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**DEPARTAMENTO DE BIOTECNOLOGÍA
Y CIENCIA DE LOS ALIMENTOS**

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Valorization of residues generated in the production of olive oil through clean technologies

PhD Thesis

Seyed Mehdi Niknam

Burgos, 2020

Valorization of residues generated in the production of olive oil through clean technologies

Valorización de los residuos generados en la producción de aceite de oliva mediante tecnologías limpias

Seyed Mehdi Niknam

Burgos, 2020

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Que el trabajo titulado "Valorization of residues generated in the production of olive oil through clean technologies", que presenta D. Seyed Mehdi Niknam para optar al Grado de Doctor por la Universidad de Burgos, ha sido realizado bajo su dirección en el Área de Ingeniería Química del Departamento de Biotecnología y Ciencia de los Alimentos de la Universidad de Burgos.

Considerando que dicho trabajo reúne los requisitos exigidos para ser presentado como Tesis Doctoral, expresan su conformidad con dicha presentación.

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CERTIFICA

Que la memoria titulada "Valorization of residues generated in the production of olive oil through clean technologies", presentada por D. Seyed Mehdi Niknam, ha sido realizada en el Área de Ingeniería Química del Departamento de Biotecnología y Ciencia de los Alimentos bajo la dirección de los Dres. D. José Manuel Benito Moreno y Dña. María Isabel Escudero Barbero y, en representación de la Comisión Académica del Programa de Doctorado, autoriza su presentación para ser defendida como Tesis Doctoral.

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Fdo.: José Manuel Benito Moreno

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SUMMARY

VALORIZATION OF RESIDUES GENERATED IN THE PRODUCTION OF OLIVE OIL THROUGH CLEAN TECHNOLOGIES

Summary of the PhD Thesis

Olive oil extraction systems can be classified into two main categories: traditional oil extraction by pressing, used for many centuries with minor modifications, and centrifugal processes, including two centrifugation systems called three- and two-phase systems. The extraction of olive oil generates large amounts of waste that have a great impact on land and water environments due to its high phytotoxicity. The most pollutant and phytotoxic waste is known as olive mill waste (OMW). During the olive oil production, almost all the phenolic content of the olive fruit (∼98%) remains in the olive mill by-products. Even though traditional pressing is a relatively obsolete technology, it is still in use for some olive oil producers. After the extraction by both pressing and two-phase centrifugation systems, a solid fraction, called olive husk, olive cake or *alperujo*, is obtained as a by-product along with an emulsion containing the olive oil that is later separated by decantation from the remaining olive mill wastewater. The three-phase system generates three fractions at the end of the process: a solid (olive husk or olive pomace) and two liquids (oil and wastewater). Some of the advantages of this system, compared to pressing, are greater automation, better oil quality and less area required for equipment; however, it also has some drawbacks, such as higher water and energy consumption, higher wastewater production, and facilities that are more expensive. Besides being a serious environmental problem, OMW represents today a precious resource of useful compounds. Phenolic compounds are hazardous to underground water and soil resources; consequently, these compounds must be removed from the OMW and recovered for further use. Furthermore, the extraction and use of phenolic compounds are economically profitable activities due to their functional properties of great interest in several applications for chemical, pharmaceutical and food industries. The quantity and type of phenolic compounds in OMW depends on the cultivar and maturity of the fruit, climatic conditions, storage time and processing technique. Clean technologies can be applied for bioactive compounds recovery because they work under moderate conditions helping in preserve their bioactivity for further uses as preservatives to extend the shelf life of foods, as nutritional supplements, or to provide them with determined functionality; thus closing the

cycle and moving towards a circular food economy. However, it should be taken into account that these bioactive compounds are unstable and usually modify the organoleptic characteristics of the foods to which they are incorporated. For this reason, their encapsulation into nanoparticles or emulsions is useful to protect them from the external environment, provided that the formulation used improves its characteristics in terms of stability, dispersibility, bioavailability and functionality in food.

The general objective of this PhD Thesis is the valorization of OMW in terms of olive pomace oil extraction and bioactive compounds recovery using clean and emerging technologies, with subsequent encapsulation of the recovered biocompounds in single and double nanoemulsions for their future use in food, cosmetic and pharmaceutical applications.

Specifically, the solid residues from the oil mill obtained from the three- and two-phase centrifugation systems were treated using conventional and ultrasound-assisted extraction methods and operation conditions for the recovery of phenolic compounds were optimized. Furthermore, solid-liquid adsorption experiments were performed on the extracts obtained from defatted oil mill solid residues. Several adsorbents and solvents were tested in the adsorption and desorption stages, in order to obtain purified and concentrated extracts and also to reach the selective isolation of desired bioactive compounds.

In addition, the oil mill wastewater (OMWW) was characterized to determine the main components of its composition, and later it was processed using membrane technologies and the optimal operating conditions were established to achieve the separation of phenolic compounds.

Finally, phenolic extracts were encapsulated in single and double emulsions by applying high-energy emulsification methods, optimizing the formulation and preparation conditions of the nanoemulsions.

Resumen de la Tesis Doctoral

Los sistemas de extracción de aceite de oliva pueden clasificarse en dos categorías principales: extracción tradicional por prensado, utilizada durante muchos siglos con pequeñas modificaciones, y procesos de centrifugación, que incluyen dos sistemas de centrifugación denominados sistemas de tres fases y dos fases. La extracción de aceite de oliva genera grandes cantidades de residuos que pueden tener un gran impacto en el medio terrestre y acuático debido a su alta fitotoxicidad. Los residuos más contaminantes y fitotóxicos se conocen como residuos de almazara (olive mill waste, OMW). Durante la producción de aceite de oliva, casi todo el contenido fenólico de la aceituna (~98%) permanece en los subproductos de la almazara. Aunque el prensado tradicional es una tecnología relativamente obsoleta, algunos productores de aceite de oliva todavía la utilizan. Tras la extracción mediante prensado o mediante el sistema de centrifugación en dos fases, se obtiene como subproducto una fracción sólida, denominada alperujo, junto con una emulsión que contiene el aceite de oliva y que se separa posteriormente por decantación del resto de las aguas residuales de la almazara. El sistema de centrifugación en tres fases genera tres fracciones al final del proceso: una sólida (orujo) y dos líquidas (aceite y aguas residuales o alpechín). Algunas de las ventajas de este sistema, en comparación con el prensado, son la mayor automatización, mejor calidad del aceite obtenido y menor área requerida para el equipamiento; sin embargo, también tiene algunos inconvenientes, como son un mayor consumo de agua y energía, mayor generación de aguas residuales, e instalaciones más caras.

Los residuos de almazara, además de constituir un problema ambiental grave, son un recurso abundante de compuestos útiles. Los compuestos fenólicos son peligrosos para las aguas subterráneas y el suelo; en consecuencia, estos compuestos deben eliminarse de los residuos de almazara y recuperarse para su uso posterior. Además, la extracción y uso de compuestos fenólicos son hoy en día actividades económicamente rentables debido a que sus propiedades funcionales son de gran interés en diversas aplicaciones de la industria química, farmacéutica y alimentaria.

La cantidad y el tipo de compuestos fenólicos presentes en los residuos de almazara depende de la variedad y madurez del fruto, las condiciones climáticas, el tiempo de almacenamiento y la técnica de procesado. La recuperación de compuestos fenólicos presentes en estos residuos puede realizarse mediante el uso de tecnologías limpias, ya que al funcionar en condiciones de operación moderadas, ayudan a preservar las propiedades bioactivas de estos

compuestos y su aplicación posterior como conservantes para extender la vida útil de los alimentos, como suplementos nutricionales o para proporcionar determinada funcionalidad, cerrando así el ciclo alimentario y avanzando hacia una economía circular en el sector.

No obstante, conviene tener en cuenta que estos compuestos bioactivos son inestables y suelen modificar las características organolépticas de los alimentos a los que se incorporan. Por este motivo, su encapsulación en nanopartículas o emulsiones es útil para protegerles del entorno, siempre y cuando la formulación empleada mejore sus características en cuanto a estabilidad, dispersabilidad, biodisponibilidad y funcionalidad en los alimentos.

El objetivo general de esta Tesis Doctoral es la valorización de los residuos de almazara en términos de extracción de aceite de orujo de oliva y recuperación de compuestos bioactivos mediante tecnologías limpias y emergentes, con posterior encapsulación de los biocompuestos recuperados en nanoemulsiones simples y dobles para su futuro uso en alimentación, cosmética y aplicaciones farmacéuticas.

