RECOVERY AND PURIFICATION OF NATURAL ANTIOXIDANTS FROM OLIVE MILL WASTEWATER CONCENTRATES BY A SUSTAINABLE PROCESS

by

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To my dear mum…

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ABSTRACT

RECOVERY AND PURIFICATION OF NATURAL ANTIOXIDANTS FROM OLIVE MILL WASTEWATER CONCENTRATES BY A SUSTAINABLE PROCESS

Olive mill wastewater (OMWW) resulting from olive oil production is characterized with high organic load and phytotoxicity due to the presence of phenolic compounds (PCs). Antioxidant properties of PCs make this waste a potential resource for some industries. Hence, instead of spending effort to treat the toxic components, the recovery of value-added compounds from OMWW can be sustainable waste management solution providing economic benefits. This study was aimed to find out a recovery process for antioxidant PCs mainly hydroxytyrosol (HTyr). The concentrated OMWW samples by Mechanical Vapor Recompression (MVR) evaporator at two different rates, OMWC1 and OMWC2 were subjected to solid phase extraction (SPE) by using different synthetic polymeric resins stands alone (Process I) and as integrated to aqueous two-phase extraction (ATPE) (Process II). Process I and II with non-ionic PAD 950 yielded higher total PCs recovery from both OMWCs while the performance of Amberlyst A26 resin in Process II was better for HTyr recovery. The recoveries of HTyr were 21.77 and 34.31 mg/g from more concentrated OMWC2 by Process II whereas 4.88 and 3.86 mg/g HTyr were recovered from OMWC1 by the application of Process I with PAD 950 and Amberlyst A26 resins, respectively. The first extraction stage of Process II with ATPE system minimized the coextraction of non-target compounds and in overall Process II provided the eliminations of 85-90% carbohydrates and almost complete proteins from the extract of OMWC2. The antioxidant activities of the extracts achieved from more productive Process II exhibited variation depending the applied activity assays.

ÖZET

ZEYTİNYAĞI KARASU KONSANTRELERİNDEN SÜRDÜRÜLEBİLİR İŞLEM İLE DOĞAL ANTİOKSİDANLARIN GERİ KAZANIMI VE SAFLAŞTIRILMASI

Zeytinyağı üretiminden kaynaklanan atık zeytin karasuyu, yüksek organik yük miktarı ve içeriğindeki fenolik bileşiklerin neden olduğu fitotoksisite ile karakterize edilir. Antioksidan özelliklere sahip fenolik bileşikler, bu atığı endüstriyel alanda kullanım için potansiyel bir kaynak haline getirmektedir. Bu nedenle, toksik bileşiklerin arıtılması için çaba harcamak yerine, atık zeytin karasuyunda bulunan faydalı bileşiklerin geri kazanımı, sağladığı ekonomik yararlar ile sürdürülebilir atık yönetimi için bir çözüm olabilir. Bu çalışmanın amacı başlıca hidroksitirosol olmak üzere fenolik bileşikler için bir geri kazanım süreci araştırmaktır. Mekanik buhar sıkıştırmalı evaporatörde iki farklı oranda konsantre edilen atık zeytin karasuları, tek başına (Süreç I) ve iki fazlı sıvı ekstraksiyona entegre olarak (Süreç II), farklı sentetik polimerik reçinelerin kullanıldığı katı faz ekstraksiyon işlemine tabi tutulmuştur. İyonik olmayan PAD 950 reçinesi ile Süreç I ve II, her iki konsantre atık zeytin karasuyundan yüksek oranda toplam fenolik bileşiklerin geri kazanımını sağlarken, Amberlyst A26 reçinesi ile Süreç II daha iyi hidroksitirosol geri kazanım performansı göstermiştir. Süreç II' nin daha konsantre atık zeytin karasuyundan hidroksitirosol geri kazanımı 21.77 ve 34.31 mg/g iken, az konsantre atık zeytin karasuyundan 4.88 ve 3.86 mg/g hidroksitirosol, Süreç I'in PAD 950 ve Amberlyst A26 reçineleri ile uygulanmasıyla geri kazanılmıştır. Süreç II'nin ilk ekstraksiyon adımı olan iki fazlı ekstraksiyon sistemi ile hedef olmayan bileşiklerin eş zamanlı ekstraksiyonunu en aza indirilmiştir ve Süreç II, genel olarak daha konsantre atık zeytin karasuyundan %85- 90 oranında karbonhidrat ve neredeyse tüm proteinlerin elenmesini sağlamıştır. Daha verimli olan Süreç II'den elde edilen ekstrakların antioksidan aktiviteleri, uygulanan aktivite testine bağlı olarak değişiklik göstermiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Unit
A	Adsorption Efficiency	%
BOD_5	Biological Oxygen Demand	g/L
COD	Chemical Oxygen Demand	g/L
D	Desorption Efficiency	%
D_{ov}	Overall Desorption Efficiency	%
EC	Electrical Conductivity	mS/cm
EY	Extraction yield	%
m	Mass	g
R	Recovery Efficiency	%
TDS	Total Dissolved Solid	g/L
TOC	Total Organic Carbon	g/L
TS	Total Solid	g/L
TSS	Total Suspended Solids	g/L
w	Mass fraction	%

Abbreviation	Explanation
Å	Angstrom
AAE	Ascorbic Acid Equivalent
ABTS	2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ATPE	Aqueous Two-Phase Extraction
ATPS	Aqueous Two-Phase System
BSA	Bovine Serum Albumin
CO_2	Carbon Dioxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EtOH	Ethanol
EtOAc	Ethyl acetate
Fe	Iron
g	Gram
GAC	Granular Activated Carbon
GAE	Gallic Acid Equivalent
GE	Glucose Equivalents

H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulfuric Acid
H_2O	Water
HCl	Hydrochloric Acid
h	Hour
HTyr	Hydroxytyrosol
K	Partition Coefficient
Kg	Kilogram
K_{ow}	Octanol/Water Partition Coefficient
K_p	Predicted partition coefficient oil/water
KHP	Potassium hydrogen phthalate
KH_2PO_4	Monopotassium phosphate
K_2CO_3	Potassium carbonate
K_2HPO_4	Dipotassium Hydrogen Phosphate
K_3PO_4	Potassium phosphate
L	Liter
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
Log K_{ow}	Octanol-Water Partition Coefficient
M	Molarity
µm	Micrometer
µM	Micromolar
mg	Milligram
mL	Milliliter
MeOH	Methanol
Mg	Magnesium
min	Minute
MVR	Mechanical Vapor Recompression
N	Normality
NaOH	Sodium Hydroxide
NaH_2PO_4	Sodium dihydrogen phosphate
$(NH_4)_2SO_4$	Ammonium Sulphate
nm	Nanometer
OH^-	Hydroxide
OM	Olive Mill
OMW	Olive Mill Waste

OMWC	Olive Mill Wastewater Concentrate
OMWW	Olive Mill Wastewater
OOWW	Olive Oil Washing Water
P	Phosphate
pKa	Acid Dissociation Constant
PC	Phenolic Compound
ppb	Parts per billion
rpm	Revolutions per minute
SPE	Solid Phase Extraction
TP	Total Phenols
Tyr	Tyrosol
v	Volume
V_r	Volume Ratio

1. INTRODUCTION

Olive processing is one of the main and ancient agricultural industry in Mediterranean countries. The utilization of olive oil has been increasing steadily in worldwide during last decades with the growing attention to Mediterranean diet (Takaç and Karakaya, 2009). Hence, the production of olive oil has been increased rapidly in response to growing demand of olive oil (Ochando-Pulido et al., 2016).

The waste generated from olive oil production which is performed by two primary extraction systems: batch (press) and continuous processes depended on the oil separation methods. However, each extraction process generates olive mill wastewater (OMWW), and semi-solid or slurry waste (pomace), with different characteristics and volumes. As a result of switching from the traditional batch press system to the continuous centrifugation-based systems, the quantities of OMW have shown a substantial increase. Water consumption of three-phase centrifugal system which results from the washing of the olives and olive oil is in the range of 750-980 L per ton of processed olive that is more than twice that of the traditional system (Khdair et al., 2019). This increment causes annual generation of high amount of OMWW and the overconsumption of potable water (Ochando-Pulido et al., 2016; Ochando-Pulido et al., 2018).

OMWW typically exhibits an acidic characteristic, strong odour, intensive dark brown to black colour, high organic load, and high electrical conductivity. The organic content of OMWW includes recalcitrant compounds such as lignins, tannins, long-chain fatty acids, and also toxic PCs; therefore, it has poor biodegradability and high phytotoxicity (Azbar et al., 2004; Paraskeva and Diamadopoulos, 2006; Takaç and Karakaya, 2009; Ochando-Pulido et al., 2016; Souilem et al., 2017).

The management of OMW is the major environmental issue in Mediterranean countries owing to its large quantity and toxic property that require the application of a treatment process prior to disposal. The land application of OMW rich in nutrient considered as a natural fertilizer is beneficial to practise for soil structure and crops growth; however, the nature of OMW can cause serious environmental damages not only on water resources but also on atmosphere and soil. In Turkey like in some Mediterranean countries, storage lagoons are currently used to reduce the volume of OMW. However, this treatment approach requiring large area leads to various environmental problems e.g., the release of extremely strong odor, underground seepage, the attraction of insects, moreover the

accumulation of sludge at the bottom of lagoon. Although several treatment methods including physicochemical, biological, thermal as well as combined process have been investigated to reduce the pollution load of OMWW (Takaç and Karakaya, 2009), there is still a requirement for applicable treatment and management of OMWW in a cost-effective way for olive oil industries (Paraskeva and Diamadopoulos, 2006; Azbar et al., 2004; Saadi et al., 2007; Ochando-Pulido et al., 2016). Since conventional treatment processes have some disadvantages with high cost and energy consumption, and generation of secondary pollution, scientists have been spent great efforts in order to develop economically and environmentally feasible strategies for OMW such as valorization and energy-production processes to enable the recovery of high valuable raw compounds and reuse the purified effluent for industrial purposes (Rahmanian et al., 2014; Souilem et al., 2017; Ochando-Pulido et al., 2018).

OMW contains about 98% of the phenols present in olive fruit and more than 50 different PCs have been currently identified in this waste (Obied et al., 2007; Rahmanian et al. 2014; Galanakis and Kotsiou, 2017). OMWW is considered a natural and cost-effective source of biologically active value-added compounds mainly PCs with antioxidant, anti-inflammatory and antimicrobial properties (Agalias et al., 2007; Dermeche et al., 2013; Kaleh and Geißen, 2016; Pinelli et al., 2016). Depending upon the quality and maturity of cultivated olives, climatic conditions, soil composition, and oil extraction techniques, the PCs composition of OMWW can be different in addition to the occurrence of specific biophenols (Obied et al., 2005a). Among PCs, hydroxytyrosol (HTyr), tyrosol (Tyr), and oleuropein are the most abundant biophenols (De Marco et al., 2007; Rahmanian et al., 2014; Galanakis and Kotsiou, 2017). HTyr with advanced antioxidant and bioactivity properties than other PCs has high market value for nutraceutical and pharmaceutical industries; however, the production of synthetic HTyr is expensive. Therefore, the recovery of PCs including HTyr from OMWW can be an economical approach to respond to the demand of forementioned industries and reduce the pollution load of OMWW industries (De Marco et al., 2007).

For the recovery of total PCs from OMWW, several techniques including membrane separation, solvent extraction, chromatographic separations, centrifugation, and coagulation have been applied individually or in combination (Agalias et al., 2007; De Marco et al., 2007; Gortzi et al., 2007; Takaç and Karakaya, 2009; Bertin et al., 2011; Ferri et al., 2011; Scoma et al., 2012; Bedouhene et al., 2014; Rahmanian et al., 2014; Zagklis et al., 2015; Frascari et al., 2016; Víctor-Ortega et al., 2016a; Yangui et al., 2017; Çelik, 2018; Ochando-Pulido et al., 2018). Even though these techniques can remove PCs from OMWW and minimize the waste generation, the requirement of complicated equipment, membrane materials, and the toxic and large quantity of solvents is an obstacle in front of

economically feasible processes for the achievement of natural antioxidants from OMWW (Takaç and Karakaya, 2009; Çelik, 2018). Additionally, current recovery processes are insufficient in terms of the large volume of OMWW. From this point, the concentration of OMWW by MVR evaporator can be efficient and economical pre-treatment process for the recovery techniques.

The main objective of the proposed study was to investigate two different extraction processes for the selective recovery of natural antioxidants with focus on HTyr from OMWW generated from the two-phase centrifugal system while eliminating complex organic matrix components. SPE with different synthetic polymeric resins stands alone and as integrated to ATPE were the extraction processes applied to concentrated OMWW. MVR evaporator concentrated the OMWW at two different ratios before the extraction processes. The effects of extraction processes on the recovery of antioxidants from two different types of OMWC was evaluated.

2. LITERATURE REVIEW

2.1. Olive Oil Production and Waste Management

2.1.1. Olive Oil Extraction Systems and OMWW Production

Olive oil production is carried out with different processes, typically feeding of olive fruit, leaf removal, olive washing, oil crushing, and malaxation, and oil extraction. The physical processes are crushing of olive and malaxation, and extraction of oil that is separation of the oily phase from residual water by the application of pressure or centrifugation are used in order to procure olive oil from olive fruit, respectively. Two main extraction systems used in olive processing are traditional batch (press) and modern continuous processes, which involve two-phase or three-phase centrifugal systems (decanter) based on the separation method of oil. (Azbar et al., 2004; Takaç and Karakaya, 2009; Souilem et al., 2017).

Traditional pressing is ancient olive oil production system; however, it is still favorable in some mills (Souilem et al., 2017) (Figure 2.1).

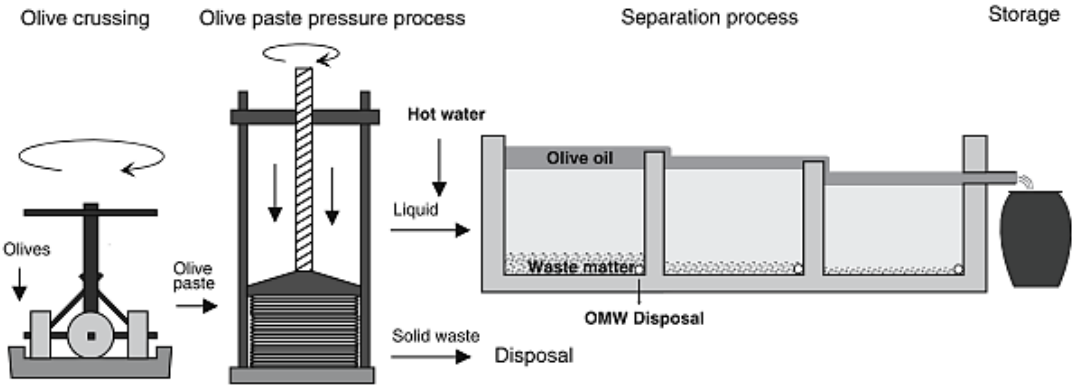


Figure 2.1. Batch (press) oil extraction system (Kapellakis et al., 2008).

The process begins by washing of olive fruit with cold water. After crushing olive fruit with using stone milling, the olive paste is lay out on natural fiber disks placed into the press to extract oily liquid phase (residual oil and vegetation water) by compaction. Warm water, added from the sides of the disks, is used for effective distillation of oil (Kapellakis et al., 2008). A solid waste called as olive husk or kernel is produced as a by-product throughout oil production process. Extraction process ends up with separation of the olive oil from added water as well as vegetation water. Olive

husk can be used as additive matter for soap industry or burned for energy utilization (Azbar et al., 2004).

Although traditional pressing produces olive oil in high quality, olive processing capacity is restricted. By the reasons for the demand to process huge amounts of olives, to achieve higher yields of olive oil, and to reduce labour, traditional press system is replaced with the modern continuous centrifugal extraction system (Souilem et al., 2017) shown in Figure 2.2.

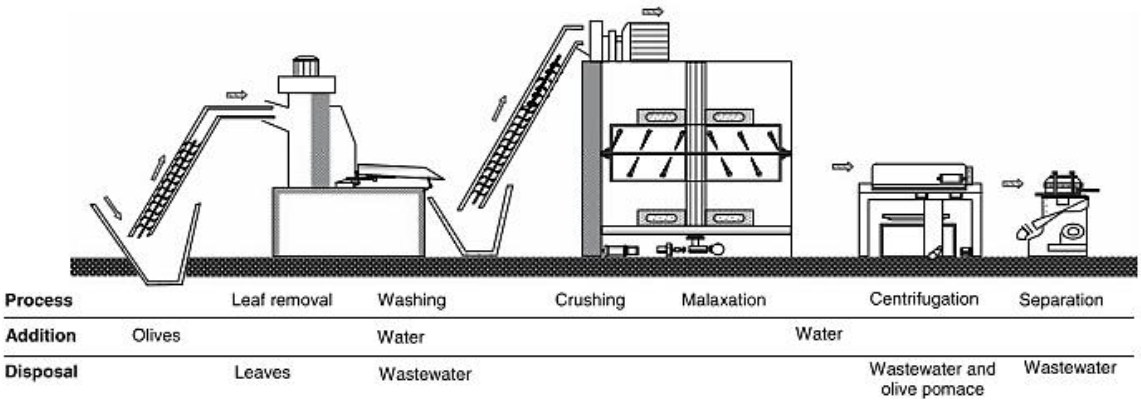


Figure 2.2. Modern continuous centrifugal oil extraction system (Kapellakis et al., 2008).

In the continuous centrifugal extraction system, after washing, crushing, and malaxation stages, continuous separation of the oil phase is accomplished by horizontal centrifuge (decanter), consisting of a conical-cylindrical bowl. Horizontal centrifuge enables continuous and automatic operation. The continuous centrifugal processes are currently widely used production systems due to higher modernity and productivity. Although continuous centrifugal extraction process has various advantageous e.g., elimination of contamination, higher oil production with high quality, improved process control, decrease labour costs, and the need smaller area, the consumption of water and energy, generation of higher wastewater which contains value-added compounds are the drawbacks of this process (Kapellakis et al., 2008; Dermeche et al.,2013; Ochando-Pulido et al., 2016; Souilem et al., 2017). Continuous oil extraction processes are classified as two-phase and three-phase centrifugation systems which depend on the density differences of olive paste components (e.g., oil, water, and insoluble solids) as well as water requirement described in Figure 2.3 (Alburquerque et al., 2004; Dermeche et al., 2013; Jiménez-Herrera et al., 2017 Souilem et al., 2017).

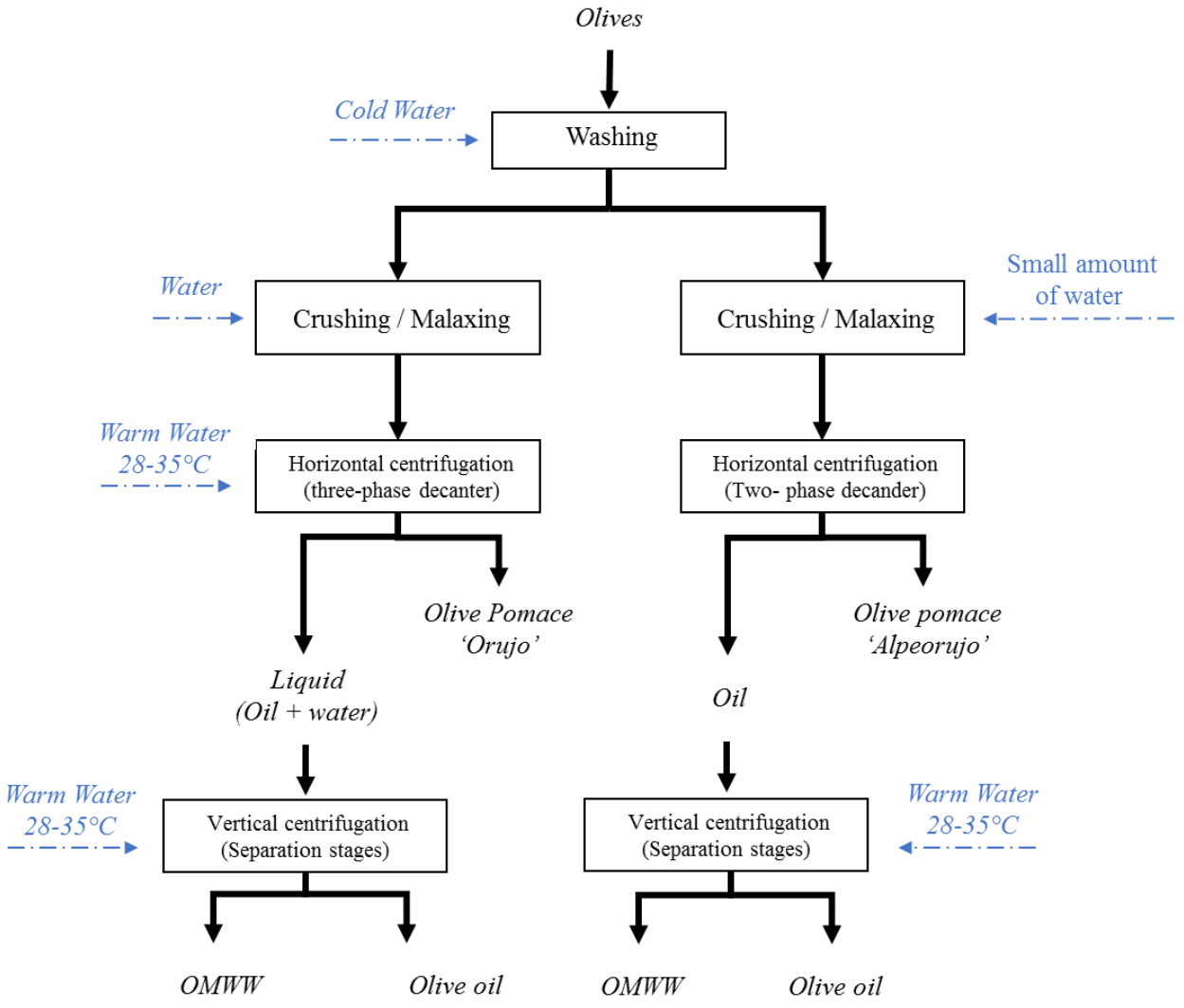


Figure 2.3. Comparison of the three and two-phase centrifugation systems for olive oil extraction.

In two-phase systems, the olive paste is divided into two-phases (olive oil and semi-solid or wet pomace) (Vlyssides et al., 2004). The added amount of water during crushing and malaxation stages is not much and depends on the vegetation water content of olive fruit (Dermeche et al., 2013). Since wastewater production together with water and energy requirements can be reduced, two-phase system is accepted as "ecological". In comparison to the three-phase system, two-phase continuous system has potential for 75% OMW reduction, 80%, water and 20% energy savings (Azbar et al., 2004). Although two-phase system minimizes water consumption and wastewater production, the management of two-phase OMW, called "*alpeorujo*", has a great challenge owing to higher moisture content (about 60%) since drying process requires high energy that increases operational cost (Alburquerque et al., 2004; Azbar et al., 2004; Roig et al., 2006).

OMWW produced from three-phase extraction and traditional pressing systems, include both vegetable water and process water added at different stages of extraction process (Roig et al., 2006).

The estimated annual OMWW from olive oil industry is 7 to over 30 million m^3 (Dermeche et al., 2013). This wastewater is generated only in short period of the year (November–February). The amount of OMWW in the olive oil industry ranged from 0.5 to 1.5 m^3/ton of olives depending on the extraction processes. While traditional press process produces 0.5- 1m^3/ton wastewater, modern three-phase and two-phase centrifugation processes yield 1- 1.5m^3/ton and ~ 0.2m^3/ ton wastewater (Alburquerque et al., 2004; Paraskeva and Diamadopoulos, 2006). However, the OMWW of tradition process is considerably concentrated.

