PURIFICATION OF ANTIOXIDANTS FROM OLIVE OIL MILL WASTE BY AQUEOUS TWO-PHASE EXTRACTION SYSTEMS

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Submitted to the Institute of Environmental Sciences in partial fulfillment of
the requirements for the degree of
Master of Science
in
Environmental Sciences

Boğaziçi University
2021

Dedicated to my mother and father

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest graditude to my thesis supervisor Prof. Dr. Işıl Balcıoğlu for her enthusiasm, kindness and encouragement during my master studies. Without her endless support this dissertation would not have been possible. I would like to thank my jury members Prof. Dr. Nilgün Cılız and Assoc. Prof. Dr. Selin Şahin Sevgili for their valuable critics and suggestions.

I would like to thank my friends in Institue of Environmental Sciences, Göksu Çelik, F. Öykü Sefiloğlu, Sena Doğan, Müge Niğdeli and Ece Özön for their friendship and help during my laboratory studies. Special thanks to Öykü and Göksu for answering my questions all the time with kindness. I am also very thankful to Filiz Ayılmaz for her help.

I would like to thank Boğaziçi University for funding this thesis through Bogaziçi University Scientific Research Projects (BAP) under project number 19Y00P3.

I would like to thank my dear friends Mert Gerçek, Merve Akan, Özgür Sarıçan and Esra Akyüz for their sense of humour and support.

Finally, I would like to thank my family, Şennur and Osman Ayaz, for their love, support and care.

ABSTRACT

PURIFICATION OF ANTIOXIDANTS FROM OLIVE OIL MILL WASTE BY AQUEOUS TWO-PHASE EXTRACTION SYSTEMS

By-products generated during olive oil production threat to the environment due to high phenolic content. Various treatment alternatives investigated so far are not effective to eliminate toxic phenolic compounds (PCs) from this waste. In this study, instead of olive mill wastewater (OMWW) treatment the recovery of PCs was aimed considering their antioxidant properties. Hence, the separation of antioxidants from the waste does not only reduce its toxicity, but also can supply raw material for the various industries. The OMWW from two-phase centrifugal olive oil extraction process was first concentrated by mechanical vapor recompression (MVR) evaporator. Then two different aqueous two-phase extraction (ATPE) systems based on ethanol/salt having acidic and basic nature were applied to concentrated waste (OMWC). The effects of volume ratio (V_r) and tie line length (TLL) that were the variables of ATPE were investigated for the partitioning of organic components of OMWC. While phase forming ability of basic salt, K_2HPO_4 in ATPE was higher, the performance of ATPE system with acidic salt, $(NH_4)_2SO_4$ was found better for the separation of carbohydrates. Increasing TLL and V_R enhanced the extraction of total PCs. The ATPE system with 29.18% EtOH and 18.00% salt provided 93.59% total PCs recovery while the elimination rates of carbohydrates and proteins were obtained as 76.05% and 53.47%, respectively. Special attention has been devoted to hydroxtyrosol (HTyr) among PCs since it is abundant in OMWW. High HTyr (35.43 mg/g) and high antioxidant activity (32.29 mg AAE/g) determined by DPPH antioxidant assay were well correlated in ethanol extracts.

ÖZET

ZEYTİNYAĞI ATIK SUYUNDAKİ ANTİOKSİDANLARIN SULU İKİ FAZ YÖNTEMİYLE SAFLAŞTIRILMASI

Zeytinyağı üretimi sırasında ortaya çıkan yan ürünler, yüksek oranda içerdikleri fenolik bileşikler nedeniyle çevre için tehdit oluşturmaktadır. Bu atıktaki toksik fenolik bileşikleri ortadan kaldırmak için günümüze kadar araştırılan çeşitli arıtma alternatifleri etkili olmamıştır. Bu çalışmada zeytin karasuyu arıtımı yerine antioksidan özellikleri dikkate alınarak fenolik bileşiklerin geri kazanımı amaçlanmıştır. Bunun sonucu olarak, atıktan antioksidanların ayrımı sadece atığın kirleticiliğini azaltmakla kalmaz; aynı zamanda çeşitli sektörler için hammadde sağlayabilir. İki fazlı santrifüj zeytinyağı ayrım sürecinde oluşan mekanik buhar sıkıştırmalı evaporatörde konsantre edildi. Daha sonra konsantre atığa asidik ve bazik yapıya sahip iki farklı etanol/tuz sulu iki fazlı ekstraksiyon sistemleri uygulandı. Konsantre edilmiş katı atıktaki organik bileşiklerin fazlar arasında ayrımının incelenmesi için sulu iki faz ekstraksiyon sistemi değişkenlerinden hacim oranı (V_r) ve bağ çizgisi uzunluğunun (TLL) etkileri araştırıldı. Bazik tuzun (K_2HPO_4) faz oluşturma kabiliyeti sulu iki fazlı ekstraksiyon sistemi için daha iyi olmasına rağmen, asidik tuzlu ($(NH_4)_2SO_4$) sulu iki fazlı ekstraksiyon sisteminin karbonhidrat ayrılma performansı daha iyi bulundu. Artan TLL ve V_r, fenolik bileşiklerin ekstraksiyonunu arttırdı. %29.18 etanol ve %18.00 tuz içeren sulu iki fazlı ekstraksiyon sistemi, toplam fenolik bileşiklerin %93.59'unun geri kazanımını sağlarken, aynı zamanda karbonhidratların ve proteinlerin uzaklaştırılma oranları sırasıyla %76.05 ve %53.47 olarak elde edildi. Zeytinyağı karasuyunda bol miktarda bulunduğu için fenolik bileşikler içinde hidroksitirosole (HTyr) özel ilgi gösterilmiştir. Etanol özütlerindeki yüksek miktardaki HTyr (35.43 mg/g) ve DPPH antioksidant madde tayini ile belirlenen yüksek antioksidan aktivite (32.29 mg AAE/g) korelasyon halindedir.

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LIST OF SYMBOLS/ABBREVIATIONS

Abbreviation	Explanation	Unit
AAE	Ascorbic Acid Equivalent	
AMF	Alcohol Mass Fraction	%, w/w
AC	Activated Carbon	
ATPE	Aqueous Two-Phase Extraction	
ATPS	Aqueous Two-Phase System	
BOD	Biological Oxygen Demand	g/L
BSA	Bovine Serum Albumin	
CE	Collusion Energy	V
COD	Chemical Oxygen Demand	g/L
CP	Cloud Point Temperature	°C
CSA	Cajaninstilbene Acid	
CXP	Collusion Cell Exit Potential	V
DEX	Dextran	
DP	Declustering Potential	V
DPPH	2,2-diphenyl-1-picrylhydrazyl	
EE	Extraction Efficiency	%
EP	Entrance Potential	V
EtOAc	Ethyl Acetate	
EtOH	Ethanol	
EU	European Union	
GAE	Gallic Acid Equivalent	
HTyr	Hydroxtyrosol	
K	Partitioning Coefficient	
K_2HPO_4	Dipotassium Phosphate	
K_{HTyr}	Partitioning Coefficient of Hydroxytyrosol	
K_{Tyr}	Partitioning Coefficient of Tyrosol	
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectroscopy	
LLE	Liquid Liquid Extraction	
m	Mass	
MeOH	Methanol	

MF	Microfiltration	
MRM	Multiple Reaction Monitoring	
MVR	Mechanical Vapor Recompression	
NF	Nanofiltration	
NPC	Negative Pressure Cavitation	
Oleu	Oleuropein	
OMWC	Olive Mill Waste Concentrate	
OMWW	Olive Mill Wastewater	
PCs	Phenolic Compounds	
PEG	Polyethylene glycol	
Q1	Parent ion	m/z
Q3	Daughter ion	m/z
R	Removal	%
R^2	Coefficients of Determination	
RE	Recovery	%
RO	Reverse Osmosis	
ROO^-	Free Radicals	
ROS	Reactive Oxygen Species	
rT	Retention Time	
S	Slope of Tie Line Lenghts	
SMF	Salt Mass Fraction	
SPE	Solid Liquid Extraction	
SPSS	Statistical Package for Social Science	
TLL	Tie Line Lenght	
TPC	Total Phenolic Compounds	
Tyr	Tyrosol	
UF	Ultrafiltration	
V_r	Volume Ratio	
Y	Yield	
z	Charge	

1. INTRODUCTION

Although olive oil has been crucial ingredient of Mediterranean diet since ancient times, an increase in global consumption of olive oil is relatively recent (International Olive Council, 2019). The extraction of oil from olive has been performed with traditional batch and modern continuous systems which produce considerable amounts of by-products namely olive oil mill wastewater (OMWW) and solid waste (pomace). These by-products have toxic properties since about 98% phenolic content of the olive fruit remains in the waste (Rodis et al., 2002) and almost 15 million m^3 OMWW and 10 million tons olive pomace per million tons olive oil are annually produced in Mediterranean countries (Khdair and Abu-Rumman, 2020). Turkey, one of the leading countries in olive oil production, has to deal with the pollution problem of this wastewater. In order to reduce OMWW, a shift from three-phase to two-phase centrifugal systems in modern oil extraction plants has been carried out in some Mediterranean countries including Turkey. However, treatment of the wastewater is a challenging task due to high organic content with poor biodegradability.

The most common practice for the waste abatement of olive oil industry is the storage of them in evaporation pond. However, this method does not only cause the release of bad odor it can also result in seepage of pollutants into ground (Gebreyohannes et al., 2016). Furthermore, the toxic phenolics remain in the residue that is applied to soil as fertilizer owing to high mineral content as well as organics (Kokkora et al., 2015; Meftah et al., 2019); hence this waste can exert phytotoxic effects (Rinaldi et al., 2003; Paraskeva and Diamadopoulos, 2006; Koutsos et al., 2018; Meftah et al., 2019). Physiochemical processes, electrochemical and advanced oxidation have been integrated with biological processes to enhance treatment efficiency of OMWW (Paraskeva and Diamadopoulos, 2006; Justino et al., 2010; Karahan Ozgun et al., 2016). However, none of them offers economical and practical solutions (Paraskeva and Diamadopoulos, 2006; ElMekawy et al., 2013; Gebreyohannes et al., 2016; Lee et al., 2019; Khdair and Abu-Rumman, 2020) due to various reasons such as operational costs (Lee et al., 2019), inefficiency to reduce organic load and meeting treatment requirements (Paraskeva and Diamadopoulos, 2006).

Polyphenols are the most problematic component of OMWW but they have a high potential for the use in food and cosmetic industries by their antioxidant properties. Therefore, the recovery of polyphenolic antioxidants from waste has gained importance instead of searching efficient treatment methods. The reduction in the treatment cost of OMWW can have beneficial contribution of olive oil industry. OMWW contains more than 30 bio-phenolic compounds and among them hydroxtyrosol (HTyr), tyrosol (Tyr) and oleuropein (Oleu) have higher antioxidant activity (Tuck and Hayball, 2002; Takac and Karakaya 2009; Bedouhene et al, 2014). HTyr, the most abundant antioxidant, has anticancer, anti-inflamatory, antimicrobial activity as well as retino protective and skin related benefits (D'Angelo et al., 2005; Granados-Principal et al., 2010).

Liquid-Liquid Extraction (LLE), Solid phase Extraction (SPE), membrane separation and surfactant mediated Aqueous Two-phase Extraction have been investigated for the recovery of phenolic compounds from OMWW. While fouling is a common problem in membrane separation processes (Nayak and Bhushan, 2019) excessive consumption of organic solvents makes the LLE process less attractive. However, the studies performed with Ethyl acetate (EtOAc) used in LLE yield high recovery of PCs from OMWW but it is not a safe solvent to the compounds that are considered for direct human consumption as opposed to EtOH. In order to provide a selectivity in EtOH extraction the separation of organic phase can be induced by the addition of inorganic salts in ATPE systems. Alcohol-salt based ATPE offers many advantages such as reduced amount of solvent requirement, quick phase separation, and relatively cheap. Accordingly, EtOH based ATPE system placed in an integrated process with SPE was applied to two different concentrated OMWW originated from two-phase centrifugal oil extraction process and 6.6 mg/g HTyr recovery was achieved with EtOH-$(NH_4)_2SO_4$ ATPE (Celik et al., 2018).

In this research, the recovery of PCs with ATPS was investigated from OMWW similar to the previous study of Celik (Celik et al., 2018). However, OMWW used in this study was generated from two-phase centrifugal system which introduced to olive oil industry relatively recent. To investigate effects of TLL and V_r on the performance of ATPS was the main goal of this study. Following to the construction of phase diagrams of two different ATPS, namely EtOH-K_2HPO_4 and EtOH-$(NH_4)_2SO_4$, the distribution of organic compounds found in OMWC between top and bottom phases were determined at selected operation points. Together with total PCs, the presence of HTyr was investigated in the ethanol-rich top phase. Finally, correlation between estimated amounts of PCs and antioxidant activity was ascertained.

2. LITERATURE REVIEW

2.1. Generation of Olive Oil Industry Waste and its Management

Olive oil has been part of the Mediterranean diet since ancient times. Throughout the years, traditional batch press has been applied to olive fruits to obtain olive oil. During the 1970s, the traditional systems have been replaced by continuous systems due to economical and practical advances (Borja et al., 2006; Hocaoğlu et al., 2018). Nowadays merely 2% of the olive oil production facilities use traditional systems whereas the remaining facilities produce olive oil with continuous systems, namely two-phase and three-phase centrifugal systems in Turkey (Hocaoğlu et al., 2017). These oil extraction methods differ in terms of their process and by-products as can be seen from Figure 2.1 (Adapted from Zbakh and El-Abbasi, 2012).

Major portion of olive fruit oil locates in the vacuoles of mesocrapal cells as small droplets, even though it can be found in other parts of the fruit (Kapellakis et al., 2008). To reach most of the oil in the fruit, water is used at different stages of the olive oil production process such as malaxation of crushed olives in which water favors coalescence and squeezes out small oil droplets. While water is added in decantation and separation stages at batch and three-phase centrifugal systems further water addition is not required in two-phase centrifugal system (Hocaoğlu et al., 2018). Indeed, olive fruit itself rich in water (50-70%) which varies with the cultivar harvested as well as their maturity stage (Zeleke et al., 2012).

Continuous extraction process which uses centrifugation for the separation of oil instead of pressure has advantages over batch press system such as providing better oil quality and requiring less space for production with automated systems. However, two-phase centrifugal systems which emerged in 1990s reduced water consumption (El-Mekawy et al., 2013). Indeed, total volume of wastewater produced from three-phase centrifugation system is 124 L per 100 kg of olive and it is almost five-fold higher than that of two-phase centrifugation system (Borja et al., 2006). Apart from the amount of wastewater produced by different centrifugal systems, toxicity of the generated OMWW differs. OMWW produced from three-phase centrifugal systems has 110 times higher pollution load than that of two-phase centrifugal systems (Hocaoglu et al., 2017).

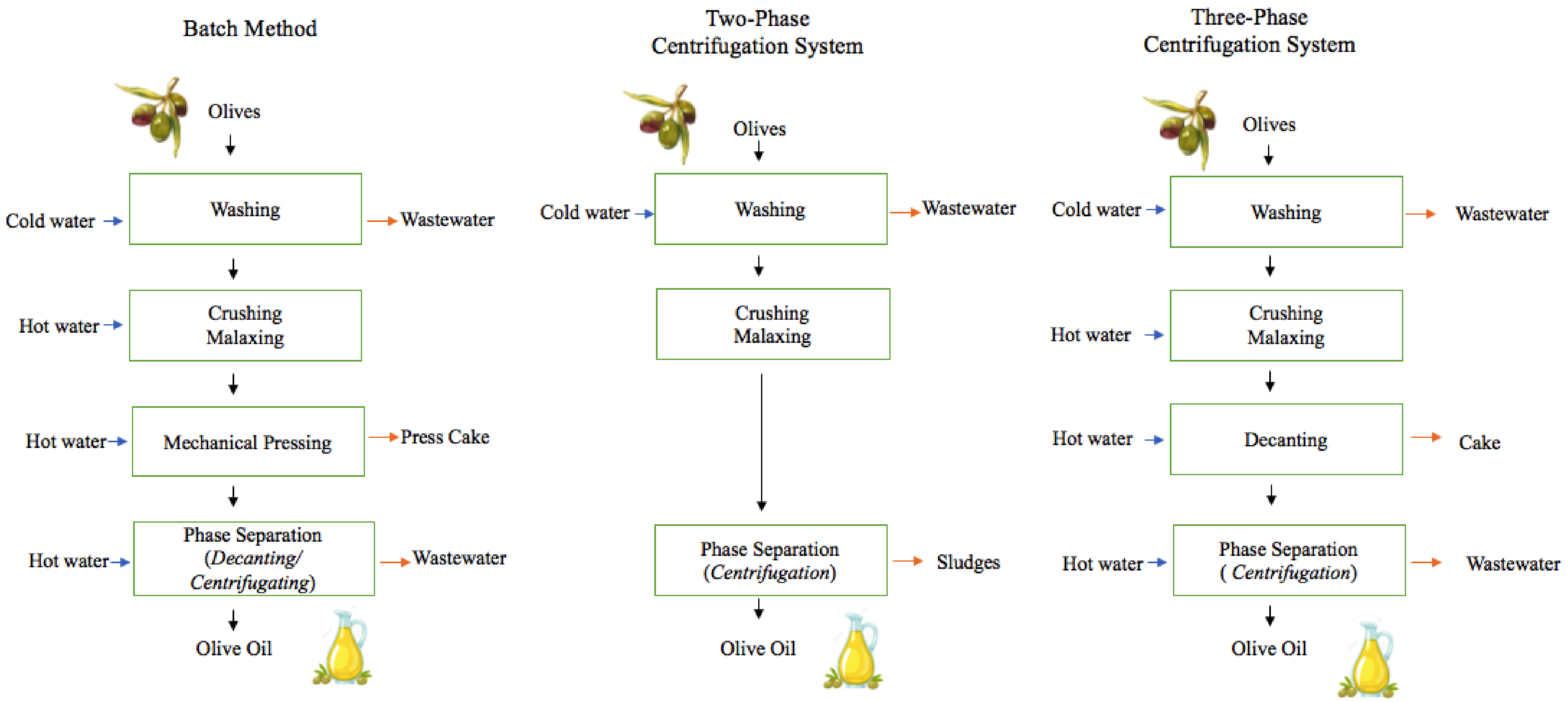


Figure 2.1. Stages and by-products of different olive oil extraction methods.

2.1.1. Characteristics of Olive Oil Industry By-products

The extraction process for olive oil generates both wastewater and solid waste with different compositions. Although the characteristics of OMWW depends on various factors such as extraction method, climate, type and maturity of olive fruit harvesting time and crop season (Paraskeva and Dianadopoulos, 2006; Ntougias et al., 2013), the wastewater of three-phase system is generally characterized by dark color, acidic pH, high salinity and conductivity, low nitrogen content and more importantly high toxicity (Azbar et al., 2004; Borja et al., 2006; Ochando-Pulido et al., 2017; Hocaoğlu et al., 2018). The organic content of OMWW is highly complex and besides sugars, nitrogenous compounds, organic acids, polyalcohols, pectins, lipids, carbohydrates, tannins OMWW includes high amount of phenolic compounds (PCs) up to 80 g/L that cause extremely high COD and BOD values (Azbar et al., 2004; Rahmanian et al., 2014). Typical values of physiochemical parameters of by-products are presented in Table 2.1 (García-Gómez, 2003; Tomati et al., 2004; Danellakis et al., 2011; Dermeche et al., 2013; Sellami et al., 2016).

Table 2.1. Physicochemical properties of olive oil manufacturing by-products.

Physicochemical Property	OMWW	Semi-solid OMWW
pH	2.2-5.6	4.9-6.8
PC (%)	0.3-0.8	0.4-2.4
TKN (g/L; g/kg)	0.5- 0.7	11.6-15.1
OC (g/L; g/kg)	20.2-39.8	551.5-626.3
Protein (%)	0.2- 4.5	2.9-7.2
Carbohydrate (%)	0.5-12.2	0.8-19.3
Lipid (%)	0.03-4.25	3.5-8.7

Both OMWW and pomace contain bioactive organic compounds. On the other hand, their toxic effects to the environment are obvious due to nondegradable organic matter content, which do not subject natural degradation (Mikdame et al., 2020). In case of uncontrolled discharge of raw OMWW on soil and water, adverse impacts are unavoidable due to its high ecotoxic and phytotoxic effects (Danellakis et al., 2011; Ntougias et al., 2013; Kavvadias et al., 2014). Basically, untreated OMWW can harm soil fertility, plants, soil micro-flora and air (Roig et al., 2006; Alhajoj and Alowaliesh, 2019). Minerals and organic compounds in OMWW cause a change in cation exchange capacity and structure of soil. Moreover, lipid fraction blocks transfer of oxygen and sunlight to the water bodies (Koutsos et al., 2018).

2.1.2. Legal Restrictions for By-products of Olive Oil Manufacturing

Similar to some members of European Union (EU) such as Spain, Italy and Greece that are leading manufacturers of olive oil, Turkey has to deal with potential environmental problems due to OMWW. However, each EU country have their own legislation for OMWW discharge which should be in the limits of Water Framework Directive (Inglezakis et al., 2012; Komnitsas and Zaharaki, 2012; Koutsos et al., 2018).

The direct discharge of OMWW into environment is forbidden in Turkey. In fact, all olive-oil producers are expected to participate the rules explained in “Technical principles to be followed in the management of wastewater formed in olive oil plants” and they have to obey the discharge limits specified by Republic of Turkey Ministry of Environment and Urbanization in Table 2.2 (Çevre ve Şehircilik Bakanlığı, 2015). However, regular control of discharge of olive oil wastes is a difficult task due to spread of small olive oil production facilities to a large geographical area in Turkey (Azbar et al., 2004).

Considering the high COD load of OMWW (80-210 g/L) (Gebreyohannes et al., 2016) it is obvious that meeting the limitations shown in Table 2.2 is a challenging task.

Table 2.2. The discharge limitations of wastewater produced from olive mill industry described by the Republic of Turkey Ministry of Environment and Urbanization.