En concreto, los residuos sólidos de la almazara procedentes de sistemas de centrifugación de tres y dos fases, se trataron mediante métodos de extracción convencionales y asistidos por ultrasonidos y se optimizaron las condiciones de operación para la recuperación de compuestos fenólicos. Además, se realizaron experimentos de adsorción sólido-líquido sobre los extractos obtenidos de residuos sólidos de almazara desgrasados. Se probaron varios adsorbentes y disolventes en las etapas de adsorción y desorción, con el fin de obtener extractos purificados y concentrados y también el aislamiento selectivo de los compuestos bioactivos deseados.

Además, se caracterizó el agua residual de la almazara (OMWW o alpechín) para determinar sus principales componentes, y posteriormente se procesó mediante tecnologías de membranas y se establecieron las condiciones óptimas de operación para lograr la separación de los compuestos fenólicos.

Finalmente, los extractos fenólicos se encapsularon en emulsiones simples y dobles aplicando métodos de emulsificación de alta energía, optimizando la formulación y las condiciones de preparación de las nanoemulsiones.

INTRODUCTION

VALORIZATION OF RESIDUES GENERATED IN THE PRODUCTION OF OLIVE OIL THROUGH CLEAN TECHNOLOGIES

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1. Introduction to olive oil production sector

Olive oil production tends to increase over the last decades as a valuable source of antioxidants and essential fatty acids in the human diet and constitutes one of the most important dietary trends worldwide. The olive oil extraction systems could be classified in two main categories: traditional pressing process [1], used for many centuries with minor modifications, and centrifugal processes, including two centrifugation systems, called three- and two-phase systems. Olive oil extraction generates huge quantities of liquid and solid wastes that may have a great impact on land and water environments because of their high phytotoxicity. The most pollutant and phytotoxic wastes are known as *olive mill waste* (OMW). During the olive oil production, almost all the phenolic content of the olive fruit (∼98%) remains in the olive mill by-products [10]. Besides being a serious environmental problem, OMW represents today a precious resource of useful compounds for recovery and valorization purposes [11–13].

2. Olive fruit processing and olive oil production technologies

Olive oil extraction involves different processes, such as leaf removal, olive washing, grinding, beating, and separation of the oil. The amount and physicochemical properties of the produced wastes and effluents depend on the method used for the extraction. Olive oil is extracted directly from the fresh fruit of olive tree (*Olea europaea* L.) using only mechanical methods, in order to maintain its natural organoleptic characteristics according to the European Commission Regulation No. 1513/2001 [14]. Olive fruits must be processed as quickly as possible after harvesting to minimize oxidation and preserve low acidity. All the operations of the olive mill can influence on olive oil quality [15, 16]. The mechanical processes used to extract olive oil from olive fruit include olives crushing, malaxation of resulting paste, and separation of the oily phase by pressure or centrifugation. In the latest one, three different systems are commonly used: the

traditional discontinuous press process, the three-phase, and the two-phase decanter centrifuge methods (Fig. I1) [4].
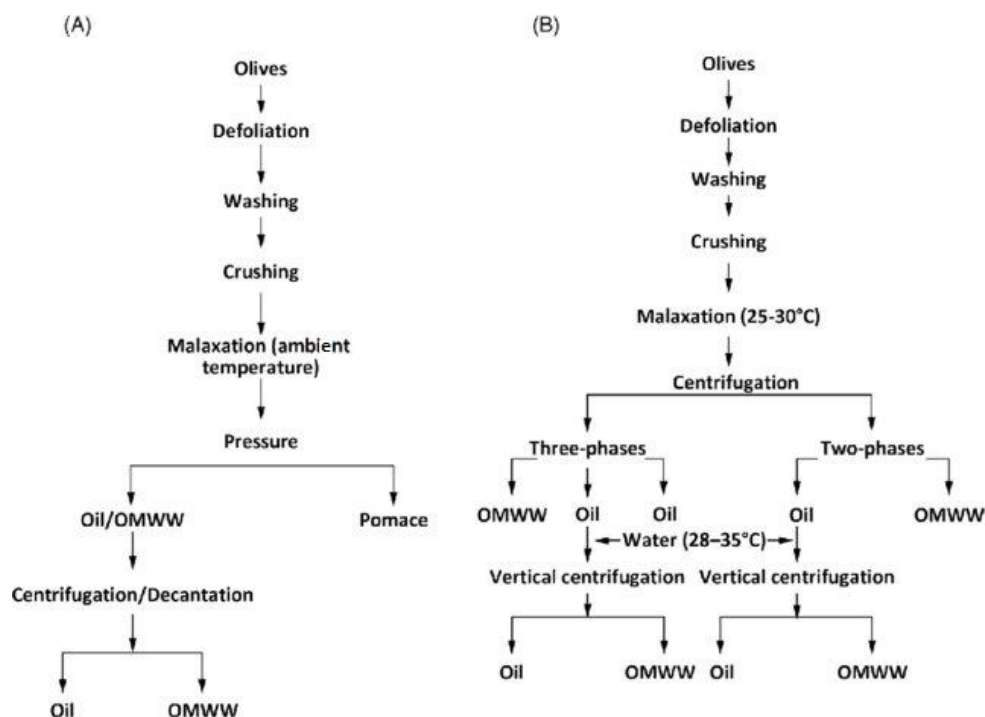
Figure I1. Traditional (A) and modern (B) processes for olive oil production (adapted from [1]).

2.1. Discontinuous press extraction system

Traditional extraction press is still used in some mills. After grinding olives in stone mills, the paste is spread on fiber diaphragms (which are stacked on top of each other) and then placed into the press (Fig. I2(A)). Hydraulic pressure is applied on the disks, thus compacting the solid phase of the olive paste and percolating the liquid phases (oil and vegetation water) [1]. This process generates a solid fraction called olive husk (or kernel), an emulsion containing the olive oil and a water phase. The olive oil is finally separated from the remaining wastewater by decantation or vertical centrifugation. The traditional process is reputed to produce a high quality olive oil, usually extra virgin olive oil. Nowadays, due to the need to process large amounts of olives and to obtain higher yields of olive oil, the evolution of the oil

extraction process has led to the replacement of traditional pressure mills with modern continuous centrifugal extraction.

2.2. Continuous centrifugal extraction systems

After washing, crushing, and malaxation steps, the mechanical oil extraction is mainly carried out using a continuous process based on centrifugation with a decanter. The decanter centrifuge is designed with screw conveyor and rotating bowl, allowing to process large amounts of olives in a short time [17]. Two types are currently used: two-phase and three-phase centrifugal decanters (Figs. I2(B) and (C)).

The continuous three-phase centrifugation process was introduced during the 1970s, in order to increase processing capacity and extraction yield and reduce labor [18]. During the three-phase process, an addition of hot water is required to wash the oil. The process yields three phases: oil phase, solid residue: olive cake (olive pulp and stones), and the olive mill wastewater which hereinafter will be referred as OMWW. Solid residue is separated from the other two phases in the decanter and will be referred as *olive pomace* in this work. The liquid phases are subsequently submitted to a vertical centrifuge in order to separate the olive oil from OMWW.

A disadvantage of this process is the large amount of produced wastewater due to the high water consumption, which is 1.25–1.75 times more than that used in press extraction [19].
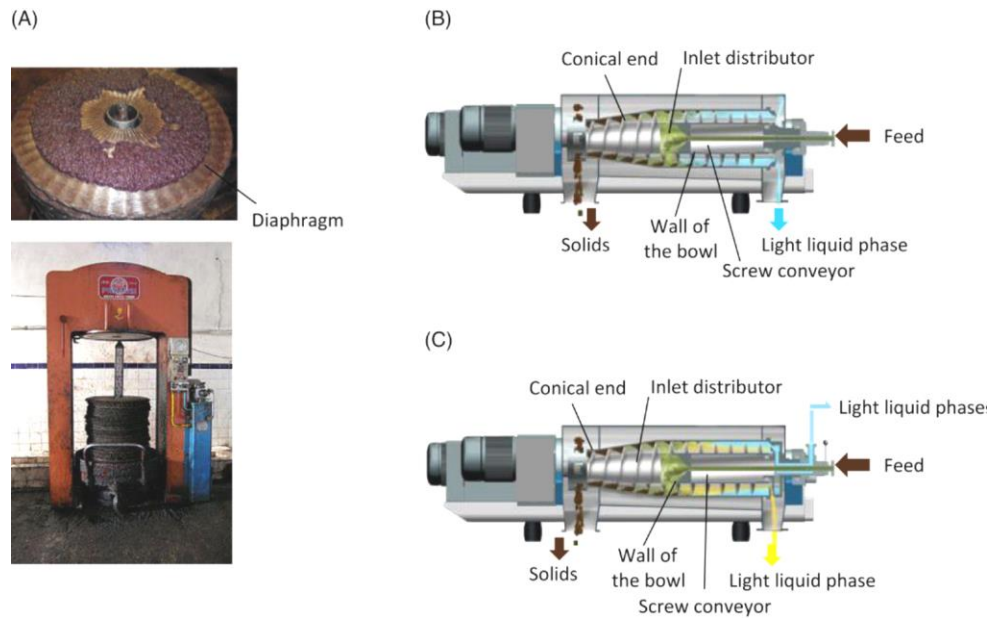
Figure I2. Extraction processes for virgin olive oil (VOO) production. (A) Press system; (B) Two-phase centrifugal decanter; (C) Three-phase centrifugal decanter (adapted from [1]).