2.1.2. General Composition and Characteristics of OMWW

Typical OMWW consists of 83-94% water, 4-16% organic compounds, and 0.4-2.5% mineral salts by weight. The organic fraction contains oil (l-14%), proteins (8-16%), polysaccharides (13-53%), organic acids (3-10%), polyalcohols (3-10%) and polyphenols (2-15%) (Ramos-Cormenzana, 1986; Cabrera et al., 1996; Davies et al., 2004).

OMWW generally possess poor biodegradability and high phytotoxicity owing to content of non-biodegradable organic compounds (e.g., long-chain fatty acids) and phenols (Azbar et al., 2004; Takaç and Karakaya, 2009; Paraskeva and Diamadopoulos, 2006; Ochando-Pulido et al., 2016; Souilem et al., 2017). OMWW typically characterized by acidic nature pH 3-6, strong spesific oil odour, intensive dark brown-blackish color caused by recalcitrant compounds such as lignins, tannins and PCs, extremely high organic load (COD: 40-220 g/L, BOD_5: 35-110 g/L and TOC: 25-45 g/L), solid matter (TSS: 1-9 g/L), high content of phenols (0.5–24 g/L), and high electrical conductivity. The characterization of OMWW was examined in various studies published in the literature and these are listed in Table 2.1.

Table 2.1. The composition of OMWW in several studies.

Source	pH	TP, g/L	COD, g/L	Protein	Carbohydrate	TS, g/L	TSS	TDS, g/L	Lipid	References
OMWW	5	8.6	53.3	-	-	39.4	-	-	-	Mekki et al., 2006
OMWW	5.24	0.70	17.34	-	-	-	11.47 g/L	-	-	Paraskeva et al., 2007
OMWW1	4.54	4.84	48.91	-	-	-	-	-	-	Scoma et al., 2011
OMWW2	4.54	2.63	55.27	-	-	-	-	-	-	
OMWW1	5.2	9.82	113	-	-	-	87 g/L	-	-	El-Abbassi et al., 2012
OMWW2	5.1	6.11	51	-	-	-	48 g/L	-	-	
OMWW	5	1.41	-	-	-		29 g/kg	-	-	Cassano et al., 2013
OMWW*	5.8	5	90	-	-	0.5	-	-	-	Kalogerakis et al., 2013
OMWW	4.91	2.27	58.62	-	-	69.84	-	26.35	1.14	Ayoub et al., 2014
OMWW	4.6	2	31.5	3 g/L	23 g/L	34	0.7 g/L	33.3	<0.1	Frascari et al., 2016
OMWW1	4.6	1.6	32	-	5.4 g/L	34	33 g/L	1	-	Pinelli et al., 2016
OMWW2	4.6	0.51	21	-	23 g/L	13	5 g/L	8	-	
OMWW3	4.9	0.80	32	-	16.2 g/L	24	-	-	-	
OMWW1	5.03	-	-	4.55 %	-	-	8.43 g/L	-	10.83 g/L	Sellami et al., 2016
OMWW2	4.75	-	-	3.74 %	-	-	7.20 g/L	-	10.71 g/L	
OMWC1	6.5		-	153.12 mg/g	57.38 mg/g	-	-	-	0.78 mg/g	Çelik, 2018
OMWC2	5.2	-	-	87.23 mg/g	129.82 mg/g	-	-	-	87.91mg/g	
OMWW	4.24	4.4	70	0.15 g/L	-	41	3.5 g/L	38	-	Frascari et al., 2018
OOWW	5.1-5.5	0.75-0.78	13.39-16.06	-	-	-	3.4-3.46 g/L	-	-	Ochando-Pulido, et al., 2020

[TP: Total Phenol, COD: Chemical Oxygen Demand, TS: Total Solid, TSS: Total Suspended Solid, TDS: Total Dissolved Solid, OOWW: Olive Oil Washing Water].
* centrifugated and filtered raw sample by 0.45 µm filter, (%): g/100g of dry matter.

Physicochemical characteristics of OMWW vary widely depending upon various factors including the quality, maturity, and type of cultivated olives, cultivation method, region of origin, soil composition, climatic conditions, and extraction methods. In Table 2.2, the physicochemical characteristics of OMWW in terms of extraction are summarized (Ochando-Pulido et al., 2016).

Table 2.2. Characteristics of OMWW produced from batch and continuous olive oil extraction processes (Ochando-Pulido et al., 2016).

Process	**COD, g/L**	**BOD_5, g/L**	**TSS, g/L**	**pH**	**EC, mS/cm**	**TP, g/L**
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_5: Biological oxygen demand, TSS: Total suspended solid, EC: Electrical conductivity, TP: Total phenols].

2.1.3. Environmental Effects of OMW

The nature of OMW causes noticeable adverse effects for all compartments of environment. This limits the discharging of OMW directly into receiving environmental systems as well as domestic wastewater collection systems (Saadi et al., 2007; Souilem et al., 2017).

2.1.3.1. The effects of OMW on atmosphere. Storage of OMWW in lagoons for natural evaporation is one of the currently used method for its disposal in olive oil-producing countries (Paraskeva and Diamadopoulos, 2006). However, the floating of oily compounds found in OMWW creates layer on the surface of the lagoon that leads to the development of anaerobic conditions and odorous compounds (e.g., phenols, sulphur dioxide, and hydrogen sulphide) release to atmosphere especially during summer season (Lagoudianaki et al., 2003; Azbar et al., 2004; Souilem et al., 2017). Consequently, this management practice of OMWW as well as the storage of OM solid waste could be responsible for the contamination of atmosphere.

2.1.3.2. The effects of OMW on soil. The controlled land application of OMW is one of the disposal strategies since the effluent can be considered as a natural fertilizer due to high nutrient (such as N, P, K, Mg and Fe) and organic matter contents (Di Bene et al., 2013; Ayoub et al., 2014). However, the application of OMW may adversely affect physical and chemical properties of soils causing the alteration of environmental conditions for the activity of biota due to the presence of toxic compounds (Tsagaraki et al., 2007; Souilem et al., 2017).

2.1.3.3. The effects of OMW on water supply. Directly disposal of untreated OMWW is strictly forbidden in most countries due to several undesirable impacts on water bodies (ground water and surface water) and aquatic life. High nutrient content of OMWW leads to eutrophication as well as the growth of pathogens hence damaging of ecological balance in natural water systems can be expected. Moreover, the presence of recalcitrant compounds (e.g., tannins and PCs) which give the dark brown color to OMWW, does not only lead to color alteration but also accumulation of them causing nuisance conditions (Paraskeva and Diamadopoulos, 2006; Tsagaraki et al., 2007; Souilem et al., 2017).

2.1.4. OMWW Treatment Methods

Environmental problems related to OMWW encountered in Mediterranean countries due to both its complex nature and the large volume made the treatment of this waste compulsory prior to disposal. In this regard, various investigations (Table 2.3) on the potential applications of physicochemical, biological, and thermal as well as combined processes have been carried out to reduce organic components of OMWW and find out efficient and environmentally safe treatment methods (Paraskeva and Diamadopoulos, 2006; Takaç and Karakaya, 2009; Dermeche et al., 2013).

Table 2.3. The treatment methods of OMWW (Souilem et al., 2017).

Category	Method	Achieved Results	References
Physical	Sedimentation, Filtration, Flotation and Centrifugation	70% COD removal, 30% oil recovery	Velioglu et al., 1987; Georgacakis and Dalis, 1993
	Micro-, Ultra-, Nano-Filtration and Reverse Osmosis	99% COD removal, but membrane fouling	Paraskeva et al., 2007; Russo 2007; Stoller and Angelo, 2006; Turano et al., 2002
	Evaporation		Masi et al., 2015
	Sedimentation		De Martino et al., 2011
	Centrifugation/ ultrafiltration		Turano et al., 2002
	Adsorption/desorption		Zagklis et al., 2015
Thermal	Evaporation, distillation	20–80% COD removal but needed further treatment	Rozzi and Malpei, 1996; Tsagaraki et al., 2007
	Combustion, pyrolysis	Toxic gases generation and high operational cost	Rozzi and Malpei, 1996; Caputo et al., 2003
	Solar Distillation		Potoglou et al., 2004; Paraskeva and Diamadopoulos, 2006
Biological	Anaerobic Processes	60-80% COD removal in 3-5 digestion days, while 90% COD removal in 25 digestion days	Dalis et al., 1996; Azbar et al., 2009; Azbar et al., 2008b
	Aerobic Processes	5–75% COD removal for few days of digestion; while 80% COD for more days	El Hajjouji et al., 2007; Velioglu et al., 1992
	Mixing and Digestion	75-90 % COD removal	Azbar et al., 2008a
	Enzymatic		

Table 2.3. Treatment technologies of OMWW (cont.).

Category	Method	Achieved Results	References
Physicochemical	Neutralization, precipitation, adsorption	30-50% COD removal individually, and 80-95% COD removal by combining precipitation with adsorption.	Kestioğlu et al., 2005; Adhoum and Monser, 2004; Sarika et al., 2005
	Oxidation and Advanced Oxidation	40-60% and 85% COD removal by simple oxidation and combined processes, respectively.	Javier Benitez et al., 2009; Chatzisymeon et al., 2009a; Chatzisymeon et al., 2009b
	Chemical oxidation	86% COD removal	Nieto et al., 2010
	Wet H_2O_2 Photocatalytic Oxidation		Azabou et al., 2010
	Electro-Fenton		Kaplan et al., 2011; Kilic et al., 2013
	Ozonation		Siorou et al., 2015
	Lime Treatment		Aktas et al., 2001
	Electrocoagulation		Hanafi et al., 2010
	Cloud Point Extraction (CPE)		Gortzi et al., 2007
	Combined		Gursoy-Haksevenler et al., 2014
Combined	Oxidation and Biological Processes	75% total PCs and 80-99% COD removal	Bressan et al., 2004

2.1.5. Olive Oil Sector and OMWW Management in Turkey

Olive sector concerning olive oil production has an importance on the economy of Turkey, who is one of the top four olive oil producer countries with 55,000 tons for olive oil export in 2018/19 season. The olive oil production in Turkey over crop years that cover the period from 1 October to 30 September for olive oil is shown in Figure 2.4. (International Olive Oil Council, 2019).

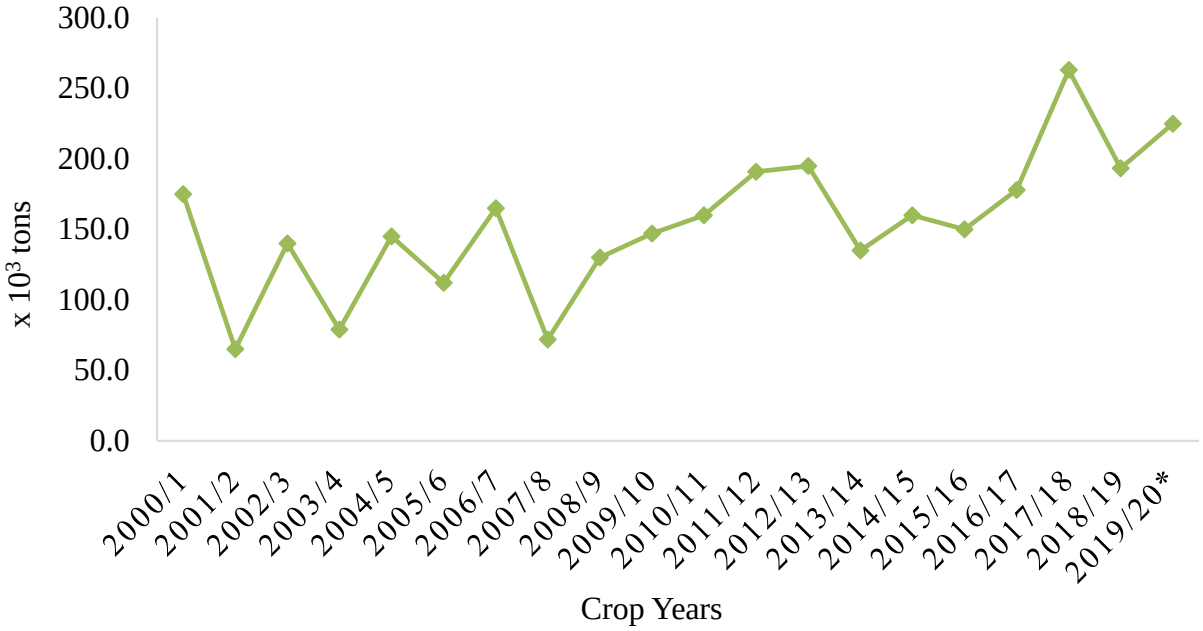


Figure 2.4. Olive oil production in Turkey over crop years (*estimated value).

Olive cultivated areas are a major agricultural land in Turkey, covering 8 million 792 decares in 2019 and total 1 million 525 tons olive was produced (Tarım ve Orman Bakanlığı, 2020). The main regions of olive cultivation are Aegean and Marmara by providing 35.8% and 16.1% of total olive production, respectively. It was reported that 75% of total cultivated olive was used for oil production (Tarım ve Orman Bakanlığı, 2020) and as a shown in Figure 2.5, Turkey was near the top of world olive oil producer list (International Olive Oil Council, 2019).

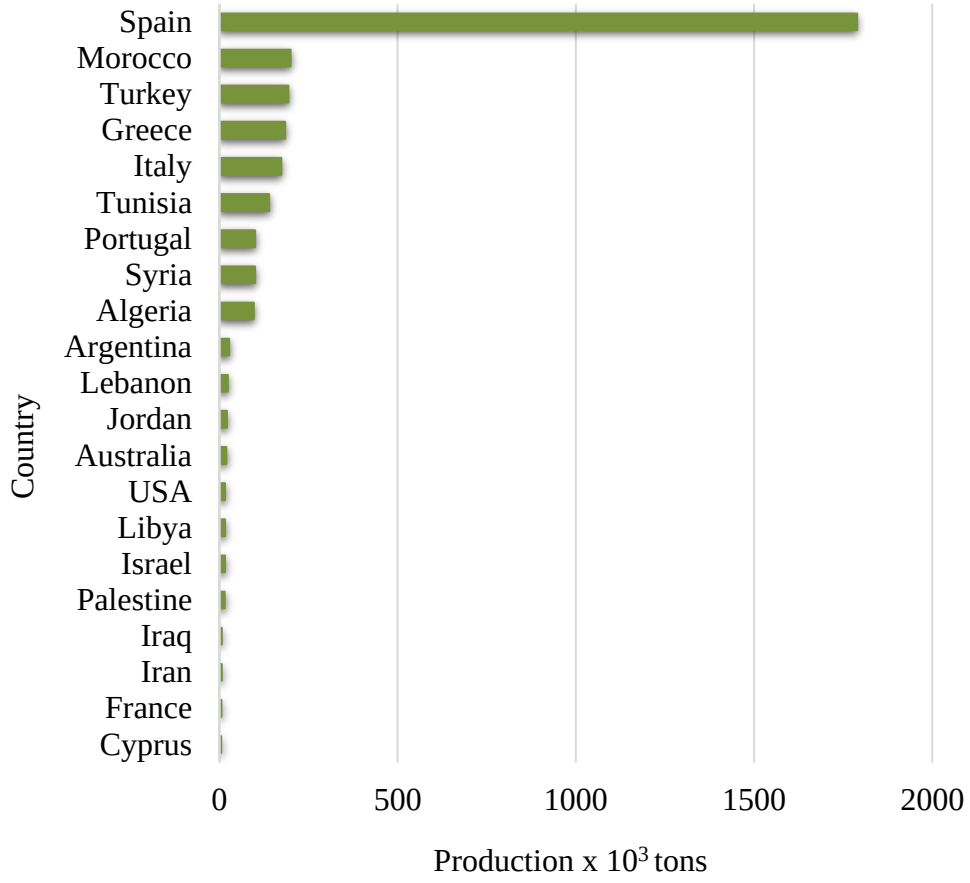


Figure 2.5. Olive oil world production in 2018/19.

In Turkey, according to 2015 data, 71% of olive oil facilities use the three-phase centrifugal system for oil extraction while 27% and 2% of those use two-phase centrifugal and traditional press systems, respectively (TUBITAK- MAM, 2015).

It was reported that about 775,000 m^3 freshwater has been used in the olive oil production and total 923,000 m^3 wastewater with 70,000 tons COD load has been produced in a season. Significant amount (89%) of OMWW is treated by evaporation ponds, while the remaining part (11%) is discharged into sewage systems. Considering the amount of OMWW wastewater in lagoons serious environmental problems are unavoidable (TUBITAK- MAM, 2015). In Turkey, direct discharge of wastewater into sewage systems is limited by water pollution control regulation. In and into receiving bodies COD, Oil & Grease, and pH of wastewater are limited with the values of 230 mg/L, 40 mg/L, and 6-9, respectively (Çevre ve Orman Bakanlığı, 2004).

Recent economic evaluation of olive oil sector's technology in Turkey revealed the possibility of switching from three-phase to two-phase olive oil production and some of olive oil production plants have started to use two-phase system. With this approach, it is estimated that used freshwater, OMWW and COD load will decrease in ratio of 60%, 80%, and 99%, respectively (TUBITAK-MAM, 2015).

2.2. Natural Phenolic Antioxidants

Phenols which possess at least one substituted hydroxyl groups (polar part) and aromatic ring (non-polar part), are found in the body of plants as a simple and complex organic compound (Dermeche et al., 2013; Galanakis et al., 2013; Rahmanian et al., 2014).

Phenols in the olive fruit or formed during olive oil extraction process have an ability to capture free radicals with strong antioxidant activities (Rodis et al., 2002; Takaç and Karakaya, 2009; Kaleh and Geißen, 2016). In addition, olive phenols have a potential for preventing chronic human diseases such as cardiovascular and inflammatory diseases, and pathological diseases e.g., cancer and atherosclerosis (Bedouhene, 2014).

Although the olive fruit is rich in PCs, only 1-2% of the amount of biphenols found in olive oil, and the remaining portion is lost with the OMW depending on the extraction process. The partitioning of biophenolic compounds between liquid and solid phases of OMW depends on their solubilities. Since olive phenols has low partition coefficients (oil/water) ranging from 6×10^{-4} to 1.5, biophenolic antioxidants favours the aqueous phase (OMWW) in which about 53% of total antioxidants release, and about 45% of total PCs remains in the solid phase (pomace). The partitioning is significantly affected by temperature and the amount of water used in the extraction process. While the higher temperature can enhance the release of biophenols into the oil, the high quantity of process water causes biophenols to be lost in wastewater. In OMWW, PCs with wide array of biological activities are found in concentration range of 0.5-24 g/L. Therefore, OMWW can be considered as natural antioxidant source (Rodis et al., 2002; Obied et al., 2005a; Paraskeva and Diamadopoulos, 2006; De Marco et al., 2007).

In general, olive fruit includes mainly phenolic acids (e.g., caffeic, *p*-coumaric, gallic, syringic, and vanillic), alcohols (e.g., tyrosol (Tyr) and hydroxytyrosol (HTyr)), secoiridoids (oleuropein), flavonoids, and lignans. More than 50 and 40 different PCs have been found in OMWW and olive

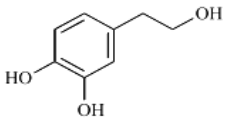
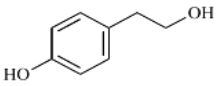
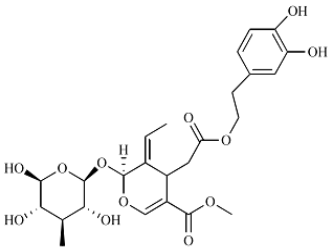
oil, respectively (Ramos-Cormenzana, 1986; Bianco et al., 2003; Obied et al., 2007; Dermeche et al., 2013; Rahmanian et al., 2014; Kaleh and Geißen, 2016; Galanakis and Kotsiou, 2017). PCs of OMWW can be classified as low-molecular weight (e.g., caffeic acid, Tyr, HTyr, *p*-cumaric acid, ferulic acid, syringic acid, protocatechuic acid) and high molecular weight compounds (e.g., tannins and anthocianins) (Ramos-Cormenzana, 1986). Actually, the presence of specific phenols in OMWW relies on quality and maturity of cultivated olives, climatic conditions, and soil composition as well as extraction techniques (Obied et al., 2005a)

Several studies investigated the phenolic composition of OMWW in last decade (De Marco et al., 2007; Leouifoudi et al., 2014; Kelebek et al., 2015; Kaleh and Geißen, 2016; Cabrera-Bañegil et al., 2017). The studies revealed that HTyr, Tyr, and oleuropein are the most abundant phenolics among more than 50 PCs found in OMWW (De Marco et al., 2007; Rahmanian et al., 2014; Galanakis and Kotsiou, 2017). The structure of these PCs with properties are listed in Table 2.4 (Obied et al.,2005a; Takaç and Karakaya, 2009; Galanakis et al., 2013; Kaleh and Geißen, 2016).

Phenolic constituents of OMW with ortho-diphenolic (catecholic) structure have antioxidant activity. Compare to oleuropein and Tyr, HTyr has superior antioxidant and radical scavenger activities (Takaç and Karakaya, 2009; Tuck and Hayball, 2002). While HTyr exists in olive fruit, it can also be generated during extraction processes by the hydrolysis of oleuropein. Depending on the maturity of olive fruit, the concentration of HTyr increases whereas oleuropein decreases (Obied et al., 2007; Tuck and Hayball, 2002). Due to possessing antibacterial, anti-inflammatory and anti-angiogenic properties HTyr is potential target for the food and pharmaceutical industries (Bedouhene, 2014; Rahmanian et al., 2014; Galanakis and Kotsiou, 2017). For the synthesis of HTyr, chemical and enzymatic processes are used but they are slow and require high cost. Therefore, the recovery of natural antioxidants from waste materials is important.

Concerning the advanced antioxidant and health-beneficial properties, and bioavailability, the recovery of PCs, particularly HTyr from OMWW is beneficial, economical, and sustainable approach and this antioxidant can be utilized in cosmetic, pharmaceutical and food industries. Consequently, the re-utilization of natural polyphenols in industrial scale has a potential to reduce the pollution load of OMWW (De Marco et al., 2007).

Table 2.4. Structure, properties, and bioactivity of the main PCs found in OMWW.

Biophenols	Chemical Structure	Properties	Bioactivity
Hydroxytyrosol		Synonyms: 3-Hydroxytyrosol, 3,4dihydroxyphenylethanol Molecular formula: $C_8H_{10}O_3$ Molecular weigth: 154.16 g/mole log K_{ow}: 0.96 pKa: 9.5 K_p (at 25°C): $4x10^{-4}$	Antioxidant, cardioprotective and antiatherogenic, chemopreventive, antimicrobial, anti inflammatory, skin bleaching.
Tyrosol		Synonyms: 4-(2-Hydroxyethyl)phenol , *p*-Hydroxyphenethyl alcohol, 4-Hydroxyphenylethanol Molecular formula: $C_8H_{10}O_2$ Molecular weigth: 138.164 g/mole log K_{ow}: 1.35 pKa: 10 K_p (at 25°C):: $9.7x10^{-2}$	Antioxidant, anti-inflammatory, antiatherogenic, cardioactive.
Oleuropein		Molecular formula: $C_{25}H_{32}O_{13}$ Molecular weigth: 540.514 g/mole log K_{ow}: -0.11 pKa: 9.7 K_p (at 25°C): $1.2x10^{-3}$	Antioxidant, cardioprotective and antiatherogenic, hypoglycemic, antihypertensive, antimicrobial and antiviral, anti-inflammatory, cytostatic, molluscicidal, endocrinal activity, enzyme modulation.

[K_p: Predicted partition coefficient oil/water, K_{ow}: Partition coefficient octonol/water].

2.3. Recovery Techniques for Natural Phenolic Antioxidants

The recovery and purification of value-added components of OMWW have gain importance recently. Since the valorization of waste instead of treatment is essential in the sustainable management of olive oil industry (Ferri et al., 2011; Gómez-Caravaca et al., 2017; Souilem et al., 2017).