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

2.1.3. Treatment Methods for Olive Oil By-products

Treatment of OMWW and pomace constitutes a remarkable subject due to its potential harm to environment especially in the Mediterranean countries who produce 97% of the olive oil (Souilem et al., 2017). Since direct disposal of effluents to the land and water bodies causes serious environmental issues, various treatment methods have been investigated. However, treatment of olive oil by products with efficient methods is hard due to complex nature of the effluent with high organic load and PC content (Souilem et al., 2017; Hocaoğlu et al., 2018).

Storage of OMWW in lagoons to evaporate the wastewater is the common practice in most Mediterranean countries. However, this practice that requires available large area can cause obnoxious odor, rapid increase in number of insects and seepage of pollutants into the ground (Gebreyohannes et al., 2016). Even though usage of OMWW as fertilizer has been suggested due to its nutritional content, its negative impact on plant growth and seed germination makes this practice out of choices (Paraskeva and Diamadopoulos, 2006). In case of pomace, thermal drying is the most common practice; however, this method is not economically feasible due to the requirement for high energy consumption needed for drying process (Roig et al., 2006).

Various treatment methods have been offered for olive mill waste by-products. These methods can be listed as physiochemical methods (floatation, adsorption, sedimentation, settling, sand, membrane filtration and coagulation-flocculation, ion-exchange and electrocoagulation), chemical methods (oxidation with electrochemical oxidation, Fenton oxidation etc.), and biological methods (anaerobic and aerobic processes, biofiltration) (Khdair and Abu-Rumman, 2020). Biological treatment is not favorable in OMWW treatment due to the high amount of PC and long chain fatty acid content which inhibit some microbial activities (El-Mekawy, 2013, Lee et al., 2019). Utilizing the physiochemical or biological treatment methods alone are not sufficient to reduce the organic load and they are partially compatible with treatment requirements (Paraskeva and Diamadopoulos, 2006). Therefore, the combination of these methods was investigated in the literature (Paraskeva and Diamadopoulos, 2006; Justino et al., 2010; El Meckawy et al., 2013; Alhajoj and Alowaiesh et al., 2019; Lee et al., 2019; Khdair and Abu-Rumman, 2020). However, combined treatment such as Fenton's reagent process with anaerobic digestion or chemical-biological treatment (Amor et al., 2015; Karahan Ozgun et al., 2016 are not economically feasible due to high need of capital and operational expenses (Lee et al., 2019).

Possible treatment methods for the pomace are drying, thermal treatment and biological treatment (aerobic and anaerobic) (Tekin and Dalgic, 2000; Doula et al., 2017; Missaoui et al., 2017; El Achkar et al., 2018; Kostas et al., 2020). Drying has many operational disadvantages such as high energy demand, investment and operating costs. Additionally, problems occur during the drying process as a result of high moisture content of pomace since most of the energy consumed for the drying of pomace and hence energy recovery is not possible. Even though combustion and pyrolysis have advantages such as reduction in the volume of waste and contribution to the energy recovery, these methods also require expensive facilities and can cause gaseous emission of toxic compounds. Even though biological treatment offers low emission and consumption of energy, abundance of PCs in pomace affects adversely anaerobic digestion, similar to the OMWW (Doula et al., 2017).

As a result, performances of various treatment methods are arguable due to practical and economical regards (Paraskeva and Diamadopoulos, 2006; Khadir and Abu-Rumman, 2020). Hence more sustainable and environmentally friendly approaches should be considered for the treatment of OMWW.

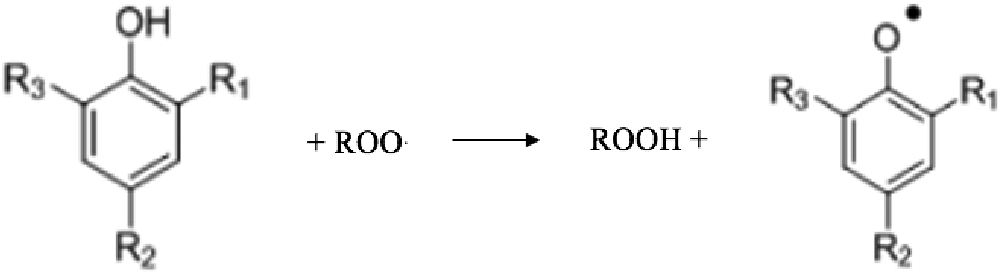
2.1.4. Valorization of Olive Oil Industry Waste

OMWW and pomace have long been considered as merely waste and minimizing/preventing their negative effects on the nature was the primary goal (Galanakis and Kotsiou, 2017). Hence, researchers aimed to enhance treatment and detoxification methods for reducing detrimental environmental effects of OMWW and pomace. However, ineffectiveness of offered treatment alternatives has led to a shift in the research perspective from the treatment of wastewater to valorization of valuable components of the waste (Gebreyohannes et al., 2016; Souilem et al., 2017; Jimenez-Lopez et al., 2020). In that matter, utilization of OMWW and pomace have been investigated in a wide range such as production of olive waste based adsorbents, food products and additives, cosmetic uses, textile dying and recovery of valuable compounds (Dermeche et al., 2013; Bhatnagar et al., 2014; Rodrigues et al., 2015; Roselló-Soto et al., 2015; Caporaso et al., 2017).

Extracted PCs from olive oil by-products can be used as additives in food industry owing to health benefits or in cosmetic industry due to its collagen-production promoting effect (Souilem et al., 2017). Recovery of PCs from OMWW and pomace by focusing on beneficial biomolecules has prime importance to meet growing demand of these compounds in various industries.

2.1.5. Phenolic Compound Profile of Olive Oil Industry Waste

PCs in OMWW and pomace are bioactive compounds with antioxidant, anti-inflammatory, angiogenic, antibacterial and antiviral functions (Tuck and Hayball, 2002; Bedouhene et al., 2014). Basically, PCs are a group of chemical compounds which all contains at least one or more hydroxyl group (-OH) and at least one aromatic hydrocarbon group. PCs are capable of reaction with reactive organic species (ROS) and free radicals ($ROO^{\cdot}$) through donating their hydrogen from phenolic hydroxyl group as shown in the following reaction.



(2.1)

The reaction of PCs with ROS minimizes deteriorating action of free radicals in organisms; hence they have vital role in human health. OMWW and pomace have a broad group of PCs such as hydroxytyrosol (HTyr), tyrosol (Tyr), oleuropein (Oleu), oleocanthal, apigenin etc. Main groups of PCs can be listed as phenyl alcohols, secoiridoids, phenolic acids/ aldehydes, flavonoids, phenylethanoid glycoside and *p*-Coumaric acid derivatives (Araújo et al., 2015). OMWW is considered a powerful biophenolic source, thanks to its content having more than 30 biophenolic structures. Especially, HTyr, Tyr and Oleu have higher antioxidant properties among them (Tuck and Hayball, 2002; Takac and Karakaya, 2009; Bedouhene et al., 2014). High antioxidant property of HTyr is attributed to its o-diphenolic structure which capable of react with radicals via intermolecular hydrogen bonding (Visioli et al., 1998). Besides the ability of HTyr to involve the processes of gene expression and life cycle of the cell, neuroprotective, cardioprotective, anti-cancerogenic and anti-obesity effects have been shown in literature (Bertelli et al., 2020).

There are many *in vivo* and *in vitro* studies which reveal beneficial effects of Oleu, HTyr and Tyr. Numerous benefits of Oleu, HTyr and Tyr such as antioxidant and radioprotectice effects, scavenging of ROS, anti-diabetic effects, lipid-lowering and inhibition of tumor growth have been proven (Cavaca et al., 2020). Olive pomace involves caffeic acid, *p*-Coumaric acid, vanillic acid, verbascoside, elonoic acid, catechol and rutin besides HTyr, Oleu and Tyr and their amount exceeds those of in OMWW (Nunes et al., 2016). Main PCs detected in OMWW and chemical structures of some of them presented in Table 2.3 and Figure 2.2, respectively (Obied et al., 2005; Kiritsakis et al., 2020).

Table 2.3. Major biophenolic compounds found in OMWW.

Phenolic Compounds	**Molecular Formula**	**Molecular weight (g/mole)**
Benzoic Acid and Derivatives		
3-Hydroxybenzoic acid *p*-Hydroxybenzoic acid	$C_7H_6O_3$	138.12
3,4-Dihydroxybenzoic acid Gentisic acid	$C_7H_6O_4$	154.12
Vanillic acid	$C_8H_8O_4$	168.15
Gallic acid	$C_7H_6O_5$	170.12
Syringic acid	$C_9H_{10}O_5$	198.17
Cinnamic Acid and Derivatives		
o-Coumaric acid *p*-Coumaric acid	$C_9H_8O_3$	164.16
Caffeic Acid	$C_9H_8O_4$	180.16
Ferulic Acid	$C_{10}H_{10}O_4$	194.18
Sinapinic Acid	$C_{11}H_{12}O_5$	224.21
Phenly Ethyl Alcohol		
Tyrosol (*p*-HPEA) or 2-(4-Hydroxyphenyl)ethanol	$C_8H_{10}O_2$	138.16
Hydroxytyrosol (3,4-DHPEA) or 3,4-Dihydroxyphenylethanol	$C_8H_{10}O_3$	154.16
Lignans		
(+)-Pinoresinol	$C_{20}H_{22}O_6$	358.40
(+)-1-Acetoxypinoresinol	$C_{22}H_{24}O_8$	416.40
Flavones		
Apigenin	$C_{15}H_{10}O_5$	270.24
Luteolin	$C_{15}H_{10}O_6$	286.24
Flavonols		
(+)-Taxifolin	$C_{15}H_{12}O_7$	304.25
Hydroxyisochromans		
1-Phenyl-6,7-dihydroxyisochroman	$C_{15}H_{14}O_3$	242.27
1-(3-Methoxy-4-hydroxy)phenyl-6,7-dihydroxyisochroman	$C_{16}H_{16}O_5$	288.29
Aglycons Secoiridoids		
Oleuropein aglycon or 3,4-DHPEA-EA	$C_{10}H_{22}O_8$	378.40
Ligstroside agylcon or *p*-HPEA-EA	$C_{19}H_{24}O_8$	380.39

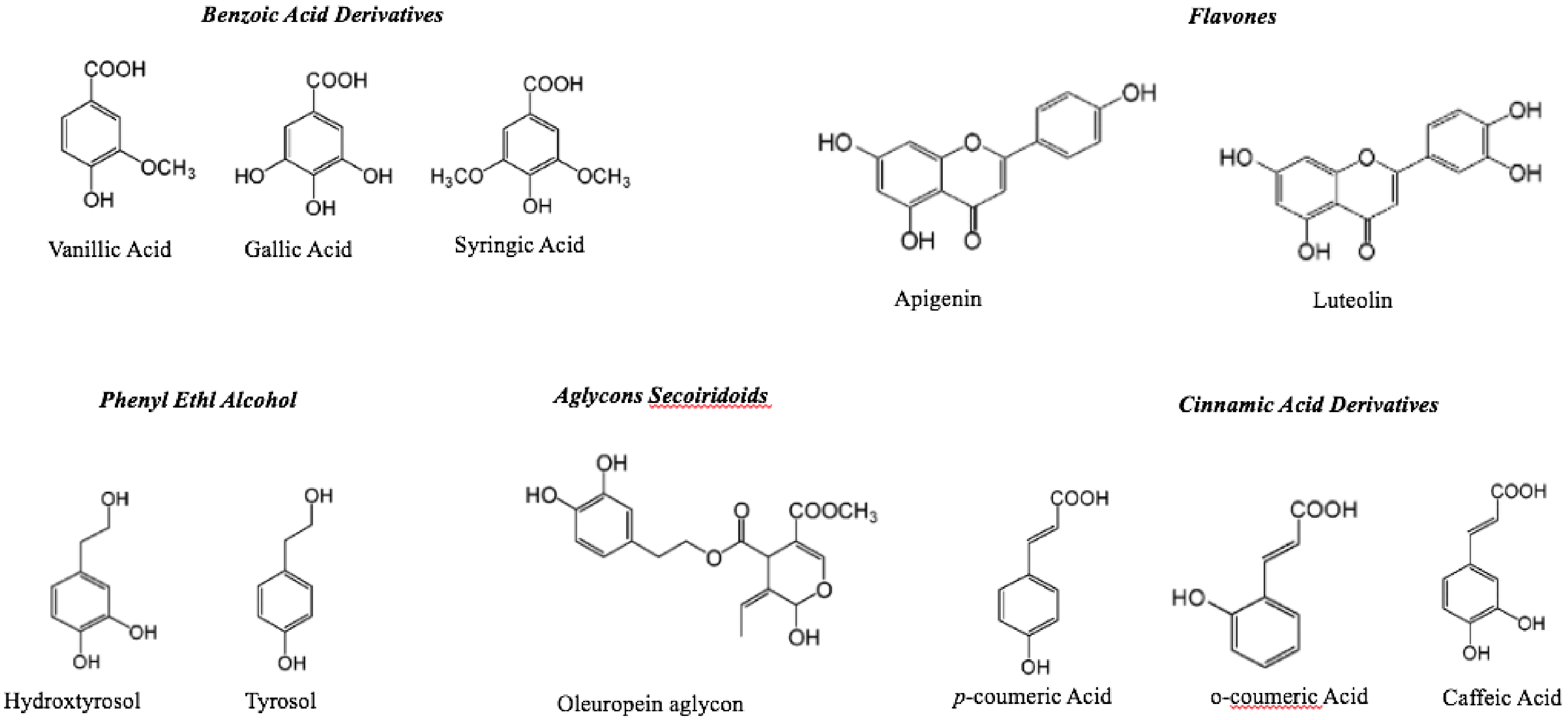


Figure 2.2. Chemical structures of some of PCs found in OMWW.

2.2. Antioxidant Recovery Methods from Olive Oil Industry Waste

Different methods have been developed for the recovery of antioxidants from olive oil industry by-products. A literature review on membrane separation, solid phase extraction liquid-liquid extraction (LLE) and aqueous two-phase extraction (ATPE) has been compiled below.

2.2.1. Membrane Separation

Various types of membranes such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO) membranes used mostly in the sequential order have been suggested for the recovery, purification and concentration of PCs from OMWW and aqueous extracts of olive mill solid waste (Paraskeva et al., 2007; Takac and Karakaya, 2009; Cassano et al., 2018; Conidi et al., 2019; Tundis et al., 2020). Among membrane operations, MF and UF are usually employed as pre-treatment steps whereas NF and RO are generally used for fractionation and concentration of permeates generated by MF and UF (Tundis et al., 2020).

Membrane separation requires simple equipment and reduced number of processing steps. Moreover, membrane technologies provide advantages by eliminating the use of solvents and adsorbents that require regeneration and by serving at mild operation conditions (temperature and pressure) which protects bioactive compounds. However, maintenance cost of the system is high due to clogging of the membrane and reduction of flux that limits its application (Nayak and Bhushan, 2019). It has been considered that presence of suspended solids, organic pollutants, and colloidal matter are the main components of OMWW that cause the fouling of membrane system (Ochando-Pulido et al., 2015). It has been known that interaction of PCs with other compounds like polysaccharides may cause formation of larger particles and cause difficulties in the membrane systems (Castro-Muñoz et al., 2018). Even though some methods (pretreatment of feed solutions, membrane surface modification, membrane cleaning etc.) have been proposed for the elimination of fouling problems, it still limits the application of membrane processes to OMWW.

2.2.2. Solid Phase Extraction

SPE is another alternative method for the recovery of PCs from OMWW and selective uptake of PCs can be optimized by choosing appropriate sorbents, conditions such as pH of effluent, sorbent dosage, contact time etc. and pretreatment. Various polymeric adsorbents have been used for this purpose (Agalias et al., 2007; Zagklis et al., 2015; Frascari et al., 2016; Kaleh and Geißen, 2016; Pinelli et al., 2016; Wang et al., 2017; Frascari et al., 2019; Vavouraki et al., 2020) and they provide an important advantage over activated carbon (AC), which requires thermal regeneration due to strong adsorption, and the recovery of target compounds is not achieved (Anbia and Amirmahmoodi, 2011). The PC recovery efficiencies of polymeric adsorbents can attain to 99.9% with a multistage process (Agalias et al., 2007). Frascari and colleagues utilized the combination of SPE using Amberlite XAD and FPX-66 resins with centrifugation and microfiltration to reduce the clogging of the resin column with the solid content of OMWW (Frascari et al., 2016). It was shown that the combined application of polymeric adsorbents (XAD4, XAD16 and XAD7HP) besides their individual use provided 74% of recovery of TPs from OMWW (Zagklis et al., 2015). Frascari and co-workers suggested the integration of anaerobic digestion and resin adsorption with XAD16N for the recovery of PCs (Frascari et al., 2019). In addition to polymeric adsorbents, some other adsorbents are also used for the recovery of PCs from OMWW such as amine modified mesoporous silica adsorbents (TRI-SBA-15 and TRI-P-10), which exhibited remarkably higher efficiencies than that of AC (Yangui et al., 2017). Recently, Sahin and colleagues revealed that graphene oxide is a promising adsorbent for the recovery of PCs from OMWW with more than 67% and 85% of TPC and HTyr recovery, respectively (Sahin et al., 2020). It should be noted the recovery of biophenols from sorbent may require more than one solvent solvent (Kaleh and Geißen, 2016) and low pH value (Bertin et al., 2011; Kaleh and Gaißen, 2016).

2.2.3. Liquid-Liquid Extraction

This extraction method is commonly applied by using organic solvents such as ethyl acetate (EtOAc), ethanol (EtOH) and a mixture of EtOH and diethyl ether. Prior to the solvent extraction acidification can be necessary to enhance the efficiency of LLE by increasing the solubility of PCs and promoting the hydrolysis of Oleu to HTy (Allouche et al., 2004; De Marco et al., 2007). A pH value between 2 and 3 considered as optimum range for the enhancement of extraction rate (Allouche et al., 2004; Zafra et al., 2006; Lafka et al., 2011) and the distribution coefficient of the PCs increases at acidic pH during LLE (Allouche et al., 2004). Additionally, the removal of solids from OMWW has been suggested as a pretreatment (Lesage-Meessen et al., 2001; De Leonardis et al., 2007; Khoufi et al., 2008; Tercan and Seker, 2012; Kalogerakis et al., 2013; Sellami et al., 2016; Jiménez-Herrara et al., 2017). Depending upon lipid content of olive by-products washing with *n*-hexane (De Marco et al., 2007; De Leonardis et al., 2007; Lafka et al., 2011; Bedouhene et al., 2014; Leouifoudi et al., 2014; Leouifoudi et al., 2015; Sellami et al., 2016; Abu-Lafi et al., 2017) or with petroleum ether (Cioffi et al., 2010) could be applied as pretreatment. An exhaustive literature review done on LLE of antioxidants from olive by-products by using organic solvents is presented in Table 2.4.

Since the determination of optimum extraction conditions such as solvent nature, solvent to OMWW ratio, the number of extraction stages, and pH of OMWW are crucial for successful LLE, the effects of these parameters on the recovery rate of PCs were investigated in the literature. As shown in Table 2.4, among various solvents EtOAc is the most effective solvent with high selectivity towards low and medium molecular weight PCs (Lesage-Meessen et al., 2001). Even though the performance of EtOAc is satisfactory in many studies, EtOAc is not food grade solvent. As clearly shown in Table 2.4, the required amount of solvent for extraction of PCs is more than the amount of OMWW that is the major disadvantage of the extraction method. Many of the organic solvents possess danger since they can cause fire and explosion and also act as air pollutant and contributes to global warming (Chemat and Vian, 2014). Reducing the consumption of hazardous solvents is one of major aims of EU for the 2010-2050 period (Bubalo et al, 2018). Also, formation of emulsions and difficulties with automation of the system are considered as other disadvantages (Poole, 2003).

It was shown that the extraction performance of PCs could be increased by the application of ultrasound assisted LLE. However, methanol (MeOH) was used as solvent in this study to achieve the recovery of PCs (Klen and Vodopivec, 2011). Although this application provided faster kinetics, high consumption of solvent is the main disadvantage. In order to reduce the volume of the extraction solvent, OMWW was freeze dried before ultrasound assisted LLE. Considering the polar nature of PCs, the extraction with CO_2 instead of polar solvents such as EtOH or MeOH has been investigated (Lafka et al., 2011; Araújo et al., 2015). Still, large volumes of solvents needed for LLE makes this method expensive and environmentally undesirable.

Table 2.4. Solvent extraction of PCs from OMWW and pomace.

Type of OMWW Systems	Extraction Solvent	Solvent/Sample Ratio	Pretreatment	Extraction Performance	Reference
Two-phase and three-phase centrifugal system	EtOAc	1:1 (v/v) Two times	Filtration Centrifugation Acidification pH=3	28.8% (Three-phase system) 42.2% (Two-phase system) TP recovery	Lesage-Meessen et al., 2001
Three-phase centrifugal system	EtOAc	2:1 (v/v) Three times	Acidification pH=2	85.5% HTyr recovery	Allouche et al., 2004
Discontinuous process	EtOAc	2:1 (v/v)	Acidification pH=2	85.5% HTyr recovery	Fki et al., 2005
Three-phase centrifugal system	EtOAc	1:2 (v/v) Three times	Heated Centrifugation Filtration Hexane washing	0.5 mg of TP/g OMWW	De Leonardis et al., 2007
Three-phase centrifugal system	EtOAc	1:1 (v/v) Four times	Acidification pH=2 Hexane washing	1.2 g HTyr/L OMWW	De Marco et al., 2007
Discontinuous process	EtOAc	-	Centrifugation Acidification pH=2	90.0% recovery of mono PCs	Khoufi et al., 2008
Two-phase centrifugal system	EtOH	5:1 (v/w) Four times	Acidification pH=2 Hexane washing Filtration	95.3% PCs recovery	Lafka et al., 2011
-	EtOH	15 (v/w)	Centrifugation Filtration	3.5% PCs recovery	Tercan and Seker, 2012
Three-phase centrifugal system	EtOAc	2:1 (v/v)	Filtration Centrifugation	0.247 g HTy/L OMWW	Kalogerakis et al., 2013
-	EtOAc	Four times	Acidification pH=2 Centrifugation Hexane washing	0.148 mg GAE/mg	Bedouhene et al., 2014
Three-phase centrifugal system	EtOAc	1:1 (v/v) Two times	Hexane Extraction Centrifugation	-	Leouifoudi et al., 2015
Three-phase centrifugal system	EtOAc	Four times	Centrifugation Filtration Acidification to pH=2 Hexane washing	125.07 mg/L (HTyr), 29.64 mg/L (Tyr)	Delisi et al., 2016
Three-phase centrifugal system	EtOAc	Four times	Acidification pH=2 Centrifugation Hexane washing Filtration	3.69 g CAE/L	Sellami et al., 2016
-	EtOAc	2:1 (v/v)	Hexane Extraction Filtration Centrifugation	-	Abu-Lafi et al., 2017
Two-phase centrifugal system	EtOAc	1:1 (v/v) Twice	Sonication Acidification pH=2	-	Jiménez-Herrera et al., 2017
-	EtOH	1:2 (v/v)	Concentration Centrifugation	0.43 g/L GAE (TPC)	Venturi et al., 2017

2.2.4. Surfactant Mediated Aqueous Two-Phase Extraction

Micellar (surfactant meditated) ATPE is a relatively new technique has been used for the recovery of PCs from OMWW in various studies as shown in Table 2.5. Aqueous solutions of many non-ionic surfactants (e.g Tween 80, Genapol X080, Triton X-100 and Triton X-114) at low volume percentage (4-12%) have been employed for the treatment of OMWW. The influence of various parameters (e.g surfactant type and concentration, equilibration time, salt addition, pH value, ionic strength and centrifugation time) on the phase separation; hence the recovery efficiency of PCs has been investigated (Paleologos et al., 2005; Katsoyannonos et al., 2006).