In recent years olive mill technology has been oriented to save water during the extraction stage. Thus, at the end of the 1991/1992 olive oil campaign, a new centrifugation system was developed that reduced by 75% the olive mill wastes. This system was launched to the market with the labeling of "ecological", because of the reduction in water consumption, and of "two-phase", because it produced two fractions: a solid one (called in different ways: alperujo, olive wet husk, wet pomace or two-phase olive mill waste) and a liquid one (olive oil).

In Spain the new two-phase system quickly replaced the three-phase method. Therefore a new waste, with peculiar physico-chemical properties, was generated in huge quantities leading to serious management problems. This sudden replacement has not happened in other countries, probably due to the management difficulties arisen with the new waste. In Europe, besides Spain, only Croatia has a high proportion of two-phase systems.

Usually, the olive pomace from the three-phase system had a second oil extraction with organic solvents after its drying. However, when two-phase olive mill waste was intended to be treated similarly, great difficulties arose due to its high moisture and carbohydrate concentration. Thus, the new waste tends to stick to the furnace walls blocking the gaseous stream and causing an explosion hazard [5,20]. Two phase centrifugation system uses only wash water and delivers oil (liquid phase) and a very wet substrate (referred to as TPOMW hereinafter) using more efficient centrifugation technology [19]. This process has a reduced environmental impact due to the lower water demand and waste amounts produced, but it requires extra energy for drying prior to olive kernel oil extraction [4,21].

2.3. Continuous olive oil production line

The first step in the olive oil extraction process is olive fruit cleaning and removal of stems, leaves, twigs, and other debris left with the olives. Washing is also aiming to remove pesticides and dirt [22].

Crushing step aims to break down the cellular membranes of olive fruits and thus release small drops of oil from the vacuoles [10]. This operation produces a mixture of two distinct liquid phases (raw oil and water) and an extremely heterogeneous solid phase (pit, skin, and pulp fragments). Crushing can be considered a critical point affecting the quality of the produced olive oil, especially in terms of phenolic content and volatile compounds [23].

Two enzymes, polyphenol oxidase and peroxidase, are highly concentrated in the olive kernel. Thus, crushing operation allows a direct contact of these enzymes with phenolic compounds and induces their oxidation, which results in lower phenolic concentration in the oil. At this phase, the main hydrophilic phenols of virgin olive oil (VOO) (e.g., secoiridoid aglycons) are generated by the hydrolysis of oleuropein, demethyloleuropein, and ligstroside, catalyzed by endogenous β-glucosidases [24].

Malaxation consists of a slow and continuous kneading of the olive paste in order to facilitate the cohesion of small oil droplets obtained during crushing step, leading

to separation of the oil from the water phase. This step is essential to achieve high oil extraction yields. Several researchers have investigated the effect of malaxation parameters on the quality of produced oil. It wsa found that malaxation time and temperatures greatly affect the olive oil quality, especially the aroma and phenolic profiles [25,26]. Aliakbarian et al. [27] demonstrated that increasing the malaxation time from 90 to 150 min decreased the nutritional quality of olive oil, mainly due to the increased oxidation of phenolics. In order to obtain a high quality and extraction yield of olive oil, a low malaxation temperature and a process time between 30 and 45 min are recommended [28].

The centrifugation process is based on the density differences of olive paste components (olive oil, water, and insoluble solids). Decanters are constituted of a cylindrical conical bowl drum where a screw feeder is rotating at differential speed [12]. Separation is conducted due to the centrifugal force developed in the drum. Olive paste could be treated either by two-phase centrifugal decanter or a three-phase centrifugal decanter. In the two-phase decanter, the product is separated into a liquid phase (oil) and a solid phase (kernel fragments, pulp, and vegetable water). In the three-phase centrifugal decanter the product is separated into a light liquid phase (oil), a heavy liquid phase (water), and a solid phase (kernel fragments and pulp). The oil phase is discharged by gravity in both centrifugal decanters. In three-phase decanter, the water phase is discharged using a centripetal or gravity pump, whereas the solid phase is discharged in the drum conical terminal of the decanter after being pushed by the screw feeder. The two-phase process requires no dilution during the malaxation step. However, in the three-phase process, large amounts of water are added to the paste. After malaxation, the olive oil is either completely free or in the form of small droplets inside microgels, or emulsified in the aqueous phase. Water addition during centrifugation enhanced the release of the oil fraction locked in the microgels [29].

In two-phase olive mills, water is only used during the washing of the olives prior to crushing and not in the olive oil extraction process. Hence, there are only two streams produced after the centrifugation process: the olive oil and the olive pomace. However, the olive pomace from two-phase olive mills has higher moisture

content compared to the one generated from three-phase or pressing mills, and the transportation and processing costs are higher. Although the production cost is lower in two-phase olive mills since the processed volumes are lower due to the non-addition of water, there is a 7% less yield in the production of olive oil compared to the three-phase mills. There is also a small amount of OMWW produced due to the use of water during the oil washing unit. Approximately 5 tons of olive leaves are also generated together with the olive pomace in all types of olive mills [30,31].

Oil clarification is the final cleaning step of olive oil. It aims to separate the residual water and impurities existing in the extracted oil from the decanter. This operation is necessary to avoid fermentation, hydrolysis, and oxidation reactions that could cause the alteration of the sensory properties of olive oil [32].

3. Wastes and wastewater generated during olive oil production

Avraamides and Fatta [33] conducted an study in Cyprus evaluating water consumption at various stages of olive oil production. The authors reported that a total of 3914 L of fresh water are consumed for the production of 1 L of olive oil. However, during olive processing, only 3.51 L of water are consumed for every liter of olive oil produced. This stage produces 4.34 kg of OMWW and 2.07 kg of solid waste (olive pomace) for every liter of olive oil produced [33]. The water dilution of the olive paste affects the partition of hydrophilic phenols between oil and water and enhances their release in the water phase. The reduction of water dilution during the three-phase process also leads to an increase of the phenolic concentration in the olive oil [34].

Olive mill wastes are the major environmental concern of olive oil industry, as leaves represent only 5% of olives' weight in oil extraction. Indeed, the annual world production of OMW is estimated to be from 10 to over 30 million m^3 [35]. The amount and the physicochemical characteristics of the generated wastes depend on the used oil extraction system, the processed fruits, and the operating conditions (added water, temperature, etc.). A limited amount of solid wastes (leaves and small

twigs) is produced during the cleaning of the olives prior to milling. OMWW is the main waste from three-phase extraction systems and traditional mills. It is constituted by vegetable water of the fruit and the water used in different stages of oil extraction. Besides, the three-phase extraction systems generate a solid waste that is used to extract olive kernel (pomace) oil.

Table I1. Composition of the olive mill solid and liquid wastes [9].