In literature studies, the great efforts have been spent to improve the recovery of PCs from OMWW by using separation processes individually or their combinations such as membrane filtration, centrifugation, chromatographic procedures, and extraction (Takaç and Karakaya, 2009). Among the recovery techniques, extraction methods can provide selective separation of PCs from phenol-rich sources while decrease the coextraction of matrix components (Obied et al., 2005a).

The integration of liquid-liquid extraction with solid phase separation can be suggested a sustainable approach to the recovery and purification of PCs (Galván D'Alessandro et al., 2013). The main purpose of this combination is to obtain high quality product, and to improve recovery yield, hence the process would be economic by increasing the value of recovered product and reducing energy consumption of the equipment and process time (Schügerl and Hubbuch, 2005; Hu et al., 2014; Pradal et al., 2018).

2.3.1. Liquid- Liquid Extraction

Liquid-liquid or solvent extraction is widely used simple alternative method for the recovery of PCs at both pilot and commercial scale (Allouche et al., 2004). Physical partitioning of low and high molecular weight PCs between two different solvents depends upon their dissolution rates. Type of solvent as well as he extraction temperature and time strongly affects the performance of extraction (Obied et al., 2005a; Galanakis et al., 2013; Galanakis et al., 2017).

PCs are partly polar compounds and favor polar solvents (Obied et al., 2007; Galanakis et al., 2017). The polarity (hydrophilicity) of these compounds that is described by the octanol/water partition coefficient (K_{ow}) generally depends upon the number of OH^- functional groups in their structure (Yangui et al., 20117). However, the K_{ow} values of phenols plant origin does not only upon the number of OH^- functional groups as deduced from Table 2.5.

Table 2.5. Polarity and characteristic groups of phenols (Galanakis et al., 2013).

Phenols	**Log K_{ow}**	**-OH groups**	**–COOH groups**	**–OCH_3 groups**
Hydroxycinnamic acids				
Cinnamic acid	1.98	0	1	0
p-Coumaric acid	1.54	1	1	0
Caffeic acid	1.15	2	1	0
Ferulic acid	1.42	1	1	1
Sinapic acid	1.29	1	1	2
Rosmarinic acid	2.07	4	2	0
Hydroxybenzoic acids				
p-Hydroxybenzoic acid	2.27	1	1	0
Protocatechuic acid	0.82	2	1	0
Gallic acid	0.47	3	1	0
Vanillic acid	1.35	1	1	1
Syringic acid	0.95	1	1	2
Hydroxyphenylacetic acids				
p-Hydroxyphenylacetic acids	1.15	1	1	0
Tyrosol	1.35	1	1	0
Hydroxytyrosol	0.96	2	1	0
Oleuropein	-0.11	2	2	2

A wide range of organic solvents such as water, ethanol, methanol, ethyl acetate, n-butanol, diethyl ether, n-propanol, and tert-butyl methyl ether, acetone, hexane, acetonitrile, dichloromethane, methyl isobutyl ketone, methyl ethyl ketone, and hydro-alcoholic solutions have been investigated for the extraction of PCs from OMW (Allouche et al., 2004; Obied et al., 2005a; Takaç and Karakaya, 2009; Rahmanian et al., 2014). It has been noted that the performance of PC extraction increases with increasing polarity of solvents (Allouche et al., 2004). While low and medium molecular weight PCs and phenolic acids can be recovered with diethyl ether and ethyl acetate, high molecular weight PCs can be effectively extracted by using more polar solvents (e.g., hydro-alcoholic solutions). Accordingly, Visioli et al. (1999) showed the selectivity of ethyl acetate for low and medium molecular weight PCs in comparison to high molecular weight PCs that remain in the water phase. As a shown in Table 2.6, ethyl acetate extraction was commonly used and provides high recovery of particularly HTyr, and Tyr from an acidified OMWW (Lesage-Meessen et al., 2001; De Leonardis et al., 2007; De Marco et al., 2007).

Table 2.6. Solvent extraction of PCs from OMW by various type of solvents and achieved results in several studies.

Waste	Extracted Component	Solvent	Achieved Results	Reference
OMWW[1] OMWW[2]	PCs	Ethyl acetate	Vanillic acid, caffeic acid, HTyr Tyr, dialdehydic form of decarboxymethyl oleuropein aglycon, ligstroside, verbascoside, luteolin, luteolin-7-glucoside in extract. 2.5 g Tyr /L in the extract from an acidified OMWW[2] 1.1 g Tyr /L in the extract from a crude OMWW[1].	De Marco et al., 2007
Raw OMWW	HTyr	Ethyl acetate	85.46% HTyr recovery, 1.225 g HTyr /L.	Allouche et al., 2004
OMW[3]	PCs	Aqueous ethanol (30% v/v) Ethyl acetate Aqueous ethanol (50% v/v) Aqueous propanol (50% v/v) Aqueous acetonitrile (50% v/v) Aqueous acetone (50% v/v)	20.3 mg GAE/g 9.1 mg GAE/g 32.9 ± 0.9 mg GAE/g 42.6 ± 1.1g mg GAE/g 41.4 ± 1.3 mg GAE/g 35.2 ± 0.8 mg GAE/g PCs recovery.	Obied et al., 2005b
Raw OMWW	PCs	Ethyl acetate	1.225 g HTyr /L, 0.345 g Tyr /L, 0.256 g Caffeic acid/L, 0.07 g Ferulic acid/L in OMWW extract.	Bouaziz et al., 2008
OMWW[4]	PCs	Ethyl acetate	2 g/L total PCs in extract, 50 mg PCs/100 g of OMWW yield, 0.34 kg/m^3 HTyr and 0.083 kg/m^3 Tyr recovered from OMWW.	De Leonardis et al., 2007
Raw OMWW Olive pomace	PCs	Ethanol	3.5% and 0.65% of PCs extracted from the solid fraction of OMWW and olive pomace.	Tercan and Şeker, 2012

[1: pre-treated wastewater by n-hexane, 2: acidified and pre-treated wastewater by n-hexane, 3: Prolonged freeze-drying of the waste, 4: boiled and pre-treated wastewater by n-hexane].

Table 2.6. Solvent extraction of PCs from OMW by various type of solvents and achieved results in several studies (cont.).

Waste	Extracted Components	Solvent	Achieved Results	Reference
Raw OMWW	PCs	Ethanol Ethanol/diethyl ether (1:2 v/v)	0.43 g GAE/L and 0.292 g GAE/L total PCs from OMWW.	Venturi et al., 2017
OMWW1[5] OMWW2[5]	PCs	Ethyl acetate	2.48 g PCs/L crude OMWW1 and 3.69 g PCs/L from acidified OMWW1, 0.56 g PCs/L from crude OMWW2 and 0.98 g/L from acidified OMWW2.	Sellami et al., 2016
OMWW[6]	PCs	Ethyl acetate	0.148 mg GAE/mg from OMWW extract.	Bedouhene et al., 2014
OMWW[7]	PCs	Ethyl acetate	5.27-10.1 g GAE/L total PCs from extract.	Leouifoudi et al., 2014
Raw OMWW	HTyr, Tyr, PCs	Ethyl acetate Diethyl ether Chloroform/ isopropyl alcohol (7:3 v/v)	0.247 kg HTyr, 0.062 kg Tyr and 3.44 kg total PCs 0.103 kg HTyr, 0.042 kg Tyr, 2.8 total PCs 0.166 kg HTyr, 0.047 kg Tyr, 3.38 total PCs from 1 m^3 OMWW.	Kalogerakis et al., 2013
OMW	PCs	Ethyl acetate	36% total PCs recovery after post-coagulation–flocculation. 0.277 kg/m^3 HTyr and 0.058 kg/m^3 Tyr recovered from OMWW.	Papaphilippou et al., 2013
OMWC1[8] OMWC2[8]	HTyr	Ethanol	6.6 g HTyr/kg from OMWC1 and 4.6 g HTyr/kg from OMWC2.	Çelik, 2018

[5,7: pre-treated by hexane, 6,8: acidified by HCl and pre-treated by hexane].

On the other hand, a mixture of methanol or ethanol/water with varying alcohol proportions has been investigated as alternative of forementioned solvents for extraction of total PCs from olive mill pomace, and water/methanol mixture was found more suitable solvent than ethyl acetate, ethanol, propanol, acetone, acetonitrile for the extraction of wide range of PCs (Obied et al., 2005a).

The solubility of total PCs generally better in polar solvent (e.g., ethanol and methanol), whereas phenolic acids (e.g., gallic, cinnamic, and coumaric acids) favour in water, dichloromethane, and acetone, respectively. Particularly, HTyr and Tyr, most abundant PCs found in OMWW, are more solubilized in water, alcoholic solvents such as ethanol and methanol, followed by acetone and ethyl acetate; however, the solubility of these PCs is low in diethyl ether and dichloromethane (Galanakis et al., 2013).

Most of the organic solvents are undesirable for industrial application due to the toxic and nonedible properties that cause to increase health, environmental, and safety problems (Galanakis et al., 2017). The environmental impact of antioxidant extraction with different types of employed solvents was investigated by Kalogerakis et al. (2013), and it was concluded that organic solvents threats to the human health and the environment in terms of the emission into atmosphere and the diffusion into the aqueous environment with global warming potential. In Fact, ethanol and hydroethanolic mixtures have been selected as the most suitable solvents in recent studies for the extraction of PCs from OMW since these organic solvents are non-toxic, cheap, and reusable. Also, the final extracts/ products can be directly used in the food and pharmaceutical industry due to the food grade nature, non- toxicity, and low-cost (Tsakona et al., 2012; Rahmanian et al., 2014).

In addition to the type of solvent, Galanakis et al. (2013) also studied the effect of temperature on performance of extraction by using seven different solvents (water, ethanol, methanol, acetone, dichloromethane, ethyl acetate, and diethyl ether). The results of the study performed at three different temperatures revealed activity coefficients of PCs in employed solvents increase with the increment of temperature from 298.15 to 333.15 K.

2.3.1.1. Liquid- Liquid extraction with Aqueous Two-Phase alcohol/salt system. ATPE is novel type of liquid–liquid extraction technique and gained great attention recently by providing preliminary purification of the value-added compounds to be extracted (Wu et al., 2014; Caldeira et al., 2019). Besides, ATPE has several advantages compared to conventional technique e.g., reduction of

separation steps and achieving higher efficiency hence reducing the overall cost of operation (Reis et al., 2014; Oliveira et al., 2018).

Among different ATPE systems, hydrophilic organic solvent (short-chain alcohols e.g., methanol, ethanol, 1-propanol, and 2-propanol) and inorganic salt (e.g., ammonium sulphate, potassium phosphate and sodium citrate) based systems provide advantages since low viscosity of phases enables their rapid separation and reduces energy consumption. (Gündüz, 2000; Reis et al., 2014; Tan et al., 2014). Additionally, target compounds can be recovered in alcohol-rich phase in which alcohol is easily separated and can be recycled besides the recycling of the salt in ATPE system (Ooi et al., 2009; Wang et al., 2010; Caldeira et al., 2019).

Recently, the application of this ATPE has been investigated for extraction and purification of natural compounds, such as rutin (Reis et al., 2014), anthocyanins (Liu et al., 2013a; Wu et al., 2014), lipase (Ooi et al., 2009), ascorbic acid (Reis et al., 2012), fucoxanthin (Gómez-Loredo et al., 2014), PCs (Xavier et al., 2017; Çelik, 2018), anthraquinones derivatives (Tan et a., 2012), flavones and sugars (Liu et al., 2013b) with a single-step procedure.

In ATPE system, phase separation is achieved by the addition of salts that exhibit low solubility in alcohol and leading them "salting-out" (Albertsson, 1986). Water molecules partition from alcohol-rich top phase to salt- rich bottom phase and simultaneously alcohol molecules with target compounds partition into the top phase.

Type and concentration of both alcohol and salt, pH value, temperature are the system properties that affect phase-separation mechanism (Rosa et al., 2010; Yuan et al., 2011; Tan et al., 2012). In the study of Wang et al. (2010) found that the increment of alcohol and salt concentration, and the decrement of pH value (from 9 to 3.5) exerted positive effect on the separation performance of aqueous two- phase system.

Recent studies shows that ethanol–ammonium sulphate is a commonly used and system among different types of ATPE systems (Liu et al., 2013a). The performance of ATPE system exhibited variations depending upon the target polyphenolic compound and matrix subjected to extraction. For the extraction of anthocyanins from grape juice by using ethanol/salt based ATPE, ethanol/(NaH_2PO_4) system yield higher recovery of target compound than that achieved with

ethanol/$(NH_4)_2SO_4$ system (Wu et al., 2014). This result was attributed to the more acidic condition created with NaH_2PO_4 (pH~ 3.7) compared to that achieved with $(NH_4)_2SO_4$ (pH~ 5.2).

In another study (Tan et al., 2012), chlorogenic acid was extracted from ramie leaf with ATPE systems including various alcohols (methanol, ethanol, 1-propanol, and 2-propanol) and salts (K_3PO_4, $(NH_4)_2SO_4$, NaH_2PO_4, K_2CO_3). Ethanol with low toxicity and cost and K_3PO_4 with higher the phase-forming ability were system components of ATPE led to higher recovery of target compound. In addition, in this study the extraction efficacy of ethanol/NaH_2PO_4 system was higher than that of ethanol/$(NH_4)_2SO_4$ system similar to the results of previous study. Actually, the salts provided acidic conditions were preferred in extraction not only for yielding higher performance but also for the stability of target compound.

As opposed to the studies of Tan et al. (2012) and Wu et al. (2014), high extraction of PCs from eucalyptus wood was achieved with ethanol/$(NH_4)_2SO_4$ system among systems including phosphate salts and their mixtures (K_2HPO_4; KH_2PO_4/K_2HPO_4) (Xavier et al., 2017). Similar to forementioned study, ethanol/$(NH_4)_2SO_4$ ATPE system exhibited higher performance for the extraction of PCs from OMWC compared with ethanol/K_2HPO_4 system (Çelik, 2018).

The effect of temperature also was investigated in the forementioned studies. Wu et al. (2014) revealed that the increment of temperature to 25 °C provided and faster phase separation and the efficient extraction of target compounds. Since increasing temperature enhanced the solubility of salt, water molecules efficiency partition from ethanol-rich phase to salt-rich phase. However, a further increment of temperature (in range of 15-55 °C) deteriorated the recovery of GA due to the degradation of anthocyanins. Similarly, Tan et al. (2012) achieved the maximum extraction efficiency was achieved at 25 °C (in range of 25-45 °C).

ATPE system was also used for the separation of proteins besides the separation of polyphenolic compounds from various matrices. As it is known that proteins are incompatible with organic solvents and alcohol/salts based ATPE system can be used for separation of proteins from applied matrix that can reduce of coextraction with PCs (Walter et al., 1985). In this respect, ATPE can be considered as an integrated method that provide the selective partitioning and purification of total PCs (Rosa et al., 2010).

2.3.2. Solid Phase Extraction

SPE is other extraction method with simplicity and low cost. The extraction ability of various synthetic resins for the recovery of PCs from OMW has been investigated in literature (Table 2.7).

Table 2.7. Literature on the resin extraction of PCs from OMWW.

Type of Adsorbent	Eliminated Component	Type of Elution Solvent	Achieved Results	Reference
XAD7HP XAD16	PCs	Cyclohexane/ EtOAc/MeOH /H_2O	>95% HTyr &Tyr removal by the combination of XAD7HP and XAD16	Agalias et al., 2007
XAD7 XAD16 IRA96 Isolute ENV+	HTyr & Tyr	Acidified EtOH (0.5% HCl, v/v)	Process Productivity by ENV+ 84%,	Bertin et al., 2011
XAD7 XAD16 IRA96 Isolute ENV+	PCs	Acidified EtOH (0.5% HCl, v/v)	Adsorption: 76% by IRA96 Desorption: 82% by IRA96,	Ferri et al., 2011
XAD16	HTyr	EtOH	Adsorption: 81.4% Desorption: 52.7% Removal: > 60%	Scoma et al., 2011
XAD4 XAD16 XAD7HP	PCs	Water, EtOH and acetone	Adsorption: >80% by XAD4 >60% by XAD16 Desorption: 80% with acetone	Zagklis et al., 2015
XAD16	PCs	Acidified EtOH (0.5% HCl, v/v)	Adsorption: 87- 88% Desorption: 65- 74%	Frascari et al., 2016
A26 IRA-67	PCs	-	Adsorption: 98% by A26 57% by IRA-67	Víctor-Ortega et al. (2016a)
XAD16	HTyr	Acidified EtOH (0.5% HCl, v/v)	Selectivity of HTyr 80-82 %	Çelik, 2018
A26 Dowex 66 XAD4	PCs	-	Adsorption : 98.9% by A26 72.4% by Dowex 66 70.5% by XAD4	Ochando-Pulido et al., 2018

Besides synthetic resins, various adsorbents such as activated carbon (GAC) (El-Shafey et al., 2005; Ena et al., 2009; Azzam et al., 2010) sand (Achak et al., 2009), Azolla plant (Ena et al., 2009), natural zeolite (Padovani et al., 2013) and silica (Yangui et al., 2016) have been used the treatment of OMWW. However, the selectivity and reversibility of adsorption are two important factors for the recovery target analytes in SPE. In this sense, forementioned adsorbents lead to poor results compared to non-ionic and ionic resins that exhibit high physicochemical stability (Carmona et al., 2006; Zagklis et al., 2015). Macroporous cross-linked polymeric structure of synthetic resins has favorably high adsorption capacity and selectivity (Zhenghao et al., 2004; Bulut and Aydın, 2006).

While IRA96 resin exhibited high performance with 76% PC adsorption (Ferri et al., 2011), XAD16 and XAD4 resins provided more than 60% and 80% recovery of PCs from OMWW (Zagklis et al., 2015). High recovery rate of HTyr 80-82% was also achieved with XAD16 resin (Çelik, 2018). In addition to forementioned resins, the performance of A26 ion exchange resin was reported as 98.9% for the recovery of biophenols (Ochando-Pulido et al., 2018).

In addition of the type of resins, various operational parameters (e.g., pH, temperature, contact time, and dosage of resin) can influence the performance of SPE. Depending upon pH, the interactions of solute with resin and the uptake mechanism of PCs can be different (Carmona et al., 2006). The uptake of PCs on ionic and non-ionic resins (R) can be described by the following equations (Caetano et al., 2009)

a) Exchange of phenolate anion (PO^-) with the counter-ion (OH^-) of the resin:

$$R^+OH^- + PO^- \Longleftrightarrow R^+ PO^- + OH^- \quad (2.1)$$

b) Adsorption of the molecular phenol (POH) on the free polymeric surface (R):

$$POH + R \Longleftrightarrow RPOH \quad (2.2)$$

According to Ahmaruzzaman (2008), an increase in pH value of solution to certain value has a positive influence for the adsorption of PCs while further increase leads to decrease of adsorption on non-conventional resins e.g., waste products, natural materials, and bio-adsorbents. In Addition, Ochando-Pulido et al. (2018) evaluated the performance of syntenic resins (e.g., strong-base anionic Amberlyst A26, and the weak anionic Dowex 66) for the recovery of PCs from OMWW at various pH (in range of 2.5- 8.5), and it was achieved that the uptake efficiency of PCs (from 32.8% to 95.4%)

on Amberlyst A26 was increased by increasing the pH value from 2.5 to 5.5 due to adsorption mechanism (equation 2.2) with the dominance of molecular phenolic species. However, further increase in pH value to 8.5 led to only 73.7% of uptake. Víctor-Ortega et al. (2016a) also attributed to the high PC uptake on Amberlyst A26 at acidic pH due to neutral form of these compounds.

On the other hand, Carmona et al. (2006) indicated that the uptake of PCs occurs as molecular adsorption and ion exchange as well as both mechanisms may be accessible in the same resin sites depends on initial pH value. In the study, Amberlite IRA-420, strong-base anion exchanger exhibited higher phenol removal performance at pH value between 9 and 14 (by combination of both adsorption and ion exchange) and remained constant lower than 8 (by adsorption). Similar result was found in the study of Zhu et al. (2011) with using prepared strongly basic anion exchange resin with Cl^- anion (MCl) at pH 11-12.6. The achieved results were explained with the abundance of phenolate species at alkaline pH described equation 2.1.

Temperature is another factor that has a critical impact on resin uptake of PCs as well as pH value of solutions. Prementioned studies also revealed that the adsorption of dissociated phenol (phenolate ion) was endothermic (equation 2.1) while the molecular adsorption of PCs was exothermic (equation 2.2). Accordingly, the uptake of PCs with a strongly basic anion exchange resin, MCl decreased by increasing the temperature from 10 to 40 ºC at pH 6.6 whereas at alkaline pH (12.6) an opposite effect of temperature was observed (Zhu et al., 2011). Similar results were obtained for Amberlyst A26, Dowex 66 and Amberlite XAD4 resins (Ochando-Pulido et al., 2018). A narrow temperature range was chosen considering the stability of PCs and the performances of all forementioned ionic and non-ionic resins were higher at 25 ºC compared to those obtained at 15 ºC and 35 ºC at pH 5.5 where molecular PCs predominate.

Besides the different operational conditions, it is well expected that physical properties of resins such as, surface area, and particle size can be important factors for the uptake of PCs. However, Huang et al. (2007) and Yangui et al. (2016) indicated in their studies that different types of surface area, particle size, and pore volume are not critical impacts for the recovery of phenols. On the other hand, the chemical structure of resin has major importance (Soto et al., 2011). The hydrogen bonding between phenol and the oxygen of the surface functional groups of resins also is an important factor for the adsorption of phenol as revealed in forementioned studies.

In SPE, the elution stage has considerable importance on the overall recovery of PCs as well as the uptake of them. Above mentioned investigations (Table 2.7), methanol, ethanol, acidified ethanol, ethanol/water mixture, and acetone were used as solvent for the elution of PCs from resin surface. Acidified ethanol provided 82-96% desorption of OMWW's PCs retained on XAD7, XAD16, ENV+, and IRA96 resins (Bertin et al., 2011; Ferri et al., 2011). Similarly, ethanol was found as suitable solvent compared to water and acetone for the selectively elution of OMWW's PCs adsorbed on non-ionic XAD7HP in addition to XAD4 and XAD16 resins (Zagklis et al., 2015) while acetone led to the removal of carbohydrates (100%) besides PCs (80%).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Olive Mill Wastewater Concentrates

OMWW used in this study was taken from an olive oil mill utilized two-phase centrifugal extraction system located in Bandırma, Turkey in the period of December 2018- January 2019. Before use in the experiments, OMWW was concentrated at two different ratios by using mechanical vapor recompression (MVR) operation on site. In the thesis, these concentrates of OMWW will be mentioned as OMWC1 and OMWC2. While OMWC1, which was in liquid form, was used for a comparative evaluation of resin performance, OMWC2 which was sticky solid due to high evaporation rate was employed in the integrated extraction process including resin and aqueous two-phase extraction for the recovery of polyphenolic antioxidants. Both waste samples were stored at 4+ °C to ensure their stability during the study.

3.1.2. Polyphenolic Antioxidant

HTyr (3,4-Dihydroxyphenylethanol, $C_8H_{10}O_3$) as the target antioxidant was analyzed in OMWCs considering the abundance of it in OMWW (Rahmanian et al., 2014). For the quantification in OMWC, the standard solutions of HTyr (≥ 98 % purity from Sigma Aldrich) were prepared.

3.1.3. Adsorbents

Six different commercial resins, purchased from Dow chemical company and Purolite, having different physical and chemical characteristics (Table 3.1), have been used: Amberlite XAD16N®, Amberlite XAD4™, PuroSorb™ PAD 610®, PAD 900®, PAD 950®, non-ionic resins, and an ionic resin Amberlyst A26™. The working pH range of all resins is reported as 0-14 by manufacturers. Physical appearance of all activated resins used in this study is shown in Figure 3.1(a-d). In order to recover the PCs from OMWW, PuroSorb™ PAD 610, PAD 900 and PAD 950, were used for the first time in this study.

Table 3.1. Physicochemical properties of selected resins.