Table 2.5. Surfactant mediated ATPE of PCs from OMWW.

Pretreatment	**Addition of Salt**	**Surfactant**	**Extraction Performance (RE, %)**	**Factors Effecting Efficiency**	**Reference**
Hexane washing	-	Triton X114 (6%, v/v)	60.0	Number of extraction stages, concentration of the surfactant	Katsoyannos et al., 2006
Acidification (pH=2.5–3.5)	NaCl (%5, w/v)	Genapol-X080 (2%, v/v)	89.5	Number of extraction stages, concentration of the surfactant	Gortzi et al., 2008
Acidification (pH=2.5-3.5)	NaCl (20%, w/v)	Tween 80 (5%, v/v)	94.4	Type of surfactants, number of extraction stages, concentration of the surfactant	Katsoyannos et al., 2012
Ultrafiltration	-	Triton X100 (10%, w/w)	66.5	Temperature, concentration of surfactant	El-Abbassi et al., 2014
Ultrafiltration	$CaCl_2$	Genapol X080 (7%, w/v)	30.0	Concentration of surfactant, pH of the solution, equilibrium temperature, effects of salts	Raiti et al., 2015
-	-	Genapol X080 (10%, w/v)	68.0	pH of solution, equilibrium temperature, incubation time and type of surfactant	Kiai et al., 2018

The effects of surfactant concentration and temperature are widely investigated factors in studies presented in Table 2.5. Katsoyannos et al. (2012), investigated the effect of Tween 80 in the concentration range of 1.0-25.0% and the surfactant concentration higher than 5% (v/v) did not enhance the recovery rate of PCs (Katsoyannos et al., 2012). Similarly, in the study of Stamatopoulos et al. (2014), increasing Tween 80 concentration from 4% to 11% (w/v) did not provide a further improvement for the recovery of PCs from olive leaf extract (Stamatopoulos et al., 2014).

The separation of water-rich and surfactant-rich phases is induced by a shift in temperature. Generally, the optimum temperature for cloud point extraction is 15-20°C that is higher than the cloud point of specific surfactant (Ma et al., 2012; Raiti et al., 2015). Effect of temperature (25-90°C) on the efficiency of extraction process was investigated for different surfactants in the literature as can be seen from Table 2.5. While increasing temperature enhances the solubility of PCs and recovery of them (El-Abbasi et al., 2014), there is a possibility for the decomposition of bio-phenolic antioxidants (Ma et al., 2012).

2.3. Aqueous Two-Phase Extraction of Biomolecules

Aqueous two-phase extraction systems, as introduced to the literature by Albertson, offers two different phases which have different chemical environments for the partitioning of biomolecules (Albertson, 1986; Rito-Palomares and Benavides, 2017). Essentially, ATPS is based on liquid-liquid fractionation technique that would consists of the combination of polymer/surfactant/ionic liquid/hydrophilic alcohols and salts which induce the formation of two phases under specific conditions (Iqbal et al., 2016; Nadar et al., 2017; Rito-Palomares and Benavides, 2017). Selective partitioning of target compounds in one phase and non-target compounds in the other phase can be obtained with ATPE process as shown in Figure 2.3. Main advantages of these systems are fast separation, mild environment, no need for pretreatment, energy efficiency and better quality of extracts due to reduced destruction of matrix components (Hatti-Kaul, 2001; Zhang et al., 2017).

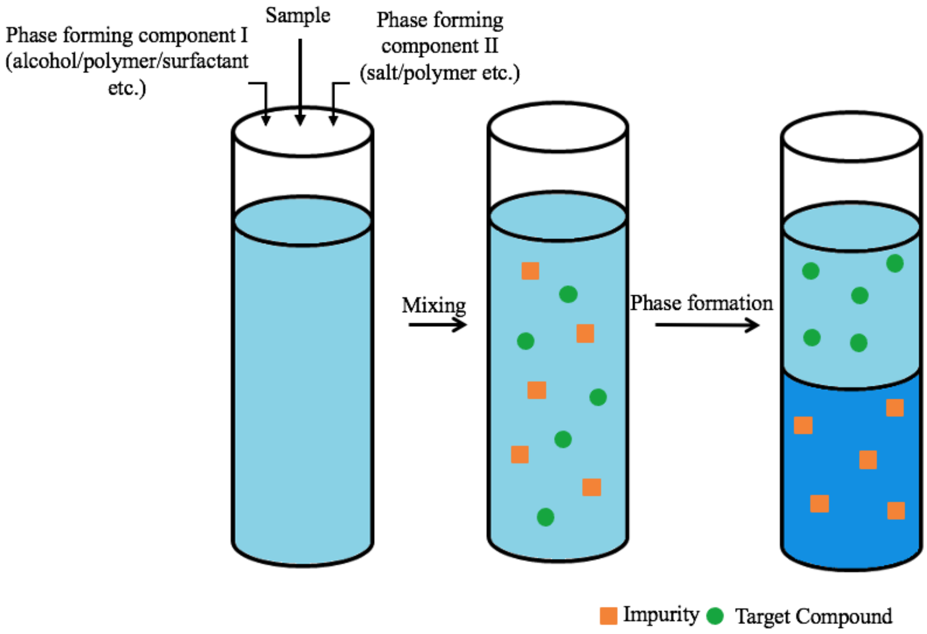


Figure 2.3. Representation of partitioning of biomolecules in top and bottom phases of ATPSs.

Partitioning of sample components subjected to extraction in ATPS depends on many factors such as mass fraction of system components, volume ratio (V_r), Tie Line Length (TLL), pH and temperature of the system (Ruiz-Ruiz et al., 2012; Rito-Palomares and Benavides, 2017) as well as hydrophobicity, molecular conformation and isoelectric point of target compound (Nadar et al., 2017).

Prior to preparation of ATPS, construction of phase diagram which shows binodal curve is essential and this diagram is necessary to define mass fractions in different working areas (Figure 2.4). Binodal curve defines the boundary of one-phase and two-phase forming areas (Iqbal et al., 2016) as can be seen in Figure 2.4. Therefore, the amount of phase components (%, *w/w*) to form two phases and the concentrations of phase components in each phase can be deduced from phase diagram. In other words, it provides data for the design of ATPE process (Raja et al., 2011).

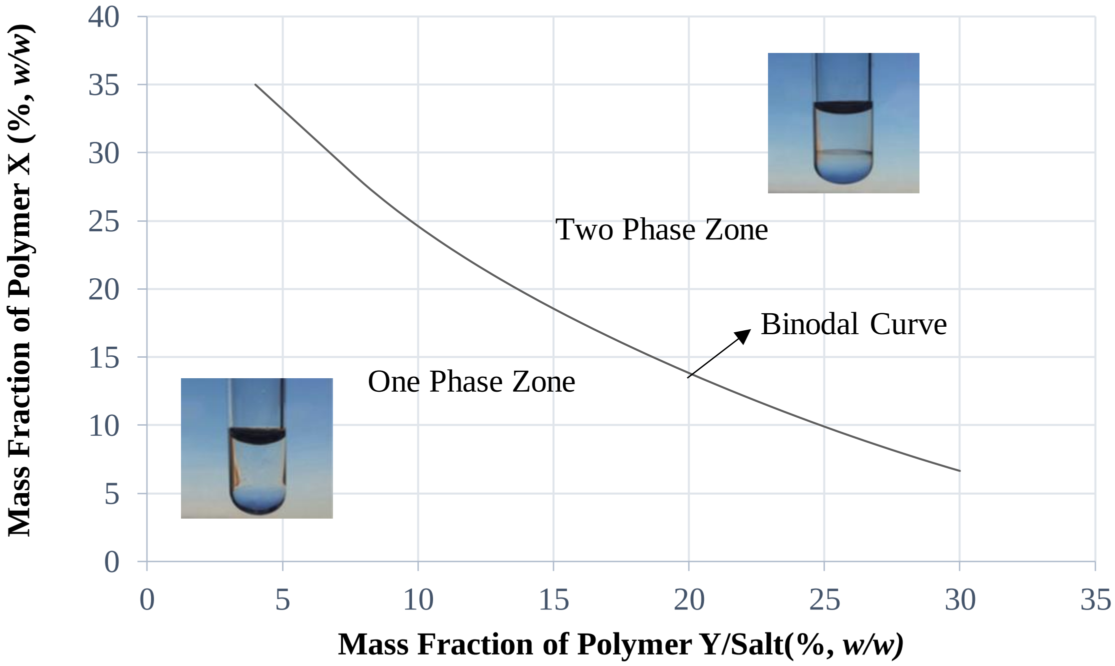


Figure 2.4. Representation of binodal curve.

Various investigations have been focused on effects of pH and temperature on binodal curve of different polymer/surfactant/alcohol and salt systems in the literature (Wang et al., 2011; Ghahremani and Rahimpour, 2013; Chakraborty and Sen, 2016; Arzideh et al., 2018; Nascimento et al., 2018). Achieved results show that the effects of pH and temperature on binodal curve depend on the type of phase components. For instance, an increase in biphasic area is obtained with increasing temperature for polymer-surfactant based ATPS while an opposite effect is observed for polymer-

polymer systems. On the other hand, no significant change in binodal line was observed with an increase in temperature for alcohol-salt based ATPSs (Chakraborty and Sen, 2016).

In equilibrium diagram, tie line connects two nodes on binodal curve and locates on two-phase area of the binodal curve. Points located on the same TLL have different system mass fractions, but they have identical top and bottom compositions (Rito-Palomares and Benavides, 2017). Therefore, TLL is a tool which is usually used as a design parameter for ATPS to evaluate the partitioning of compounds (Hatti-Kaul, 2001). TLL can affect partitioning of biomolecules by hydrophobicity difference between phases and interfacial tension. When TLL increases, mass fractions of phase forming components increase. Hence, reduction of water availability makes the system more hydrophobic (Nadar et al., 2017).

A detailed information about different types of ATPE, micelle based, polymer-based and alcohol-salt based, is presented in the following sections.

2.3.1. Micelle Based Aqueous Two-Phase Extraction

In micelle based ATPS separation of phases can be achieved by heating the system to a temperature acknowledged as cloud point temperature (CPT) (Villegas et al., 2016) and additionally, inorganic salts could facilitate the phase separation by reducing cloud point temperature (Gortzi et al., 2008). Before heating the micellar system, non-ionic surfactant endures as a monomer in aqueous solution. When the temperature of the solution exceeds cloud point temperature, hydrogen bonds between surfactant and water molecules are broken. There molecules form micelles which consist of hydrophobic tail and hydrophilic head part as shown in Figure 2.5. However, without increasing the temperature only salt addition can provide the formation of micelles. In this case, phase separation is completed via salting-out effect and hence time needed for phase separation reduces due to absence of heating process (Raiti et al., 2015; Mortada, 2020). The performances of salts for enhancing phase formation depend on their salting out ability that obeys Hofmeister series (Hatti-Kaul, 2001). In micellar based ATPE, NaCl is commonly used salt at varying concentrations (Rito-Palomares and Benavides, 2017).

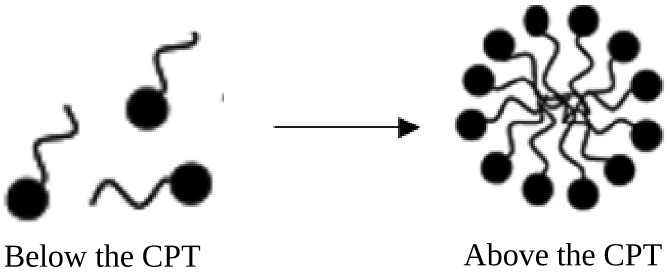


Figure 2.5. Formation of micelles after cloud point temperature.

Advantages of micellar ATPE are simplicity, shorter extraction time, limited equipment requirement and use of non-toxic compounds. Thus, safer environment for the recovery of biological materials can be obtained (Gortzi et al., 2008; Rito-Palomares and Benavides, 2017). Besides, surfactant regeneration and reuse are the economical merit of micellar ATPE and various methods such as ultrafiltration, pH or temperature adjustment have been investigated in literature (Gortzi et al., 2008; Ghouas et al., 2016). On the other hand, there are some limitations for the application of micellar ATPE such as difficulties in detection of analytes due to complex nature of surfactants and temperature constrainment during phase separation for thermally labile compounds (Arya et al., 2019). Besides, it is not compatible with some biocompounds such as thermo-sensitive enzymes (Nadar et al., 2017).

2.3.2. Polymer based Aqueous Two-Phase Extraction

In polymer based ATPE, polymer/electrolyte solution or polymer/low molecular weight solvents are employed for the formation of two-phase system. The components commonly used are dextran (DEX) and polyethylene glycol (PEG) (Rito-Palomares and Benavides, 2017). In PEG-DEX systems, hydrophobic PEG phase constitutes the top phase while bottom phase consists of hydrophilic dextran. The major bottlenecks of the polymer based ATPE are high viscosity resulted from the nature of the polymers and incapability for the large-scale applications. However, replacement of dextran with cheaper compounds such as polyacrylic acid, maltodextrin, starch etc. or usage of polymer-salt systems instead of two polymers make this system more environmentally friendly (Goja et al., 2013; Rito-Palomares and Benavides, 2017). The type of the salt must be carefully selected since it can manipulate the hydrophobic interaction among the components of ATPE (Nadar et al., 2017). In the polymer-salt systems, PEG-phosphate systems are commonly used in various studies (Babu et al., 2008; Naganagouda and Mulimani, 2008; Chethana et al., 2014; Simental-Martínez et al., 2014; Ramesh and Murty, 2015**;** Ramakrishnan et al., 2016; Xavier et al., 2016) due to their low viscosity and quick separation ability (Rito-Palomares and Benavides, 2017).

Various parameters such as mass fraction (%, w/w) of polymer and salt, molecular weight and size of polymer, and pH are effective factors in the partitioning of the biomolecules (Iqbal et al., 2016; Panadare and Rathod et al., 2018). For instance, an increase in molecular weight of polymer can reduce necessary amount of phase-forming polymer in the system and the partitioning coefficient of biomolecule can change by the variation system hydrophobicity (Iqbal et al., 2016). Therefore, nature and molecular weight of polymer should be considered to achieve better separation of biomolecules. However, high viscosity and low polarity of polymers, slow segregation of phases and difficulties in recycling of the phase components are the main drawbacks of polymer-polymer ATPS (Cienfuegos et al., 2017). Additionally, recovery of low molecular weight compounds is limited and their isolation from phases are partially obtained in PEG-DEX ATPS (Simental-Martínez et al., 2014).

2.3.3. Alcohol-Salt Based Aqueous Two-Phase Extraction

Alcohol-salt based ATPS can be formed by mixing of immiscible aliphatic alcohol and salt solution. This system which consists of alcohol-rich top phase and salt-rich bottom phase provides many advantages such as having cheap components unlike polymer or surfactant systems, high polarity, low viscosity, easy mixing, quick and easy solvent separation (Rito-Palomares and Benavides, 2017). On the other hand, alcohol-salt based ATPS may cause the accumulation of biomacromolecules at the interface and loss of activity of compounds (Ooi et al., 2009). However, these advantageous are not valid for the low molecular weight compounds which also easily dissolve in the alcohol-rich phase of ATPS (Simental-Martínez et al., 2014).

Alcohol-salt ATPSs have been used for the extraction of PCs from various plants such as mulberry, grape seed, marigold flower etc. (Table 2.6). To enhance efficiency of extraction, various assistance methods such as microwave, ultrasound etc. have also been used (Dang et al., 2013; Ma et al., 2013; Zhang et al., 2013; Wang et al., 2015; Yang and Lü, 2016; Dordevic and Antov, 2017; Hou et al., 2017; Fu et al., 2019; Zhou et al., 2019; Zhao et al., 2020). As can be seen from Table 2.6, the effects of various factors such as type and mass fractions of phase forming components, sample load, temperature, pH, extraction time, TLL and volume ratio (V_r) on the performance of PCs have been investigated in the literature.

Table 2.6. Extraction of PCs with alcohol-salt based ATPE.

Components of ATPS	**Natural Source**	**Extraction Performance**	**Factors Effecting Extraction Efficiency**	**Reference**
EtOH-$(NH_4)_2SO_4$	Mulberry	K: 85.1% (Antocyanins)	Mass fractions of alcohol and salt, sample load, temperature, pH	Wu et al., 2011
EtOH-$(NH_4)_2SO_4$	Artemisia argyi leaves	R: 93.2% (Flavonoids)	Type of salt, concentration of alcohol, the amount of added salt ($MgCl_2$), temperature	Xie et al., 2012
EtOH-$(NH_4)_3C_6H_5O_7$ (*microwave assisted)*	Grape seed	R:97.1% (TPC)	Mass fractions of alcohol and salt, extraction time and temperature	Dang et al., 2013
EtOH-K_2HPO_4 (*microwave assisted)*	*Dalbergia odorifera* T. Chen leaves	Y: 2.0 mg/g (Biochanin)	Mass fractions of alcohol and salt, extraction time	Ma et al., 2013
EtOH- K_2HPO_4 (*Microwave assisted)*	Pigeon pea roots	R:94.0 % (Apigenin)	Mass fractions of alcohol and salt, temperature, extraction time, solid/liquid ratio	Zhang et al., 2013
1-propanol + K_2HPO_4 /KH_2PO_4	Acerola waste	K:98.6% (Rutin)	Type of alcohol and salt, the composition of the system, sample load, temperature and addition of NaCl	Reis et al., 2014
EtOH-K_2HPO_4/KH_2PO_4	Carrot tissue	R:81.9% (Chlorogenic acid)	Type of ATPS and salt, TLL, V_R, pH	Sánchez-Rangel et al., 2014
EtOH-NaH_2PO_4	Grape juice	K:90.0% (Antocyanins)	Type of salt, mass fraction of alcohol, temperature, sample load, extraction time	Wu et al., 2014
EtOH-K_2HPO_4	Fig fruits	K:75.0% (TPC)	Mass fraction of alcohol and salt, sample loading, temperature	Feng et al., 2015
EtOH-$(NH_4)_2SO_4$ *(NPC* assisted)*	Pigeon pea leaves	R:10.2 mg/g (CSA)**	Mass fractions of alcohol and salt, temperature, extraction time, solid/liquid ratio	Wang et al., 2015
EtOH-$(NH_4)_2SO_4$ (*microwave assisted)*	*Taraxacum mongolicum*	Y:5.6% (Flavonoids)	Mass fractions of alcohol and salt, pH	Yang and Lü, 2016
EtOH-$(NH_4)_2SO_4$ (*ultrasound assisted)*	Wheat Chaff	K:64.0% (TPC)	TLL, sample load, pH, ultrasound assistance	Dordevic and Antov, 2017

Table 2.6. Extraction of PCs with alcohol-salt based ATPE. (continues)

Components of ATPS	**Natural Source**	**Extraction Performance**	**Factors Effecting Extraction Efficiency**	**Reference**
EtOH-K_2HPO_4 (*Chelatometric method assisted*)	*Folium nelumbinis*	EE: 90.4% (Flavonoids)	Extraction system, mass fractions of alcohol and salt, pH	Hou et al., 2017
EtOH-$(NH_4)_2SO_4$	Garlic	EE: 94.7% (Allicin)	Mass fractions of alcohol and salt, sample load	Li et al., 2017
EtOH-$(NH_4)_2SO_4$	Eucalyptus Wood Wastes	K:2.3 (TPC)	Type of salt, TLL, V_R, temperature	Xavier et al., 2017
EtOH-$(NH_4)_2SO_4$ (*Enzyme assisted)*	Marigold flower	***RE:95.4 % (TPC)	Mass fractions of alcohol and salt, pH	Fu et al., 2019
EtOH-$(NH_4)_2SO_4$ (*ultrasound assisted)*	*Polygonum cuspidatum*	RE:99.1% (Resveratrol)	Mass fractions of alcohol and salt	Zhou et al., 2019
EtOH-NaH_2PO_4	Haskap leaves	K: 6.6 (TPC)	Type of ATPE, TLL	Chong et al., 2020
EtOH-K_2HPO_4/KH_2PO_4	*Sedum T dendroideum*	RE: 8.0% (TPC)	V_r, TLL, sample loading, addition of NaCl	Enriquez-Ochoa et al., 2020
1-Propanol+$MgSO_4$	*Garcinia indica*	EE: 96.3 % (Antocyanins)	Mass fraction of alcohol and salt, TLL	Nainegali et al., 2020
EtOH-$(NH_4)_2SO_4$ (*Microwave assisted)*	*Ribes nigrum* L.	RE:72.4% (TPC)	Extraction method, mass fractions of alcohol and salt	Zhao et al., 2020

NPC*: Negative-Pressure Cavitation, CSA**: Cajaninstilbene Acid, ***RE: Recovery.