Parameter	Liquid residue	Solid residue		Ref.
	OMWW	Olive Pomace	TPOMW	
Pulp (%)	-	12-35	10-15	[50]
Olive stone (%)	-	15-45	12-18	[50]
Ash (%)	1	1.7-4	1.42-4	[31,50,51]
Total proteins (%)	-	3.43-7.26	2.87-7.2	[31,52,53]
Cellulose (%)	-	17.37-24.14	14.54	[31]
Hemicellulose (%)	-	7.92-11	6.63	[31]
Total carbon (%)	2-3.3	29.03-42.9	25.37	[54–56]
Organic matter (%)	57.2-62.1	85	60.3-98.5	[19,57–60]
Total organic carbon (g/L)	20.19-39.8	-	-	[19,54]
Total suspended solids (g/L)	25-30	-	-	[61,62]
Lignin (%)	-	0.21-14.18	8.54	[31]
Phosphorus (P) (%)	0.19	0.03-0.06	0.03-0.14	[50,59,63,64]
Potassium (K) (%)	0.44-5.24	0.1-0.2	0.63-2.9	[19,50,59,64,65]
Sodium (Na) (%)	0.15	-	0.02-0.1	[59,64,66]
Calcium (Ca) (%)	0.42-1.15	-	0.23-1.2	[59,63–65]
Magnesium (Mg) (%)	0.11-0.18	-	0.05-0.17	[53,64–66]
Iron (Fe) (%)	0.26±0.03	-	0.0526-0.26	[59,65,67]
Copper (Cu) (%)	0.0021	-	0.0013-0.0138	[59,64,66]
Manganese (Mn) (%)	0.0015	-	0.0013-0.0067	[59,64,66]
Zinc (Zn) (%)	0.0057	-	0.0010-0.0027	[59,63,64]

Furthermore, wet olive pomace from two-phase mills (TPOMW) is a solid waste with a strong odor and a doughy texture that makes its management and transport difficult. It is very humid and very difficult to treat. It is generally subjected to a

solar drying and subsequently used to extract oil with solvents [4,36]. The composition of olive mill wastes for continuous olive oil extraction systems is shown in Table I1.

Olive is rich in phenolic compounds and its bitter taste is basically associated with these compounds [37,38]. The important point is that just about 2% of its phenolic content enters the oil phase during oil extraction while about 45% of olive's phenolic compounds remains in the olive-waste cake [37]. Phenolic compounds of olive-waste cake are solid, colorless, water soluble with considerable environmental resistance and they decompose hardly. Therefore, entrance of waste cake from olive oil extraction plants, and in fact the entrance of their phenolic compounds to underground water and soil is hazardous. Consequently, these compounds should be extracted and removed from the olive waste cake [39]. Furthermore, extraction and production of phenolic compounds are economically important due to their applications in chemical, pharmaceutical and food industries.

Olive cake is considered a rich source of phenolic compounds with a wide array of biological activities [10]. The quantity and type of phenolic compounds in olive cake depends on the cultivar and maturity of the fruit, climatic conditions, storage time and processing technique [40–42]. The major phenolic compounds present in olive cake are hydroxytyrosol [43], oleuropein [44], tyrosol [40], caffeic acid [45], p-coumaric acid, vanillic acid [46], verbascoside, elenolic acid [44], catechol [41] and rutin [47].

The organic fraction of OMW contains, among other components, 2–15% of phenolic compounds divided into low-molecular weight (tyrosol, hydroxytyrosol, *p*-coumaric acid, ferulic acid, syringic acid, protocatechuic acid, etc.) and high molecular weight compounds (tannins, anthocyanins, etc.) as well as catechol-melaninic polymers [48]. It is characterized by a dark color (caused by lignin polymerization with phenolic compounds), increased acidity (pH about 5), and high electrical conductivity. The physicochemical characteristics and mineral content of olive mill wastes are shown in Table I2.

Table I2. Physicochemical characteristics of the olive mill solid and liquid wastes [9].

Parameter	Liquid residue	Solid residue		Ref.
	OMWW	Olive Pomace	TPOMW	
pH	2.24-5.9	-	4.9-6.8	[59,76–78]
Chemical Oxygen Demand (COD) (g/L)	30-320	-	-	[78,79]
Biological Oxygen Demand (BOD) (g/L)	35-132	-	-	[79,80]
Dry matter (%)	6.33-7.19	87.1-94.4	-	[58,64,81,82]
Lipids (%)	0.03-4.25	3.5-8.72	3.76-18	[31,50,64,67,76,83]
Total phenols (%)	0.63-5.45	0.2-1.146	0.4-2.43	[2,31,50,65]
Total sugars (%)	1.5-12.22	0.99-1.38	0.83-19.3	[2,19,31,84]
Total nitrogen (%)	0.63	0.2-0.3	0.25-1.85	[50,64,85]

The inorganic content of OMW is mainly composed of metals. Major elements in this wastewater are potassium (0.73–8.6 g/L), followed by calcium and sodium (0.03–1.1 and 0.05–0.8 g/L, respectively). Metals are important both from nutritional and toxicological viewpoints. Some metals, particularly iron, copper, and zinc, are essential to plant metabolism. It should be pointed out that OMWW had a high potential to be used as fertilizer if its polluting power was controlled [4]. In the past years, several researchers have studied the presence of contaminants (pesticides) in the pomace [49], the detoxification of this residue by the use of microorganisms [68] or the reuse of olive pomace as metal ion adsorbent [69]. A suitable use of these olive oil residues could not only improve the economic status of olive oil producers but could also reduce such an environmental problem. Thus, it is of great interest to evaluate the possibility of recovering an extract enriched with phenolic compounds, from a low-cost and widely available by-product, especially in the Mediterranean area [70].

4. Environmental effects of olive mill wastes

Olive mill wastewaters are often disposed in evaporation ponds or various environmental receptors causing strong odor nuisance, soil contamination, plant growth inhibition, natural streams pollution as well as severe effects to the aquatic fauna and to the ecological status [71]. OMWW is one of the most polluting effluents produced by the agrofood industries due to its high organic load and a wide range of contaminants, including organo-halogenated pollutants, fatty acids, phenolic compounds, and tannins [72,73].

Three types of olive processing technologies are currently used worldwide, producing different type and amount of wastes, that is, the traditional olive-pressing process, the three-phase, and the two-phase extraction processes [4]. The approximate input-output data for these three types of technologies are presented in Table I3. The high phenolic nature of OMWW and its organic content make it highly resistant to biodegradation [74]. OMWW shows poor biodegradability and high phytotoxicity due to the presence of phenolic compounds. Likewise, the presence of reduced sugars can stimulate microbial respiration and lower dissolved oxygen concentrations [35].

4.1. Effects on atmosphere

Olive mills generate gas emissions that cause considerable odor grievances. Many low-boiling organic compounds and volatile organic acids produce characteristic odors that can be smelled around the olive mills. OMWW is generally discharged into natural waters or on the land and/or stored in poorly engineered evaporation ponds. This effluent undergoes a natural fermentation and emanates pungent gases, such as phenols, sulfur dioxide, and hydrogen sulfide. This fact leads to a considerable odor pollution particularly during oil production season [75].

Table I3. Approximate input-output data for olive oil production processes [2–4].

Production Process	Input	Output
Traditional pressing	Olives = 1 ton Wash water = 0.1-0.12 m^3 Energy = 40-60 kWh	Oil ≈200 kg Solid waste = 200-400 kg (25% water + 6% oil) Wastewater ≈ 400-600 kg (88% water + solids and oil)
Three phase centrifugation	Olives = 1 ton Wash water = 0.1-0.12 m^3 Fresh water for decanter = 0.5-1 m^3 Water to polish the impure oil ≈ 10 L Energy = 90-117 kWh	Oil = 200 kg Solid waste = 500-600 kg (50% water + 4% oil) Wastewater = 1000-1200 kg (94% water + 1% solids and oil)
Two phase centrifugation	Olives = 1 ton Wash water = 0.1-0.12 m^3 Energy = 90-117 kWh	Oil = 200 kg Solid waste = 800-950 kg (60% water + 3% oil) Wastewater = 85-110 kg

4.2. Effects on water resources

OMWW disposal leads to significant effects on natural water bodies, too. These impacts are mainly related to concentration, composition, and seasonal production. The most visible effect is the discoloring of streams and rivers. This color change is mainly due to the oxidation and subsequent polymerization of tannins giving darkly colored phenols [83]. The high reduced sugars content in OMWW stimulates microorganism growth, lowering dissolved oxygen concentrations in water, and thus decreases the share available for other living organisms. Other deteriorating effects can result from the high phosphorus concentration, which accelerates the growth of algae and leads to eutrophication (devastating the entire ecological balance in natural streams). Conversely to carbon and nitrogen (which flee after

degradation as carbon dioxide and atmospheric nitrogen), phosphorus cannot be degraded. This means that phosphorus is taken up merely to a small level through the food chain, plant-invertebrates-fish-prehensile birds. The presence of considerable amount of nutrients in OMWW supplies an appropriate medium for pathogens to multiply and infect streams, which have stern consequences to aquatic life, and humans, coming in contact with infected water [4].