Resin	Property	Matrix	Surface Area (m^2/g)	Pore Volume (mL/g)	Pore Diameter (Å)	Particle Size (µm)
Amberlite®XAD16N	Adsorbent	Styrene-divinylbenzene	900	1.82	200	560-710
Amberlite ™ XAD4	Adsorbent	Macroreticular crosslinked aromatic polymer	≥ 750	-	100	490-690
Amberlyst ™ A26	Ion exchanger (OH^-)	Styrene-divinylbenzene	30	0.2	290	560-700
PuroSorb ™ PAD610*	Adsorbent	Polymethacrylic/ divinylbenzene	490	1.2	300	350-1200
PuroSorb ™ PAD900*	Adsorbent	Polystyrene cross-linked with divinylbenzene	850	1.9	220	350-1200
PuroSorb ™ PAD950*	Adsorbent	Polymethacrylic	450	0.6	120	350-1200

* Purolite® Adsorbents Brochure, 2020.

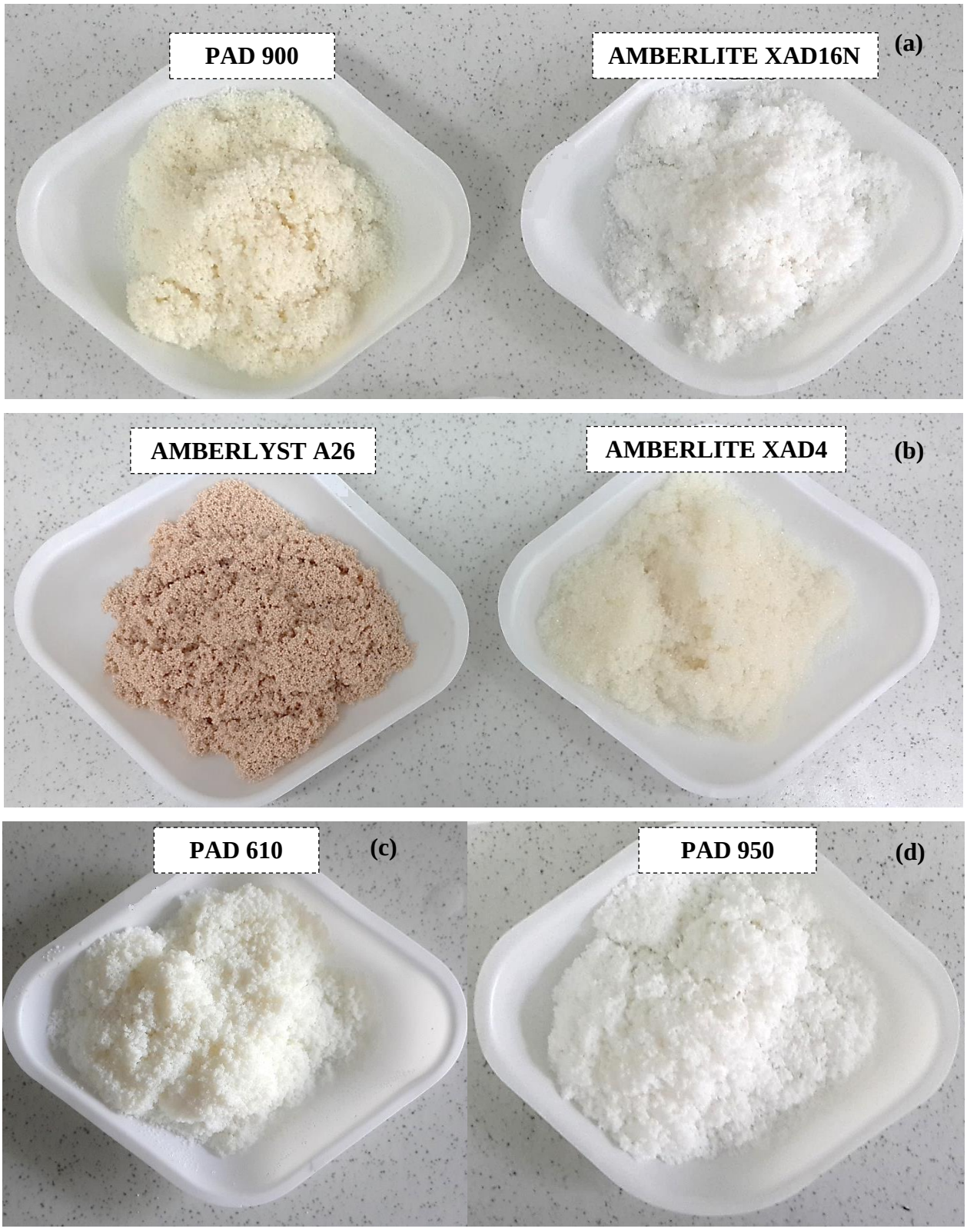


Figure 3.1. Physical appearance of the resins used in the study.

3.1.4. Other Chemicals

Chemicals that were employed during the research are listed in Table 3.2.

Table 3.2. List of all chemicals substances that were used in experiments.

Chemical Name	**Molecular Formula**	**CAS number**	**Experimental Use**	**Supplier**
Gallic acid	$C_7H_6O_5$	149-91-7	Total Phenol Analysis	Merck
Folin-Ciocalteu Reagent	$C_{10}H_5NaO_5S$	521-24-4	Total Phenol Analysis	Merck
Sodium Carbonate	Na_2CO_3	497-10-8	Total Phenol Analysis	Merck
Hydrochloric acid, 37%	HCl	7647-01-0	pH Adjustment/ Resin Activation	Riedel de Haën
Sodium Hydroxide	NaOH	1310-73-2	pH Adjustment	Riedel de Haën
Bovine Serum Albumins- BSA		9048-46-8	Protein Analysis	Sigma-Aldrich
Bradford Reagent			Protein Analysis	Sigma-Aldrich
L-Ascorbic Acid	$C_6H_8O_6$	50-81-7	Antioxidant Activity (DPPH)	Sigma-Aldrich
Methanol	CH_3OH	67-56-1	Antioxidant Activity (DPPH)	Sigma-Aldrich
2,2-diphenyl-1-picrylhydrazyl	$C_{18}H_{12}N_5O_6$	1898-66-4	Antioxidant Activity (DPPH)	Sigma-Aldrich
Trolox	$C_{14}H_{18}O_4$	53188-67-1	Antioxidant Activity (ABTS)	Sigma-Aldrich
2,29-azinobis-(3 ethylbenzothiazoline -6-sulfonic acid)	$C_{18}H_{24}N_6O_6S_4$	30931-67-0	Antioxidant Activity (ABTS)	Sigma-Aldrich
Sodium Persulphate	$Na_2S_2O_8$	7775-27-1	Antioxidant Activity (ABTS)	Sigma-Aldrich
Sulfuric acid	H_2SO_4	7664-93-9	COD/ Carbohydrate	Sigma-Aldrich
Mercury (II) Sulphate	HgSO4	7783-35-9	COD	Sigma-Aldrich
Silver Sulphate	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
n-Hexane	C_6H_{14}	110-54-3	Oil-Grease Analysis	ISO-LAB
Ethanol	EtOH	64-17-5	ATPE/ Resin Activation	ISO-LAB
Ammonium Sulphate	$(NH_4)_2SO_4$	7783-20-2	ATPE	ISO-LAB

3.2. Methods

SPE stand alone and as integrated to ATPE were applied to OMWC for the recovery of antioxidant PCs by the elimination of other organic components of wastewater. In Figure 3.2, the flow chart of the procedure used for OMW is shown.

The first stage of the process included the water content reduction of OMWW by using a pilot scale MVR evaporator. The performance of various resins was screened by batch adsorption/desorption experiments. In the following steps, two different extraction options were investigated: SPE was applied as stand-alone on OMWC1 (Process I) and ATPE combined with SPE was performed on OMWC2 (Process II). Prior to process I, OMWC1 was subjected to pre-treatment step. Afterwards, the selected resins with high sorption performance were used in Process II.

3.2.1. Pre-treatment of OMWC1

Prior to the batch adsorption test and the extraction of the PCs, the pre-treatment of OMWC1 was carried out by (i) acidification and (ii) centrifugation.

Freshly acidified OMWC1 with HCl (0.1 and 1 M) was centrifuged at 5000 rpm for 15 min to remove the insoluble particles hence blockage of resin pores during sorption could be prevented.

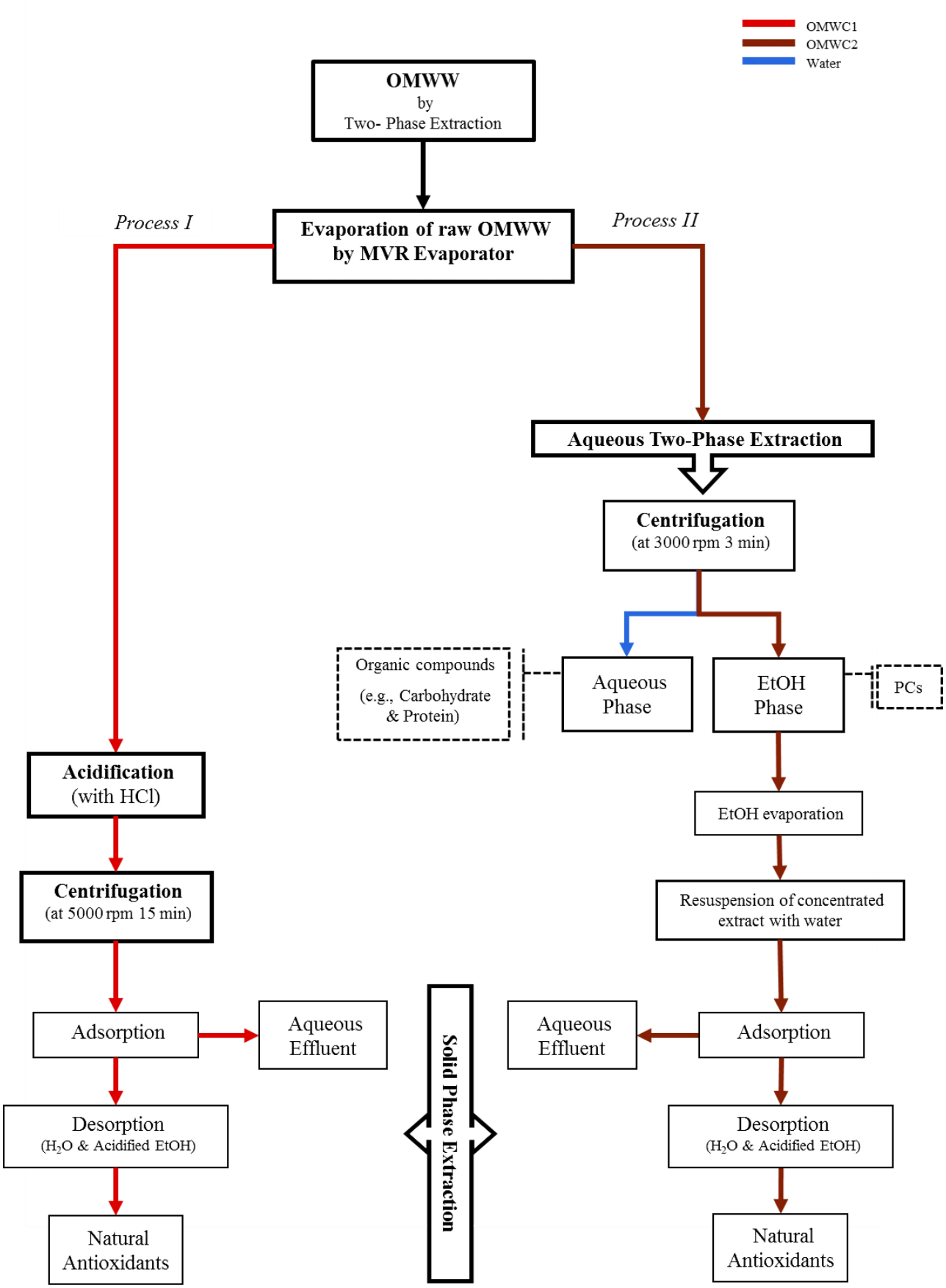


Figure 3.2. Methodology for the selective recovery process of phenolic antioxidants from OMWW.

3.2.2. Solid Phase Extraction of OMWC

The recovery of total PCs and HTyr from pre-treated OMWC1 (Process I) and the further purification of total PCs and HTyr from OMWC2 (Process II) were studied by using SPE process.

The performances of the six resins were evaluated with batch sorption experiments at different conditions. The effects of resin dosage and initial pH of samples on the sorption efficiency were studied for each selected resin. Prior to the use of selected resins in all batch experiments described herein, a cleaning procedure was applied in order to remove any monomer attached on the resin matrix. First, 5 mL acidified EtOH (0.5% 0.1 M HCI, v/v) was added to per gram of resin placed in a rotary shaker for 8h and then rinsed several times with deionized water to remove residual ethanol thoroughly. Subsequently, the resins were dried overnight at 105 °C in a laboratory oven and they were stored in desiccator until use. A moisture analyzer (Kern DBS) was used to quantify the moisture content of dried resin before sorption experiments.

3.2.2.1. Adsorption experiment. The uptake capacity of each resin at three different dosages (30, 60, 90 g/L) was determined with 25 mL pre-treated OMWC1 placed in Erlenmeyer flasks. Batch equilibrium experiment was performed in a rotary shaker operated at 140 rpm and ambient temperature for 3h. The samples were taken at different time intervals (every 10 min for the first operating hour, then every hour), and they were centrifuged at 10000 rpm for 5 min to separate residual resin particles.

In addition to the effect of resin dosage, the effect of pH on resin performance was studied in the range of 3.5 to 8.5 for the recovery of PCs from OMWC1 regarding with complex organic compounds, bounded to PCs. The pH value of OMWC was adjusted with 0.1 and 1 M NaOH or HCl solutions. The performance of each resin was evaluated in terms of adsorption efficiency (A) of target components of OMWC by using following equation (3.1) (Lin et al., 2012):

Adsorption Efficiency:

$$A, \% = \frac{C_0 - C_e}{C_0} \text{ x } 100 \qquad (3.1)$$

where C_0 and C_e are the initial and equilibrium concentrations of the components, respectively.

3.2.2.2. Desorption experiment. The recovery of the adsorbed components was achieved by desorption. Two different eco-friendly organic solvents were used sequentially for this purpose; water that favors the removal of carbohydrates from the resin surface, and then acidified aqueous EtOH for the elution of PCs. In this context, spent resin was rinsed with 20 mL deionized water two times, and this step was followed by treatment of resin with 20 mL acidified aqueous EtOH (0.5% HCI 0.1 M, 70% EtOH, v/v) for 3h at 30 °C. The desorption of target components of OMWC (D) was calculated by following equation (3.2) (Lin et al., 2012):

$$D, \% = \frac{C_d x V_d}{(C_0 - C_e) x V_i} \times 100 \quad (3.2)$$

where C_0, C_d and C_e are the initial, desorbed and equilibrium concentration of target components, V_d and V_i are the volume of desorbed sample solution and initial sample solution, respectively.

An overall Desorption ratio (D_{ov}), which refers to the desorption obtained in three stages, was determined by replacing the parameter C_d with the sum of the final target component concentration calculated at the end of each stage.

3.2.3. Aqueous Two- Phase Extraction of OMWC

The alcohol- salt-based ATPE system was applied to OMWC2 as the first step of integrated extraction process (Process II). EtOH as solvent and $(NH_4)_2SO_4$ as salt were the components of ATPE.

First the equilibrium diagram of EtOH/$(NH_4)_2SO_4$ ATPE system was constructed by cloud point titration method. In this context, OMWC2 placed in a test tube was dissolved in deionized water. The mixture of sample and salt solution was titrated with EtOH until reaching cloud-point which indicates the biphasic mixture Then the aliquots of deionized water were added to the turbid mixture until turbidity disappeared. At each stage, the weight of mixture was recorded. This procedure was repeated to collect binodal curve data. The composition of each point was determined by using an analytical balance. The mass balance of salt and EtOH in the system calculated by following equations:

$$w_1 = \frac{m_1}{m_1 + m_2 + m_3} \quad (3.3)$$

$$w_2 = \frac{m_2}{m_1+m_2+m_3} \quad (3.4)$$

w_1: mass fraction of EtOH
w_2: mass fraction of $(NH_4)_2SO_4$
m_1: weight of EtOH
m_2: weight of $(NH_4)_2SO_4$
m_3: weight of sample

In order to extract total PCs with ATPE system, 5mL diluted OMWC2 (13 g/L) sample and the appropriate amounts of $(NH_4)_2SO_4$ and EtOH were added to centrifuge tube to obtain a system with 10 g weight. Afterwards, the salt/EtOH/sample solution centrifugated at 3000 rpm for 3 min, then let stand at ambient temperature to separate two-phases. The top EtOH rich phase, was carefully collected in a flask of rotary evaporator and EtOH was evaporated. The residual of the extract was resuspended by the addition of deionized water and then it was filtered through 0.45 μm syringe filter.

3.2.4. Analytical Methods

Analytical methods for the quantification of PCs and other components of OMWC as well as the characterization are described in the following sections.

3.2.4.1. Total Phenol content. Total PCs content of samples was determined by colorimetric Folin-Ciocalteu method (Singleton and Rossi, 1965; ISO 14502-1, 2005). After mixing of 0.5 mL sample and 2.5 mL Folin-Ciocalteu Reagent (0.2 N), greenish yellow colour was developed in assay tubes as a result of the electron-transfer reaction between PCs and Folin-Ciocalteu reagent (Sánchez-Rangel et al., 2013). In order to maintain basic condition (pH ~10), 2 mL sodium carbonate solution (7.5%, w/v) was added to sample mixture 3 min just after later the addition of Folin-Ciocalteu Reagent. The sample was incubated in a water bath at 50 °C for 5 min and subsequent it was cooled to ambient temperature. A color alteration from greenish yellow to blue was developed in the sample that is proportional to PCs concentration (Figure 3.3).

The absorbance of the sample at 765 nm wavelength was measured by using a spectrophotometer (Shimadzu UV-1208) against blank reagent prepared by addition of 0.5 mL deionized water instead of sample. Gallic acid was the standard of this test and it was used to prepare a calibration curve in

concentration range of 10 to 80 mg/L (Appendix A.1). The results were expresses as Gallic Acid Equivalent (GAE).

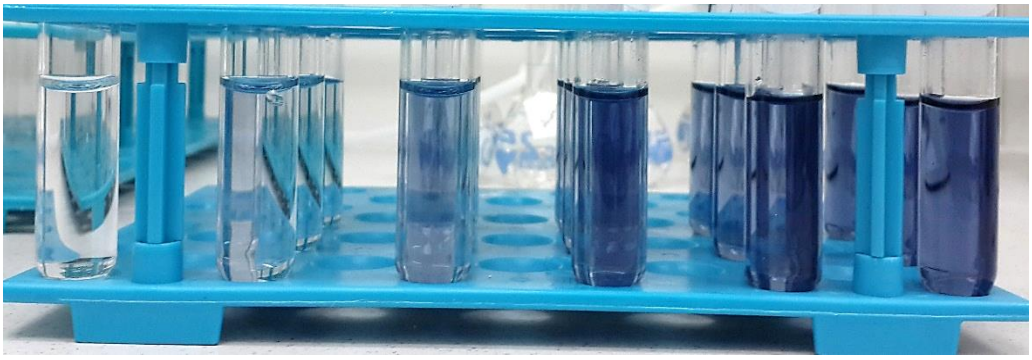


Figure 3.3. Gallic acid standards for total PCs analysis by Folin-Ciocalteu method (Concentrations of standards from left to right, 0, 10, 20, 40, 60, 80 mg GAE/L).

3.2.4.2. Antioxidant activity. The antioxidative activity of OMWC extracts was determined by two different colorimetric methods namely 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (Brand-Williams et al., 1995) and 2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet}+$) decolorization assay (Re et al., 1998).

The DPPH assay which is easy and rapid method depends on the estimation of the scavenging capacity of phenolic antioxidants for the stable organic nitrogen radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH•) (Sánchez-Moreno, 2002). For this test, DPPH standard solution in MeOH (0.004%, w/v) with dark violet color was prepared daily and stored in the dark until analysis. The addition of 0.1 mL of sample to 3.9 mL DPPH standard solution and then incubation in the dark at ambient temperature for 30 min allowed the scavenging reactions shown with equations 3.5 and 3.6 (Prior et al., 2005) . Consequently, a color alteration from deep to pale violet occurred (Figure 3.4) and the quantification of antioxidant activity was performed by measuring the absorbance at 517 nm wavelength with Shimadzu UV-1208 spectrophotometer.

$$DPPH^{\bullet} + \text{Antioxidant} \longrightarrow DPPH\text{-}H + A\bullet \quad (3.5)$$

$$DPPH^{\bullet} + R\bullet \longrightarrow DPPH\text{-}R \quad (3.6)$$

L-ascorbic acid was the standard and a calibration curve in the concentration range of 10 to 150 mg AAE/L was prepared (Appendix A.2). The results were expressed as ascorbic acid equivalents (AAE).

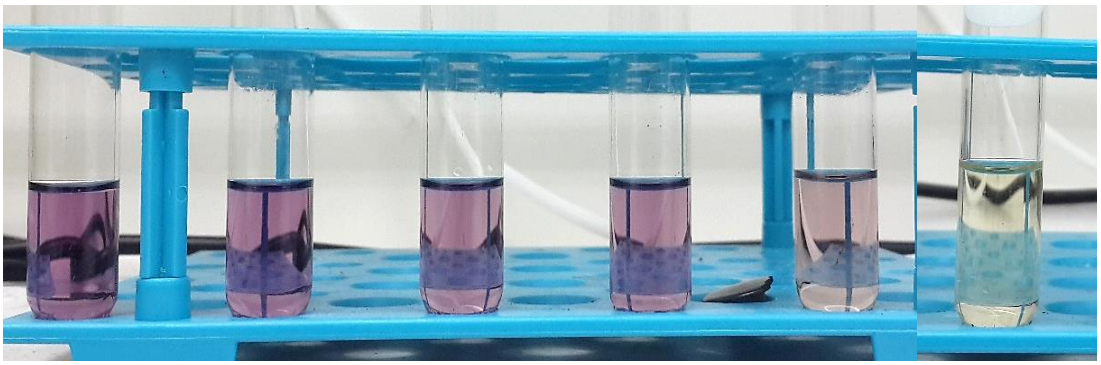


Figure 3.4. Ascorbic acid standards for antioxidant activity analysis by DPPP assay (Concentrations of standards from left to right, 10, 20, 40, 50, 100, 150 mg AAE/L).

The antioxidant activity was also determined by the colorimetric ABTS test according to Re et al. (1998). ABTS assay depends on the decolorization technique that induced by antioxidative compounds.

Prior to analysis, ABTS working solution was prepared by dissolving ABTS reagent in water (7 mM final concentration). The stock solution of ABTS included the working solution of ABTS and sodium persulfate solution (140 mM final concentration) was kept in the dark at ambient temperature overnight (almost 16 h) before use. In the same day of the analysis, an ABTS radical solution is prepared by the dilution of ABTS stock solution in MeOH at 30° C. The absorbance of this solution was 0.70 ±0.02 at 734 nm while MeOH was the reference.

For the measurement of the antioxidant activity, 4.0 mL of diluted $ABTS^{\bullet+}$ solution and 40 µl of OMWC extract were mixed and this mixture was incubated at 30 ºC in a dark environment for 4 min. After the mixing of solution, the decrease in absorbance values were recorded immediately at 734 nm wavelength. Total spent time for analysis from incubation to reading should not exceed 7 min. Blank reagent was run daily for the control of the assay by following same procedure with 40 µl water instead of extracts, and the water/MeOH mixture was used as reference spectrophotometric measurement (Shimadzu UV-1208). The calibration curve was prepared by dissolving stock standard of Trolox dissolving in MeOH in the concentration range of 0 to 1440 µM (Appendix A.3). The decolorization of stock standard is shown in Figure 3.5. Antioxidant activity of extract was expressed relative to Trolox equivalents.