System parameters that affect extraction efficiency in ATPS is crucial in order to enhance yield, recovery and partition coefficient of the extracted matter and reduce impurities. Partition behavior in ATPS depends on salting-out and hydrophobicity in ATPS (Gómez-Loredo et al., 2014). In alcohol-salt ATPS, chemical compounds with weak polarity have a tendency to accumulate in alcohol-rich top phase whilst those with stronger polarity shifts to salt-rich bottom phase (Yang and Lü, 2016). Hence, hydrophilic compounds such as protein, polysaccharides, organic acids are expected to partition towards to the bottom phase while hydrophobic or amphiphilic compounds partition towards to the top phase (Gómez-Loredo et al., 2014; Feng et al., 2015; Qin et al., 2017).

As can be seen from Table 2.6, effect of mass fraction of phase forming components, alcohol and salt, is widely investigated in the literature. Indeed, mass fractions of alcohol and salt not only affect extraction performance but also determine whether formation of two phase (Wu et al., 2011;

Zhang et al., 2013). When the salt mass fraction was fixed and alcohol mass fraction increases, volume of top phase increases (Dang et al., 2013). Similarly, an increase in salt mass fraction causes reduction in the volume of top phase. Then, molecules concentrate towards either salt or alcohol phases according to their polarities (Ma et al., 2013).

TLL is an important factor which affects extraction performance. In general, higher TLL values enhance the partitioning of various phenolic compounds. For instance, the extraction yield of chlorogenic acid and hydroxycinnamic acid derivatives from carrot tissue investigated in the TLL range of 30-50 has the highest value in EtOH- potassium phosphate ATPS at TLL=50 (Sánchez-Rangel et al., 2014). Similar trend was observed for the partitioning of PCs from towards top phase and sugars towards bottom phase for the recovery of PCs and removal of sugars from wheat chaff sample with EtOH-$(NH_4)_2SO_4$ ATPS. Highest recovery of PCs (67.0%) was attained at highest TLL (TLL=60.1) (Dordevic and Antov et al., 2017). Xavier and co-workers also obtained highest partitioning with highest TLL (TLL=57) for the extraction of PCs from eucalyptus wood with EtOH-$(NH_4)_2SO_4$ ATPS (Xavier et al., 2017). The explanation behind this trend is the higher salting out effect at high TLL. Hence, major polarity difference between top and bottom phases results in more easily transfer of compounds to the phase of interest (Dordevic and Antov et al., 2017; Xavier et al., 2017). On the other hand, for the EtOH- NaH_2PO_4 ATPS, higher PC yields were obtained with relatively low TLL value (TLL=33.60) (Chong et al., 2020).

Besides TLL, temperature is also crucial factor for the partitioning of biomolecules in ATPS. Xavier and colleagues investigated effect of temperature (25-45-65 °C) on recovery of PCs and results showed that even though best recovery of PCs is obtained at highest temperature (65 °C), selectivity of PCs decreased due to increasing solubility of extracted compounds (Xavier et al., 2017). Feng and co-workers showed that distribution of PCs and sugars did not change significantly depending on temperature (between 30-10 °C) for EtOH-K_2HPO_4 ATPS (Feng et al., 2015). Similarly, Wu and colleagues revealed that temperature (between 25-50 °C) had almost no effect on distribution of anthocyanins between top and bottom phases in EtOH-$(NH_4)_2SO_4$ ATPS (Wu et al., 2011). Hence, temperature of 20°C or 25°C was chosen as a working condition in various studies in literature (Guo et al., 2012; Chen et al., 2013; Qin et al., 2017).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Olive Mill Wastewater Concentrates

The wastewater used in this study was originated from an olive oil extraction plant utilized two-phase centrifugal system. The wastewater collected in the the December 2018- January 2019 period was concentrated by using a pilot scale mechanical vapour recompression evaporator (MVR) on site. Solid concentrated sample (OMWC) has a very sticky nature, dark colour and distinguishable odour. It was stocked in the dark at +4 °C to protect its stability until chemical analyses were performed.

Due to the sticky nature of OMWC, heating to 35-40 °C was necessary for the weighting of the sample.

3.1.2. Other Chemicals and Standards

Since the OMWW is rich in HTyr and Oleu they were selected as target antioxidants in this study. Fresh stock solutions of HTyr and Oleu (≥98% purity, Sigma- Aldrich) were prepared in MeOH as 5 mg/ mL and 1 mg/mL concentrations, respectively and they were stored at -20°C until use in LC/MS-MS analysis as standards. The physicochemical properties of target antioxidants are shown in Table 3.1.

Table 3.1. Physicochemical characteristics of HTyr and Oleu.

Antioxidant	**Molecular Weight (g/mol)**	**Water Solubility (g/L)**	**pK_a**
HTyr	154.2	17.4	9.45
Oleu	540.5	0.73	4.94

All chemicals together with their intended use in the experiments are listed in Table 3.2.

Table 3.2. Chemical substances used in the experiments.

Chemical Name	**Molecular Formula**	**CAS number**	**Experimental Use**	**Supplier**
Ethanol	C_2H_5OH	64-17-5	ATPE	Isolab
Ammonium Sulfate	$(NH_4)_2SO_4$	7783-20-2	ATPE	Sigma-Aldrich
Dipotassium hydrogen phosphate	K_2HPO_4	7758-11-4	ATPE	Merck
Sodium Carbonate	Na_2CO_3	497-10-8	TP	Merck
Gallic Acid	$C_7H_6O_5$	149-91-7	TP	Merck
Folin- Ciocalteu Reagent	$C_{10}H_5NaO_5S$	521-24-4	TP	Merck
Anthrone	$C_{14}H_{10}O$	90-44-8	Carbohydrate Analysis	Sigma-Aldrich
Alpha-D-Glucose	$C_6H_{12}O_6$	492-62-6	Carbohydrate Analysis	Sigma-Aldrich
Sulfuric Acid	H_2SO_4	7664-93-9	Carbohydrate Analysis	Sigma-Aldrich
Bradford Reagent	-	-	Protein Analysis	Sigma-Aldrich
Bovine Serum Albumin	-	9048-46-8	Protein Analysis	Sigma-Aldrich
Galacturonic Acid	$C_6H_{10}O_7$	685-73-4	Pectin Analysis	Sigma-Aldrich
Sodium Tetraborate	$Na_2B_40_7. 10H_2O$	12267-73-1	Pectin Analysis	Merck
m-Hydroxydiphenyl	$C_{12}H_{10}O$	580-51-8	Pectin Analysis	Sigma-Aldrich
Sodium Hydroxide	NaOH	1210-73-2	Pectin Analysis	Riedel-de Haën
2,2-Diphenyl-1-picrylhydrazyl	$C_{18}H_{12}N_5O_6$	1898-66-4	Antioxidant Activity	Sigma-Aldrich
L-Ascorbic acid	$C_6H_8O_6$	50-81-7	Antioxidant Activity	Sigma-Aldrich
Formic Acid	HCOOH	64-18-6	LC-MS/MS	Sigma-Aldrich
Water	H_2O	7732-18-5	LC-MS/MS	Merck
Ammonium Formate	HCO_2NH_4	540-69-2	LC-MS/MS	Sigma-Aldrich
Sodium- Lauroyl Sarcasine	$C_{15}H_{28}NNaO_3$	137-16-6	LC-MS/MS	Amresco
Methanol	CH_3OH	67-56-1	LC-MS/MS	Merck
Hydroxytyrosol	$C_8H_{10}O_3$	10597-60-1	LC-MS/MS	Sigma-Aldrich
Oleuropein	$C_{25}H_{32}0_{13}$	32619-42-4	LC-MS/MS	Sigma-Aldrich

3.2. Methods

3.2.1. Extraction of Phenolic Antioxidants from OMWC by Using Aqueous Two-Phase System

Two different alcohol-salt based ATPSs, namely EtOH-$(NH_4)_2SO_4$ and EtOH-K_2HPO_4, were used for the extraction of phenolic antioxidants from OMWC in this research. For this purpose, first phase diagram of each ATPS was determined. Then, TLLs were determined based on binodal data. Finally, different volume ratios on the TLLs were selected to evaluate effects of TLL, V_r, and type of ATPS on the partitioning of PCs and impurities such as proteins and carbohydrates.

3.2.1.1. Determination of phase diagrams and tie line length. EtOH and two different salts with acidic and basic nature, namely ammonium sulfate $(NH_4)_2SO_4$ and hydrogen phosphate, KH_2PO_4 were the selected components of ATPS. Even though several methods proposed for construction of binodal curve such as turbidimetric titration method, node determination etc., cloud point method has been used as a common practice in literature (Chakraborty and Sen, 2016). Therefore, the phase diagrams of EtOH-$(NH_4)_2SO_4$ and EtOH-K_2HPO_4 systems were determined by using cloud point titration method (Fu et al., 2015). In brief, after the preparation of aqueous salt solution with known concentration, EtOH was slowly added to the solution until it becomes turbid. Mixing of the solution was ensured with a magnetic stirrer. The weight of the solution was recorded to calculate the mass fraction of each component at the cloud point. Then, water was added to turbid solution until it becomes clear and procedure was repeated to obtain sufficient data to construct binodal curve. Mass fractions of alcohol and salt (%, w/w) were calculated by equations 3.1 and 3.2 (Rito and Palomares, 2017).

$$\text{Mass Fraction of alcohol } (\%, w/w) = \frac{Weight\ of\ alcohol(g)}{Weight\ of\ solution(g)} \tag{3.1}$$

$$\text{Mass Fraction of salt } (\%, w/w) = \frac{Weight\ of\ salt(g)}{Weight\ of\ solution(g)} \tag{3.2}$$

where weight of solution is the sum of weights of water, alcohol and salt.

After having experimental data, binodal curve was fitted by three-parameter or four-parameter empirical equations shown in Table 3.3. A non-linear equation initially developed by Merchuk et al. (Merchuk et al., 1998) for PEG4000-phosphate system and it was modified with several researchers to define different ATPSs including alcohol-salt, polymer-salt, polymer-surfactant, surfactant-salt and ionic liquid.

Table 3.3. Correlations used for binodal curve.

Type of ATPS	**Equations/Equation number**	**References**
Alcohol-salt, polymer-salt, polymer surfactant, surfactant- salt, ionic liquid based ATPE	$Y_B = a \exp[b(X_B^{0.5}) - c(X_B^3)]$ $Y_T = a \exp[b(X_T^{0.5}) - c(X_T^3)]$ (3.3)	Merchuk et al., 1998; Xie et al., 2010; Reis et al., 2011; Liu et al., 2012; Liu et al., 2013a; Madadi et al., 2013; Ghahremani and Rahimpour, 2013; Taghavivand and Pazuki, 2014; Fu et al., 2015; Chakraborty and Sen, 2016; Santos et al., 2016; Li et al., 2016; Arzideh et al., 2018; Caldeira et al., 2019; Leong et al., 2019
Alcohol-salt, polymer-salt, polymer surfactant ATPE	$Y_B = a + b(X_B^{0.5}) + c(X_B)$ $Y_T = a + b(X_T^{0.5}) + c(X_T)$ (3.4)	Murugesan and Perumalsamy, 2005; Liu et al., 2013a; Caldeira et al., 2019
Alcohol-salt, polymer-salt, polymer surfactant ATPE	$\ln Y_B = a + b(X_B^{0.5}) + c(X_B) + d(X_B^2)$ $\ln Y_T = a + b(X_T^{0.5}) + c(X_T) + d(X_T^2).$ (3.5)	Katayama and Miyahara, 2006; Liu et al., 2013a; Khayati and Shahriari, 2016; Li et al., 2016; Nascimento et al., 2018; Caldeira et al., 2019; Lu et al., 2020
Alcohol- salt based ATPE	$Y_T = a_1\exp(-X_T/b_1) + a_2\exp(-X_T/b_2) + c$ $Y_B = a_1\exp(-X_B/b_1) + a_2\exp(-X_B/b_2) + c$ (3.6)	Wang et al., 2009; Wang et al., 2010; Wang et al., 2011; Nainegali et al., 2019

In these equations, X and Y are the mass fractions of phase forming components, respectively; T and B refer top and bottom, respectively; a, b, c and d are constants of the equations. Non-linear regression of the experimental binodal curve of both EtOH-$(NH_4)_2SO_4$ and EtOH- KH_2PO_4 systems was done with curve fitting tool of Matlab software (version: R2018a, The Mathworks, 2018) and fitting parameters were obtained.

The TLLs associated with each phase diagram were determined by a simple gravimetric method (Merchuk et al., 1998). For this purpose, some points having different mass fractions of salt and alcohol in two-phase area of the phase diagram were selected. Total weight of ATPS was fixed to 10 g, and mass of water, alcohol and salt were determined accordingly. After the preparation of ATPS, phase separation was quickly observed. Then, top and bottom phases were carefully separated and each of them was precisely weighed. Eventually, each tie-line was determined by the application of lever arm rule which states a relationship between top phase composition and the overall system composition (Merchuk et al., 1998). The weight fractions of top and bottom phase components were calculated with the following equations:

$$Y_T = (Y/\alpha) - ((1-\alpha)/\alpha)Y_B \qquad (3.7)$$

$$X_T = (X/\alpha) - ((1-\alpha)/\alpha)X_B \qquad (3.8)$$

where $\alpha = \frac{weight\ of\ the\ top\ phase}{weight\ of\ the\ mixture}$

The slope of tie line (S) and TLL were calculated by using the weight fractions of system components in top and bottom phases;

$$S = (Y_T - Y_B)/(X_T - X_B) \qquad (3.9)$$

$$TLL = \sqrt{\Delta Y^2 + \Delta X^2} \qquad (3.10)$$

The values of X_m (weight of salt, g) and Y_m (weight of alcohol, g) were already known from the working points on the binodal curve, and the value of α was determined with phase separation experiment. X_T, X_B, Y_T and Y_B, weight fraction of the salt and the alcohol in each phase, were resolved via equation solve tool of Matlab. A flow chart for the determination of binodal curve and TLLs is presented in Figure 3.1.

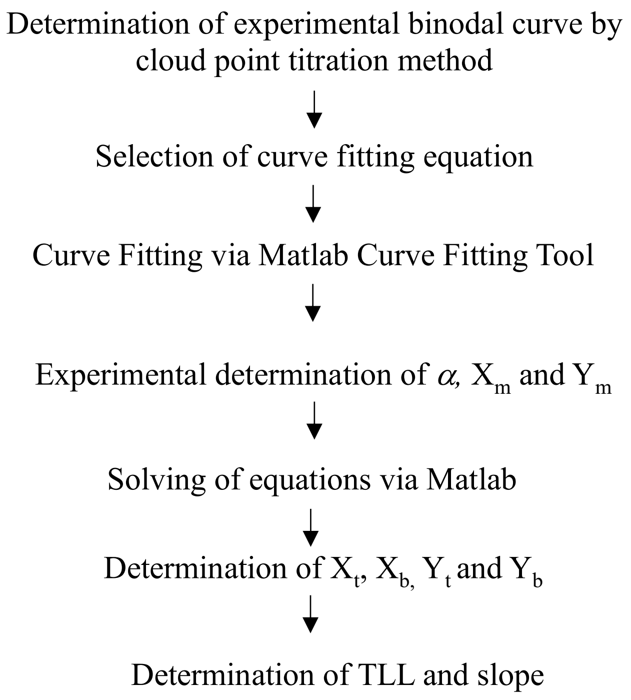


Figure 3.1. Determination of TLL and slope of TLL.

3.2.1.2. Experimental procedure of aqueous two-phase systems. For the preparation of each system, 0.074 g of solid sample was weighted in a falcon tube. After the addition of deionized water, aqueous sample was vortexed for 5 min at 2030 rpm. Subsequently, first EtOH then $(NH_4)_2SO_4$ or KH_2PO_4 were added to system and after the addition of each chemical, the mixing of the system was performed with a vortex for 15 min at 2030 rpm. Followingly, the systems were centrifuged at 3000 rpm for 5 min to achieve phase separation. For the evaluation of top and bottom phase components, they were separated carefully with a pipette and the volume of each system was noted. EtOH was removed from the top phase by using rotary evaporator at 47 °C and residue was resuspended with deionized water. Then both resuspended residue and bottom phase were filtered through a syringe filter (0.22 µm Sartarious). A summary of experimental procedure of ATPS is given in Figure 3.2.

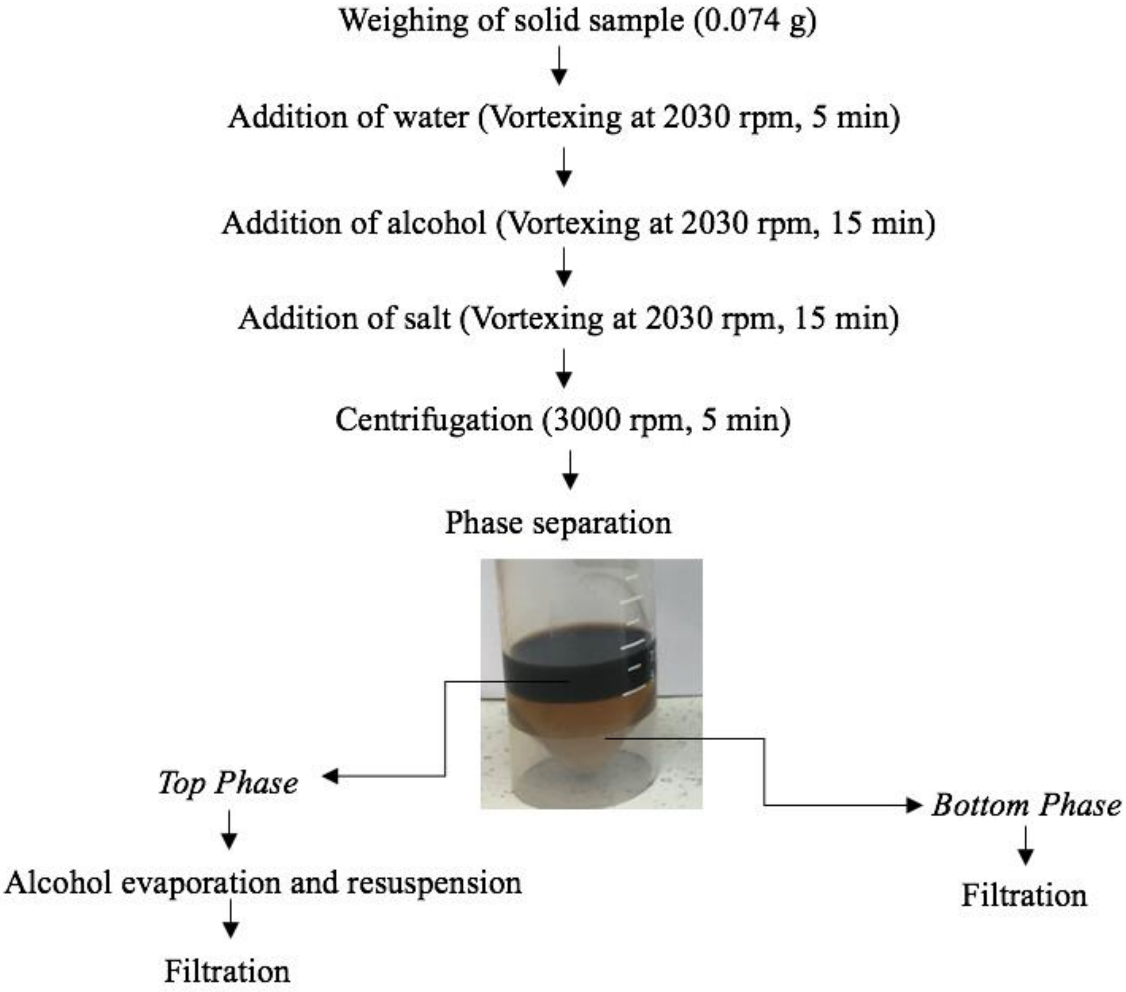


Figure 3.2. Experimental Procedure of ATPE.

3.2.2. Analytical Methods for the Components of OMWC

The characterization of OMWC sample and the performance of ATPE were accomplished by the estimation of TPC, proteins, carbohydrates, and pectins. Additionally, HTyr and Oleu contents of OMWC were examined as well as pH and moisture of the samples.

3.2.2.1. Total phenolic content. TPC of the samples was determined via colorimetric method which is known as a convenient, simple, and reproducible assay for routine quantification of PCs (Singleton and Rossi, 1965; Huang et al., 2005). This method based upon the reduction of a phosphomolybdate /phosphotungstate complex found in Folin-Ciocalteu reagent by phenolics to blue reaction products which exhibited a maximum absorption at 765 nm as shown in equation 3.11.

$$\text{Mo (IV)} + e^{-}\ \text{(antioxidant)} \longrightarrow \underset{(\lambda_{max}\text{: 765 nm})}{\text{Mo (V)}} \qquad (3.11)$$

In TPC analysis, first, the aliquots of aqueous solution of OMWC or extract (0.5 ml) was mixed with 0.2 N Folin-Ciocalteu reagent (2.5 mL); then, sodium carbonate solution (2.0 mL, 7.5 %, w/v) was added to obtain basic medium (pH almost 10) since oxidation of phenolic compounds with this reagent occurs under alkaline conditions (Huang et al., 2005). Finally, before cooling to room temperature the assay tubes were incubated in a water bath at 50 °C for 5 min.

The absorbance values of samples at λ=765 nm were determined against a blank sample with a spectrophotometer (Shimadzu UV-1208). All measurements were recorded in triplicates. Gallic acid was the standard and a calibration curve with five points was constructed in the concentration range of 10 mg/L to 80 mg/L (Appendix A.1). In Figure 3.3, colour intensity of the gallic acid standards is shown.