4.3. Effect on crops and soil

OMWW contains large amount of organic substances, including phenolic compounds that may have negative impact when applied to soil. Because of the high concentrations of organic matter and mineral nutrients, OMWW could be a promising fertilizer for cropping systems. Ayoub et al. [86] recommended the application of 10 L OMWW/m^2 to improve soil fertility and olive plant performance. Indeed, a significant increase in shoot growth, photosynthesis, fruit set, and fruit yield without any negative effects on oil quality parameters was observed. Besides, the concentrations of K, organic matter, phenolic compounds, and total microbial count were significantly increased in OMWW-treated soil [86]. OMWW application shows short-term negative effects on soil chemical and biological properties, but it can be considered negligible after a suitable waiting period [87].

5. Sustainable management methods of olive mill wastes

The difficulties of OMWW treatment are mainly related to [88]:

1. Its high organic loading´
2. Seasonal operation
3. High territorial scattering, and
4. The presence of non-biodegradable organic compounds like long-chain fatty acids and phenols.

Many different processes have been suggested to treat OMWW, namely lagooning or direct watering on fields, cocomposting, physicochemical methods (flotation and settling, coagulation, oxidation by O_3 and Fenton reagent, flocculation, filtration, sedimentation, dilution open evaporating ponds, and incineration), ultrafiltration/reverse osmosis, chemical and electrochemical treatments, and manufacture into animal food [89]. Although the environmental problem is more intense for the disposal of OMWW, the earlier mentioned techniques are typically proposed to treat all kind of olive oil processing effluents. In an attempt to categorize the proposed methodologies of OMW management, three categories can be denoted:

1. Waste reduction via olive production systems conversion (i.e., two-phase instead of three-phase continuous systems).
2. Detoxification methods aiming at the reduction of impact of the pollution load to the recipient.
3. Recovery or recycling of OMW compounds.

Physical processes are typically applied as a pretreatment step to remove the contained solids. Thermal processes are used to remove the contained water and condense the waste streams, however are ineffective due to the required operational cost. Despite their effectiveness, advanced oxidation processes are also very expensive. Biological treatment requires a longer lag phase, whereas physicochemical methods, such as neutralization and precipitation, are relatively cheaper, but they cannot diminish completely the pollution load of OMWW. Following these considerations, the researchers have proposed several combined treatments (chemical, physical, and biological). However, none of these treatments have found a widely accepted application since they have not been proved to be sustainable in a long-term base [89]. The most common treatment methods are denoted in Table I4.

Table I4. Treatment methods of olive mill wastes [2,3,5,6]

Method	Notes	Advantages	Disadvantages
Wastewater			
Biological treatment	Reduction of BOD and COD. Enables the removal of organic and inorganic suspended solids. Anaerobic process is used to remove organic matter in high concentration streams by converting organic compounds to methane and carbon dioxide. Aerobic process is used on lower concentration streams to further remove residual organic matter and nutrients.	Anaerobic technology has clear advantages in comparison to aerobic processes including higher removal efficiency, lower excess sludge production (20-fold lower than the aerobic process) and low space requirements. It produces biogas, a valuable by-product that can be used to cover the mill energy demand..	Pretreatment of wastewater is needed, for example, sedimentation-filtration.. The presence of oil and antimicrobial phenolic compounds create a challenge for biological treatment. Due to high investment costs and complex process management, this technology is suited for industrial-scale olive mills or as a centralized treatment facility serving several olive mills.
Composting	It is one of the main technologies used for recycling wastewater and transforming it into a fertilizer. Wastewater is added repeatedly during the thermophilic phase.	Valorization of waste and production of natural fertilizers. This process allows the return of nutrients to cropland.	Wastewater has to be removed before composting (e.g., using simple precipitation units, by adsorption on a solid substrate).
Membrane filtration	Microfiltration, ultrafiltration, nanofiltration, and reverse osmosis can be applied on olive mill wastewaters. The concentrate can be sent to incineration or landfilled.	Optimal COD reduction. Since conventional biological treatment is not always sufficient, other processes, such as membrane filtration (e.g., reverse osmosis or ultrafiltration) are required.	Hardly suited for treatment of OMW in small-scale oil mills due to the high investment and operational costs, high energy demand, and rather complex process control that requires highly qualified personnel.
Chemical-physical treatment	Removal of non-biodegradable dissolved organic pollutants by adsorption, precipitation, and flocculation, characterized by the addition of specific chemicals: activated carbon, polyelectrolytes or flocculants/coagulants, such as $FeCl_3$, $AlCl_3$, $FeSO_4$ and $Ca(OH)_2$.	High removal efficiency. Low investment and operational costs, better suited for small-scale olive mills.	Large quantities of produced sludge and subsequent disposal problems.
Natural evaporation in open storage ponds	The most common management method.	Low investment is required; the approach is favored by the climatic conditions in Mediterranean countries.	It needs large areas and causes several problems such as bad odors, infiltration, and insect proliferation. Evaporation of OMW results in the production of sludge which is typically disposed of in landfill sites. Most studies targeting revalorization of OMW sludge focus on composting.
Forced evaporation	Water separation by using a multistage evaporation system enables heat recovery. The concentrate has to be treated, prior to its disposal, generally by biological treatment, such as aerobic digestion and activated sludge process.	Heat recovery possible.	High energy demand, considerable air emissions, and complex control process that requires qualified personnel. High operational costs are associated. It is a solution suited for industrial-scale oil mills only.

Solid and semi-solid waste (olive kernel, olive husks/cake and wet olive pomace)			
Drying	Heat is transferred to waste by means of hot gases (drum dryers, belt dryers, and fluidized-bed dryers).	The resulting dried waste may be incinerated for energy production, reused in agriculture, or landfilled, while air emissions must be treated appropriately.	High-energy demand, but the resulting final product can be reused for the production of the required energy. High investment and operating costs are associated to drying plants; moreover, trained and qualified personnel is required to ensure trouble-free operation. The high moisture content of the wet olive pomace causes technical problems during drying in traditional husk-extraction mills. These problems initiated research for new drying methods.
Thermal treatment	The treatment provides the possibility of energy recovery while mixtures of OMW with other organic wastes can be incinerated as well. After the second extraction of the wet olive pomace, the exhausted olive cake is usually used as fuel in the husk mill to obtain thermal or electric energy through combustion.	Possible energy recovery	Very expensive, considering the high cost of the combustion chambers and the associated air pollution control systems. Most of the energy obtained by combustion is used for drying of the fresh wet olive pomace and therefore the total energy recovery is low.
Biological treatment	Both aerobic (composting) and anaerobic (fermentation) processes can be used. Bulking material such as wood shavings has to be added during waste composting to achieve proper moisture level and good aeration of the pulp. Unlike wastewater, the wet olive pomace is added at once at the beginning of the composting process.	Fairly low air emissions and insignificant consumption of energy and resources. As far as the fermentation process is concerned, energy and space requirements are very low, while biogas production enables energy recovery.	Compared to composting, problems often arise in process control, hence qualified personnel is required. Moreover, this technology is affected by higher investment costs. The toxicity of phenolic compounds present in wet olive pomace affects anaerobic digestion. Due to its semi-solid state, pomace needs to be mixed with bulking agents, such as straw, cotton-waste, poplar sawdust, and bark chips, before composting. The physical characteristics of wet olive pomace cause difficulties during composting by forced aeration systems and mechanical turning is often preferred.

Zagklis et al. [90] conducted a sustainability and benchmarking study of OMWW treatment methods, showing that the most effective processes in terms of organics reduction are membrane filtration, electrolysis, supercritical water oxidation, and photo-Fenton. Lower environmental impact was found with anaerobic digestion, coagulation, and lime processes, while the lowest-cost methods are composting and membrane filtration, owing to the added-value of composts and phenolic compounds, respectively [90].

Several techniques (individually or in combination) have been proposed to recover phenols from OMW. These techniques include solvent extraction, chromatographic

separations, centrifugation, membrane processes and more recently high voltage electrical discharges, pulsed electric field, and ultrasounds [91]. Membrane separation processes are among the key physicochemical and nondestructive techniques applied for the separation of macro- and micromolecules in food waste streams [92,93].