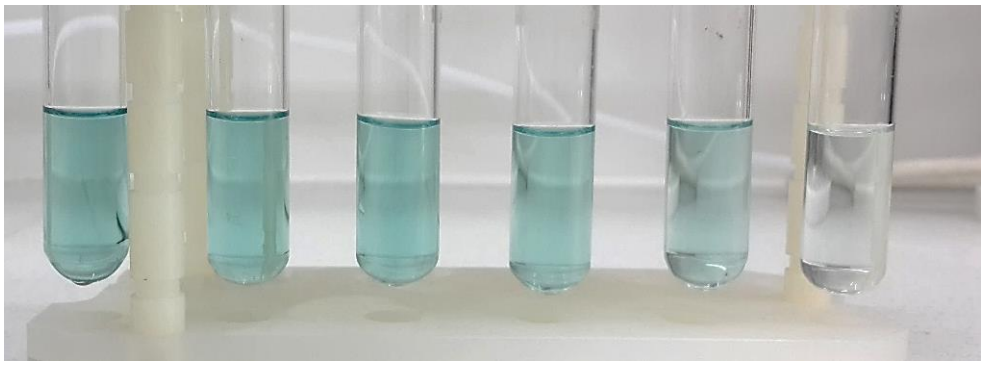


Figure 3.5. Trolox standard used in antioxidant activity analysis by $ABTS^{\bullet+}$ assay (Concentrations of standards from left to right, 0, 90, 180, 360, 720, 1440 μM).

3.2.4.3. LC-MS/MS analysis. HTyr concentration in the extracts and samples was determined by a triple quadrupole mass spectrometer, AB Sciex QTRAP 4500 LCMS/ MS system combined with Eksigent Ekspert Ultra-LC 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 at temperature of 550 °C (TEM) with using turbo spray ion source. In LC-MS/MS system chromatographic conditions were optimized for HTyr standard with gradient elution program through a reversed phase column to obtain optimum separation conditions for the samples.

The calibration curve of HTyr was constructed with fresh prepared standards at four different concentrations (10, 25, 100, and 200 ppb) from HTyr stock solution (Appendix B.1).

3.2.4.4. Protein content. The protein content of OMWCs and their extracts were determined according to Bradford method (Bradford, 1976). In this test, 5 mL of Bradford reagent was added to 0.1 mL filtered samples (0.45 μm filter, Sartorius™). After mixing, assay tubes were kept at ambient temperature for 5 min. for the formation of protein- dye complex which can stay in the solution up to 1h. During reaction, brownish red color of the samples was immediately converted to blue. The color was proportional binding of the dye to proteins and the absorbance value of samples was recorded at 595 nm wavelength by using the spectrophotometer (Shimadzu UV-1208). Bovin Serum Albumin (BSA) was the standard and a calibration curve in concentration range of 0.01 to 2 g/L was prepared to predict the protein concentration in OMWC and extracts (Appendix A.4).

3.2.4.5. Carbohydrate content. The carbohydrate content of OMWCs and their extracts were estimated according to Anthrone method (Yemm and Willis, 1954; Pons et al., 1981). The Anthrone reagent which was freshly prepared before analysis by dissolving 200 mg Antrone in 100 mL concentrated sulfuric acid (2 g/L) was mixed with filtered samples (0.45 μm filter, Sartorius™) at 1:4

ratio (v/v) by using vortex mixer to ensure uniform dispersion. Carbohydrates present in the samples are hydrolyzed to simple sugars in hot acidic condition provided by incubation in boiling water (at 100°C) for 10 min. After the completion of the reaction, the samples having blueish green depending on the concentration of carbohydrate were cooled to ambient temperature and the absorbance values of the samples were determined at 630 nm wavelength. Alpha-D-glucose was the standard of this test and a calibration curve in the concentration range of 10 -100 mg/L was prepared (Appendix A.5). The obtained results were expresses in terms of Glucose Equivalents (mg GE/L).

3.2.4.6. Solid content. The total solid (TS), total suspended solid (TSS) and total dissolved solid (TDS) content of OMWC1 were determined by using gravimetric method (APHA, 1998).

TS and TDS contents in samples were quantified according to the following equation (3.7).

$$\text{TS of TDS, g/ L} = \frac{\text{(A-B)x 1000}}{\text{sample volume, mL}} \qquad (3.7)$$

where A is weight of dried residue and dish, g and B is weight of dish, g.

For the determination of TSS filtered aqueous solution of OMWC was subjected to same procedure applied to TS or TDS. TSS content was calculated with equation 3.7 in which A is the sum of weight of filter and the dried residue retained on the filter papers, and B is weight of filter paper.

3.2.4.7. Chemical Oxygen Demand (COD) analysis. COD value of OMWC1 was determined by closed reflux colorimetric method according to a standard method (APHA, 1998). For this purpose, 2.5 mL OMWC1 sample filtered through 0.45 μm filter (Sartorius™) and diluted OMWC1 was well mixed with 1.5 mL of digestion solution, and 3.5 mL of sulfuric acid reagent. The mixture was digested in pre-heated block digester at 150 ºC for 2h. The absorbance values of the samples were recorded by a spectrophotometer (HACH DR/2010) at 600 nm wavelength against blank reagent. Potassium hydrogen phthalate (KHP) was the standard to prepare calibration curve (Appendix A.6).

3.2.4.8. Oil-Grease analysis. Oil-grease content of OMWC was determined by the partition-gravimetric method (APHA, 1998). Briefly, 15 mL samples pre-acidified with 1M HCl (36.5%) to pH 1-2 were centrifuged twice at 2000 rpm for 15 min after the addition of 15 mL n-hexane. Before the evaporation of solvent, solution was mixed thoroughly for about 2 min. The layers of sample and

extraction solvent were separated by centrifugation. Top phase containing oil was transferred into a flask of rotary evaporator for evaporation of n-hexane at 70 °C. The oil content was calculated by the following equation (3.8).

$$\text{Oil content g/L} = \frac{\text{(A-B)x 1000}}{\text{Sample volume, mL}} \quad (3.8)$$

where A is weight of flask + oil content of sample, g and B is weight of flask with constant weight, g.

3.2.4.9. pH measurement. pH value of OMWCs and extracts were determined with WTW pH 330 pH-meter.

3.2.4.10. Moisture content. The moisture content of OMWC sample was determined automatically by using Kern DBS Version 1.0 Electronic Moisture Analyser operating at 120 °C. For OMWC2 one g sample was used for the moisture analysis.

3.2.5. Error Analysis Method

A Chi-square test was used in order to perform an error analysis and evaluate the suitability of kinetic models to the experimental results.

The sum of square errors (SSE) is determined by following equation (3.9):

$$\text{SSE} = \sum \frac{\left(q_{e,\,exp-}\, q_{e,calc.}\right)^2}{q_{e,calc.}} \quad (3.9)$$

where $q_{e,exp}$ is the experimental data of the equilibrium capacity (mg/g), $q_{e,calc}$ is the calculated equilibrium capacity obtained by the model (mg/g).

4. RESULTS AND DISCUSSION

4.1. Characteristics of Olive Mill Waste Concentrates

The main physicochemical characteristics of raw OMWCs were determined to evaluate the recovery efficiency of total PCs as well as for the decision of process stages. Prior to the extraction of polyphenolic antioxidants, OMWW was exposed to MVR evaporation through different detention periods generating two different OMWCs: OMWC1 was liquid with 77.14 g/L TS content, while OMWC2 was sticky solid with 1.40% moisture content. The characteristics of these OMWCs are summarized in Table 4.1.

Table 4.1. Physicochemical characterization of raw OMWCs.

Parameter	**OMWC1**	**OMWC2***
pH	4.75	5.49
Total Phenolic Compounds	6.08 g/L	75.43 mg/g
Protein	0.41 g/L	112.00 mg/g
Carbohydrate	4.56 g/L	27.64 mg/g
COD	96.95 g/L	-
TS	77.47 g/L	-
TSS	2.80 g/L	-
TDS	68.22 g/L	-
Oil	1.87 g/L	-
Moisture (%)	-	1.40

*13 g OMWC2/L.

As shown in Table 4.1, each OMWCs has different characteristics depending on the rate of evaporation operation. While both OMWCs demonstrated a mildly acidic nature, slightly higher acidity of OMWC1 could be explained by the presence of volatile compounds e.g., volatile acids in the waste matrix. Table 4.1 also shows that the contents of organic compounds, namely PCs, proteins, and carbohydrates are considerably high in OMWC2.

4.2. Recovery of Total PCs from OMWC1 by SPE Process

The application of SPE process to OMWC1 that was pretreated by acidification was investigated with ionic and nonionic synthetic resins (Process I) for the recovery of total PCs, mainly HTyr.

4.2.1. Acidification of OMWC1

It is known that proteins can be precipitated at pH 2-3 (Obied et al., 2005a) and under acidic conditions, several PCs can also be liberated from the complex organics found in OMW (Rubio-Senent et al., 2013). With these facts in mind, OMWC1 was acidified to a pH value of 3.00±0.1 prior to SPE process to provide the release PCs into the liquid phase and to precipitate nontarget organic components (e.g., proteins) bounded to polymeric PCs from the concentrated sample. In accordance with the previous studies in relevant literature (Ceccon et al., 2001; De Marco et al., 2007; Lafka et al., 2011; Rubio-Senent et al., 2013; Leouifoudi et al., 2014; Sellami et al., 2016; Çelik, 2018), HCl (0.1 and 1 M) was used for the acidification. The achieved results for the effect of acidification on total PCs and proteins found in OMWC1 are showed in Table 4.2.

Table 4.2. Effect of acidification on the precipitation of proteins and the liberation of total PCs.

Organics	Raw OMWC1	Acidified OMWC1
PCs (g/L)	6.08	5.60
Proteins (g/L)	0.41	0.18

As clearly seen in Table 4.2, partial precipitation of proteins was achieved under acidic condition. However, acidification did not cause an increase in the concentration of PCs as opposed to the literature findings (Rubio-Senent et al., 2013).

4.2.2. Total PCs Extraction with Synthetic Resins

The screening of resins for the uptake of total PCs was performed with batch experiments by using OMWC1. Amberlite XAD16N®, Amberlite XAD4™, PuroSorb™ PAD 610®, PAD 900®, PAD 950®, and Amberlyst A26™ were six different commercial resins used in this study. Purosorb™ PAD series, reported by the manufacturer to be specific for polyphenol compounds (Purolite® Adsorbents Brochure, 2020), have not been used for the waste from olive oil industry. However, Amberlite XAD16N and XAD4, and Amberlyst A26 were the resins investigated for the recovery of PCs from

OMWW in previous studies, in which encouraging results were achieved (Agalias et al., 2007; Bertin et al., 2011; Ferri et al., 2011; Scoma et al., 2011; Zagklis et al., 2015; Frascari et al., 2016; Çelik, 2018).

4.2.2.1. Effect of resin dosage on total PCs extraction. For the extraction of PCs from acidified OMWC1 (pH=3), a suitable dosage of the resins was determined with the experiments in which resin dosage was varied from 30 to 90 g /L and all other conditions were kept constant. It is worthy to mention that the selected dosage range in this study depends upon the results of recent studies (Bertin et al., 2011; Zagklis et al., 2015; Ochando-Pulido et al., 2018). The adsorption efficiency of total PCs achieved within 3h contact time was calculated by equation 3.1 and the results are shown as a function of dosage of each tested resin in Figure 4.1.

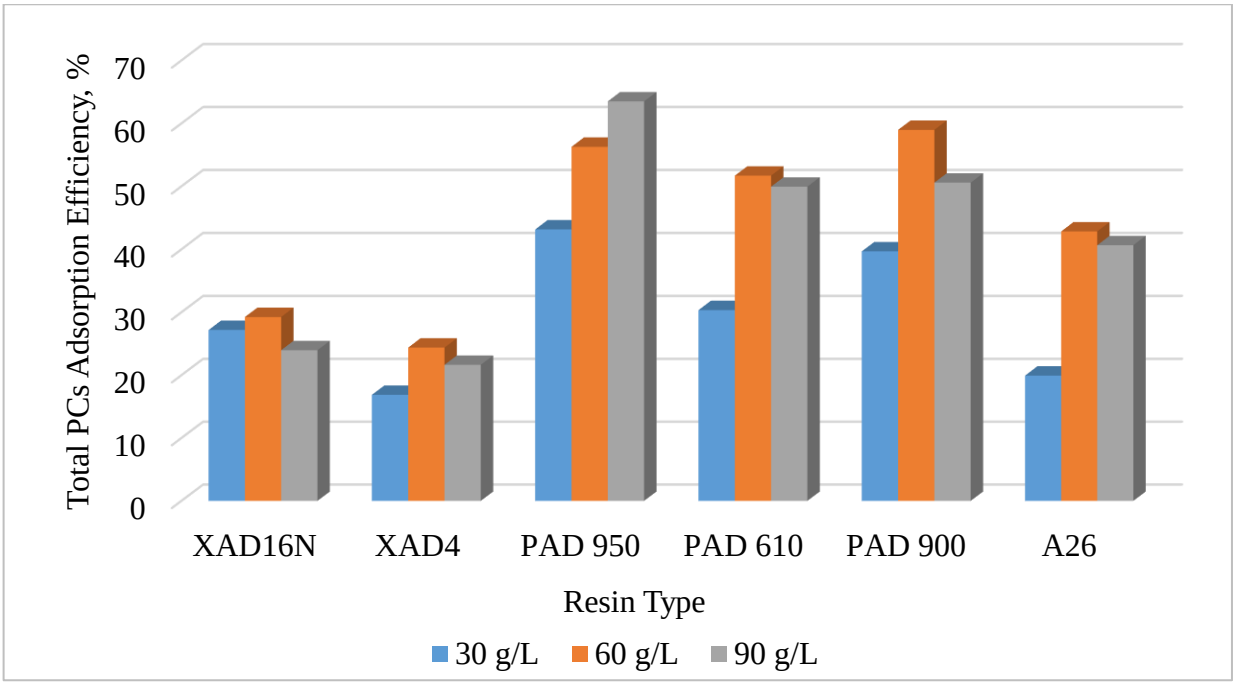


Figure 4.1. Effect of different resins dosages on the adsorption efficiency of total PCs at ambient temperature (pH= 3 and contact time= 3h).

As shown in Figure 4.1, total PCs extraction from OMWC1 ranged from 17% to 64%. The performance of non-ionic PAD series was higher compared to those of Amberlite series, and Amberlyst A26. The results achieved in this study with Amberlite XAD16N and XAD4, and Amberlyst A26 were not higher than those of literature studies in which OMWW with 0.76–3.37 g/L PCs content was used (Bertin et al., 2011; Zagklis et al., 2015; Ochando-Pulido et al., 2018). It is known that the removal percentage of phenols is highly dependent upon initial concentration of the compound and high concentration of PCs causes a reduction in the adsorption percentage although

adsorption capacity exhibits an enhancement (Víctor-Ortega et al., 2016b). With these facts in mind, it can be concluded that highly concentrated OMWW (Table 4.1) that was used in this study could cause competition for the target compound adsorption.

An increase in resin dosage from 30 g/L to 60 g/L has a positive influence on the adsorption efficiency due to the majority of active sites. However, further increase of the dosage of all resins except PAD 950 above 60 g/L caused a slight abatement in adsorption efficiency. Similar to the results of this study, Ochando-Pulido et al. (2018) reported that the lower quantity of Amberlyst A26, Dowex 66, and Amberlite XAD4 resins were not enough for sufficient uptake of total PCs. On the other hand, similar abatement in the adsorption efficiency of Amberlite XAD4 was observed at high (114 g/L) resin dosage while Amberlyst A26 did not exhibit such behaviour in OMWW (Ochando-Pulido et al., 2018).

4.2.2.2. Effect of resin properties on total PCs extraction. It is known that the differences in physical and chemical properties of a resin e.g., specific surface area, pore size, pore distribution, polarity, and functional groups have an influence on the adsorption ratio of the target compound (Yangui et al., 2017).

As stated before, among all investigated resins, PAD series generally exhibited higher adsorption ratio for total PCs (Figure 4.1). This achieved results could be attributed to the high pore volume with consistent pore size distribution (Purolite® Adsorbents Brochure, 2020). PAD 900 with large surface area (850 m^2/g), which is almost two times higher than those of PAD 610 and 950 provided high total PC uptake. Additionally, due to polystrenic matrix of PAD 900, strong hydrophobic interaction with molecules having aromatic groups (Purolite® Adsorbents Brochure, 2020) could be expected. However, quite similar results were obtained for total PCs adsorption rate with PAD 900 and 950 at 60g/L dosage of resin. Although ionic Amberlyst A26 is characterized by extremely low surface area (30 m^2/g) among all resins (Table 3.1), the adsorption efficiency of total PCs was found higher than non-ionic resins Amberlite series (XAD16N and XAD4). This fact could be explained by styrene divinylbenzene matrix of A26 (Table 3.1) that provided a higher affinity of total PCs to resin surface.

Amberlite series (XAD16N and XAD4) (Figure 4.1) provided significantly lower performance for uptake total PCs. This result could be explained for XAD16N with non-polar properties (Bertin et al., 2011) that show a low affinity for most naturally occurring PCs having high polarity (Table 2.5). Although Amberlite series was found as convenient for extraction of PCs in previous literature

studies (Table 2.7), these resins also have an affinity to organic substances besides total PCs that causes the competition between total PCs and other organics. Carbohydrates, one of the abundant components of OMWC1, could reduce the performance of neutral resins for PC uptake (Kammerer et al., 2010). Therefore, both resins achieved lower adsorption efficiency for the recovery of total PCs than other examined resins.

4.2.2.3. Adsorption kinetics of total PCs. Adsorption kinetics is one of the main factors for the practical application of a resin. In order to compare to the performance of employed resins, adsorption kinetic experiments were carried out with pre-treated OMWC1 throughout 3h. At certain time intervals, the adsorption capacity (q_t) of each resin for total PCs was calculated with the following equation (4.1):

$$q_t = \frac{C_0 - C_t}{W} \times V_i \qquad (4.1)$$

where C_0 and C_t are the concentrations of total PCs at time 0 and t, respectively, V_i is volume of initial sample solution, and W is dry weight of the resins. The adsorption capacity of each resin for total PCs is represented as a function of time in Figure 4.2.

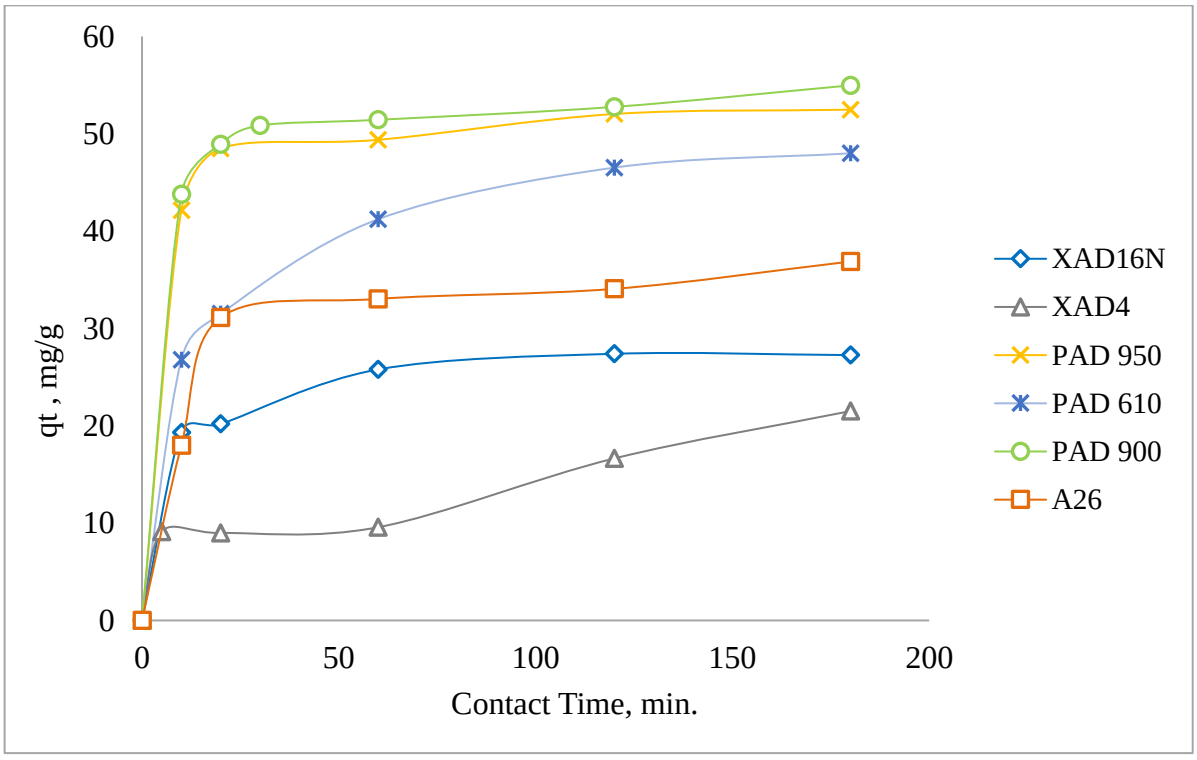


Figure 4.2. Adsorption kinetics of total PCs from pre-treated OMWC1 with six different resins at 60 g/L dosage.

As shown in Figure 4.2, PAD 950 and PAD 900 resins exhibited quite similar results and almost 90% of equilibrium PCs uptake was achieved within first 20 min. Similarly, rapid uptake of total PC was obtained with A26 before reaching equilibration although the adsorption capacity of this resin was lower than those of PAD 900 and PAD 950. On the other hand, Amberlite XAD16N reached equilibration within first one hour. These results could be attributed to high initial total PC concentration of OMWC1 (Table 4.1) that causes a high driving force for mass transfer to the resin surface (Víctor-Ortega et al., 2016a). However, the adsorption capacities of XAD4 and PAD 610 resins increased as the contact time increased further.

In order to analyse the adsorption mechanism, the pseudo-first order (Lagregren, 1898), pseudo -second order (Ho and McKay, 1999), simple Elovich (Chien and Clayton, 1980), and intra-particle diffusion (Weber and Morris, 1963) kinetic models, which are commonly used in kinetics studies, were applied to experimental data. The linear correlation coefficients (R^2) and the sum of squares error (SSE) values were calculated to evaluate the reliability of these models. The equations of these kinetics models are listed in Table 4.3.

Table 4.3. Adsorption kinetic model equations.

Kinetic Model	**Equation**	**Plot**	**k**	**q_e**
Pseudo First Order	$\ln(q_e - q_t) = \ln q_e - k_1 t$	ln (q_e-q_t) vs. t	k_1	$e^{intercept}$
Pseudo Second Order	$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e}$	t/q_t vs. t	1/ (qe^2x intercept)	1/slope
Simple Elovich	$q_t = a + b\ln(t)$	q_t vs. lnt	b	a
Intra-particle diffusion	$q_t = k_p\sqrt{t} + C$	q_t vs. $t^{0.5}$		

In the kinetic equations, qt (mg/g) is the adsorbed amount of target compound at time t; qe (mg/g) is the adsorption capacity at equilibrium; k_1, k_2 and k_p are the rate constants of pseudo-first-order, pseudo-second order and intra-particle diffusion kinetics models, respectively; C is the constant in particle diffusion kinetics model that inform the thickness of boundary layer i.e., the larger value of the intercept means the greater effect of boundary layer (Amin, 2009); b (mg/g.min) is the initial adsorption rate constant and a (mg/g) is the extent of surface coverage and activated energy for the chemisorption in Elovich model. The experimental and predicted adsorption capacities of resins with kinetic models together with kinetic constants for the adsorption of total PCs are listed in Table 4.4.

Pseudo-first order and Simple Elovich kinetic models exhibited a poor fitting for the prediction of adsorption process with the resins except PAD 610 owing to low linear correlation coefficients (R^2) and noticeably high SSE values as shown in Table 4.4.