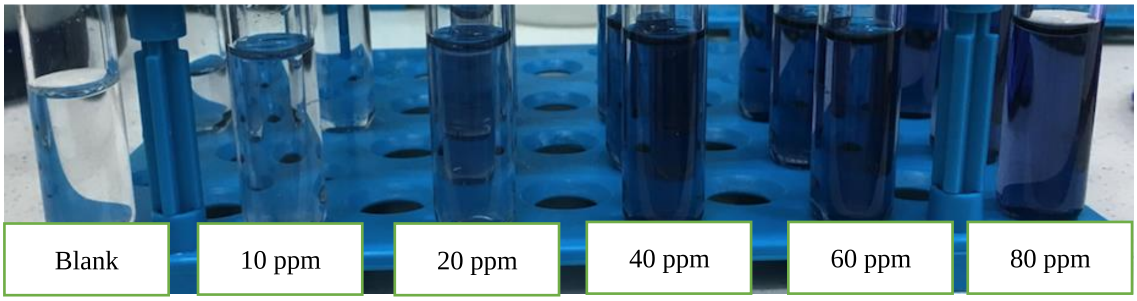
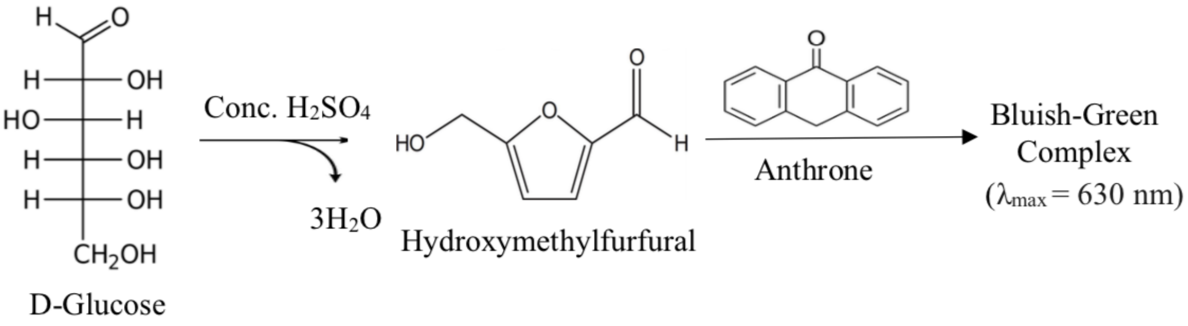


Figure 3.3. Gallic acid standards at different concentrations used in TPC analysis.

The results of TPC in samples were expressed as Gallic Acid Equivalents (GAE). Highly reproducible results for standards ($R^2 > 0.9996$) and samples were obtained.

3.2.2.2. Carbohydrate content. The carbohydrate content of OMWC and extracted samples from ATPSs was determined with the Anthrone Method, which provides rapid colorimetric estimation of carbohydrates either free or present in polysaccharides (Jagannathan et al., 2010). Under the test conditions carbohydrates are converted to glucose and dehydration of carbohydrates with concentrated H_2SO_4 forms “Furfural”, which condenses with Anthrone reagent (equation 3.11). Finally, the formation of a green colour complex was determined by measuring the absorbance at λ=630 nm.



(3.12)

After mixing the sample with the reagent which consists of 200 mg Anthrone reagent in 100 mL concentrated sulfuric acid at 1:4 volume ratio it was rested in an ice bath to prevent instant temperature increase. Before measuring absorbance values, the assay tubes were cooled to room temperature for five minutes to complete the reaction. Alpha-D-glucose was used as calibration standard and the results were expressed as glucose equivalents (mg glucose/g of extract). In this test, Anthrone reagent was prepared daily and used within 12 h (Yemm and Willis, 1954). The calibration curve for the standards prepared in 10-100 mg/L concentration range (Figure 3.4) is presented in Appendix A.2.

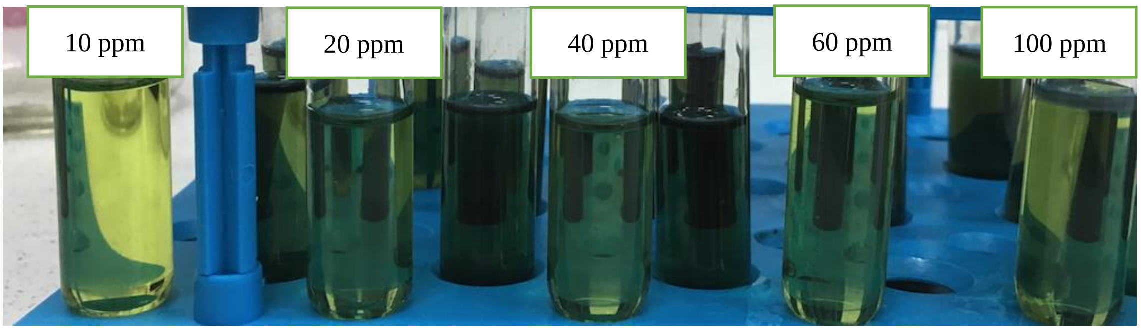
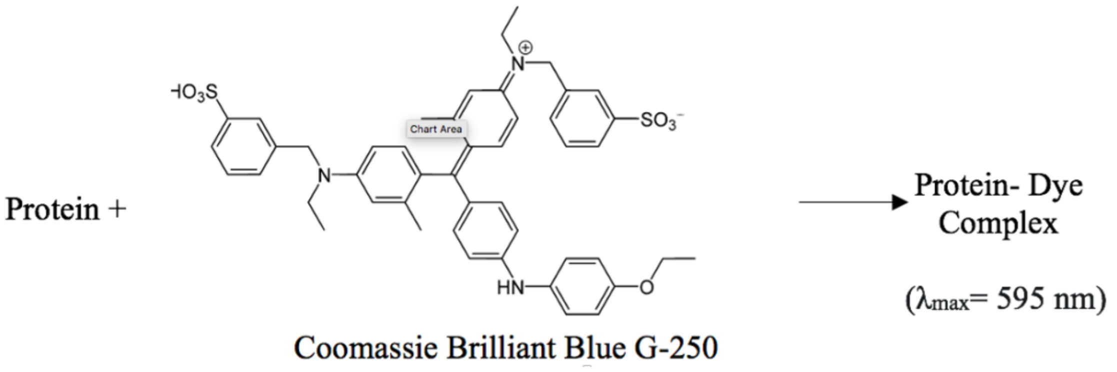


Figure 3.4. Alpha-D-Glucose standards at different concentrations used in carbohydrate analysis.

3.2.2.3. Protein content. Bradford Method (Bradford, 1976) was used to determine protein content of the samples. Bradford reagent contains the dye Coomassie Brilliant Blue G-250 which can be exist in two different colors: red and blue. When it binds to the protein fraction of the sample (equation 3.13) and it causes a color change from red to blue, which gives highest absorbance value at λ=595 nm.



(3.13)

For the estimation of protein content, 100 μl of sample and 5 mL of Bradford reagent were vortexed and assay tubes were stored at room temperature for 5 min in the dark. Then, absorbance values recorded at λ=595 nm in triplicates. The calibration curve was constructed with bovine serum albumin (BSA) protein standards in the concentration range of 0.01-1 g/L (Appendix A.3). Color change of BSA standards in protein analysis is presented in Figure 3.5.

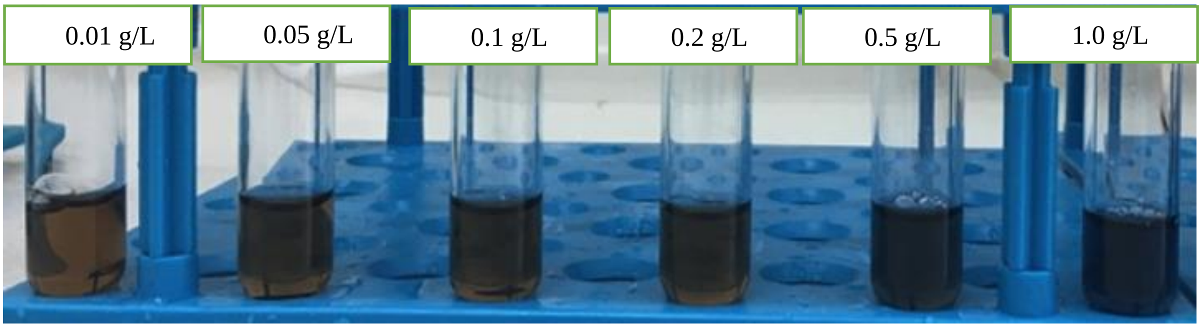


Figure 3.5. BSA standards at different concentrations used in protein analysis.

3.2.2.4. Pectin content. For the estimation of pectins in OMWC, m-hydroxydiphenly-sulfuric acid assay was used (Blumenkrantz and Asboe-Hansen, 1973). This analysis is based on the reaction of m-hydroxydiphenly with pectin derivatives (e.g. galacturonic acid) in the presence of concentrated acid generating pink color as presented in Figure 3.6.

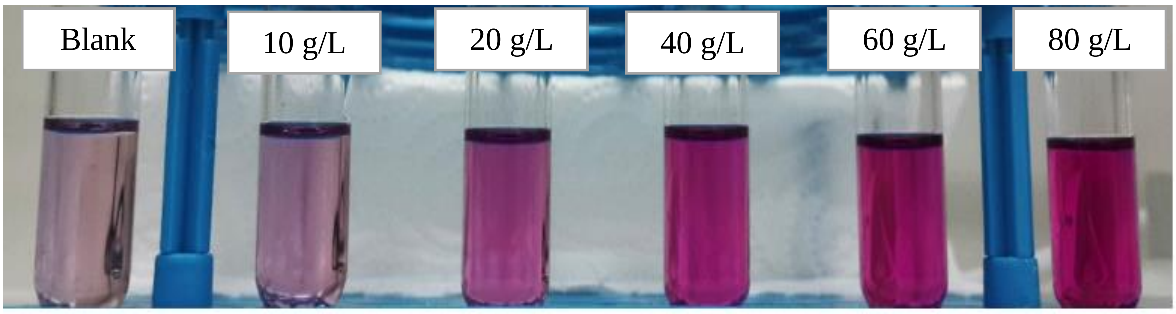


Figure 3.6. Galacturonic Acid standards at different concentrations used in pectin analysis.

For the estimation of pectin content, 0.5 mL of sample mixed with sulfuric acid-tetraborate solution (0.0125 M). After holding in ice bath to prevent rapid raise in temperature, samples were transferred to oil bath at 100 °C for 5 min. After cooling to room temperature, 0.05 mL of m-hydroxydiphenyl (0.15%) and NaOH (0.5%) solutions were added to the samples and blank sample, accordingly. It should be noted that m-hydroxydiphenyl solution was protected from light (Ibarz et al., 2006). Then, each cuvette was allowed to sit for 20 min to allow to bubbles dissipate. Finally, absorbance values were recorded at λ=520 nm with zeroing the device with deionized water (Gebreyohannes et al., 2013; Blumenkrantz and Asboe-Hansen, 1973).

For the calibration curve, 10-80 g/L Galacturonic acid standards were prepared. The calibration curve for the pectin content is shown in Appendix A.4.

3.2.2.5. Determination of total antioxidant activity by DPPH method. The antioxidant activity of OMWC samples were estimated with 2,2 diphenyl-1-picrylhrazyl (DPPH) method which based on the determination of free-radical-scavenging capacity of the antioxidants with the radical DPPH$^-$ (Blois, 1958). In its radical form DPPH has maximum absorption at λ=517 nm. However, after its chemical reaction with antioxidant or radical compounds, the colour disappears. Lone N atom in DPPH is reduced by the antioxidants as shown in Figure 3.7 (Brand-Williams et al., 1995; Ozcelik et al., 2003). Therefore, lower absorbance values imply higher antioxidant activity.

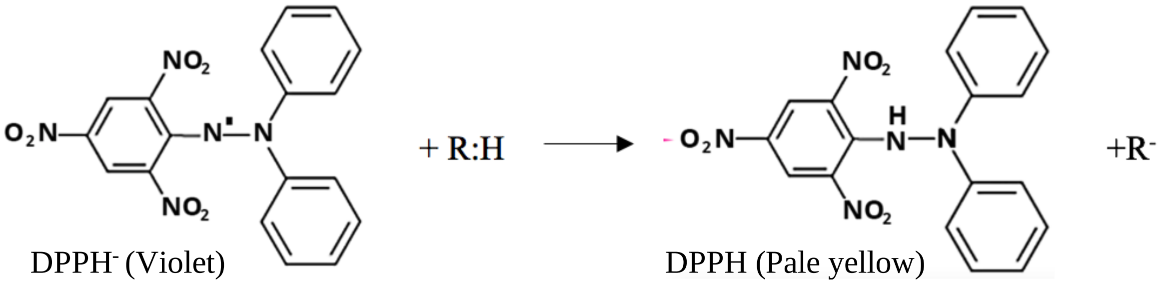


Figure 3.7. Reaction mechanism of DPPH with antioxidant compounds (R:H and $R^{\cdot}$ are antioxidant radical scavenger and antioxidant radical, respectively).

To determine antioxidant capacity, 3.9 mL of DPPH solution in MeOH (1x 10^{-3} M) was mixed vigorously with 0.1 mL of L-ascorbic acid standards (50-150 mg/L concentrations) at room temperature and allowed to stand for 30 min in the dark for reaching the constant absorbance as recommended (Rodríguez et al, 2005). Then, absorbance values recorded at λ= 517 nm. A calibration curve was prepared with L-ascorbic acid standards prepared in the concentration range of 50-300 ppm (Figure 3.8).

It should be noted that methanolic solution of DPPH is highly sensitive to the light as well as oxygen and impurities. Therefore, its protection from light was guaranteed by covering the flask of solution with aluminum foil (Mot et al., 2011). Colour change in standards of ascorbic acid is shown in Figure 3.8 and calibration curve is presented in Appendix A.5.

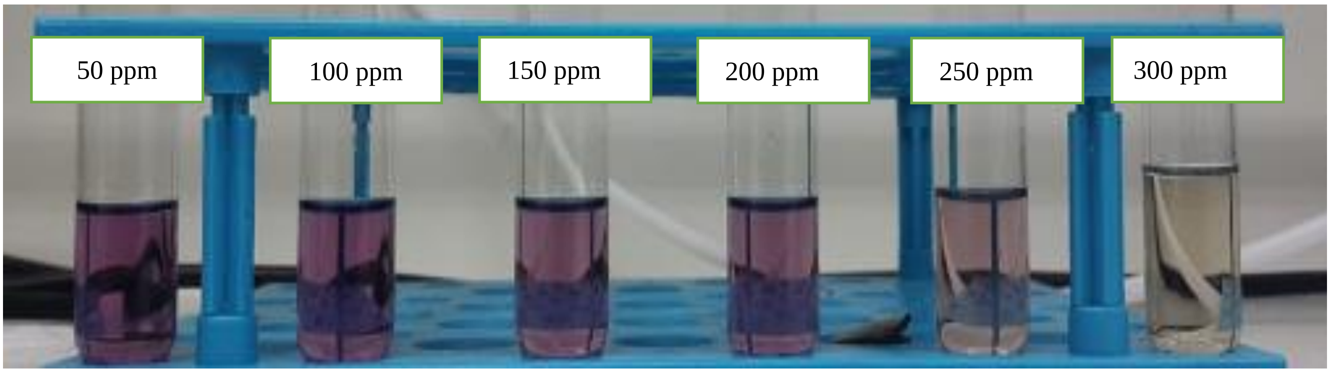


Figure 3.8. Ascorbic Acid standards at different concentrations used in DPPH antioxidant activity analysis.

__3.2.2.6. Quantification of HTyr and Oleu.__ The analyses of two selected polyphenolic antioxidants were carried out with liquid chromatography with tandem mass spectrometry (LC-MS/MS). To determine HTyr and Oleu concentration of the OMWC samples, AB Sciex QTRAP 4500 LC-MS/MS system which united with Eksigent ekspert ultra LC system 110 was used. Multiple reaction monitoring (MRM) was performed in MS/MS system. The chromatographic analysis was implemented with Phenomenex Kinetex C18 column at 40 °C. The Analyst software (AB Sciex) and Multiquant 3.0.1 (AB Sciex) were used for instrumental control and quantification purposes, respectively.

For the calibration of antioxidants, 10, 25, 50, 100 and 200 ppb fresh solutions of HTyr and Oleu were prepared from their stock solutions and Sodium- Lauroyl Sarcasine was used as internal standard. First, parent ion (Q1) of antioxidants was determined. Followingly, MRM parameters which are declustering potential (DP), entrance potential (DP), collusion energy (CE), and collusion cell exit potential (CXP) were determined with “compound optimization” mode and resulted data were shown in Table 3.4. Retention time (rT) of each analyte was determined after completion of MRM optimization. Then, ions which have the highest intensity amongst all, two daughter ions (Q3s) were detected.

Table 3.4. Optimised MS/MS conditions for antioxidant analysis.

Chemical	**Q1 (m/z)**	**Q3 (m/z)**	**rT**	**DP (V)**	**EP (V)**	**CE (V)**	**XCP (V)**	**Ion mode**
HTyr	152.9	122.8	2.77	-65	-10	-20	-3	Negative
HTyr	152.9	122.8	2.77	-65	-10	-20	-9	Negative
Oleu	539.1	274.6	3.66	-105	-10	-34	-7	Negative
Oleu	539.1	377.1	3.66	-105	-10	-34	-7	Negative

Calibration curves obtained for the quantification of HTyr and Oleu are presented in Appendix B.1 and B.2, respectively.

3.2.2.7. pH. The pH values of OMWC samples dissolved in water or aqueous EtOH was determined with WTW 330 pH meter.

3.2.2.8. Moisture content. The moisture content of OMWC sample was determined with Kern DBS version 1.0 Electronic Moisture analyzer by setting the temperature to 120 °C. The weight of OMWC sample was weighted 0.5 g and the measurement was completed within 15 min measurement.

3.2.2.9. Weight determination. Precisa XB 22A analytical balance with 0.0001 g (0.1 mg) precision was used to determine weight of OMWC, salt, and chemicals used in analyses.

3.2.2.10. Statistical analysis. In order to examine correlation between partitioning coefficients of TPCs, HTyr, and Oleu, IBM SPSS (Statistical Package for Social Science) 23 and Bivariate Correlation Test (Pearson's Correlation) were used.

4. RESULTS AND DISCUSSION

4.1. Characteristics of Olive Mill Waste Concentrates

OMWC used in this study was solid with 1.62% moisture content. Prior to focus on extraction of bio-phenolic antioxidants from OMWC, preliminary investigations were done to evaluate the release of OMWC components both into water and EtOH at room temperature by vortexing the samples at 2030 rpm for 15 min. While aqueous concentrations of TPCs, carbohydrates, and proteins are presented as a function of water to dry weight of concentrated sample ratio in Figure 4.1, the aqueous concentrations of these components are shown as a function of EtOH concentration (0-100%, v/v) in Figure 4.2.

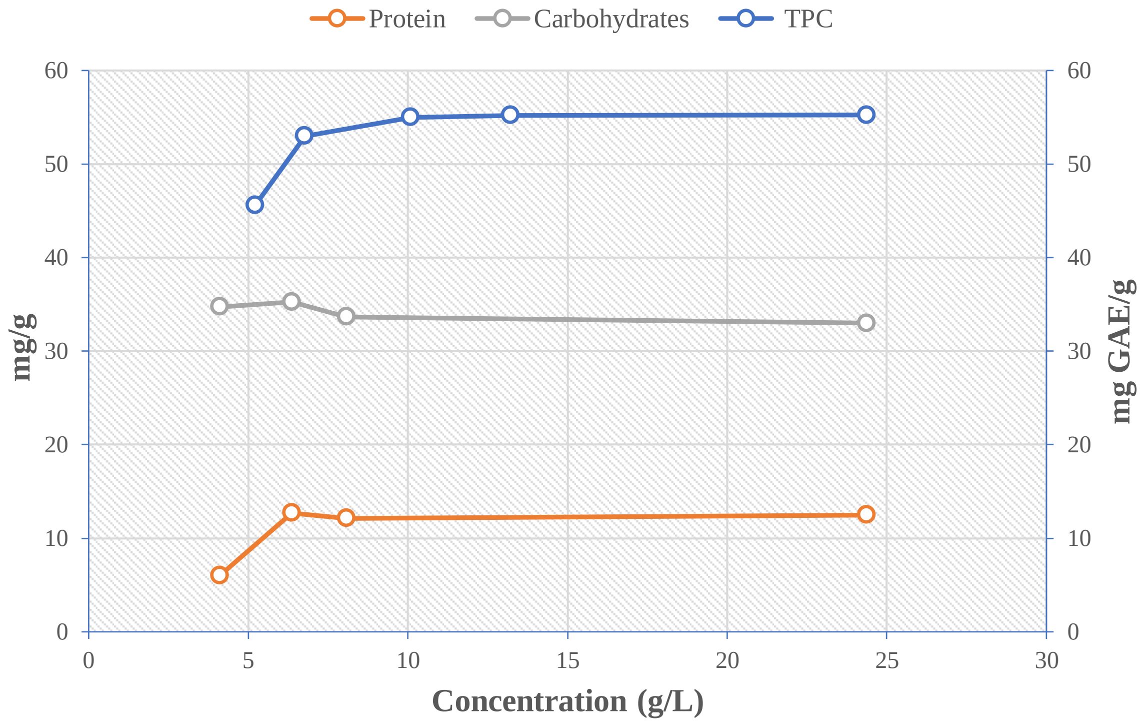


Figure 4.1. Concentrations of TPC (mg GAE/g), proteins (mg BSA/g) and carbohydrates (mg Glucose/g) at different sample to water ratios.

As can be seen in Figure 4.1, TPCs, proteins and carbohydrates are readily soluble in water at solid/solvent ratio of 13 g as dry weight/L. Considering these results, the effect of EtOH concentration on the release of TPCS, proteins, and carbohydrates in aqueous phase was determined with this sample to solvent ratio. After having the evaporation of the alcohol and the resuspension of residue with distilled water the aqueous concentrations of OMWC components were determined in filtered samples (Figure 4.2).

