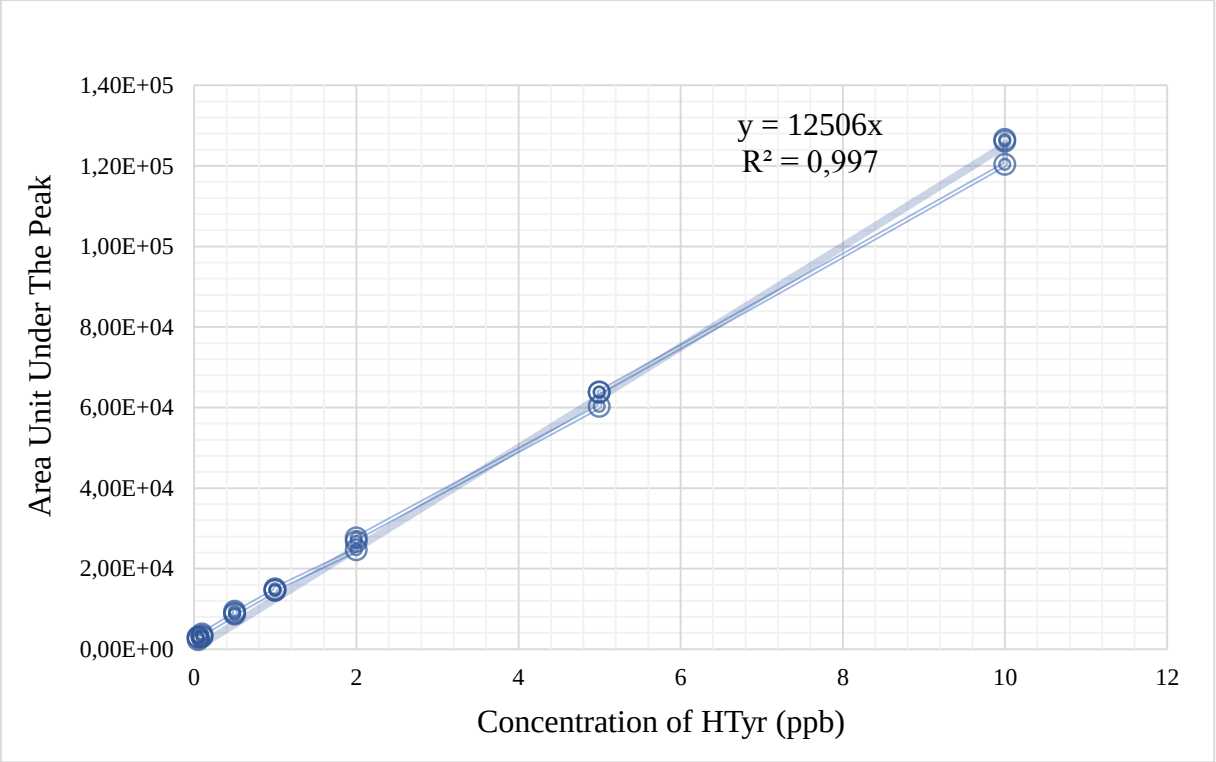


Figure A.2. Calibration curve of Tyr by HPLC.



APPENDIX B: LC-MS/MS CALIBRATION CURVES OF HTYR

Figure B.1. Calibration curve of HTyr by LC-MS/MS.



APPEXDIX C: CALIBRATION CURVES OF SAMPLE CHARACTERIZATION METHODS

Figure C.1. Calibration curve of gallic acid by Folin-Ciocalteu method for total phenols analysis.