On the other hand, it is observed that pseudo-second order kinetic model was well fitted to experimental data of total PCs with considerably high correlation coefficients ($R^2 = 0.99$) for ionic and non-ionic resins except XAD4 as shown in Figure 4.3. Moreover, the experimental equilibrium adsorption capacities of the resins ($qe_{,exp}$) are close to the theoretical $qe_{,cal}$ values estimated from the model as shown in Table 4.4. For this model, rate constant (k_2) values were found in the ranges of 0.0014–0.025 g/mg·min. Similarly, Víctor-Ortega et al. (2018) found that pseudo-second order kinetic model is well fitted model for the recovery of phenol from model aqueous solution. However, it is known that the rate constant decreases with an increasing initial phenol concentration. Considering this fact, 6 g/L initial phenols concentration in OMWC1 could explain almost 1.5-fold lower k_2 value than the study of Víctor-Ortega et al. (2016b) in which the removal of phenol from was performed aqueous solution (0.2 g/L) by using Amberlyst A26.

As a result of kinetic data evaluation with intraparticle diffusion model, the plots of PAD series resins exhibited multi-linearity characteristic and they did not pass passed through the origin (Figure 4.4). Thus, intraparticle diffusion was not a rate-limiting step and surface adsorption was dominant. The adsorption of total PCs occurred in two sequential stages on PAD series: the first linear portion describes the macropore diffusion (phase I) while the second linear portion might be mainly owing to micropore diffusion (phase II) (Weber and Morris, 1963).

Table 4.4. Kinetics models and rate constants for the adsorption of total PCs with polymeric resins at 60 g/L dose.

		Pseudo First Order Model				Pseudo Second Order Model			
	$q_{e,exp.}$ (mg/g)	k_1 (min^{-1})	$*q_{e,cal.}$ (mg/g)	R^2	SSE	k_2 (g/mg·min)	$*q_{e,cal.}$ (mg/g)	R^2	SSE
Non- ionic									
XAD16N	27.26	2.49×10^{-2}	11.34	0.8266	717	2.48×10^{-2}	27.62	0.9951	3.67
XAD4	21.51	0.82×10^{-2}	13.59	0.4818	397	1.39×10^{-3}	22.72	0.851	13.94
PAD 610	48.16	2.67×10^{-2}	31.84	0.9608	145	1.53×10^{-3}	51.28	0.9989	0.96
PAD 900	54.97	2.00×10^{-2}	14.27	0.5738	2217	4.92×10^{-3}	55.56	0.9993	0.43
PAD 950	53.09	2.58×10^{-2}	17.27	0.7073	1157	4.44×10^{-3}	53.48	0.9989	1.48
Ionic									
A26	36.87	1.80×10^{-2}	17.51	0.7035	750	3.10×10^{-3}	37.88	0.996	8.03

		Simple Elovich				Intraparticle diffusion					
	$q_{e,exp.}$ (mg/g)	b (mg /g.min)	a (mg/g)	R^2	SSE	k_{1p} ($mg.min^{0.5}$ /g)	R^2	SSE	k_{2p} ($mg.min^{0.5}$ /g)	R^2	SSE
Non- ionic											
XAD16N	27.26	2.06	17.97	0.1868	3.78	4.20	0.7523	0.68	0.27	0.7512	0.01
XAD4	21.51	2.77	3.37	0.5157	6.04	1.21	0.7106	5.17	2.11	0.9991	0.004
PAD 610	48.16	7.79	9.01	0.9738	0.26	2.96	0.9787	0.02	1.22	0.9437	0.03
PAD 900	54.97	3.28	38.02	0.8861	0.18	3.10	0.9669	0.02	0.61	0.9527	0.01
PAD 950	53.09	3.51	35.20	0.8065	0.76	2.45	0.8387	0.13	0.56	0.9038	0.01
Ionic											
A26	36.87	4.59	12.71	0.7549	3.13	9.92	0.9616	0.14	0.66	0.8911	0.02

*calculated data of the equilibrium capacity obtained by the model.

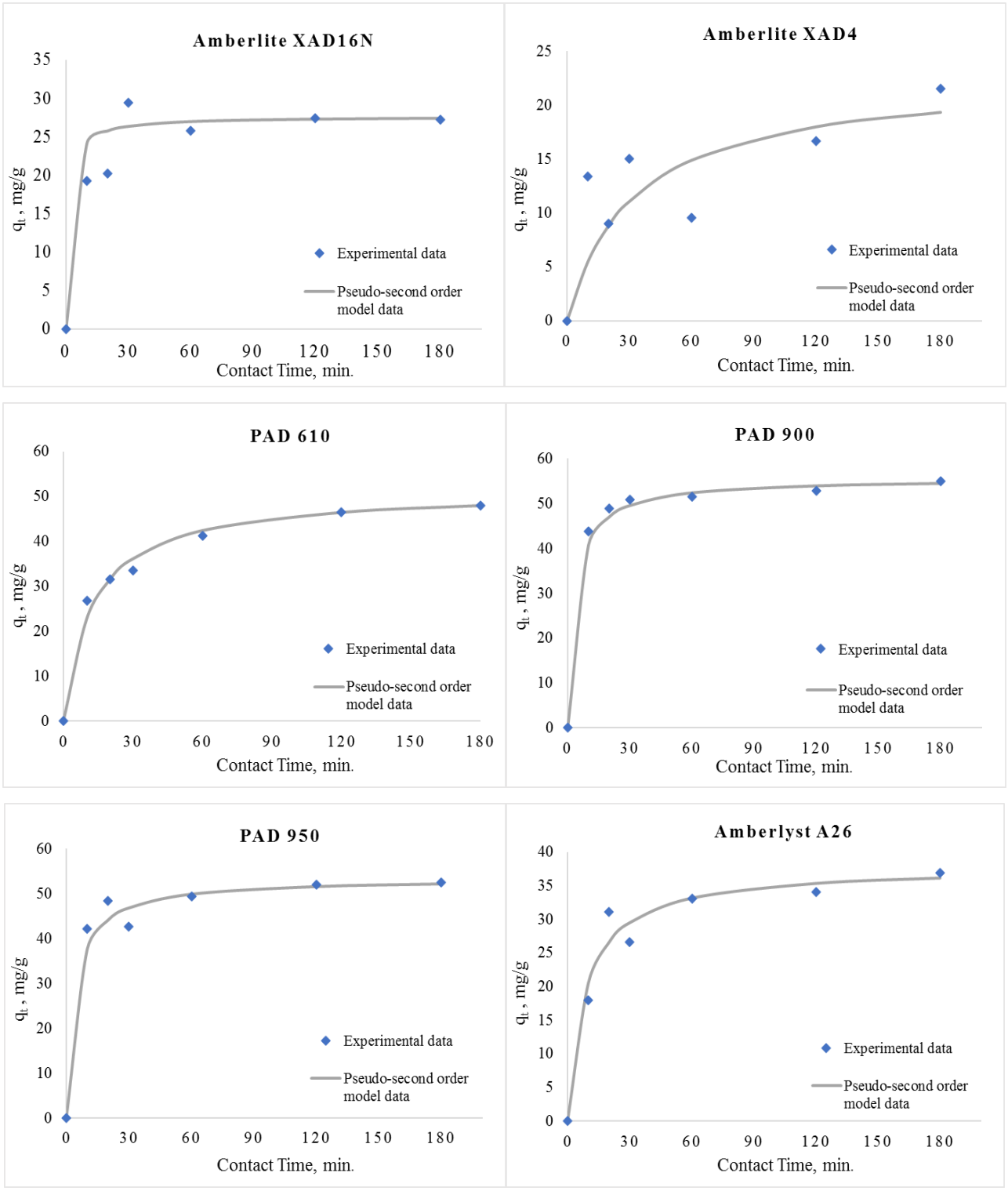


Figure 4.3. Experimental and calculated total PCs adsorption profiles for pseudo-second order kinetic model onto examined six different resins.

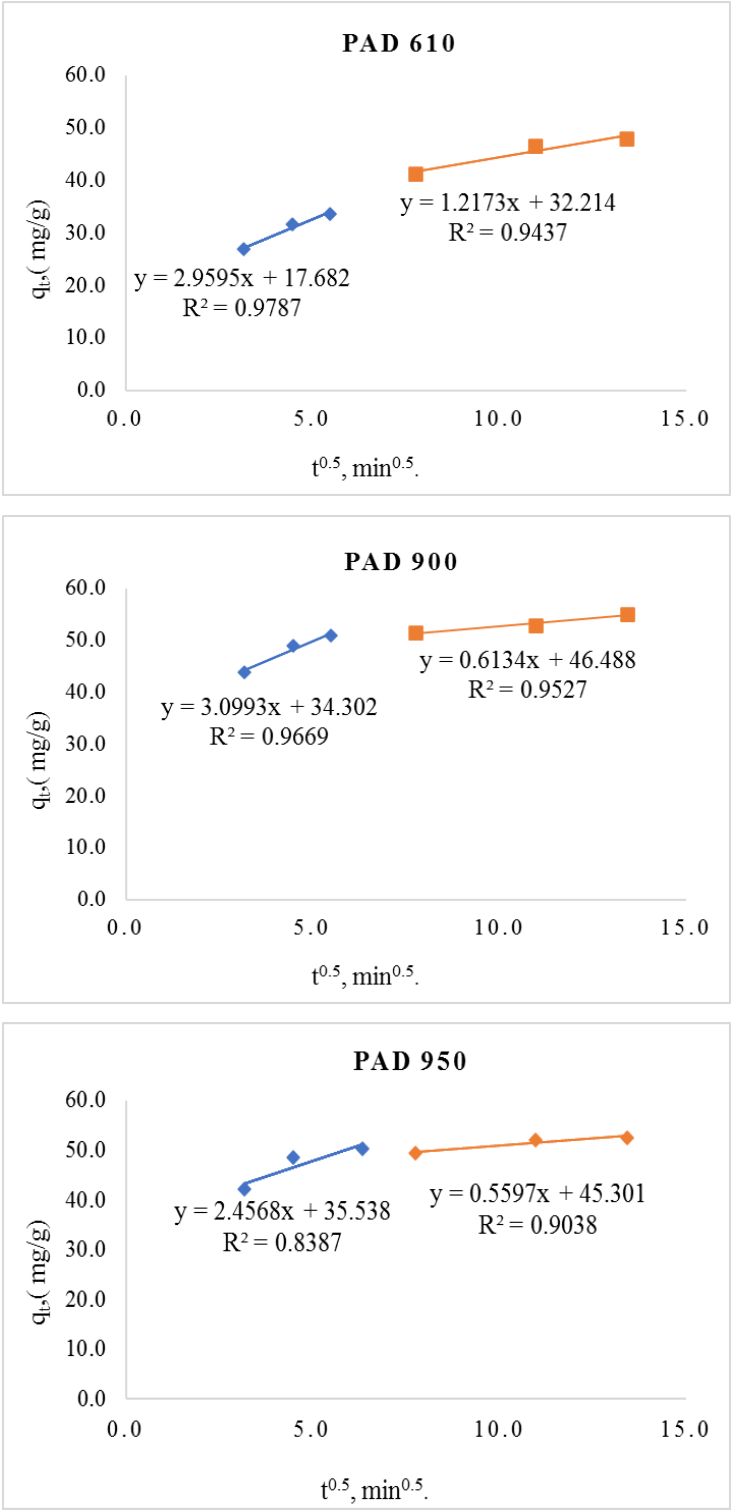


Figure 4.4. Intraparticle diffusion kinetics of total PCs on PAD series.

4.2.2.4. Effect of pH on total PCs extraction. In order to investigate the effect of OMWC1's pH on the resin performance, total PCs uptake experiments were performed in pH range of 3.5-8.5. The obtained results are represented in terms of adsorption efficiency of total PCs (%) as a function of initial pH in Figure 4.5.

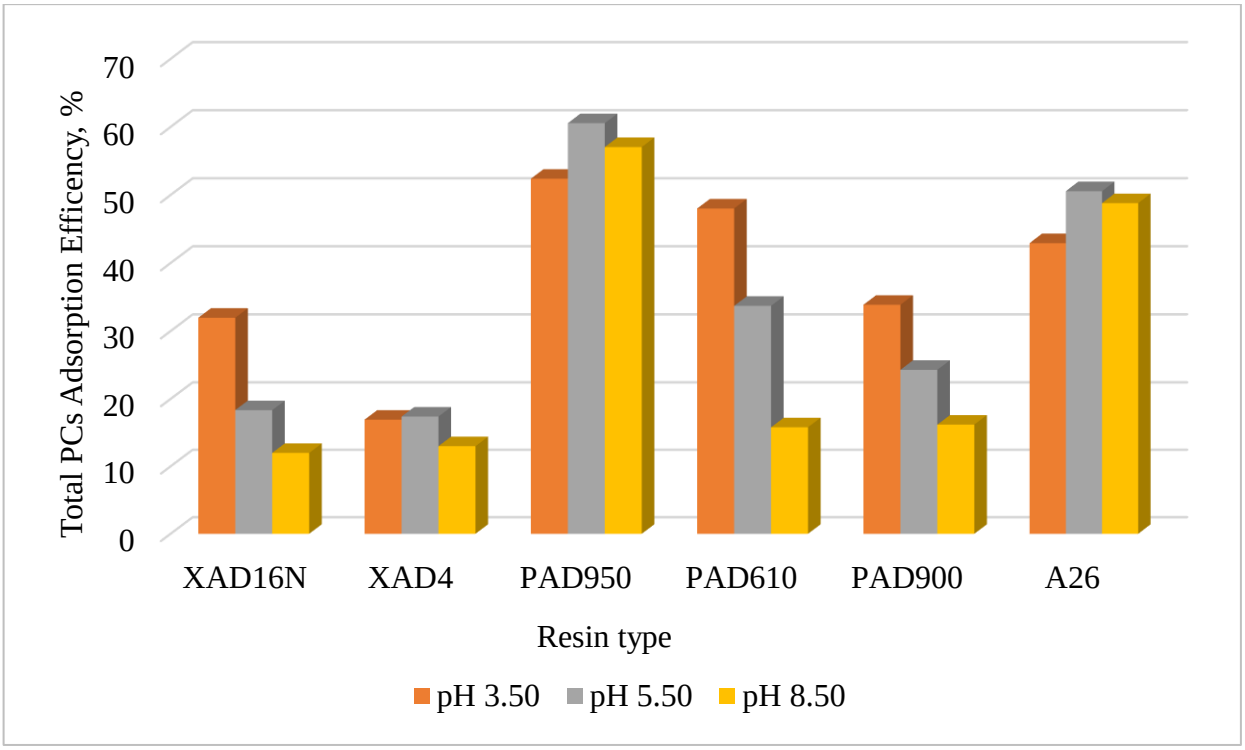


Figure 4.5. Effect of OMWC's pH on the adsorption efficiency of total PCs at ambient temperature (M_{resin}= 60 g/L and contact time= 3h).

As a shown in Figure 4.5, the effect of initial pH on the performance of A26 ionic resin was not pronounced contrary to what was expected mechanism describe in Section 2.3.2 while this effect was remarkable for non-ionic XAD16N, PAD 610 and PAD 900 resins. However, pKa values of PCs found in OMWW vary widely (Kaleh and Geißen, 2016) since these PCs have different function groups which are dissociated depending upon the pH. The adsorption of some PCs that act as weak carboxylic acid (pKa value in the range 4.0–5.0) could favour ion exchange mechanism at natural pH of OMWC. On the other hand, the adsorption with ion exchange mechanism could not be expected at alkaline pH for the PCs which do not include carboxyl functional group and have high pKa values (close to 9.9 at 25°C) (Kammerer et al., 2010; Pinelli et al., 2016). These facts could explain the reason of common observation for each investigated resin that was the deterioration of performance by increasing the pH from 5.5 to 8.5. Similar effect of pH was obtained in the study of Ochando-Pulido et al. (2018) in which A26 and XAD4 were used to recover the PCs from OMWW. Víctor-Ortega et al. (2016b) explained the phenol uptakes on Amberlyst A26 at acidic pH with their neutral.

Consequently, the achieved results of this study could indicate the simultaneous occurrence of forementioned uptake mechanisms on different resins for the extraction of total PCs from complex OMWC matrix. Since pH 5.5 was sufficient to obtain high sorption of total PCs from OMWC, the adjustment of OMWC's pH was not carried out during resin treatment for further experiments.

4.2.3. Elution of Extracted PCs

The desorption of total PCs from the resins were carried out in three stages as mentioned in Section 3.2.2.2. In the first stage (D1), water was used to eliminate carbohydrates and in subsequent stages (D2 and D3) acidified aqueous EtOH (0.5% HCl at 0.1 M, 70% EtOH, v/v) was applied to resin two times to ensure elution of adsorbed PCs. However, washing with water caused the loss of total PCs. Therefore, overall desorption of total PCs from examined resins was evaluated by combination of eluents obtained from three desorption stages. In Figure 4.6(a), the achieved desorption efficiencies at each stage are comparably presented for the investigated resins and in Figure 4.6(b) overall desorption (D_{ov}) performances are shown.

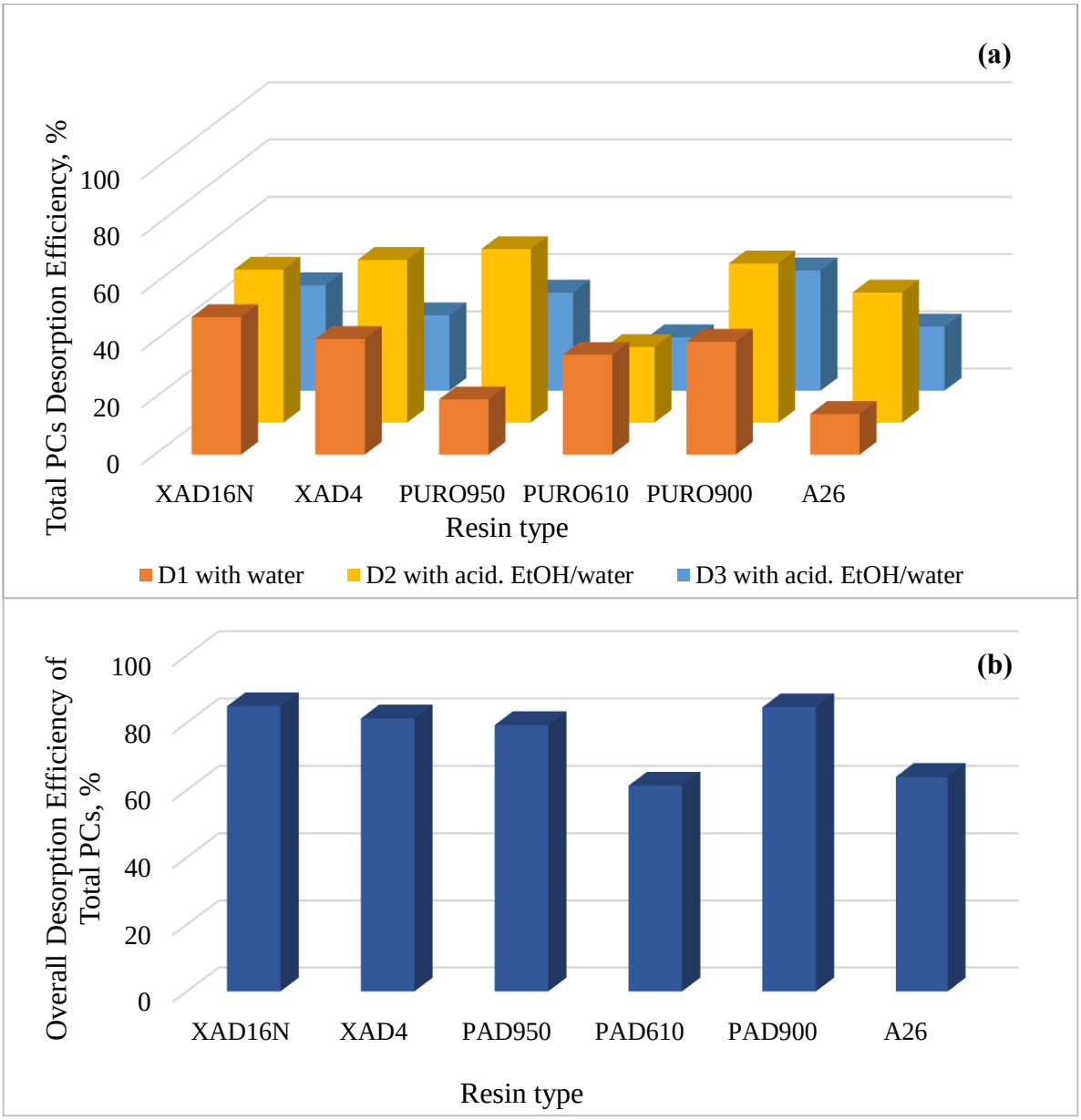


Figure 4.6. Elution of total PCs from non-ionic and ionic resins (M_{resin}= 60 g/L and pH= 5.5).

The capability of acidified EtOH for the desorption of retained PCs from the surface of investigated resins is obvious. This result is in accordance with the results of studies carried out with OMWW (Bertin et al., 2011; Ferri et al., 2011). In the study of Bertin et al. (2011), complete desorption of total PCs was achieved from ENV+ and XAD7 resin by acidified EtOH while 96% and 82% recovery were obtained from XAD16 and IRA96, respectively. In the study of Ferri et al. (2011), 80-90% of adsorbed total phenolic acids were recovered by using same desorption solvent.

4.2.4. Overall Performance of Process I

The recovery efficiency of SPE was determined by considering both initial concentrations of compounds and their overall desorption (equation 4.2) and the results are comparably presented for all investigated resins in Figure 4.7.

$$R, \% = \frac{C_{dov}}{C_0} \times 100 \qquad (4.2)$$

where R is the recovery efficiency, C_{dov} and C_0 is overall desorbed concentration of compounds from resin surface and initial concentration of target component of OMWC1, respectively.

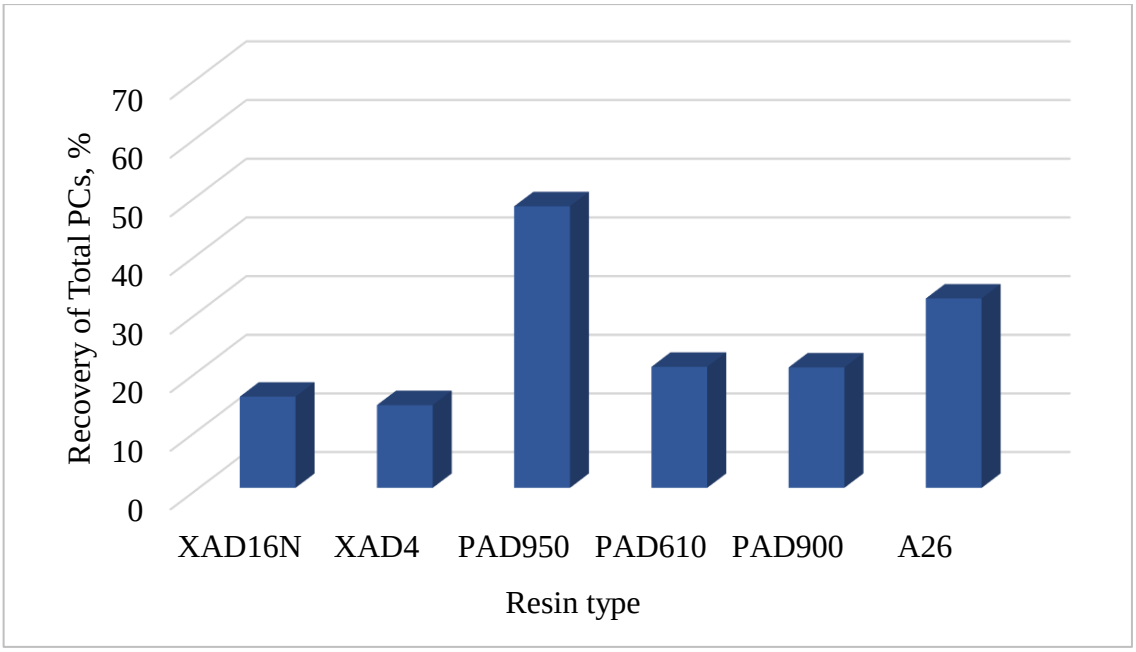


Figure 4.7. Recovery of total PCs by SPE ($C_{initial}$= 5.93 g/L).