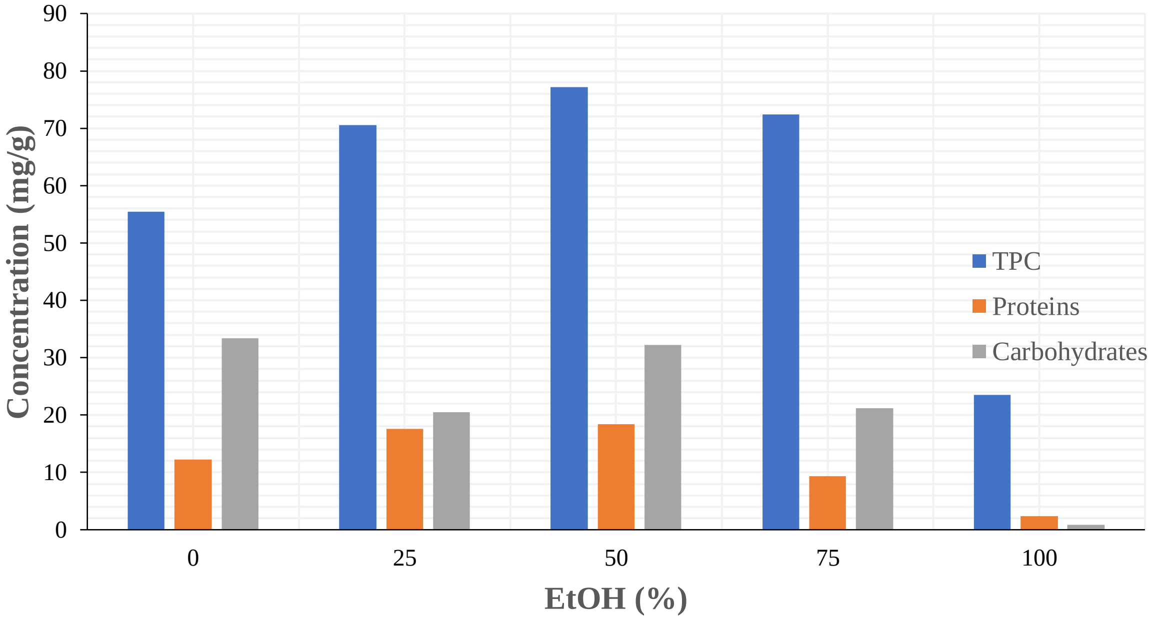


Figure 4.2. Effect of EtOH concentration on the release of TPCs, proteins and carbohydrates from OMWC. OMWC/solvent=13 g/L, temperature =20±1 °C, contact time=15 min.

As shown in Figure 4.2, the highest release of TPCs, proteins and carbohydrates from OMWC were achieved with 50% EtOH aqueous solution whereas their concentrations remarkably decreased by increasing EtOH concentration up to 100%. Similarly, in previous studies (Cacace and Mazza, 2003; Liu et al., 2013b) the maximum extraction yield of PCs from black currants and *Euryale ferox* seed shells was achieved with 50-60% EtOH concentration and increasing concentration of EtOH deteriorated the release of polar compounds including total phenolics and anthocyanins from raw samples (Cacace and Mazza, 2003). In fact, the addition of high EtOH concentration caused the formation of some precipitates in aqueous sample as shown in Figure 4.3a. This result could be attributed to the precipitation and denaturation of proteins with EtOH due to interaction of it with the hydrophobic groups of proteins found in OMWC (Yoshikawa et al., 2012). In accordance, the amount of protein released from OMWC was 18.34 mg BSA/g in 50% EtOH aqueous solution whereas it was decreased to 2.35 mg BSA/g with 100% EtOH.

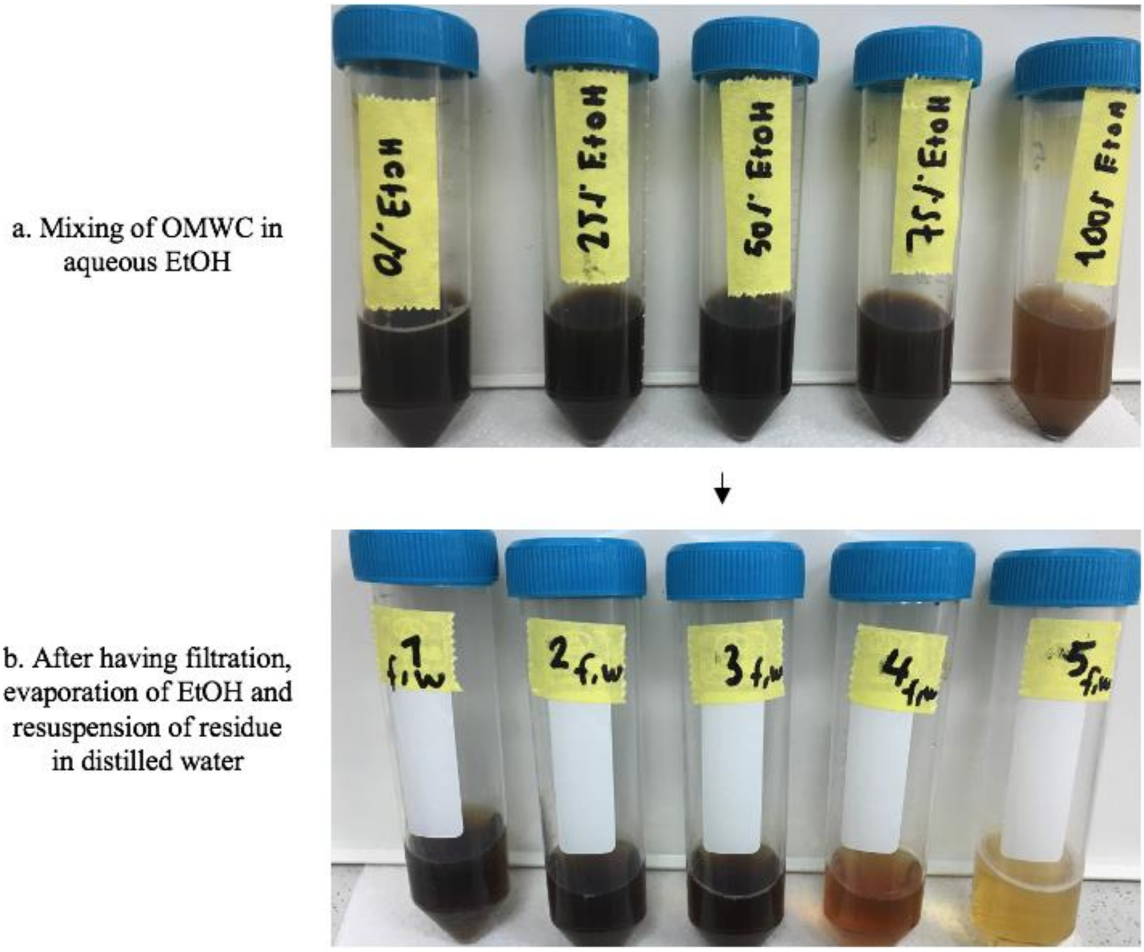


Figure 4.3. (a) OMWCs dissolved in aqueous EtOH (0%, 25%, 50%, 75% and 100%, left to the right) (b) filtered aqueous solutions of OMWCs after evaporation of EtOH.

Together with the formation of precipitates light color of the sample prepared in 100% EtOH is obvious in Figure 4.3 b compare to the others prepared with lower EtOH concentration while the color of the samples is in accordance with dissolved TPC content as deduced from Figure 4.2 and 4.3.

Aqueous solution of OMWC at 13 g/L concentration was used for characterization with some physiochemical properties (Table 4.1) that were also used to assess the performance of applied extraction of the process.

Table 4.1. Physicochemical characterization of aqueous OMWC.

*Phenolic Compounds (mg GAE/g)	57.47±0.72
*HTyr (mg/g)	13.56±0.76
*Oleu (µg/g)	542.52±1.76
*Antioxidant Activity (mg AAE/g)	60.86±03.43
*Protein (mg BSA/g)	12.46±0.22
*Carbohydrate (mg glucose/g)	33.98±2.50
*Pectin(mg GA/g)	6.83±0.98
Conductivity (mS/cm)	5.18±0.07
pH	5.36±0.04

*These analyses were performed with respect to dry weight of aqueous OMWC.

In accordance to previous studies in which acidic nature of OMWW and OMWC was reported (Bouknana et al., 2014; Sellami et al., 2016) the pH value of aqueous OMWC was 5.36. The concentrated sample used in this study was obtained from OMWW generated during two-phase centrifugal system but indeed slightly acidic characteristic of olive oil mill by- products is a common property of both two-phase and three-phase centrifugal systems while the organic content of the samples can exhibit variation depending on the oil extraction method. As shown in Table 4.1, the protein and carbohydrate contents of OMWC were estimated as 12.46 mg BSA/g and 33.98 mg glucose/g, respectively. While these values were lower than those noted in a recent study performed with the concentrated waste obtained from three-phase centrifugal system (Celik et al., 2020) the sample in this study is richer in terms of TPC content and antioxidant activity.

4.2. Design of ATPS for the Extraction of PCs from OMWC

EtOH and $(NH_4)_2SO_4$ or K_2HPO_4 were selected as the components of ATPE systems to concentrate and purify the phenolic antioxidants in this research. First, phase diagram of each system was constructed to determine the working conditions of the extraction. Experimental binodal curve was determined via cloud point titration method. Merchuk's equation which is commonly used in the studies with alcohol-based ATPS (Reis et al., 2011; Liu et al., 2013a; Fu et al., 2015; Chakraborty and Sen, 2016; Li et al., 2016; Arzideh et al., 2018; Caldeira et al., 2019; Leong et al., 2019) was selected among suggested equations in the literature (Table 3.3) since it gave higher correlation ($R^2>0.99$) for the experimental data as the results of various trials. The fitting parameters of Merchuk's equation (equation 3.3) together with coefficients of determination (R^2) are presented for EtOH-$(NH_4)_2SO_4$ and EtOH-K_2HPO_4 systems in Table 4.2.

Table 4.2. Empirical coefficients of Merchuk’s equation and the coefficients of determination obtained for EtOH-$(NH_4)_2SO_4$ and EtOH-K_2HPO_4 systems.

Binodal Curves	**a**	**b**	**c**	**R^2**
EtOH- $(NH_4)_2SO_4$	73.63	0.2467	0.00002440	0.9924
EtOH- K_2HPO_4	70.53	0.3261	0.00002132	0.9917

As it is known that both type of alcohol and salt are effective on the location of binodal curve and this was observed in Figure 4.4 for EtOH-$(NH_4)_2SO_4$ and EtOH-K_2HPO_4 systems. EtOH is more hydrophilic compared to some solvents. For instance, with 1-propanol higher amount of phase forming components (alcohol and salt) are necessary to reach two-phase area (Ooi et al., 2009). In addition to the low salt dosage requirement to achieve a two-phase system, the main reason for choosing EtOH as a solvent was it is generally recognized as safe (GRAS) substance.

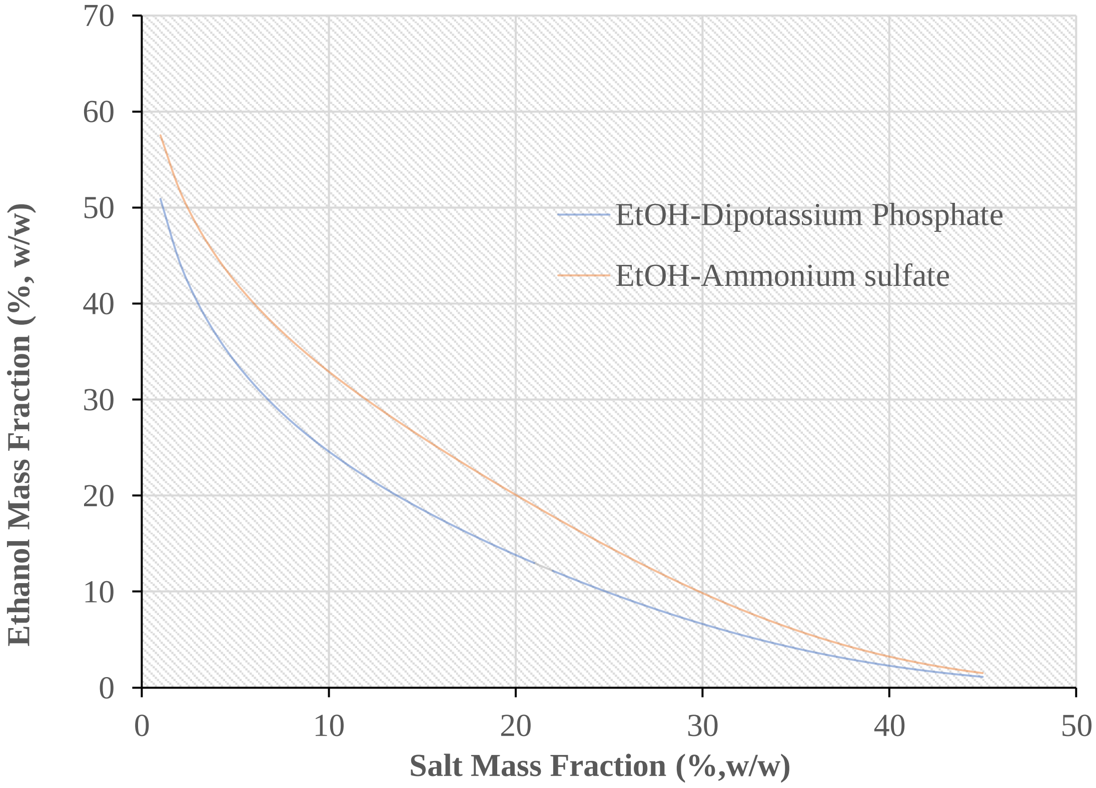


Figure 4.4. Phase diagram of EtOH- K_2HPO_4 and EtOH-$(NH_4)_2SO_4$ systems.

While the upper limits of EtOH and salt mass fraction of EtOH-K_2HPO_4 were 50% and 45%, for EtOH-$(NH_4)_2SO_4$ system, they were 57% and 45%, respectively. These results are in accordance with literature values (Li et al., 2009, Liu et al., 2013a; Zhang et al., 2018). As can be seen from Figure 4.4, phase separation in EtOH- K_2HPO_4 system occurred with lesser amount of alcohol when mass fraction of salts was equal. Therefore, phase forming ability of K_2HPO_4 found slightly better that of $(NH_4)_2SO_4$. This result could be explained with hydration of inorganic salts which can be quantified with Gibbs Free Energy of hydration (ΔG^o_{H}=-13.67 kJ/g for K_2HPO_4 and ΔG^o_{H}=-12.50 kJ/g for $(NH_4)_2SO_4$). Accordingly, K_2HPO_4 with more negative Gibbs Free Energy exhibited better salting out effect similar to the results of previous studies (Khayati and Shahriari, 2016; Zhang et al., 2018).

In order to find slope (S) and TLL values (equations 3.9 and 3.10, respectively) of the systems the weight fractions of top and bottom phase components, Y_b, Y_t, X_b, and X_t were calculated by Matlab using the experimental values of α. The results of all these calculations are presented in Table 4.3.

Table 4.3. Phase compositions and tie-line data for EtOH-(NH4)$_2$SO$_4$ and EtOH-K_2HPO_4 systems.

EtOH-$(NH_4)_2SO_4$ ATPS								
Alcohol (%, w/w)	**Salt (%, w/w)**	**α**	**Y_b**	**Y_t**	**X_b**	**X_t**	**S**	**TLL**
16.33	23.87	0.75	12.06	29.40	27.60	12.46	-1.15	23.03
19.98	20.97	0.31	13.06	35.53	26.57	8.39	-1.24	28.90
35.00	12.50	0.66	8.66	48.37	31.43	2.89	-1.39	48.90
42.15	8.44	0.81	5.91	50.41	35.35	2.36	-1.36	55.28
46.23	6.53	0.85	7.51	53.30	32.89	1.71	-1.47	55.40
41.03	9.45	0.79	5.44	50.66	35.89	2.29	-1.35	56.34
40.60	12.00	0.67	6.18	57.17	34.74	1.05	-1.51	61.11
29.90	19.08	0.46	3.64	52.38	39.15	1.90	-1.31	61.35
EtOH- K_2HPO_4 ATPS								
Alcohol (%, w/w)	**Salt (%, w/w)**	**α**	**Y_b**	**Y_t**	**X_b**	**X_t**	**S**	**TLL**
27.00	10.00	0.80	9.47	31.40	25.60	6.09	-1.12	29.35
30.00	10.00	0.76	7.05	37.40	29.31	3.77	-1.19	39.67
25.00	20.00	0.46	2.00	44.74	41.03	1.94	-1.09	57.92

The slope values of each TLL are slightly different since they are not exactly parallel to each other (Merchuk et al., 1998). TLLs of EtOH-$(NH_4)_2SO_4$ and EtOH-K_2HPO_4 systems are shown corresponding binodal curves in Figure 4.5 a and b, respectively.

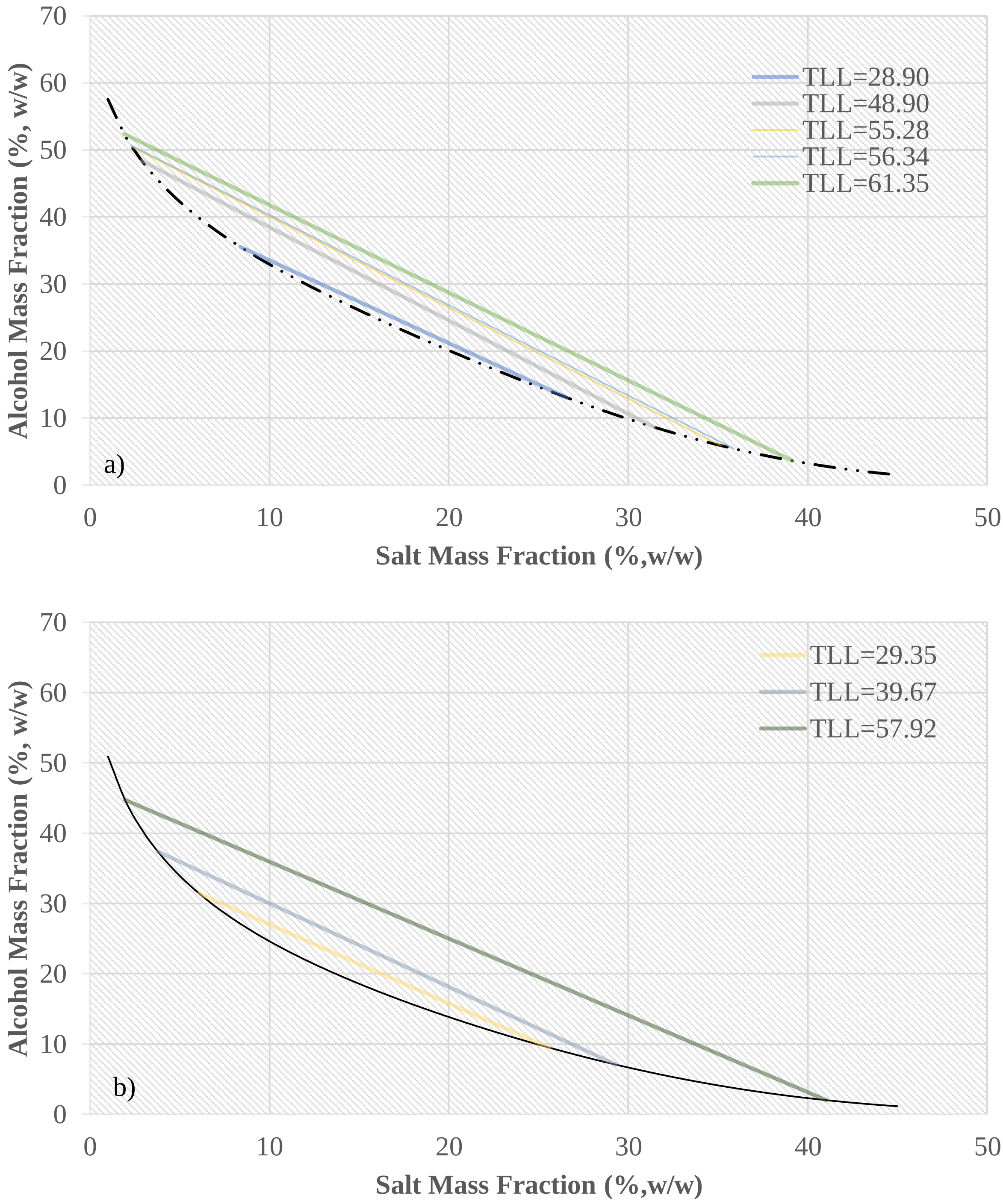


Figure 4.5. Graphical representation TLLs for (a) EtOH-$(NH_4)_2SO_4$ and (b) EtOH-K_2HPO_4 ATPS.

Then, in order to evaluate the effect of TLL and V_r (equation 4.1) on the extraction efficiency of TPCs, different operation points on two different TLL were selected for each ATPE system. The locations of these operation points are shown in Figure 4.6 a-b while the volume ratios together with mass fractions at these points are listed in Table 4.4.

$$V_r=\frac{V_t}{V_b} \tag{4.1}$$

where V_t and V_b are the volumes of top and bottom phases, respectively.

Table 4.4. Composition of selected operation points for EtOH-$(NH_4)_2SO_4$ and K_2HPO_4 ATPS.