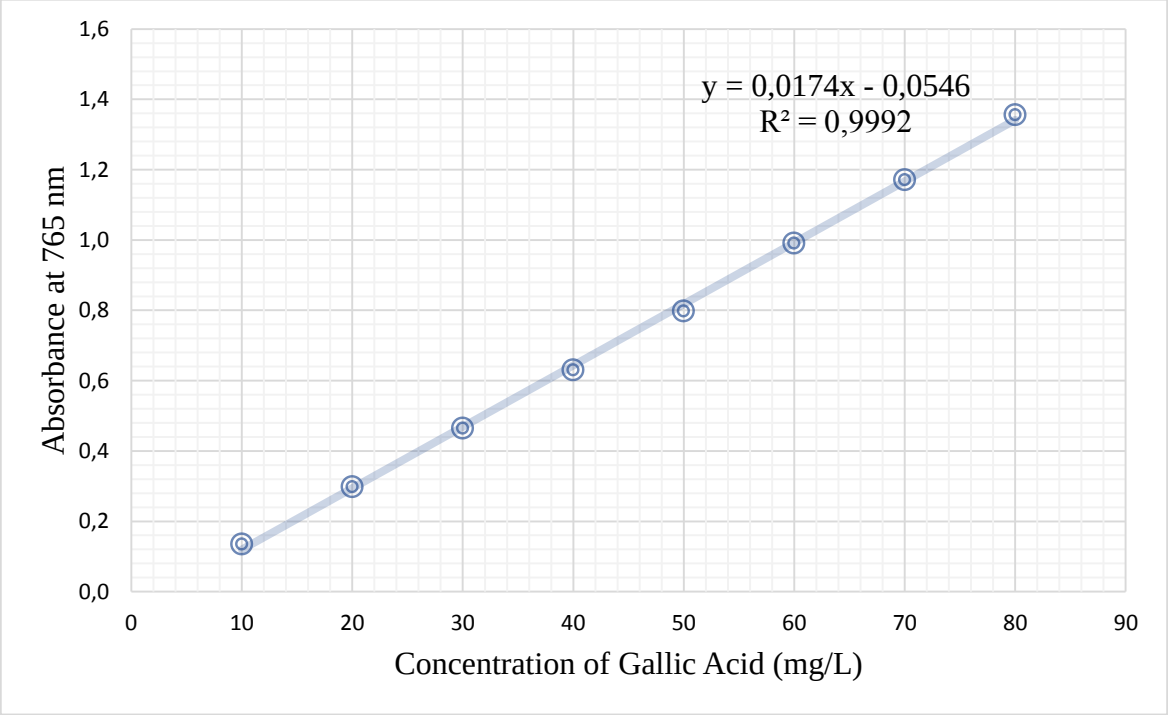


Figure C.2. Calibration curve of BAS by Bradford method for protein analysis.