As clearly seen in Figure 4.7, non-ionic PAD 950 gave highest recovery efficient of total PCs from OMWC1 among examined resins, followed by ionic A26.

To evaluate the performance of resins forementioned discussions have been performed only for total PCs. However, besides these compounds OMWC contains various organic components which could cause a competition during adsorption and could reduce the purity of eluted target compound. The potential effects of the organic components of OMWC, namely carbohydrates and proteins on the efficiency of SPE were determined during adsorption and desorption stages. The obtained results of these organic compounds with Amberlite XAD16N, PAD 950, and Amberlyst A26 resins are shown in Figure 4.8.

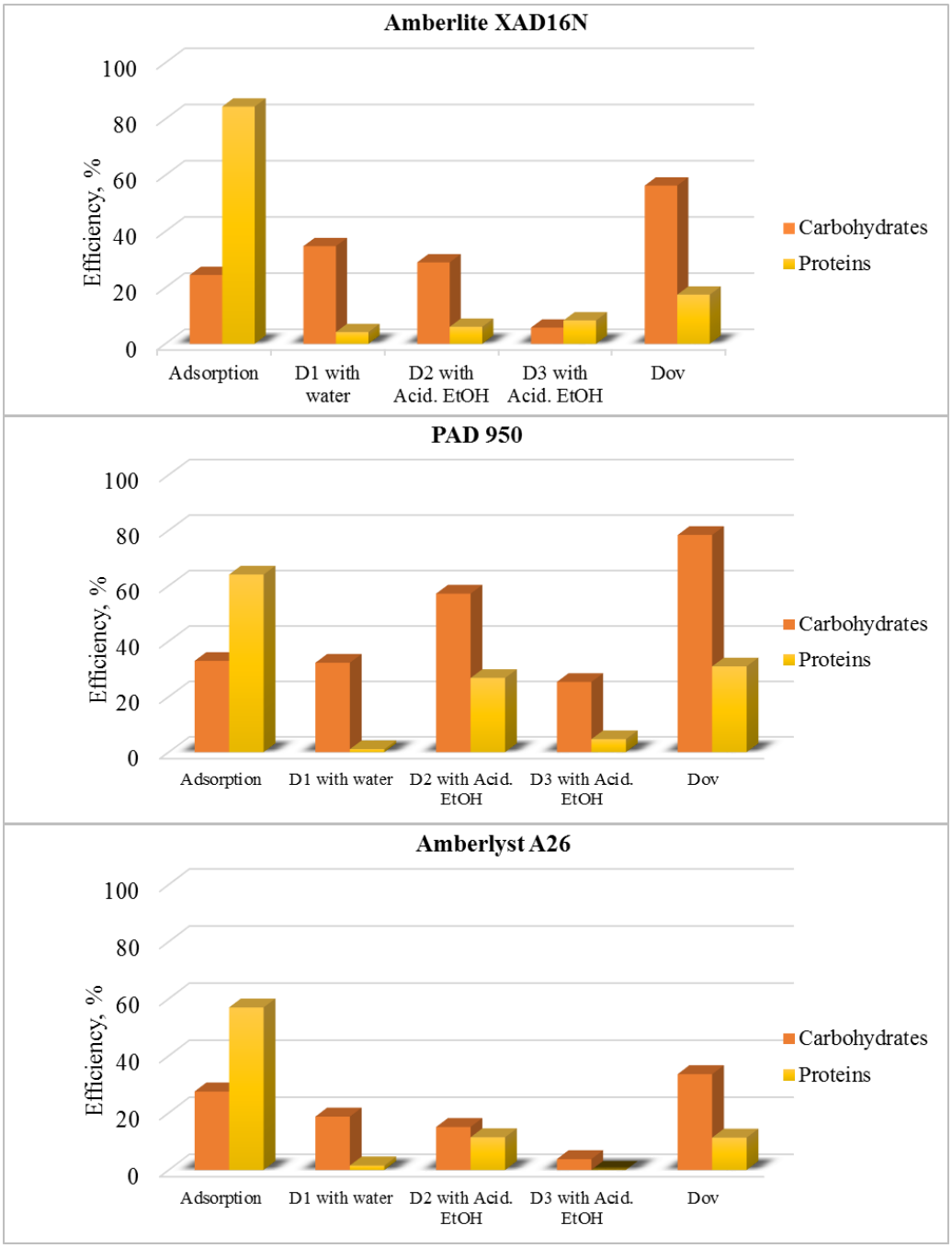


Figure 4.8. SPE efficiency of carbohydrates and proteins found in OMWC1 (M_{resin}= 60 g/L and pH= 5.5).

Considerably high sorption of proteins was achieved with non-ionic and ionic resins (Figure 4.8). Although, mass transfer of the organic compounds to the resin surface could be affected by their initial concentrations as mentioned previously the selectivity for the sorption and desorption is the critical factor on the overall performance of SPE process. In this respect, the recovery of carbohydrates and proteins by SPE were also evaluated and the results achieved for XAD16N, PAD 950, and A26 resins are presented in Figure 4.9.

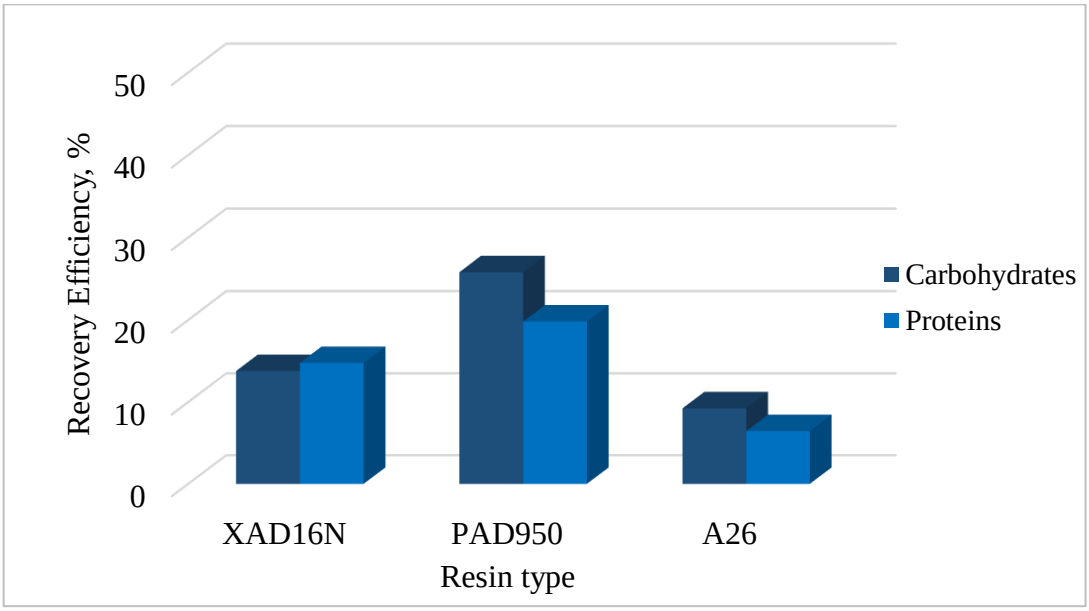


Figure 4.9. Recovery of carbohydrates ($C_{initial}$ = 4.5 g/L) and proteins ($C_{initial}$ = 0.97 g/L) by SPE.

PAD 950 resin exhibited high recovery efficiency for all investigated organic components of OMWC1. On the other hand, the lower recovery of proteins and carbohydrates by using ionic A26 resin could indicate the selectivity of this resin for target phenolic antioxidants.

The overall performance of Process I in terms of the recovery of total PCs and the separation of proteins and carbohydrates was evaluated for PAD 950, A26 and XAD16 resins. The achieved results are summarized in Table 4.5.

Table 4.5. Overall performance of Process I with PAD 950, A26 and XAD16N resins.

		SPE with PAD 950	SPE with A26	SPE with XAD16N
HTyr	Eluted C_{HTyr}, g/L	5.04	3.99	1.04
Total PCs	Adsorbed $C_{Total\ PCs}$, g GAE/L	3.59	3.00	1.09
	Eluted $C_{Total\ PCs}$, g GAE/L	2.85	1.91	0.93
	Recovery (%)	48.02	32.32	15.57
Carbohydrates	Adsorbed $C_{carbohydrate}$, g glucose/L	1.40	0.68	1.04
	Eluted $C_{Carbohydrate}$, g glucose/L	1.09	0.39	0.59
	Removal, %	74.21	90.76	86.20
Proteins	Adsorbed $C_{Proteins}$, g BSA/L	0.62	0.55	0.82
	Eluted $C_{Proteins}$, g BSA/L	0.19	0.07	0.13
	Removal, %	80.18	92.67	85.22

PAD 950 and A26 resins were found more effective for the recovery of total PCs and HTyr, while higher removal rates of carbohydrates and proteins were achieved with A26. Therefore, these resins were considered in Process II.

4.3. Recovery of Total PCs from OMWC2 by Integrated-Extraction Process

In order to improve the selective recovery of polyphenolic antioxidants, mainly HTyr from OMWC, highly concentrated olive mill waste, OMWC2 was subjected to integrated extraction process that consists of ATPE and SPE.

4.3.1. Aqueous Two-Phase Extraction of PCs from OMWC2

The first step of integrated extraction process was ATPE with EtOH and $(NH_4)_2SO_4$ which exhibited high performance for OMWC obtained from three-phase centrifugal system in a previous study (Çelik, 2018; Çelik et al., 2021).

4.3.1.1. Phase diagram of EtOH/salt ATPE system. The phase diagram of EtOH/$(NH_4)_2SO_4$ ATPE which is necessary for the design of the system was constructed in the presence of 13 g/L OMWC2. In Table 4.6, the mass fractions of system components on binodal curve are given and in Figure 4.10, equilibrium diagram together with the pictures of the systems is shown.

Table 4.6. Mass fraction of ATPE system components.

EtOH/ $(NH_4)_2SO_4$/ Water System			
Binodal Points	EtOH (%, w/w)	$(NH_4)_2SO_4$ (%, w/w)	Water (%, w/w)
1	43.5	5.7	50.8
2	28.4	13.5	58.2
3	17.2	25.5	57.4
4	4.9	39.1	56

As seen from the picture inset in Figure 4.10, the volume ratio of the top and bottom phases exhibits variation along the binodal curve which separates biphasic and monophase regions. It should be highlighted that EtOH rich phase corresponded to the top phase while the salt rich phase corresponded to the bottom phase. The phase-separation ability of the system was enhanced by the increment of salt concentration as shown in the picture. Higher amount of salt in the system led to an increase in the volume of bottom phase due to salting-out effect. However, further increment led to the formation of salt precipitates.

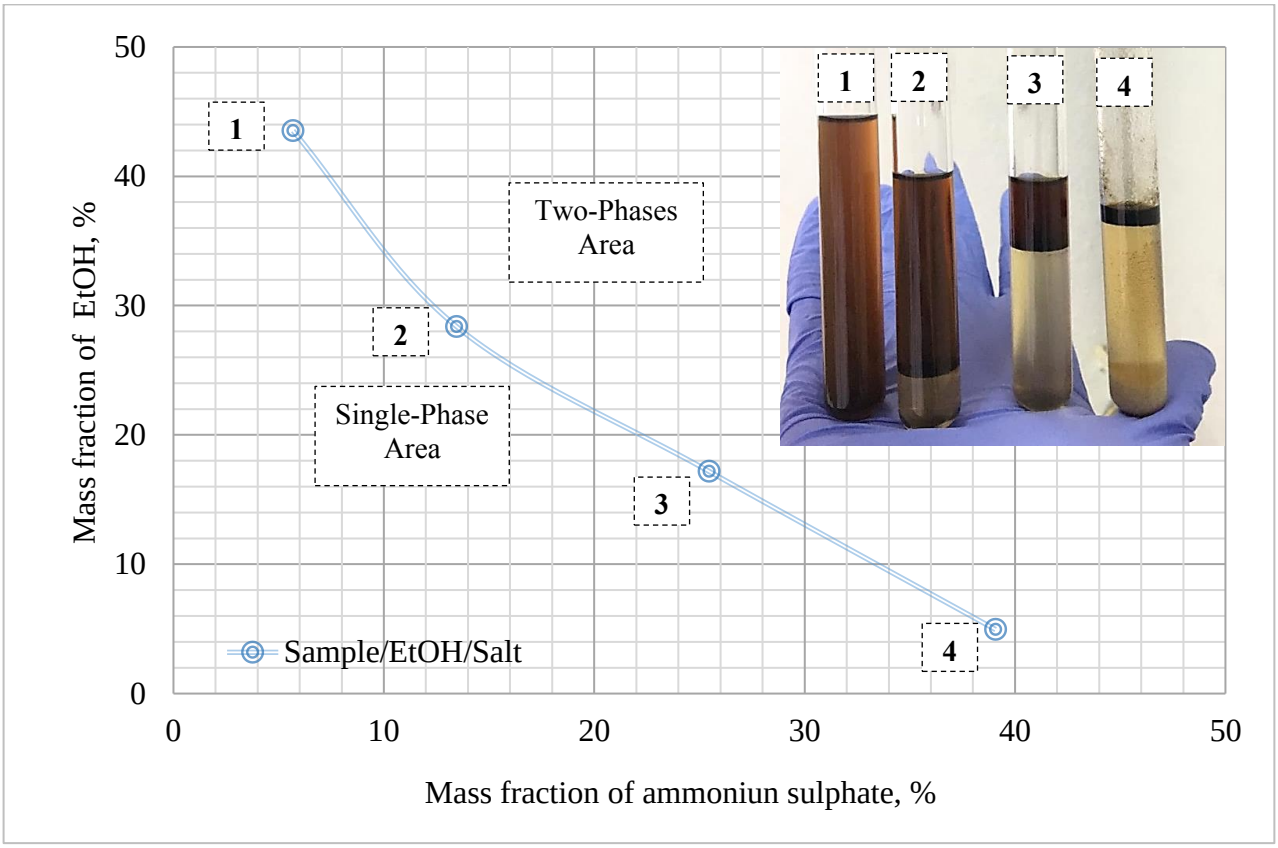


Figure 4.10. Phase diagram of EtOH/$(NH_4)_2SO_4$ ATPS with 13 g/L OMWC2.

4.3.1.2. Effect of alcohol/salt concentrations on the partitioning of total PCs. The effect of system components, alcohol, and salt concentrations, on the partitioning of the organic components of OMWC2 was investigated. Partition coefficient (K), volume ratio (V_r), and extraction yield (EY) calculated by following equations were used to evaluate the efficiency of ATPE system (Wu et al., 2014):

$$K = \frac{C_T}{C_B} \tag{4.3}$$

where C_T and C_B are the concentrations of target component of OMWC2 in the top and bottom phases, respectively.

$$V_r = \frac{V_T}{V_B} \tag{4.4}$$

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

$$EY_j, \% = \frac{(C_j x V_j)}{(C_B x V_B) + (C_T x V_T)} x100 \tag{4.5}$$

where EY_j is extraction yield of target components of OMWC2 in the top or bottom phase.

Two series of experiments were conducted in two-phase area of phase diagram (Figure 4.10). In the first series of experiments the mass fraction of $(NH_4)_2SO_4$ was decreased from 14% to 10 % at constant EtOH mass fraction, 36% while in the second series of experiments, alcohol mass fraction was increased from 30% to 36% at fixed salt mass fraction, 14%. The performances of each investigated system for the extraction of total PCs are presented in Table 4.7.

Table 4.7. ATPE system performance for total PCs recovery.

EtOH, 36% (w/w)							
System	$(NH_4)_2SO_4$ (%, w/w)	V_r	Total PCs , mg GAE/g		K	EY_j, %	
			Top	Bottom		Top	Bottom
1	14	2.1	31.74	1.60	19.8	97.7	2.3
2	12	2.7	41.11	7.20	5.7	93.8	6.2
3	10	5.7	46.18	11.61	4	95.8	4.2
$(NH_4)_2SO_4$, 14% (w/w)							
System	EtOH (%, w/w)	V_r	Total PCs , mg GAE/g		K	EY_j, %	
			Top	Bottom		Top	Bottom
1	36	2.1	31.74	1.60	19.8	97.7	2.3
4	33	2.4	35.31	7.41	4.8	91.9	8.1
5	30	5.4	41.26	8.12	5.1	92.4	7.6

[GAE: Gallic acid equivalent].

The results of the experiments reveal that higher K values of total PCs were achieved at lower V_r ratio. It could be attributed to the enhancement of salting out effect by pulling of water molecules from EtOH rich top phase to salt rich bottom phase (Wang et al., 2010; Nainegali et al., 2019). The affinity of total PCs to the top phase can also be attributed to the mildly acidic medium provided by the addition of $(NH_4)_2SO_4$ to the system since molecular form of phenols are pre-dominant at acidic pH value (Wu et al., 2014; Xavier et al., 2017). Only, 6% increment in alcohol mass fraction of the system considerably improved the partitioning of total PCs in the top phase.

An observation of semi-solid particulates at the interface of top and bottom phases could be ascribed to the precipitation of proteins found in the composition of OMWC2 since the concentration of proteins decreased in the top phase. However, the formation of higher precipitates by increasing the amount of $(NH_4)_2SO_4$ caused a decrease in total concentration of PCs in the system owing to interaction between total PCs and proteins. This fact was pronounced for increment of alcohol concentration in the system.

4.3.1.3. Partitioning of organic components of OMWC2. The purpose of ATPE system application was not only to recover phenolic antioxidants from OMWC2 but also to minimize the coextraction of non-target organic compounds. Therefore, the partitioning of carbohydrates and proteins were also evaluated in the ATPE system with 36% EtOH/ 14% $(NH_4)_2SO_4$ in which the highest partition coefficient was achieved for total PCs. In addition, the fate of HTyr which is the most abundant polyphenolic antioxidant in OMWW (Rahmanian et al., 2014) was quantified in top and bottom

phases. In Table 4.8, top and bottom phase concentrations of forementioned organic components of OMWC2 are listed together with the performance parameters of ATPE system.

Table 4.8. Partitioning of OMWC2 components in EtOH/ $(NH_4)_2SO_4$ ATPE system.

EtOH (36%, w/w) / (NH4)2SO4 (14%, w/w)						
	Phases				EY_j, %	
	Top		Bottom		K	Top	Bottom
Total PCs	31.74	mg GAE/g	1.60	mg GAE/g	19.8	97.70	2.30
HTyr	35.15	mg/g	10.37	mg/g	3.4	87.89	12.11
Carbohydrates	6.68	mg glucose/g	20.46	mg glucose/g	0.3	41.15	58.85
Proteins	6.06	mg BSA/g	4.27	mg BSA/g	1.4	75.24	24.76

[GAE: Gallic acid equivalent].

The results presented in Table 4.8, clearly reveals that total PCs exhibited remarkably higher affinity to the alcohol rich top phase compared to the non-target components of OMWC2. ATPE system provided high extraction yield for both total PCs and HTyr that were 98% and 88%, respectively. The proteins and carbohydrates in OMWC2 were partially removed from the top phase.

4.3.2. Overall Performance of Process II

In the second stage of the integrated process (Process II), SPE applied to the extract of ATPS for further purification of polyphenolic antioxidants. By taking into account the high efficiency of non-ionic PAD 950 and ionic A26 resins as mentioned in Section 4.2.4, SPE trials were performed only with these resins. The performance of Process II was evaluated by both the recovery of total PCs and HTyr, and the removal rates of carbohydrates and proteins (Table 4.9).

Table 4.9. Overall performance of Process II.

		ATPE+SPE with PAD 950	**ATPE+SPE with A26**
HTyr	Eluted C_{HTyr}, mg/g	21.77	34.30
	Recovery, %	43.30	68.28
Total PCs	Eluted $C_{Total\ PCs}$, mg GAE/g	31.46	14.41
	Recovery, %	41.70	19.10
Carbohydrates	Eluted $C_{Carbohydrates}$, mg glucose/g	4.04	2.64
	Removal, %	85.38	90.43
Proteins	Eluted $C_{Proteins}$, mg BSA/g	0.11	1.03
	Removal, %	99.90	99.08

As can be deduced from Table 4.9, integrated process was able to efficiently separate total PCs and HTyr from complex matrix of OMWC2. The performances of ionic and non-ionic resins were different in the integrated process. The utilization of PAD 950 resin in the integrated process provided higher recovery of total PCs while the process with A26 resin was selective for HTyr. Moreover, the performance of the process with A26 resin for the removal of carbohydrates was slightly higher. It should be also noted that ATPE stage of the process caused the precipitation of organic components of OMWC2.

4.4. Antioxidant Activities of OMWCs and Extracts

The antioxidant activities of the extracts of OMWC1 and OMWC2 achieved with Process I and Process II were evaluated with two different methods: DPPH and ABTS assays that are used for complex matrices and individual compounds (Henriquez et al., 2002). The antioxidant activities of OMWC samples and final OMWC extracts are comparably shown in Table 4.10. The results are also expressed in terms of ascorbic acid and Trolox equivalent which are widely used standards.

Table 4.10. Antioxidant activities of OMWC samples and their extracts achieved by Process I and Process II.

	OMWC1[1]		**OMWC2[2]**	
	DPPH, g AAE/L	**ABTS,** g Trolox/L	**DPPH,** mg AAE/g	**ABTS,** mg Trolox/g
Samples	4.50	5.72	44.44	67.85
	Process I with PAD 950		***Process II with PAD 950***	
Extracts	2.14	2.70	14.31	50.72
	Process I with Amberlyst A26		***Process II with Amberlyst A26***	
Extracts	1.53	1.65	4.68	14.69

[AAE: Ascorbic Acid Equivalent] (1; pre-treated with acidification, 2; raw sample).

High antioxidant activity consistent with the total PCs content was detected for highly concentrated OMWC2. DPPH and ABTS antioxidant activity tests exhibited relatively similar results for both OMWC1, and its extracts achieved by using two different resins. The results of these assays were also compatible with the total PCs and HTyr contents of the extracts. Process I yielded 2.85 g/L and 1.91 g/L total PCs, and 5.04 and 3.99 g/L HTyr with PAD 950 and A26 resins, respectively. On the other hand, the results of two different activity assays for OMWC2 extracts obtained by integrated process were quite different.

The antioxidant activity determined by ABTS assay was 3-fold higher than those of DPPH assay. Although similar to the extracts of OMWC1, the higher total PCs content corresponded to higher antioxidant activity in OMWC2 extracts this relationship was not observed for HTyr content of OMWC2 extracts. The evaluation of antioxidant activity could help to understand the effective separation of target polyphenolic compounds by the application of different processes. Although ionic A26 resin exhibited selective separation of total PCs from carbohydrates and proteins found in OMWC extracts by the application of Process I and Process II, antioxidant activities of OMWC extracts treated by this resin were lower than those of non-ionic PAD 950.

4.5. Overall Performance Evaluation of the Recovery Processes

The overall performances of Process I and Process II with PAD 950 which exhibited high performance for both OMWCs, were compared in terms of total PCs and HTyr recoveries, and antioxidant activities in order to deduce the most productive recovery process (Table 4.11).

Table 4.11. Comparison of Process I and Process II performances.

		Process I with OMWC1*	**Process II with OMWC2**
		PAD 950	**PAD 950**
HTyr	C_{HTyr}, mg/g	4.88	21.77
Total PCs	$C_{Total\ PCs}$, mg GAE/g	2.76	31.46
DPPH	mg AAE/g	2.07	14.31
ABTS	mg Trolox/g	2.61	50.72

[*density= 1.0334 g/cm^3].

As clearly seen from Table 4.11, Process II with PAD 950 resins was more efficient for the recovery of phenolic antioxidant than compared to Process I. Moreover, the concentration of OMWW at higher rates gave satisfactory results for the recovery of natural antioxidants from OMWW.