EtOH/ $(NH_4)_2SO_4$ /Water System				
System	**Alcohol (%, w/w)**	**Salt (%, w/w)**	**Water (%, w/w)**	**Volume Ratio (V_r)**
TLL=28.9				
1	18.00	22.57	59.43	0.3
2	25.00	16.91	58.09	1.2
3	31.06	12.00	56.94	4.2
4	32.30	11.00	56.70	7.6
TLL=55.4				
5	15.00	28.00	57.00	0.3
6	29.18	18.00	52.82	1.1
7	41.12	10.00	48.88	4.5
8	42.90	8.55	48.55	6.1

EtOH/ K_2HPO_4 /Water System				
System	**Alcohol (%, w/w)**	**Salt (%, w/w)**	**Water (%, w/w)**	**Volume Ratio (V_r)**
TLL=29.3				
1	13.52	22.00	64.48	0.6
2	17.12	19.00	63.88	1.1
3	25.91	10.97	63.12	5.4
TLL=57.9				
4	17.34	27.00	55.66	0.6
5	22.74	22.00	55.26	1.2
6	34.06	11.71	54.23	3.9

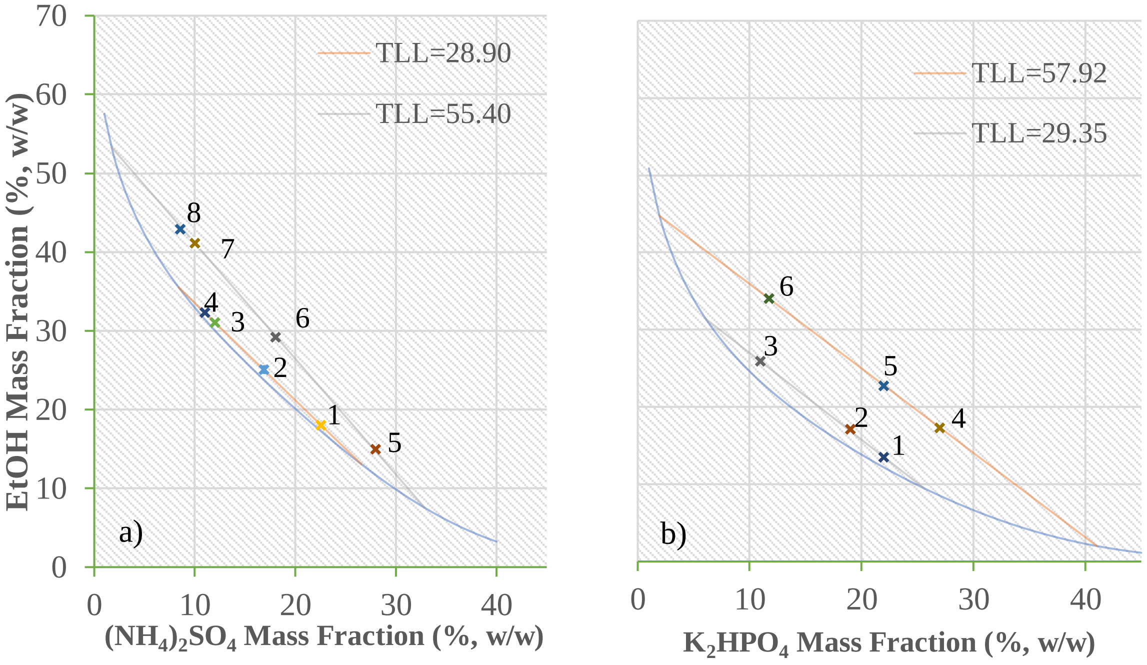


Figure 4.6. Selected operation points on phase diagram for the extraction of TPCs. (a) EtOH-$(NH_4)_2SO_4$ ATPS, (b) EtOH- K_2HPO_4 ATPS.

pH values of top and bottom phases of each system were determined (Table 4.5). It can be clearly seen from Table 4.5 that pH values of the systems depend upon the type of salt. It is known that pH is a critical factor in ATPS due to its effects on existing form of compounds, polarity and distribution of components through the phases (Zhang et al., 2019). Additionally, some reactions may favor in the acidic conditions. For instance, increasing the solubility of PCs and promoting the hydrolysis of Oleu to HTy are possible in acidic medium (Allouche et al., 2004; De Marco et al., 2007). On the other hand, it should be considered that some phenolic compounds including caffeic acid and gallic acid could be damaged when they are subjected to high pH (Friedman and Jurgens, 2000).

4.3. Partitioning of OMWC's Carbohydrates in ATPE Systems

Partitioning of carbohydrates of OMWC EtOH-$(NH_4)_2SO_4$ and EtOH- K_2HPO_4 ATPE was investigated in this research at selected operation points of EtOH-$(NH_4)_2SO_4$ and EtOH- K_2HPO_4 ATPE systems. The performance of each system for the eliminate of carbohydrates was evaluated in terms of partitioning coefficient, K (equation 4.2) and removal rate, R (equation 4.3) and achieved results together with the pH values of top and bottom phases are shown in Table 4.5.

$$K=\frac{C_T}{C_B} \tag{4.2}$$

where C_T and C_B are concentrations of the compound of interest in top and bottom phases, respectively.

$$R\ (\%) =\frac{C_i-C_T}{C_i} \times 100 \tag{4.3}$$

where C_i is the amount of non-target compound in OMWC.

Table 4.5. Carbohydrates elimination performance of EtOH-$(NH_4)_2SO_4$ and EtOH-K_2HPO_4 ATPE systems.

EtOH-$(NH_4)_2SO_4$ ATPE					
TLL	**V_r**	**pH (Top Phase)**	**pH (BottomPhase)**	**K**	**R (%)**
28.9	0.3	6.00	5.77	0.18	93.47
55.4	0.3	6.41	6.07	0.30	87.96
28.9	1.2	5.68	5.44	0.33	75.21
55.4	1.1	6.16	6.02	0.39	76.05
28.9	4.2	6.16	6.52	0.86	68.30
55.4	4.5	7.03	6.69	0.42	85.42
28.9	7.6	6.68	6.77	1.10	74.01
55.4	6.1	6.79	6.71	0.85	76.08
EtOH-K_2HPO_4 ATPE					
TLL	**V_r**	**pH (Top Phase)**	**pH (Bottom Phase)**	**K**	**R (%)**
29.3	0.6	10.13	10.12	1.27	44.82
57.9	0.6	10.28	10.76	1.32	49.28
29.3	1.1	9.58	9.59	1.62	27.82
57.9	1.2	10.27	10.10	1.34	50.96
29.3	5.4	10.19	9.68	1.88	29.02
57.9	3.9	10.11	10.44	1.71	32.61

Carbohydrates removal performance of each ATPS at selected TLL and V_r are different and type of salt is one of the crucial factors for the partitioning of carbohydrates (Table 4.5). The partitioning of carbohydrates towards top phase is decreased with increasing salt mass fractions that causes a reduction in V_r due to salting out effect (Guo et al., 2013; Chikari et al., 2020).

Relatively lower values of K (between 0.184 and 0.860) achieved with EtOH-$(NH_4)_2SO_4$ ATPE, indicated higher affinity of carbohydrates towards bottom phase but K value greater than one when TLL is 28.9 and V_r is 7.6. On the other hand, the partitioning of carbohydrates is mainly through the top in the EtOH-K_2HPO_4 ATPE. While removal rate of carbohydrates from EtOH rich phase ranged between 28-51% in EtOH-K_2HPO_4 ATPE it was reached up to 93.48% in EtOH-$(NH_4)_2SO_4$ ATPE.

Even though extraction of polysaccharides from different matrices with EtOH-salt based ATPS was investigated, the effects of TLL and V_r were not considered in earlier studies (Dong et al., 2012; Cheng et al., 2016; Chen et al., 2016; Huang et al., 2018; Lin et al., 2019; Chikari et al., 2020; Zhu et al., 2020). However, it has been known that some carbohydrates are water soluble and some others are EtOH soluble. Therefore, partitioning into either top or bottom phases can be expected depending upon the properties of extracted matrice (Chen et al., 2016; Cheng et al., 2017; Huang et al., 2018; Lin et al., 2019). It was shown that, monosaccharides such as glucose, galactose, arabinose, rhamnose galacturonic acid are rich in OMWW, whereas insoluble cellulose and hemicelluloses are found in olive pomace (Nadour et al., 2015). Additionally, arabinan-rich pectic polysaccharides which are not soluble in EtOH found in considerable amounts in OMWCs in a previous study (Coimbra et al., 2010). A detailed carbohydrate analysis can be necessary to evaluate their fate in ATPE.

4.4. Partitioning of OMWC's Proteins in EtOH-$(NH_4)_2SO_4$ ATPE Systems

Since EtOH-K_2HPO_4 ATPE did not exhibit better performance for the separation of carbohydrates from the top phase protein analysis was performed for only EtOH-$(NH_4)_2SO_4$ ATPE. Similar to carbohydrates, the performance of the system was evaluated in terms of partitioning coefficient (K) and removal (R) (equations 4.2 and 4.3). These performance parameters are shown together with top and bottom phase protein concentrations in Table 4.6.

Table 4.6. Proteins elimination performance of EtOH-$(NH_4)_2SO_4$ ATPE.

TLL	**V_r**	**Top Phase**	**Bottom Phase**	**K**	**R (%)**
		(mg BSA/g)			
28.9	0.3	3.05	9.37	0.32	75.04
55.4	0.3	2.65	9.99	0.26	78.28
28.9	1.2	2.24	7.44	0.30	81.65
55.4	1.1	5.68	1.75	3.26	53.47
28.9	4.2	6.61	1.83	3.61	45.91
55.4	4.5	10.65	0.49	21.53	12.83
28.9	7.6	10.57	0.75	14.15	13.49
55.4	6.1	9.66	0.46	21.00	20.90

The hydrophobicity EtOH-$(NH_4)_2SO_4$ ATPs was increased by increasing TLL; hence the amount of proteins in the hydrophilic bottom phase decreased. However, the best performance that was 82% was achieved with 29% EtOH and 18% $(NH_4)_2SO_4$. This result in accordance with those found in in earlier studies (Hua et al., 2013; Cheng et al., 2016) in which EtOH-salt based ATPS was investigated without considering TLL and V_r effects as system parameters. In the study of Hua et al. (Hua et al., 2013), 58% protein removal from the top phase of fruit residues extracts was achieved with 30% EtOH and 19% $(NH_4)_2SO_4$. In another study, the elimination of proteins from the extracts of *Schisandra chinensis Baill* fruit reached to 90.43% with 22% EtOH and 21% $(NH_4)_2SO_4$ (Cheng et al., 2016).

As shown in Table 4.6, total protein content of extracts exhibited some variations depending upon the composition of ATPS. Indeed, this is well expected since both $(NH_4)_2SO_4$ and EtOH are used as protein precipitation agents (Li et al., 2009; Ooi et al., 2009; Cheng et al., 2016).

4.5. Extraction performance of ATPE systems for TPCs in OMWC

The ability of EtOH-$(NH_4)_2SO_4$ ATPE system for TPC partitioning was evaluated in terms of partitioning coefficient (K) and extraction efficiency (EE) at eight different system (Table 4.4). While K of TPCs was determined with equation 4.2 similar to proteins and carbohydrates the EE of the ATPE system was calculated with below mentioned equation and all of the results represented in Table 4.7.

$$EE\ (\%) = \frac{(C_T\ X\,V_T)}{(\,C_T\ X\,V_T)+(C_B\,X\,V_B)}\ \text{x}\ 100 \qquad \text{(eq. 4.4)}$$

Table 4.7. Performance of EtOH-$(NH_4)_2SO_4$ ATPE for the recovery of TPCs.

TLL	**V_r**	**Top Phase**	**Bottom Phase**	**K**	**EE (%)**
		(mg GAE/g)			
28.9	0.3	25.59	19.68	1.30	31.18
55.4	0.3	36.59	12.43	2.94	47.98
28.9	1.2	53.31	11.77	4.53	84.80
55.4	1.1	51.27	3.78	13.58	93.59
28.9	4.2	61.54	4.11	14.98	98.42
55.4	4.5	64.87	1.61	40.34	99.46
28.9	7.6	59.14	3.02	19.61	99.34
55.4	6.1	64.88	1.61	40.42	99.60

Considerably high K and EE (%) values were obtained for TPCs with EtOH-$(NH_4)_2SO_4$ ATPE as can be seen from Table 4.7. This achieved result was in consistent with a previous study in which migration tendency of low molecular weight hydrophobic phenolic compounds towards EtOH-rich top phase was obtained with EtOH-$(NH_4)_2SO_4$ ATPE (Celik et al., 2020). Promising performance of the EtOH-$(NH_4)_2SO_4$ ATPE for the recovery of PCs with compare to the five different salts including KH_2PO_4 was shown in Guo's study (Guo et al., 2013). According to the results in Table 4.8, an increase in TLL and V_r induced positive impact on the performance of ATPE system for the recovery of TPCs. Along the same TLL with an increase in V_r, migration of more TPCs towards top phase was achieved by providing higher available volume of solvent and overcoming of saturation related problems (Gómez-Loredo et al., 2014; Kalaivani and Regupathi, 2015; Xavier et al., 2017; Celik et al., 2020; Enriquez-Ochoa et al., 2020). Apart from V_r, the impact of TLL on distribution of TPCs between phases could be attributed to higher salt and alcohol mass fractions in the ATPS. Variation in TLL causes thermodynamic phase differences, a change in hydrophobicity of the system, and a change in free available volume for the biomolecules (Borges et al., 2016). The higher TLL value results in more hydrophobic environment in the top phase due to increased salting out effect hence decreased water availability. Then, transfer of the molecules towards more hydrophobic phase could be obtained (De Souza et al., 2010; Enriquez-Ochoa et al., 2020).

4.6. Antioxidant Activity of OMWC Extracts

For the estimation of antioxidant activity in the extracts of ATPE system, DPPH method, a widely applied method for the waste of olive oil extraction process was used (Kedare and Singh, 2011; Celik et al., 2018). In Table 4.8, antioxidant activity values achieved in top phase of EtOH-$(NH_4)_2SO_4$ ATPE system at selected operation points are shown.

Table 4.8. Estimated antioxidant activity values.

TLL	**V_r**	**mg AAE/g***
28.9	0.3	15.60
55.4	0.3	15.97
28.9	1.2	32.07
55.4	1.1	32.29
28.9	4.2	34.24
55.4	4.5	46.96
28.9	7.6	44.02
55.4	6.1	38.28

*: Ascorbic Acid Equivalent.

A linear correlation between estimated values of TPCs and antioxidant activity in the extracts was obtained as shown in Figure 4.7.

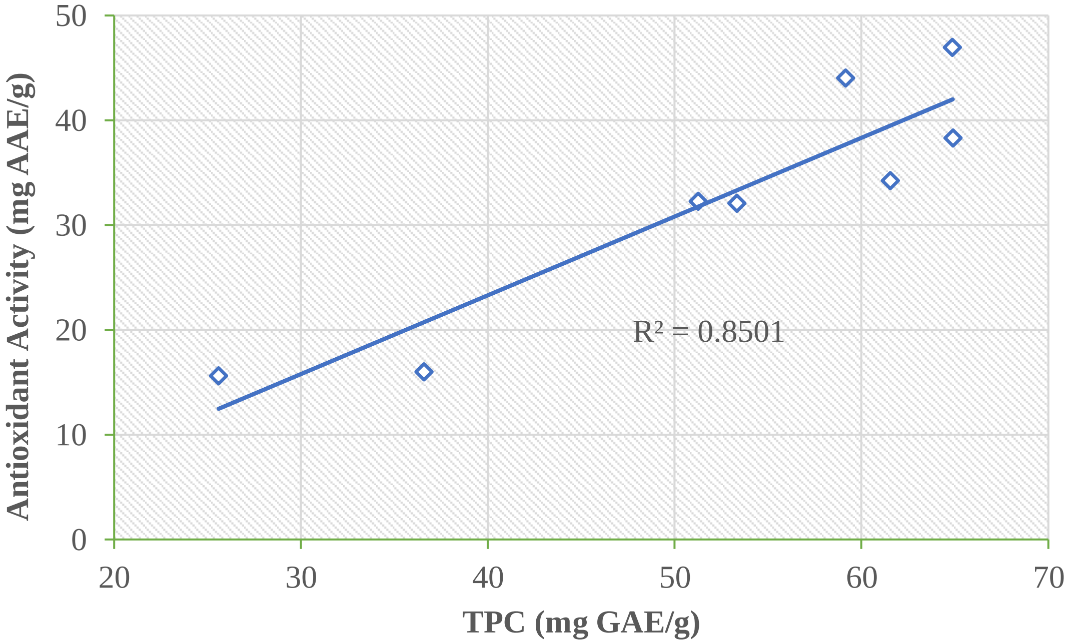


Figure 4.7. Relationship between estimated values of TPC and antioxidant activity.

Similarly, the direct relationship of TPC and DPPH antioxidant activity of various olive mill waste was shown in previous studies (Azaizeh et al., 2012; Uribe et al., 2012; Leouifoudi et al., 2015; Wang et al., 2017; Celik, 2018; Celik et al., 2020; Peršurić et al., 2020). However, it should be noted that not only TPCs but also carbohydrates and proteins in OMWCs might contribute to antioxidant activity of samples (Nadour et al., 2015).

4.7. Hydroxtyrosol and Oleuropein Contents of OMWC Extracts

The extracts of OMWC achieved with EtOH-$(NH_4)_2SO_4$ ATPE system were subjected to LC-MS/MS analysis for the quantification of HTyr and Oleu. The concentration of these polyphenols in top and bottom phases together with partition coefficients of HTyr and Oleu (K_{HTyr} and K_{Oleu}, respectively) are presented for different operational conditions of ATPE system in Table 4.9.

Table 4.9. Partitioning of HTyr and Oleu in top and bottom phases of EtOH-$(NH_4)_2SO_4$ ATPE system.

		HTyr			**Oleu**		
TLL	V_r	Top Phase (mg/g)	Bottom Phase (mg/g)	K_{HTyr}	Top Phase (µg/g)	Bottom Phase (µg/g)	K_{Oleu}
28.9	0.3	23.06	19.63	1.18	75.55	22.47	3.36
55.4	0.3	22.17	13.53	1.64	103.44	5.88	17.59
28.9	1.2	16.69	10.32	1.62	29.12	11.62	2.51
55.4	1.1	35.43	4.06	8.73	92.83	1.96	47.47
28.9	4.2	43.30	4.60	9.42	117.07	5.23	22.38
55.4	6.1	49.69	0.80	61.77	120.66	0.66	182.03

As can be seen from Table 4.9, the amount of HTyr is considerable higher than that of Oleu. Indeed, Oleu is abundant in olive leaves (Sahin and Bilgin, 2017; Da Silva et al., 2019; Lama-Muñoz et al., 2019) and its concentration in OMWW depends upon harvesting season. The conversation of Oleu to elonoic acid and HTyr can be expected at the late harvesting season of olives (Allouche et al., 2004) but the presence of it has not been reported in OMWW till now.

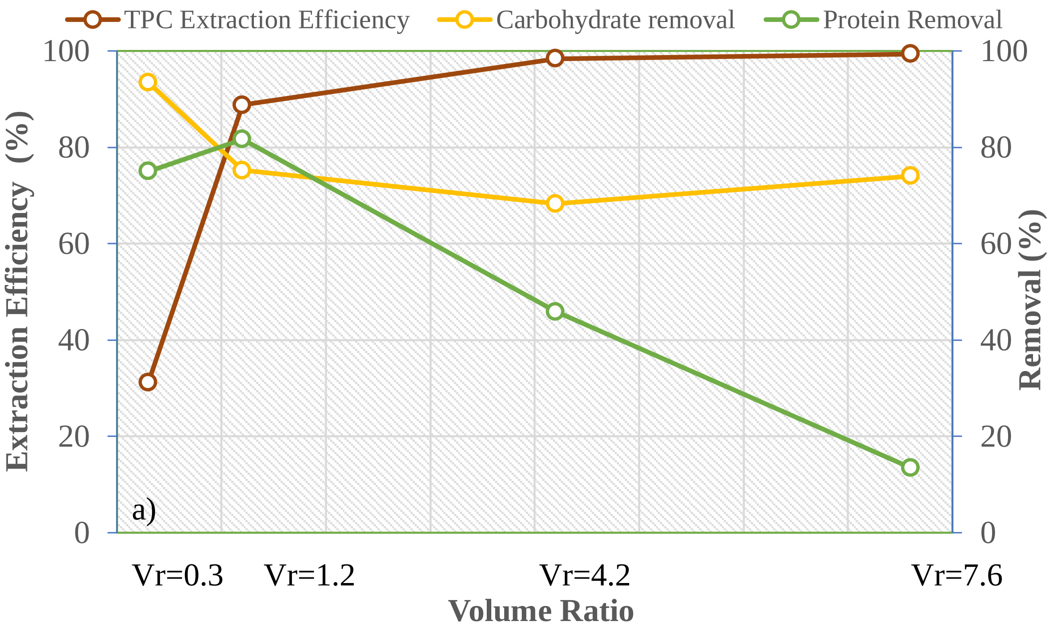
The concentrations of HTyr and Oleu were in accordance with antioxidant activity. At highest TLL (=55.4) and V_r (=6.1) HTyr and Oleu were quantified at top phase as 49.69 mg/g and 120.66 µg/g, respectively while antioxidant activity was 38.29 mg AAE/g (Table 4.8 and Table 4.9).

Correspondingly, lower antioxidant activity (15.60 mg AAE/g) was detected when HTyr and Oleu were quantified as relatively lower amounts at the top phase (23.06 mg/g and 75.55 µg/g, respectively).

The highest partitioning coefficients of HTyr and Oleu were 61.77 and 182.03, respectively. The higher of partitioning coefficient of these compounds than that of TPCs is well expected considering high solubilities of HTyr and Oleu in EtOH among various phenolic compounds found in OMWW (Galanakis et al., 2013). A correlation between K values of TPC, Htyr and Oleu was established ($R^2>0.97$) by using SPSS. From this evaluation, it can be deduced that the partitioning of TPCs can be an indicator for the behavior of low molecular weight bio-phenolic compounds namely, HTyr and Oleu.

4.8. Overall Partitioning Performance of EtOH-$(NH_4)_2SO_4$ ATPE in terms of TPCs, Proteins and Carbohydrates

Extraction efficiency of TPCs and removal of carbohydrates and proteins from top phase are shown at low and high TLL values in Figure 4.8a and b, respectively.



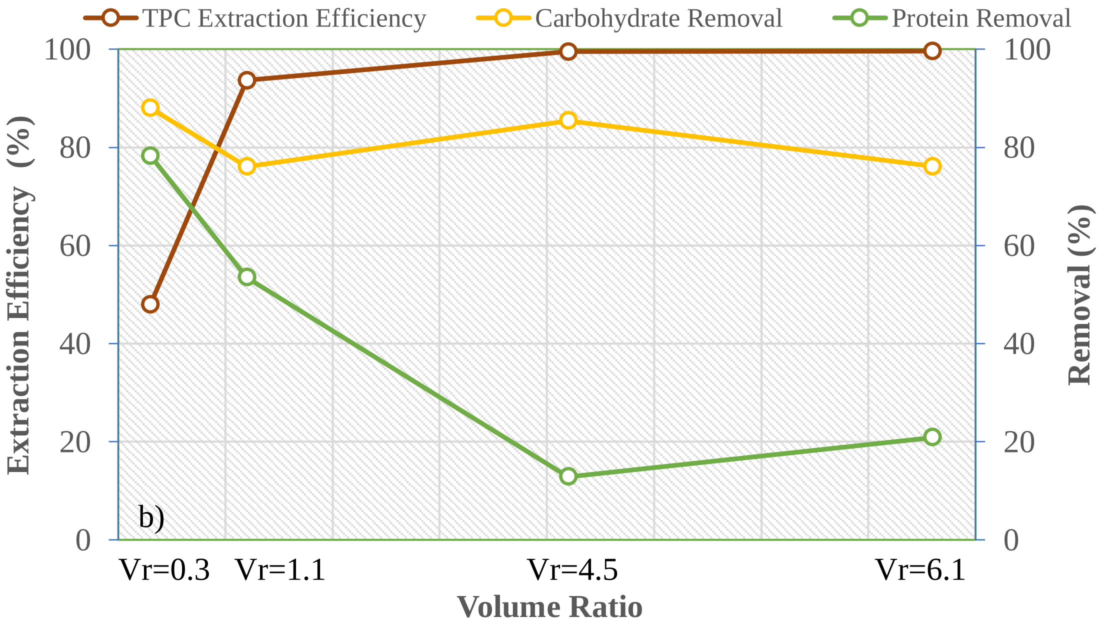


Figure 4.8. Extraction Performance of EtOH-$(NH_4)_2SO_4$ ATPE at (a) TLL =28.9 (b) TLL =55.4.