The majority of olive oil producer countries in the Mediterranean area suffer from desertification, so the water and organic matter reuse would be beneficial to improve soil fertility and control the erosion processes. Also in organic agriculture, the use of OMW as fertilizer and/or biopesticides could represent an important source of nutrients, protecting crops, and closing the cycle of residues–resources. However, phenols of OMW are too valuable to be diminished or discharged to the environment. Therefore, phenols recovery and their reuse in different products and markets should come first. To conclude, the selection of the most suitable valorization strategy depends on the social, agricultural, or industrial environment of the olive oil production plants, but more importantly, on the long-term sustainability of the solution provided. Solutions that promise full OMW treatment and at the same time financially collapse the olive oil industries have been rejected in practice, not only because of sector's denial, but also because of society's tolerance that delays the enforcement of environmental legislations implementation [4].

5.1. Potential use of OMWW for olive trees irrigation and fertilization

An alternative, environmentally friendly, and economically feasible approach to manage OMWW would take advantage of its valuable ingredients (e.g., organic matter, nutrients, and water). In this sense, OMWW could be considered as a soil amendment and natural fertilizer when applied under specific preconditions for trees irrigation (LIFE Prosodol) [94]. Italy is the leading country in using OMWW for these purposes. Capitalizing the Italian experience in reusing OMWW into olive trees orchards, an irrigation system was installed in Albenga, Italy, during the implementation of PROSODOL project. For this, a hanging drip line system at 60

cm above soil surface per each olive row was installed and pressure compensated tubes normally used in the agricultural sector for the distribution of water or the application of liquid fumigants were adopted. Such driplines have the following properties: flow: 2 L/h, distance between drips: 30 cm, pressure: 1.5 bar. Each plant was provided with 2 drips aligned with plant row roughly at 15 cm apart from olive stem.

Filtration represents the key aspect during OMWW distribution through dripline in consideration of the waste composition. Ease of distribution may vary depending on the thickness and the presence of suspended solids in OMWW. It is clear that irrigation requires additonal costs in terms of design, set up, and system management compared to an olive orchard without any irrigation system. It is also crucial to evaluate costs due to the exploitation of water in areas where it is often lacking [6].

5.2. Soil remediation techniques

The suitable soil remediation and protection plan for OMW disposal areas should include methodologies for polyphenols reduction and retention or immobilization of inorganic constituents. *In situ* bioremediation may be applied to reduce polyphenols concentration in soil, since it targets biodegradation of organic pollutants in soil by taking full advantages of the natural biodegradation process of organic molecules by soil microorganisms [95]. Furthermore, the use of clinoptilolite, a natural zeolite, as soil amendment was shown, during PROSODOL project, to be the most suitable solution to reduce inorganic soil constituents because of the well-known properties of clinoptilolite to attract, retain, and slowly release inorganic cations. Moreover, this method is of very low cost and very easy to be implemented, even by not qualified personnel [96,97]. Both methods were applied in a pilot area with very satisfactory results [6,94].

6. Recovery of bioactive compounds

The difficulties in OMWW management arise from the fact that olive oil production is seasonal (during olive harvest), so the treatment process should operate in a discontinuous mode. The large number of small olive mills, spread across the Mediterranean region, make individual on-site treatment options unaffordable [98]. Besides, the high phenolic nature of OMWW and its organic content make it highly resistant to biodegradation [74].

Like all food processing wastes, OMWW and other olive oil processing by-products have long been considered as a matter of treatment, minimization, and prevention due to the environmental effects induced by their disposal. On the other hand, similarly to all fruit processing by-products, they account as a cheap source of valuable compounds that could be recovered and used as natural food additives [99,100]. For instance, phenols are characterized by antioxidant properties with potential health benefits [101]. Thus, from the early 2000s, an increasing interest not only in safely disposing of OMWW but also in recovering valuable nutrients for nutraceutical applications has been emerged [102–106]. Perspectives originate from the huge amounts discharged in the Mediterranean and the existing technologies, which promise the recovery, recycling, and sustainability of high-added value ingredients inside food chain [89].

Natural evaporation in open-air lagoons, favored by the Mediterranean weather, is the most conventional method for OMWW treatment [107,108]. Physicochemical treatments (flocculation, advanced oxidative methods, and electrochemical processes, such as the electrocoagulation) are also used to reduce the organic load of OMWW [3]. Biological treatments, such as activated sludge, anaerobic treatments, and membrane bioreactors, have also been used for the reduction of its organic load [109]. Other alternative tested is the direct agronomic application, but its high content in phytotoxic phenolic compounds increases soil hydrophobicity and decreases water retention [110].

In an integrated biorefinery approach, aimed at producing high added-value products from OMWW, a preprocessing step to extract the remaining high value antioxidants may represent an economically interesting strategy that provides the

triple opportunity to obtain high-added value biomolecules, to increase biodegradability and to reduce phytotoxicity of the effluent.

Most of the phenolic compounds in olives and olive oil (hydroxytyrosol, tyrosol, caffeic acid, rutin, luteolin, and flavonoids) are insoluble in oil and, thereby, remain in wastewater. The water-soluble phenolic compound fractions represent 50–72% of the total phenolic compounds in olives [111]. The selective recovery of these phenolic compounds in a preprocessing step can achieve both the reduction of the intrinsic wastewater environmental toxicity and the production of high added value molecules.

6.1. Bioactive phenols

Broadly distributed in the plant kingdom and abundant in our diet, phenols are today among the most discussed categories of natural antioxidants [112]. They include one or more hydroxyl groups (polar part) attached directly to an aromatic ring (nonpolar part) and are often found in plants as esters or glycosides, rather than as free molecules. This stereochemistry distinguishes them according to their polarity variance [113]. Recent epidemiological studies have shown the inverse association between risk of cancer, cardiovascular diseases, diabetes, several age related chronic diseases, and intake of diet rich in phenols and antioxidants [114–116].

Phenols can exist inherently in the olive fruit or be generated during olive oil production [101]. OMWW contains typically from 0.5 to 24 g phenols/L, which is about 98% of the phenols present in olive fruit [10], since only 2% of them pass into the oil phase during extraction process [89]. Olive fruit contains phenolic acids and alcohols, secoiridoids and flavonoids, while more than 50 and 40 relevant compounds have been identified in OMWW and olive oil, respectively [112,117].

Phenolic acids include *o-* and *p*-coumaric, cinnamic, caffeic, ferulic, gallic, sinapic, chlorogenic, protocatechuic, syringic, vanillic, and elenolic acids [41,44,118,119]. The most typical phenolic alcohols are tyrosol and hydroxytyrosol [120,121]. The qualitative and quantitative HPLC analysis of raw OMWW is similar to olive oil, whereas hydroxytyrosol and tyrosol are the most abundant phenolic compounds, as

in olive oil [122,123]. Furthermore, OMWW contains numerous flavonoids such as apigenin, hesperidin, cyanidin flavone, anthocyanin, and quercetin [101,124,125]. OMWW phenols are well known for their unique antioxidant properties for human health that promise their reuse as additives in foodstuff and cosmetics [126]. Indeed, one of the most established activities of OMWW phenols is their ability to capture free radicals (both *in vitro* and *in vivo*).

OMWW phenols have also shown a scavenging ability against hypochlorous acid (HClO) [127] as well as ferric reducing ability [128]. Besides, several studies have reported the antimicrobial properties (i.e., against *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cinerea*, *Escherichia coli,* and *Pseudomonas aeruginosa*) of olive polyphenols [112,117,129].

Hydroxytyrosol is the most known olive phenol in spite of its beneficial properties, for example, it exerts advanced antiradical properties compared to vitamins E and C [130]. The antioxidant ability of hydroxytyrosol has been proven in the plasma and liver of rats [131], while its cardio-protective effect has been successfully assayed on human cells [132]. Together with other *o*-phenolics (e.g., oleuropein and caffeic acid) are known to exert protective effect against low-density lipoprotein (LDL) oxidation *in vitro* and appeared that it can be most effective at low concentrations to protect human erythrocytes and DNA against oxidative damages [133–135]. The beneficial effect of hydroxytyrosol as an effective hypoglycemic and antioxidant agent in alleviating oxidative stress and free radicals as well as in enhancing enzymatic defenses in diabetic rats has also been demonstrated [40]. Hydroxytyrosol-rich olive oils have been approved by the European Food Safety Authority (EFSA) for their ability to maintain healthy LDL cholesterol level and lipid antioxidation [136]. The natural hydroxytyrosol concentrates obtained from OMWW and other olive by-products (using clean technologies) may also exert antioxidant, antimicrobial, antiinflammatory, and anticarcinogenic properties superior to those observed for pure hydroxytyrosol, in equivalent concentration [137,138].