5. CONCLUSIONS AND RECOMMENDATIONS

Total PCs with the antioxidant properties found in OMWW are potential resource for some industries. In the present study, the recovery of phenolic antioxidants mainly HTyr from OMWW was investigated with the samples concentrated at two different ratios by the application of SPE (Process I) and ATPE+ SPE (Process II) processes. Based on the results of this investigation, the main conclusions are summarized below:

- The OMWCs achieved by the MVR evaporation of OMWW have different characteristics depending on the rate of evaporation operation. While both OMWCs demonstrated a mildly acidic nature, highly concentrated OMWC2 was rich in total PCs, proteins, and carbohydrates.

- Acidification of liquid concentrated sample, OMWC1, to pH= 3 as pre-treatment resulted in 56% decrease in the content of proteins as result of their precipitation.

- Screening of six different polymeric nonionic (Amberlite XAD16N and XAD4, PuroSorb PAD 610, PAD 900, and PAD 950) and ionic (Amberlyst A26) resins used for SPE of pretreated OMWC1 revealed that regardless of resin type, the pH of matrix and the dose of resin influenced the extraction rate. As opposed to the expectations, the extraction rate was not enhanced with higher dose of resin in the range of 30-90 g/L and with the higher pH in the range of 3.5-8.5.

- Pseudo-second order kinetic model generally well explained experimental sorption data. The rate constants (k_2) of total PCs in OMWC1 were in the range of 0.0014–0.025 g/mg.min with all investigated resins, at 60 g/L dose and at pH=5.5.

- The elution rate of target polyphenolic compounds from the resins performed at three stages with water and acidified EtOH was more than 60%.

- Process I with non-ionic PAD 950 and ionic Amberlyst A26 resins provided 48% and 32% recovery rates of total PCs found in OMWC1, respectively. In addition, the removal rates of carbohydrates and proteins were 74-90% and 80-92%, respectively.

- In order to enhance the recovery of phenolic antioxidants, highly concentrated OMWC2 was subjected to Process II consisted of ATPE based on alcohol/ inorganic salt and SPE with PAD950 or A26 resins. The variation of ATPE system composition highly influenced the partitioning of concentrated waste matrix components between EtOH rich top phase and $(NH_4)_2SO_4$ rich bottom phase. The partition coefficient of total PCs, K was 4 with the ATPE system having 36% EtOH and 10% $(NH_4)_2SO_4$ while an increase in salt mass fraction to 14% resulted in a K value of 19.8 by eliminating the significant portion of carbohydrates.

- In Process II, the treatment of top phase extracts of ATPE with PAD 950 or A26 resins provided high elimination rates of proteins (almost complete) and carbohydrates (85-90%) from OMWC2. Both PAD 950 and A26 resins allowed high recovery for HTyr (43-68%) than total PCs (41-19%), respectively. While Process II yielded 21.77-34.30 mg/g HTyr from OMWC2, Process I extracted 4.88 -3.86 mg/g HTyr from OMWC1 with PAD 950 and A26 resins, respectively.

- The antioxidant activities of samples and the extracts were in accordance with total PCs contents. Although ionic A26 resin provided more selective separation of organic components of OMWC compared to PAD 950 resin, antioxidant activities of the extracts obtained by Process I and Process II were lower than that of non-ionic PAD 950. As opposed to OMWC1, HTyr content of OMWC2 extracts were not consistent with antioxidant activity. This results clearly reveals that OMWC includes compounds other than HTyr having antioxidant activity. Therefore, detailed analysis of such compounds during the extraction process is important.

- This study revealed that highly concentrated OMWW can be potential resource of antioxidants and Process II with PAD 950 gave satisfactory results for the recovery of total PCs. MWR evaporation enhanced the recovery efficiency of phenolic antioxidants from OMWW. Furthermore, the concentration of the wastewater could reduce the consumption chemicals in extraction process. However, the reuse of solvent, salt, and resin in the extraction process should be evaluated to estimate the cost of recovered antioxidants that have high market value.

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APPENDIX A: CALIBRATION CURVES OF SAMPLE CHARACTERIZATION METHODS

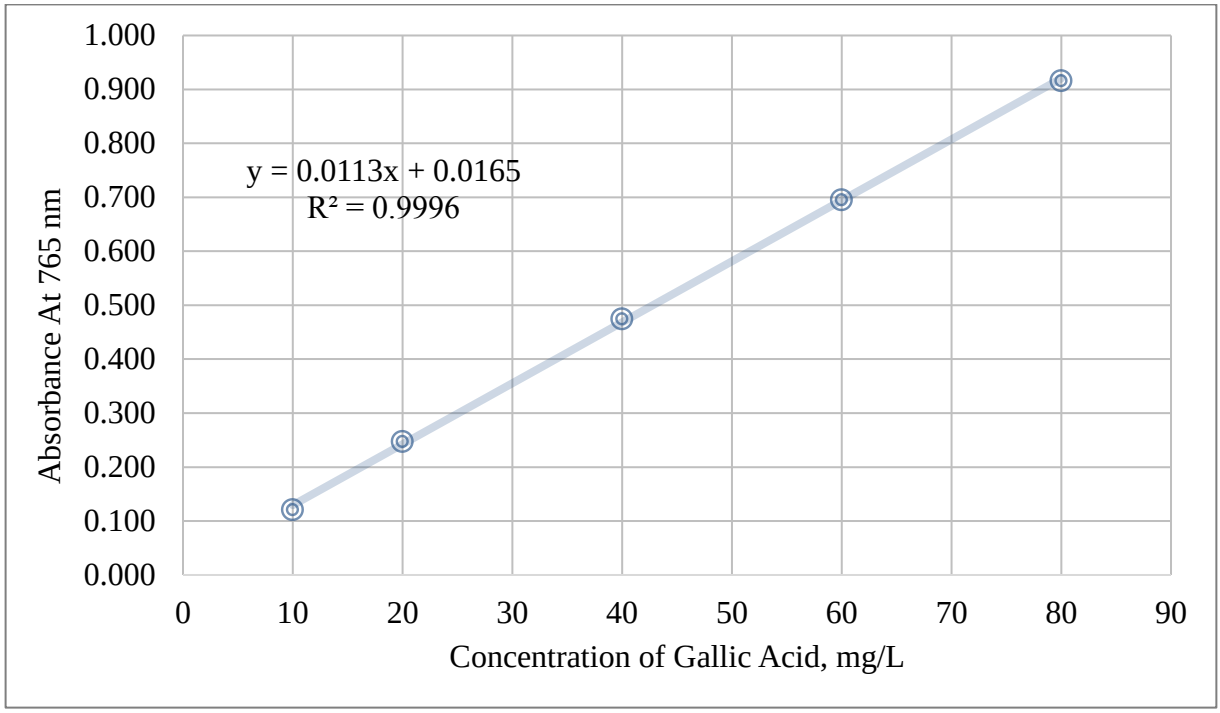

Figure A.1. Calibration curve of gallic acid by Folin-Ciocalteu method for total phenols analysis.

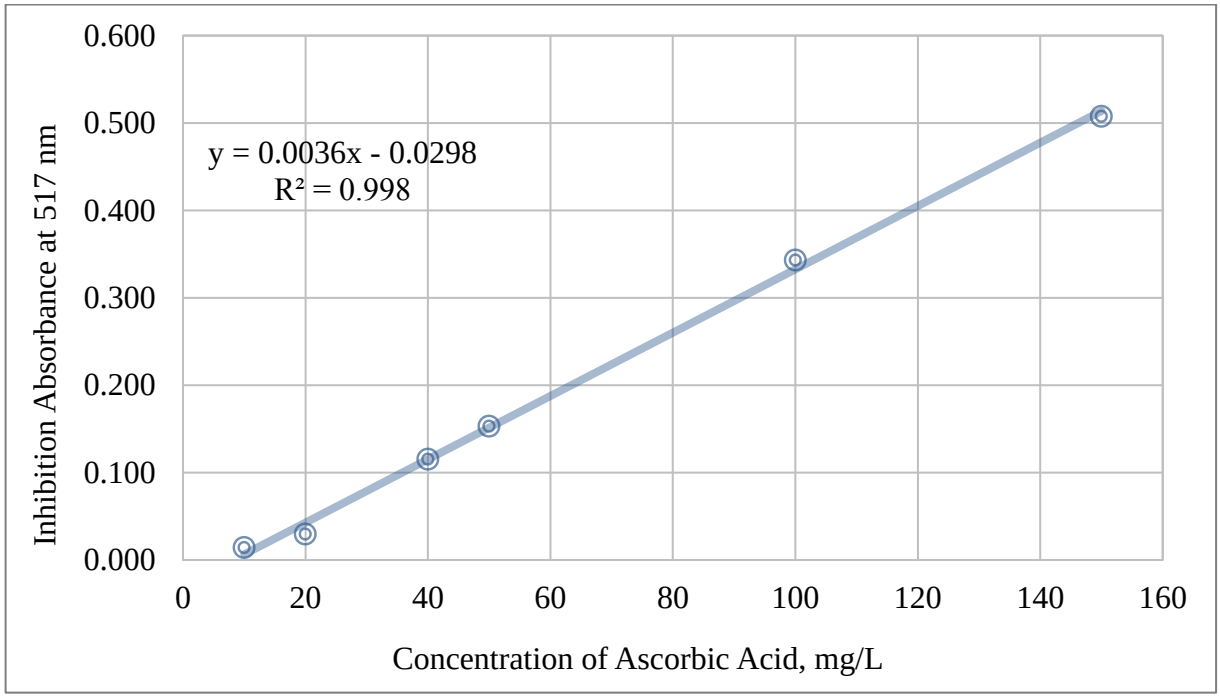

Figure A.2. Calibration curve of ascorbic acid by DPPH method for the determination of antioxidant activity.

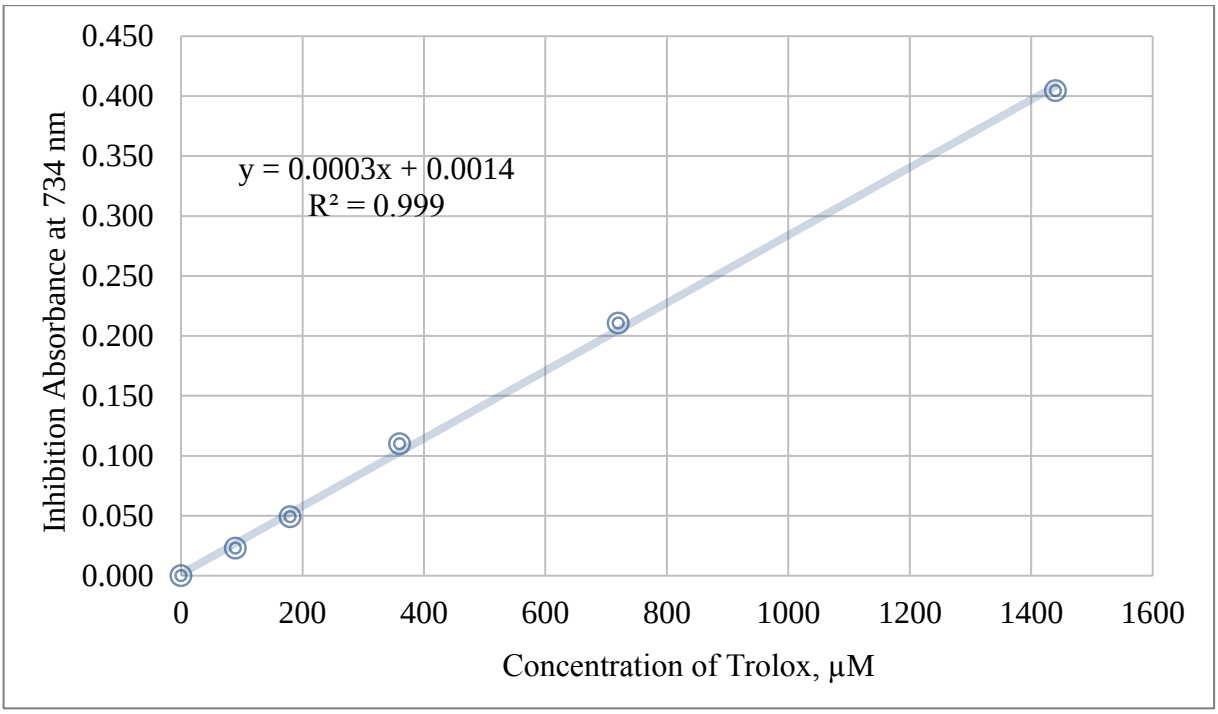

Figure A.3. Calibration curve of Trolox by ABTS method for the determination of antioxidant activity.

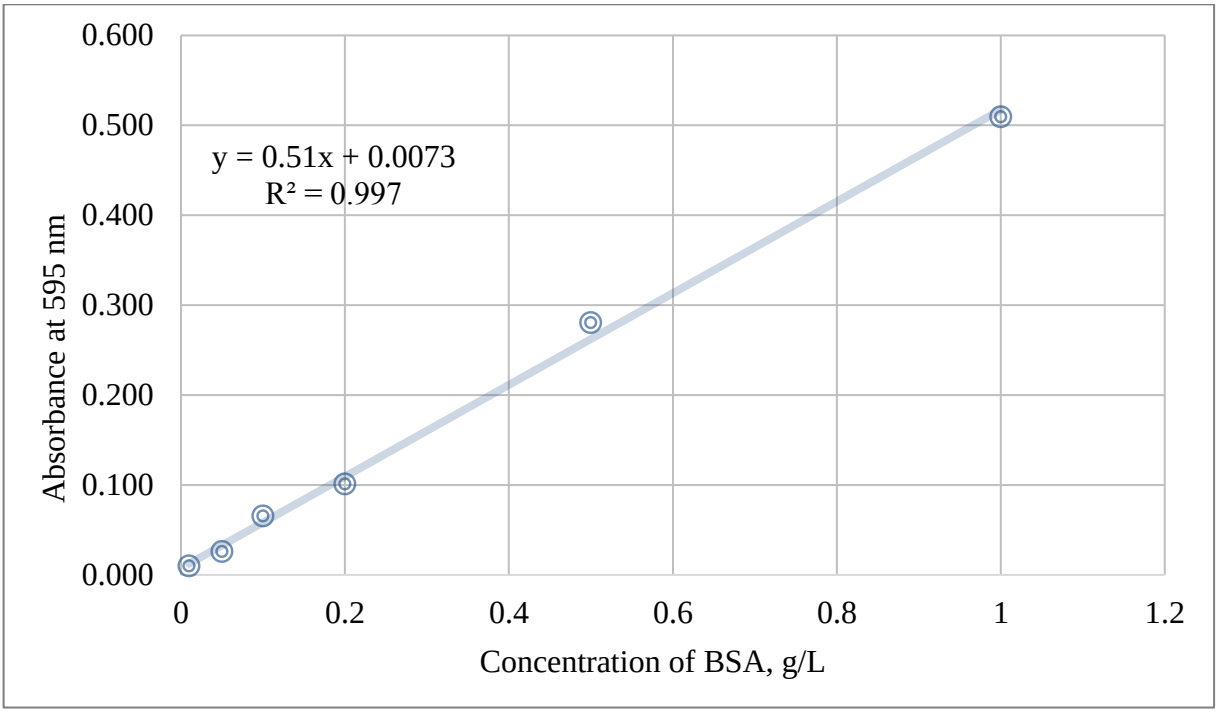

Figure A.4. Calibration curve of BSA by Bradford method for protein analysis.

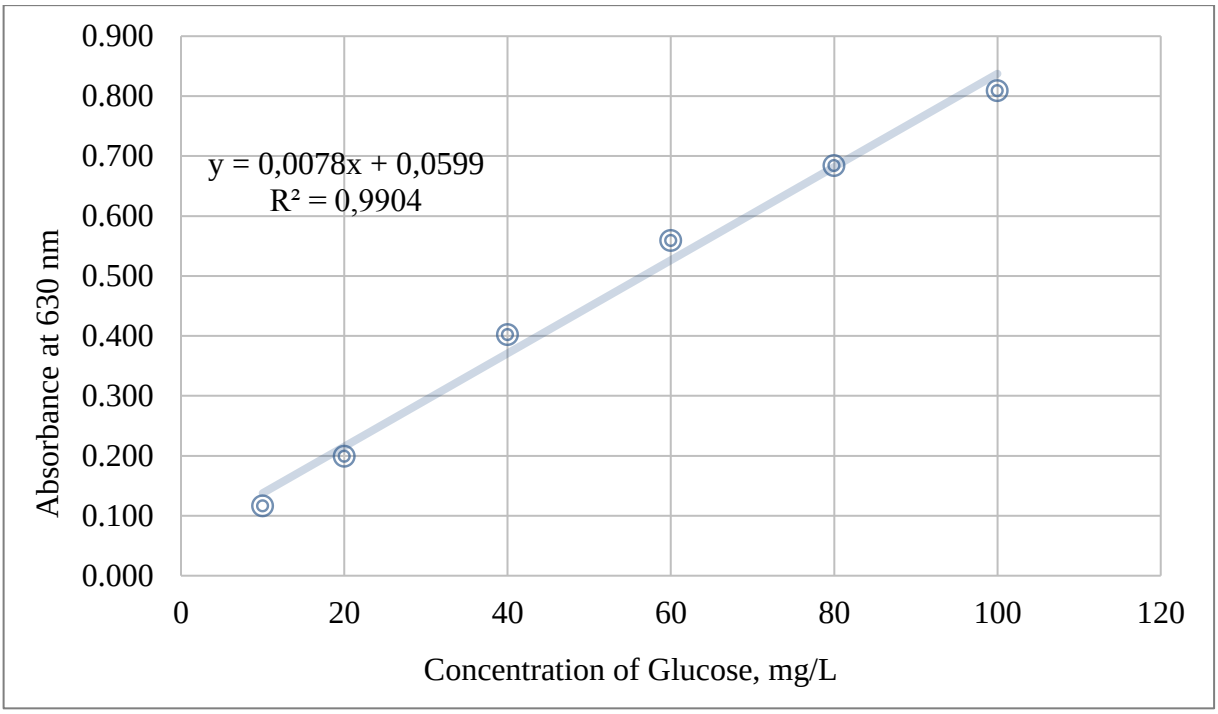

Figure A.5. Calibration curve of glucose by Anthrone method for carbohydrate analysis.

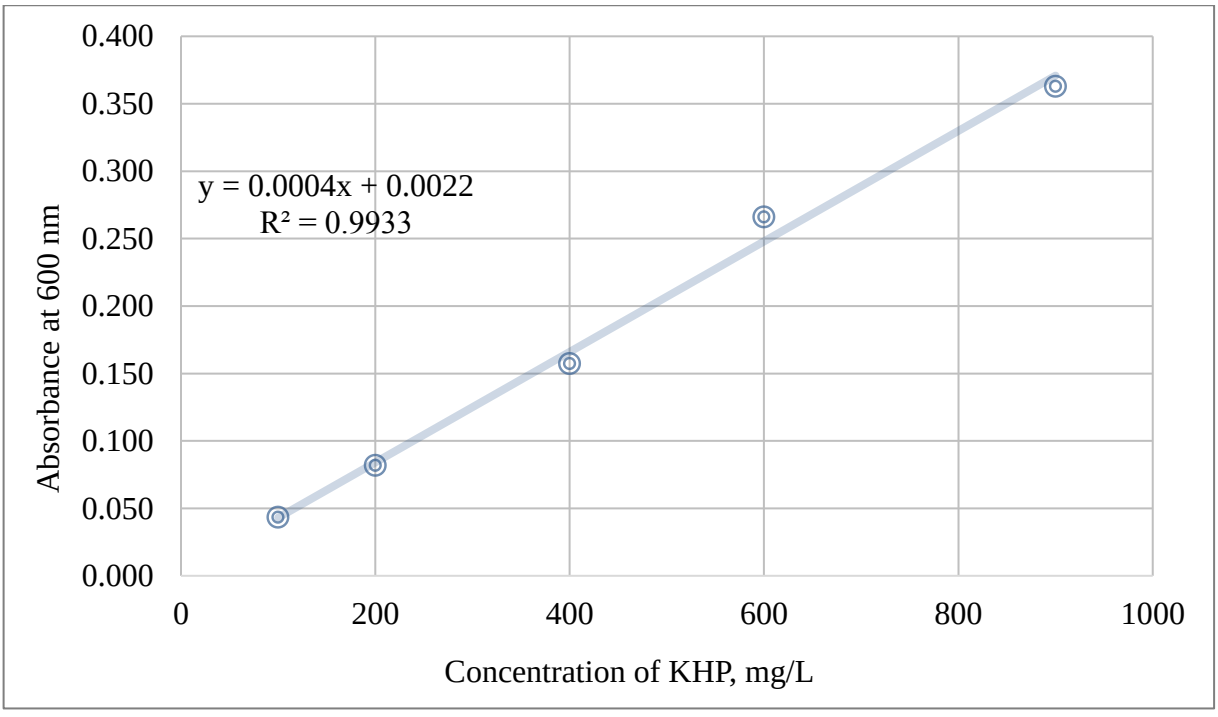

Figure A.6. Calibration curve of KHP by Closed Reflux method for COD analysis.

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

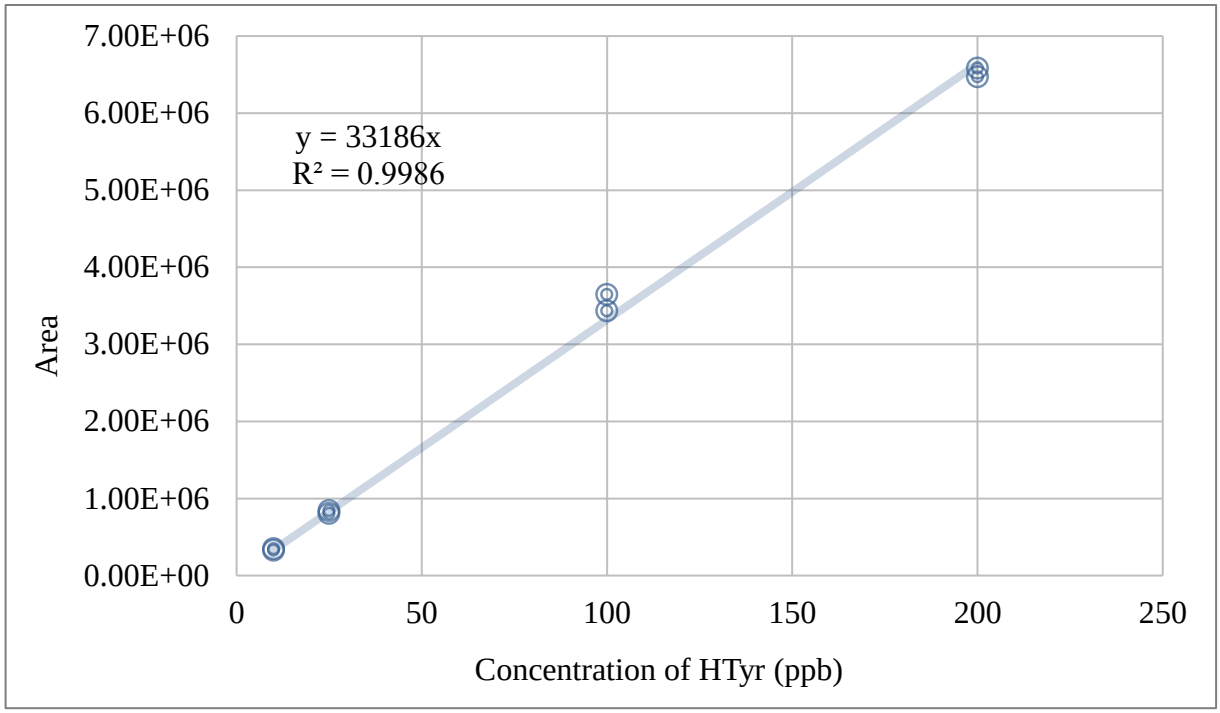

Figure B.1. Calibration curve of HTyr by LC-MS/MS.