According to the results presented in Figure 4.9a and b, better extraction performance of TPC extraction efficiency is obtained with high TLL (TLL=55.4) for EtOH-$(NH_4)_2SO_4$ ATPE and increasing V_r causes better extraction of TPCs for both TLLs. On the other hand, the removal rates of proteins and carbohydrates were higher when the low V_r was employed. Considering both extraction of TPCs into the top phase and the removal of carbohydrates and proteins as impurities from the top phase, mass fractions of 29.18% and 18.00% for alcohol and salt, respectively, resulted considerable high extraction performance.

5. CONCLUSIONS

In scope of this research, the extraction of bio-phenolic antioxidants with focus on HTyr and the removal of impurities, namely carbohydrates and proteins by using alcohol-salt based ATPE systems were investigated. The following results mark the main findings of the research.

- Concentrated waste of OMWW generated by MVR evaporation has considerably high TPC and HTyr contents.

- Pure EtOH was not suitable solvent for the extraction of TPCs from OMWC but the aqueous solution of EtOH at 50% was the best for the maximum release of the TPCs from OMWC.

- For the extraction of TPCs and the removal of matrix impurities of OMWC, two different ATPE systems, EtOH-$(NH_4)_2SO_4$ and EtOH-K_2HPO_4 with acidic and basic nature, respectively, were investigated. According to the binodal curve data, phase forming ability of K_2HPO_4 was found better that of $(NH_4)_2SO_4$ due to having higher hydration capacity.

- Although salting out performance of EtOH-K_2HPO_4 was found better that of EtOH-$(NH_4)_2SO_4$ ATPE the elimination of carbohydrates from the extract of OMWC with basic ATPE system was poor.

- EtOH-$(NH_4)_2SO_4$ ATPE with 25.0% alcohol and 16.9% salt has an ability to concentrate TPCs in alcohol rich top phase with a partition coefficient of 4.5 and extraction efficiency of 84.80%. Additionally, removal rates of the proteins and carbohydrates were 82% and 75% respectively. High removal of proteins was attributed to the presence of both EtOH and $(NH_4)_2SO_4$, which are employed as protein precipitating agents.

- A linear correlation between estimated amount of TPCs and antioxidant activity has been established. Besides, the partitioning coefficients of TPCs, HTyr and Oleu were well correlated.

- By increasing TLL and V_r of EtOH-$(NH_4)_2SO_4$ ATPE the partitioning of TPCs was enhanced due to the increased hydrophobicity and available free volume of the top phase. The extraction efficiency for TPCs reached to 99.60% with TLL=55.40 and V_R=6.1. However, the removal ratios of proteins and carbohydrates were decreased at higher V_r. Therefore, with regarding both extraction of TPCs and removal of impurities from the EtOH-rich top phase, promising performance of EtOH-$(NH_4)_2SO_4$ ATPE was obtained when high TLL (55.40) and medium V_r (1.1) was employed. Under these conditions, 93.59% extraction efficiency of TPCs was achieved with 76.05% carbohydrates and 53.47% proteins removal rates. Target bio-phenolics, HTyr and Oleu were quantified as 35.43 mg/g and 92.83 µg/g at the EtOH-rich alcohol phase, respectively and antioxidant activity was found as 32.29 mg AAE/g with DPPH antioxidant assay.

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APPENDIX A: CALIBRATION CURVES OF SAMPLE CHARACTERIZATION METHODS

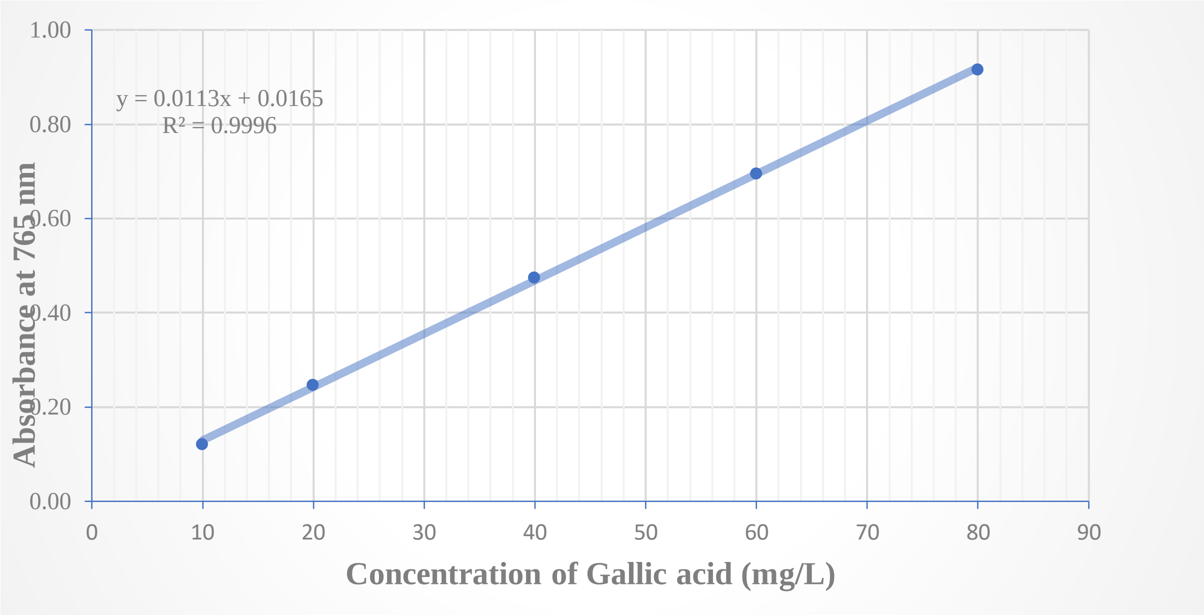

Figure A.1. Calibration curve of gallic acid by Folin-Ciocalteu method for total phenol analysis.

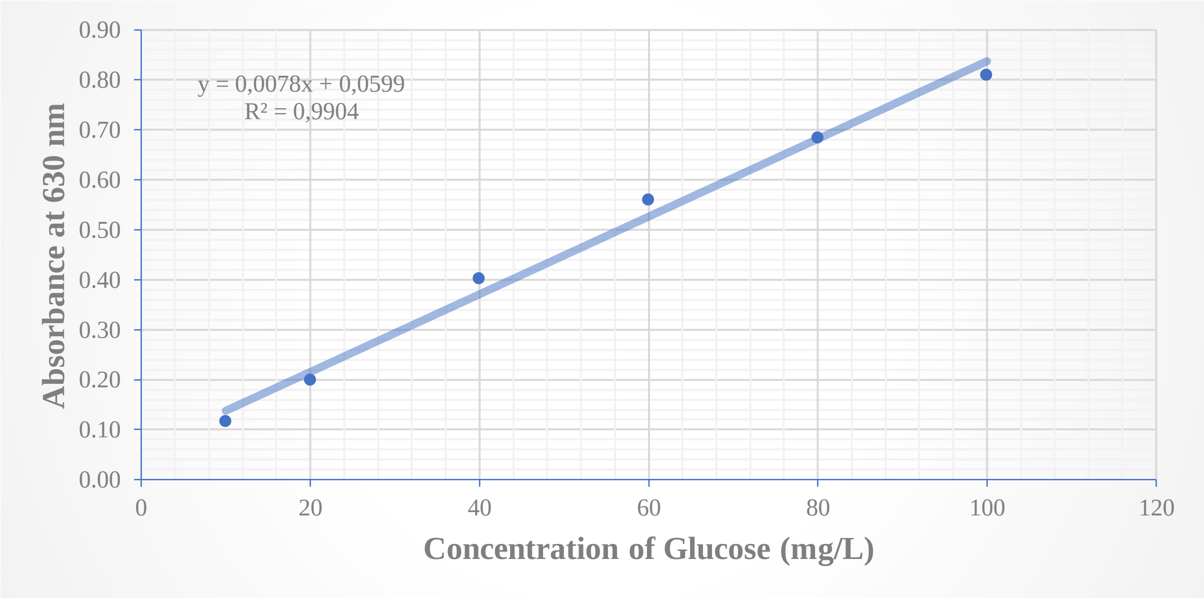

Figure A.2. Calibration curve of glucose by Anthrone method for carbohydrate analysis.

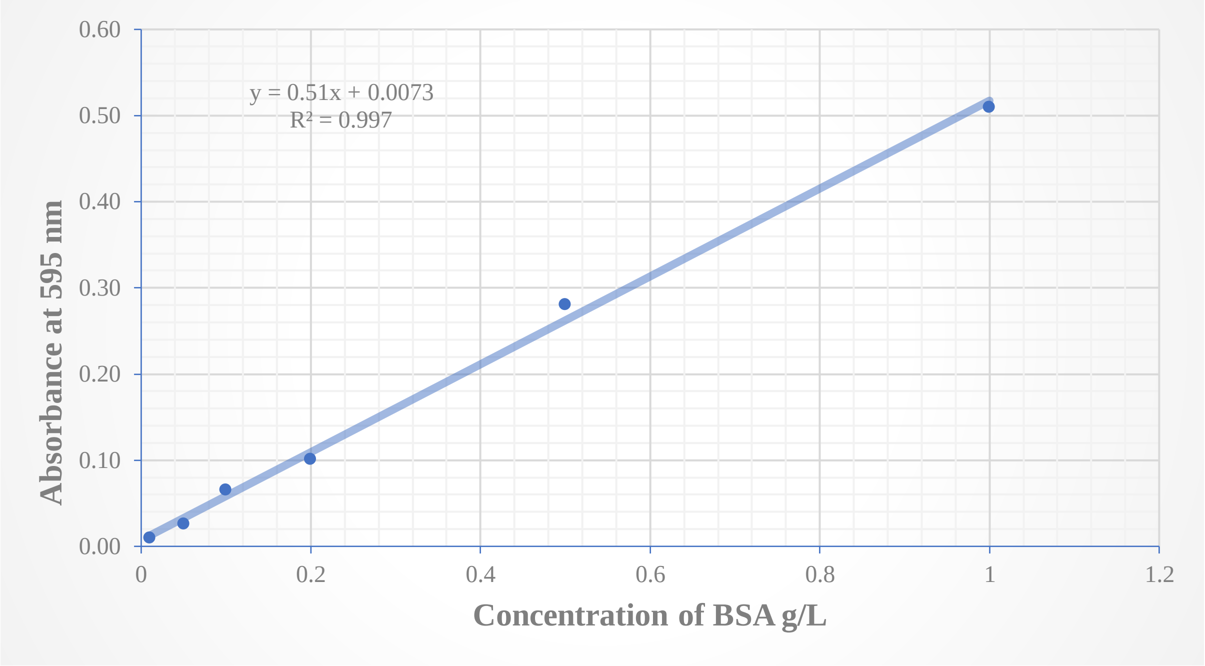

Figure A.3. Calibration curve of BSA by Bradford method for protein analysis.

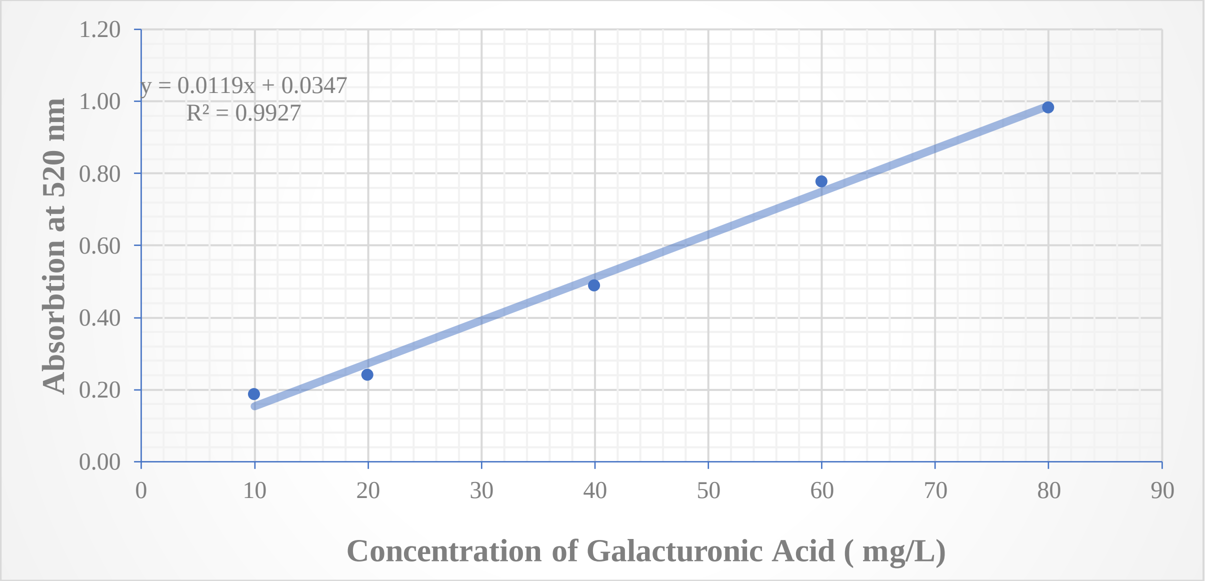

Figure A.4. Calibration curve of galacturonic acid for the determination of pectin.

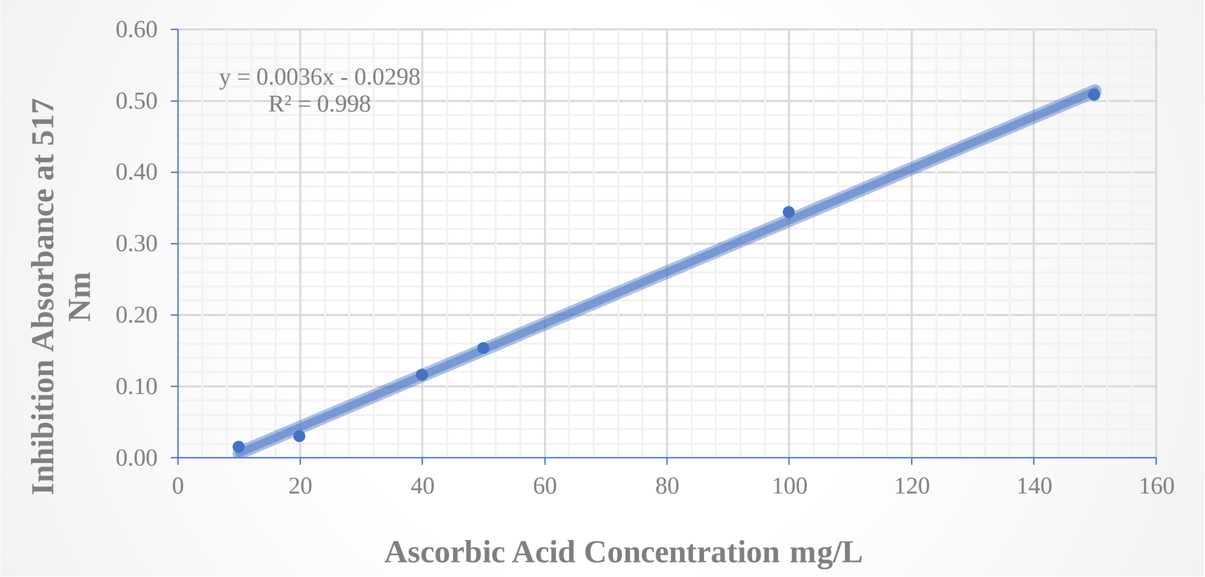

Figure A.5. Calibration curve of ascorbic acid by DPPH method for the determination of antioxidant activity.

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

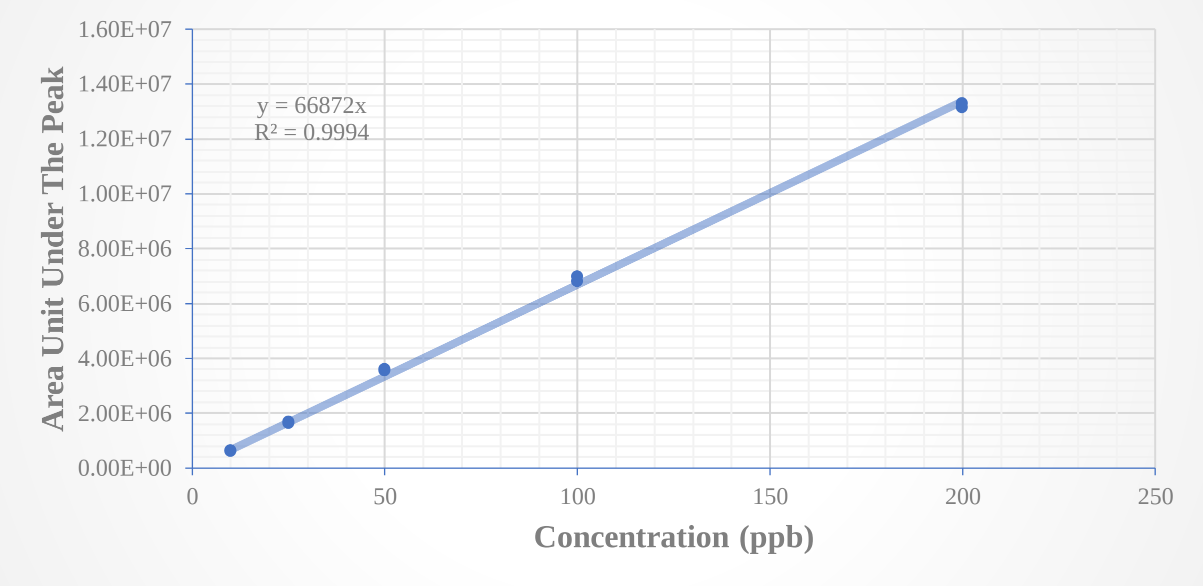

Figure B.1. Calibration curve of HTyr by LC-MS/MS.

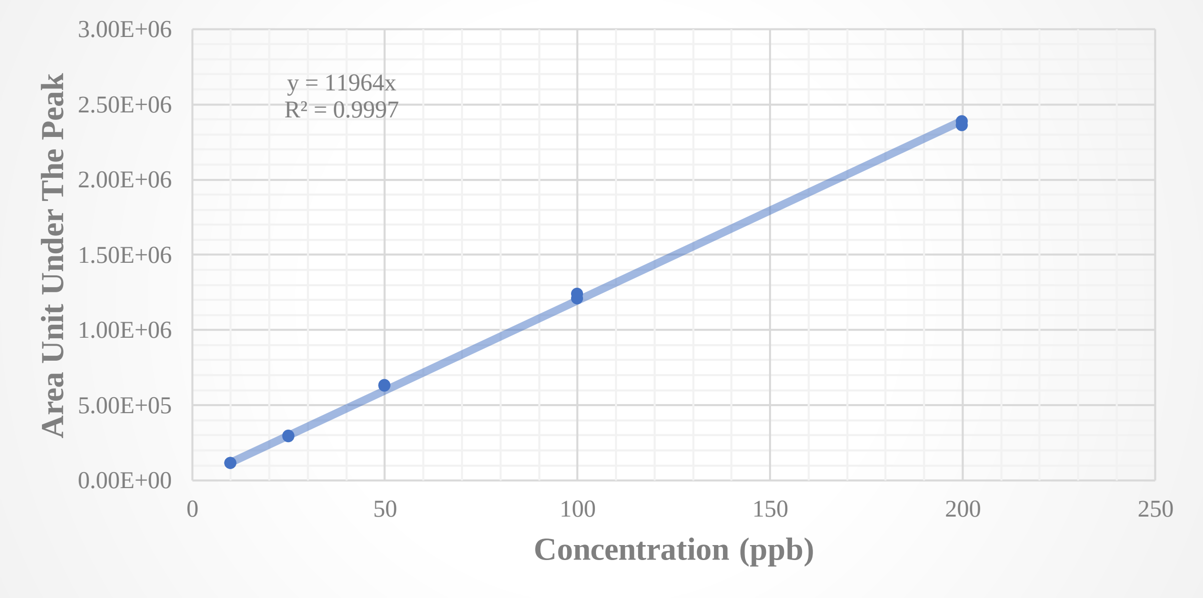

Figure B.2. Calibration curve of Oleu by LC-MS/MS.

APPENDIX C: MATLAB CODES FOR CALCULATION OF THE TLL

C.1. Calculation of TLL for EtOH- $(NH_4)_2SO_4$ ATPS

```
syms x y a b

AMF=46.2263019;
Alpha=0.84550236;
SMF=6.530477562;

[solx soly sola solb]=solve(b==(AMF/Alpha)-((1-Alpha)/Alpha)*y, a==(SMF/Alpha)-((1-
Alpha)/Alpha)*a,y==73.63*exp(-0.2467*x^0.5-0.0000244*x^3), b==73.63*exp(-0.2467*a^0.5-
0.0000244*a^3));

Xb=solx
Xt=sola
Yb=soly
Yt=solb
```

C.2. Calculation of TLL for EtOH- K_2HPO_4 ATPS

```
syms x y a b

AMF=46.2263019;
Alpha=0.84550236;
SMF=6.530477562;

[solx soly sola solb]=solve(b==(AMF/Alpha)-((1-Alpha)/Alpha)*y, a==(SMF/Alpha)-((1-
Alpha)/Alpha)*a,y==70.53*exp(-0.3261*x^0.5-0.00002132*x^3), b==70.53*exp(-0.3261*a^0.5-
0.00002132*a^3));

Xb=solx
Xt=sola
Yb=soly
Yt=solb
```

Where AMF: alcohol mass fraction, alpha: mass of top phase/ mass of mixture, SMF: salt mass fraction. For the calculation of the each TLL, values of AMF, SMF and alpha written and codes were run in Matlab.