6.2. Extraction, clarification and isolation of bioactive phenols

The main separation strategies [139] involves the use of liquid–liquid extraction (LLE) [140,141], ultrasound-assisted extraction [142–144], solvent extraction [101,145], superheated liquid extraction [143], supercritical fluid extraction [146], and more recently emerging technologies, such as high voltage electrical discharges and pulsed electric field [89,147–149]. Although LLE provides efficient results, the necessity of using large volume of organic solvents is increasing the interest to replace LLE by solid-phase extraction [150].

Membrane processes have also been proposed for the selective fractionation and total recovery of polyphenols, water, and organic substances from OMW [103, [151–155]. Using a preliminary membrane filtration followed by two ultrafiltration steps (6 and 1 kDa membranes, respectively) and a final reverse osmosis treatment, Russo [156] obtained an enriched and purified low molecular weight polyphenols extract to be used for food, pharmaceutical or cosmetic purposes. The remaining liquid can be used as fertilizer or in the production of biogas in anaerobic reactors as detailed later [7].

From another point of view, the "5-Stage Universal Recovery Process" [157] provides an integral strategy in order to combine these techniques and purify phenols prior to their final formation into a product. Following this approach, the recovery of any target compound from food by-products can be accomplished in five distinct stages that follow the principles of analytical chemistry: (1) macroscopic pretreatment, (2) separation of high-molecular from low-molecular compounds, (3) extraction, (4) purification/isolation, and (5) encapsulation or product formation [139,158]. This procedure is selected if two (at least) different components are recovered or one of them is a small molecule, for example, an antioxidant. However, when the target compound concerns a longer molecule (e.g., protein or pectin), the second stage may be omitted [138].

6.2.1. Extraction of phenols

The extraction of phenols from natural substrates is typically conducted using organic solvents that provide a physical carrier for their transportation between different phases (i.e., solid, liquid, and vapor) [126]. Liquid–liquid solvent extraction is an easy to operate technique and it can be applied even in small, family-owned olive oil mills. It is affected by the diffusion coefficient and the dissolution rate of compounds until they reach the equilibrium concentration inside the solvent, whereas the most important parameters include the type of solvent used, the extraction temperature, and time. Phenols are polar compounds that match perfectly with highly polar solvents. For example, ethyl acetate is a promising solvent for the liquid–liquid extraction of flavonoid aglycons [159–161]. Phenolic acids are easily solubilized in polar protic media like hydroalcoholic mixtures, while respective fractions can be obtained on the basis of polarity by varying alcohol concentration [162,163]. In general, methanol–water mixtures have been referred to extract phenols with the highest yield and widest array.

On the other hand, the recovery of certain phenolic terpenes has been referred to occur preferably with nonpolar solvents (i.e., hexane or petroleum ether). One of the main disadvantage of this process is that most of the organic solvents are undesirable for industrial exploitation as they are toxic, nonedible, and raise environmental, health, and safety concerns [164,165].

Ethanol has a lot of advantages: it is cheap, reusable as well as nontoxic and the corresponding extracts could be utilized directly in the beverage industry [140]. In addition, it inhibits enzyme activity at low pH conditions (pH 2–3) and precipitates larger compounds, such as dietary fiber [138].

The emergent need for more efficient technologies has given rise to a deeper interest in new non-thermal extraction techniques that promise to reduce solvent consumption, shorten the processing time, increase recovery yield, control the Maillard reactions, improve the product quality, and enhance functionality of extracts. These include ultrasound-assisted, microwave-assisted, pressurized liquid, and supercritical fluid extraction (SFE) as well as electrotechnologies (e.g., high voltage electrical discharges, ultrasound (US), or pulsed electric field) [157,166–

170]. In particular, ultrasound-assisted extraction (UAE) is based on the disruption of plant cell membranes as a result of the formation of cavitation bubbles generated via ultrasound high frequency (20 kHz) waves [171]. The optimum operating conditions (solid/solvent ratio = 50 mg/mL, 50% ethanol, and extraction time of 60 min) for UAE of phenols from OMWW have been reported by Sahin and Samli [172]. High voltage electric discharge (HVED) is an alternative extraction technique that requires low energy input (60–80 kJ/kg) to disrupt cell tissues and subsequently enhance phenols extraction from plant matrices due to direct energy release into the medium [173]. Similarly, pulsed electric field (PEF) accelerates mass transfer via tissue breakdown, which is induced by the application of a critical electrical potential to cell membranes [174].

6.2.2. Clarification or isolation

This stage aims either the purification or the isolation of the target compounds from the co-extracted impurities with membranes as indicated above. Adsorption, supported by activated carbons, resins, or polysaccharides is another attractive process that enables the isolation of selected low molecular weight phenols from dilute solutions with high capacity and insensitivity to toxic substances [126]. Removal of phenols from OMWW has also been conducted with a high yield (95%) using sand filtration and subsequent treatment with powdered activated carbon in a batch system [175]. Achak et al. [176] used another food processing by-product (banana peel) as a low-cost biosorbent to remove phenols from OMW. The adsorption rate of phenols was increased from 60% to 88% as a function of increasing banana peel dosage from 10 to 30 g/L, respectively [138].

6.2.3. Ultrasound-assisted extraction (UAE)

Ultrasound is a special type of sound wave beyond human hearing. Usually, its frequency in most common chemistry applications varies from 20 kHz to 100 MHz. Like other waves, it passes through a medium by creating compression and expansion. This process produces a phenomenon called cavitation, which means

production, growth and collapse of bubbles. A large amount of energy can be produced from the conversion of kinetic energy of motion into heating the contents of the bubble. According to Suslick and Doktycz [177], bubbles have temperature about 5000 K, pressure of 1000 atm, and heating and cooling rate above 1010 K/s. UAE has been developed based on this principle. Only liquid and liquid containing solid materials have cavitation effect. The main benefit of UAE can be observed in solid plant sample because ultrasound energy facilitates organic and inorganic compounds leaching from plant matrix [178]. Probable mechanism is ultrasound intensification of mass transfer and accelerated access of solvent to cell materials of plant parts. The extraction mechanism by ultrasounds involves two main types of physical phenomena, (a) the diffusion across the cell wall and (b) rinsing the contents of cell after breaking the walls [179]. Moisture content of sample, milling degree, particle size and solvent are very important factors for obtaining efficient and effective extraction. Furthermore, temperature, pressure, frequency and time of sonication are the governing factors for the action of ultrasound.

UAE have also been incorporated along with various classical techniques as they are reported to enhance the efficiency of a conventional system. In a solvent extraction unit, an ultrasound device is placed in an appropriate position to enhance the extraction efficiency. The advantages of UAE include reduction in extraction time, energy and use of solvent. Ultrasound energy for extraction also facilitates more effective mixing, faster energy transfer, reduced thermal gradients and extraction temperature, selective extraction, reduced equipment size, faster response to process extraction control, quick start-up, increased production and eliminates process steps [180].

6.2.4. Phenols recovery using membranes

Membrane techniques have been developed for the treatment of liquid wastes from food industries [181]. They are considered as clean technologies and can be used not only for wastewater purification, but also to recovering valuable compounds. Pressure-driven membrane technologies (i.e. microfiltration (MF), ultrafiltration

(UF), nanofiltration (NF) and reverse osmosis (RO)) are the most widely used for industrial applications: feed solutes larger than the membrane pore size are retained by the membrane in the *concentrate* or *retentate* stream, whereas solvent and smaller molecules will pass through the membrane to the *permeate* stream. Their advantages include small area-requirement, low energy consumption, no phase change, water-reuse, by-products recovery, stabilization of effluent, and absence of organic solvents. Moreover, membrane processes allow treatment to be adjusted with regard to the particular wastewater. However, up today, membrane technologies have not been proved to be completely efficient in spite of OMWW treatment, mainly because of their high operational cost (due to the continuous discharge or cleaning of membranes) and other phenomena, such as concentration polarization and membrane fouling. The latest phenomena reduce the permeate flux and cause repeated shutdown for membrane cleaning and washing. Concentration polarization and fouling depend on several factors, mainly membrane characteristics and feed solution properties [103,105]. A common problem is that OMWW is loaded almost directly (after the removal of solid particles) to the UF units. However, OMWW contains an increased amount of high molecular weight solutes (e.g., pectin, protein) that interact with the membrane surface together with the polar micromolecules (e.g., phenols, sugars), thus developing the earlier phenomena. Although they cannot fully solve the environmental problem of OMWW disposal, membrane technologies comprise a valuable tool in terms of food and ingredient's separations [104,182,183], and thus provide important solutions when implemented this way [138].