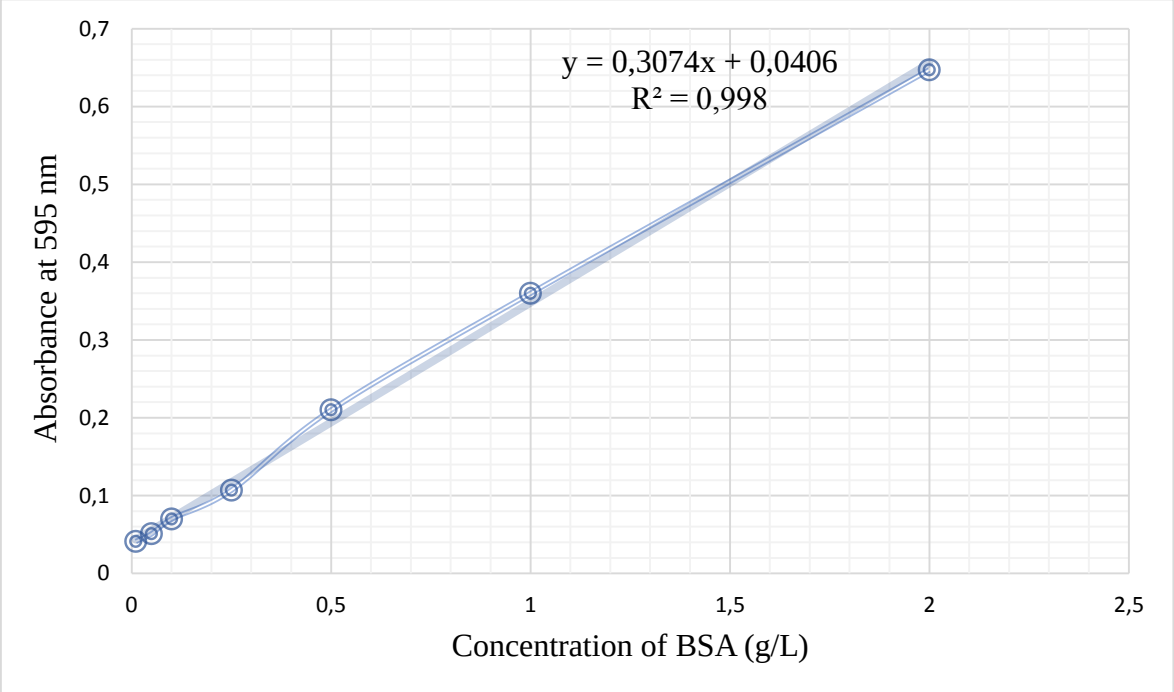


Figure C.3. Calibration curve of glucose by Anthrone method for carbohydrate analysis.

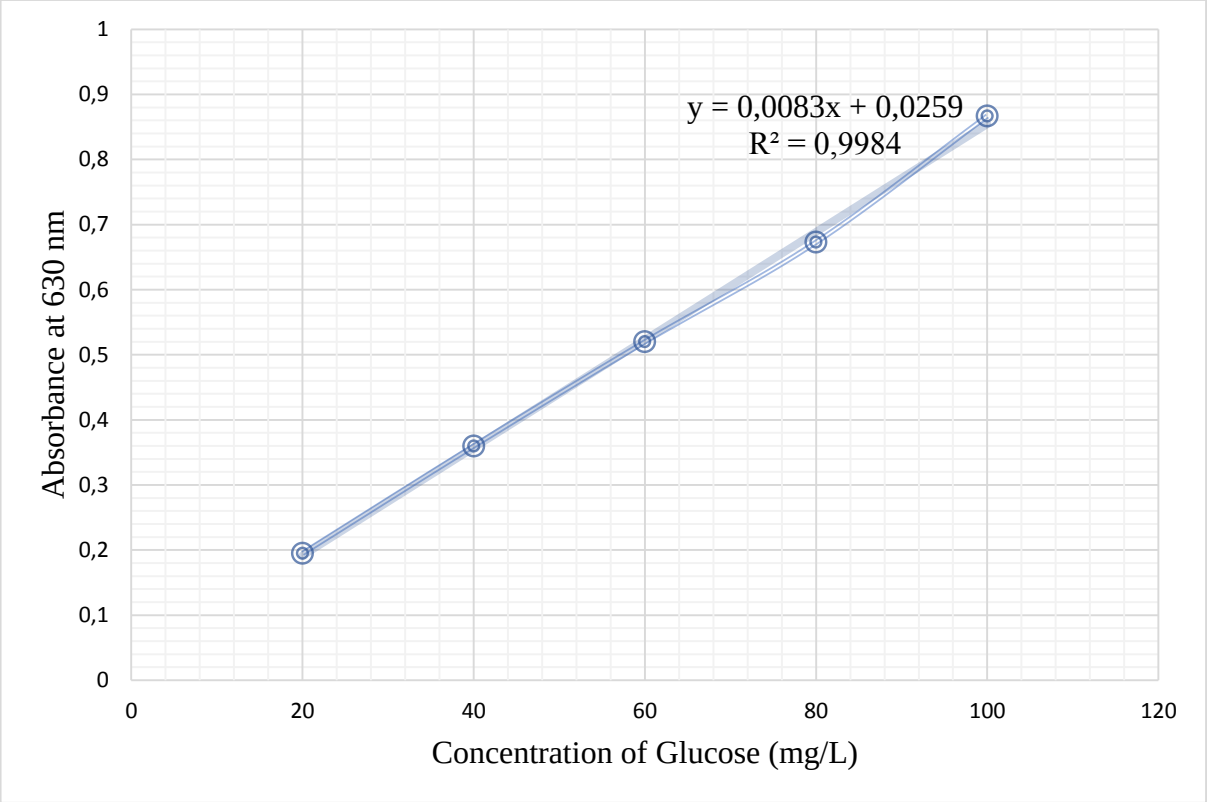


Figure C.4. Calibration curve of ascorbic acid by DPPH method for the determination of antioxidant activity.