As a first step, OMWW is pretreated (e.g., by centrifugation or common filtration) to remove suspended solids, reducing its chemical oxygen demand (COD) and making it easier to handle. MF and UF can also be applied as pretreatment stages and their permeates can be treated by NF and/or RO to generate concentrated solutions of phenolic compounds, suitable for food, pharmaceutical, and cosmetic applications. The final NF and RO permeates can be safely discharged in aquatic systems or used for irrigation purposes [138].

Turano et al. [184] presented an approach for OMWW treatment, including a preliminary centrifugation step, in which the suspended solids are removed, and a selective UF step of the centrifuge supernatant. The centrifuge operated at 4000 rpm for 10 min, whereas UF was performed at 25 ºC and 1.5 bar using a polysulfone flat-sheet membrane, with a molecular weight cut-off (MWCO) of 17 kDa. The final UF permeate contained 0.2– 0.3 g/L phenols depending on the feed concentration, and 90% COD reduction was achieved.

Pizzichini and Russo [185] suggested a sequential fractionation of OMWW constituents by a combined centrifugation/MF/UF/NF/RO treatment. The process starts with pH adjustment to a value between 3 and 4.5 to prevent the oxidation of phenols by deactivating the polyphenol-oxidase enzyme, promoting the transformation of oleuropein into hydroxytyrosol and creating the optimal conditions for subsequent enzymatic treatment. Moreover, organic compounds are degraded through enzymatic hydrolysis so that their degradation products can be easily separated via the upcoming centrifugation. After centrifugation, OMWW is subjected to MF where ceramic membranes (0.1 to 1.4 μm pore size and 0.35 m^2 surface area per ceramic block) were used. The obtained MF permeate is treated by UF using spiral-wound polymeric membranes (1–20 kDa cut-off) made of one of the following materials: polysulfone, polyethersulfone, polyamide, or regenerated cellulose acetate. UF permeate is further treated by NF using spiral-shaped polymeric membranes made of composite polyamide or nylon (cut-offs ranging from 150 to 250 Da). Finally, the obtained NF permeate is subjected to RO, using spiral-shaped polymeric membranes made of composite polyamide. This final operation yields a liquid RO concentrate rich in phenols (30 g/L) and a purified water permeate. Through this operation setup, 12 L of concentrate is obtained from 200 L of OMWW.

Villanova et al. [186] focused on the recovery of tyrosol and hydroxytyrosol from OMWW. The process included rough filtration (RF), MF, UF, NF, and RO, using a pilot plant with 3 units. The first unit consisted of seven RF modules ranging from 4.76 to 0.033 mm. The second one consisted of two modules, which were both equipped with a 120 kDa MF membrane. The final unit consisted of four different

modules in the following sequence: two UF membranes with cut-offs of 120–20 kDa and 20–1 kDa, an NF membrane (1000–350 kDa cut-off), and an RO membrane (350 kDa cut-off). According to the described process, the final RO concentrate contained more than 1 g/L of hydroxytyrosol and more than 0.6 g/L of tyrosol, which could be later isolated with purity higher than 98% by reversed-phase chromatography on a preparatory column.

Abdel-Shafy et al. [187] used integrated MF/UF/NF membrane systems for the recovery and concentration of phenols from OMWW followed enzymatic (using β-glucosidase) and acid hydrolysis in order to release higher concentration of pure hydroxytyrosol. OMWW containing 2.5 g/L of phenols was directly subjected to the MF unit without any preliminary centrifugation. The MF membrane was made of polyvinyl-fluoride (with mean pore size of 200 nm) and resulted in 77.6% and 34.8% reduction of total suspended solids (TSS) and COD, respectively, with a phenols concentration of 2.3 g/L in the MF retentate. The UF system was equipped with a ceramic membrane (20 kDa cut-off) and 44.9% and 100% removal for COD and TSS, respectively, were obtained, whereas phenols concentration in the retentate was 2.0 g/L. In the NF unit step COD was reduced from 33 to 6.9 g/L and TSS was completely removed; phenols concentration in NF retentate reached 2.4 g/L. Finally, a concentration of 1.3 g/L of pure hydroxytyrosol was obtained by β-glucosidase acid hydrolysis of NF retentate.

6.3. Recovery and purification of phenolic compounds by adsorption

Adsorption technology is one of the most commonly applied processes for the recovery of polyphenols from plant extracts and is gaining increasing importance in food industry [188]. The use of synthetic resins as adsorbents has several advantages such as relatively low operation costs, simple handling, and long resin shelf-life resulting from its regeneration and reuse ability [189]. Compared with alternative technologies, adsorption is attractive for its relative simplicity of design, operation and scale-up, and its potential selectivity to toxic substances. Besides, adsorption avoids toxic solvents and minimizes the transformation of the target

substances [190]. Among the disadvantages of adsorption technology are the limited overall purification efficiency [191], the loss of capacity observed after the first regeneration and solvent regeneration which has to be developed and/or adapted. A method for gathering hydroxytyrosol by means of a two-step chromatographic treatment was suggested [192]. Likewise, an aqueous fraction of hydroxytyrosol from an aqueous phenolic extract was recovered [133]. Researchers used Amberlite XAD or Duolite to obtain extracts rich in antioxidants while in some patents applied Amberlite XAD was followed by Sephadex LH-20 or Amberlite XAD/Lewatit EP [193,194].

6.3.1. Operational conditions affecting adsorption and desorption

The influence of the major operational variables on the adsorption process should be known in order to achieve high recovery rates and economical production. The theoretical and practical principles of adsorption–desorption are well established [195–199]. The effects of the most influential variables have been extensively treated and reviewed for activated carbons [200] and low cost adsorbents [201]. The following sections cover only representative information on the influence of selected variables. Surface area, functionality, porosity, irregularities, strongly bound impurities, internal porous structure and particle size influence the physical adsorption. Phenol molecules may form hydrogen bonds with surface functional groups, and the aromatic ring determines the magnitude of hydrophobic interactions [202]. Resin performance is strongly affected by chemical structure and surface functionality. Polyphenol binding to ion exchange resins is affected by interactions between functional groups, by hydrophobic interactions and hydrogen bonding [203]. The interactions of solutes with a strong-base anion exchanger followed different mechanisms at different pH: both adsorption and ion exchange were important under alkaline conditions, whereas phenol desorption was predominant at acidic pH [204,205]. Ionic strength and pH are key factors influencing the adsorption of phenolic compounds. At acidic pH, the uptake of phenolics by

different adsorbents is enhanced because phenols are non-dissociated and the dispersion interactions predominate [190,200,206–209].

6.4. Market of recovered phenols from olive processing by-products

Nowadays, several patents have been obtained for the extraction of target phenolic compounds (e.g., oleuropein and hydroxytyrosol) from olive oil processing by-products [8]. In most cases, a slow acidic hydrolysis (for 2–12 months) of the initial substrate is proposed with a final aim of converting more than 90% of the present oleuropein to hydroxytyrosol [138]. Phenolea Complex is a natural hydrophilic extract (without using any kind of organic solvent) obtained directly from OMWW. After the milling process, OMWW is collected, pretreated and sequentially subjected to tangential MF (using ceramic membranes) and vacuum evaporation of the permeate [210]. The commercial recovery of hydroxytyrosol from wet olive pomace has been proposed using chromatographic columns filled with two resins: a nonactivated ionic and an XAD-type nonionic. Hydroxytyrosol has shown improved advanced antiradical properties compared to vitamins E and C, and thus could be used as functional supplement and food preservative in bakery products. More specifically, OMWW is pretreated with acid and then an incubation process follows aiming at the conversion of oleuropein to hydroxytyrosol [138].

In general, olive pulp extracts have been approved by FDA which GRAS (generally recognized as safe) status (GRN No. 459) to be used as antioxidants in numerous foods (e.g., baked goods, beverages, and dressings) up to a level of 3 g/kg in the final food [8].

7. Encapsulation

Microencapsulation, developed approximately 60 years ago, is defined as a technology of packaging solids, liquids, or gaseous materials in miniature, sealed capsules that can release their contents at controlled rates under specific conditions [211,212]. The packaged materials can be pure materials or a mixture, which are also called coated material, core material, actives, fill, internal phase or payload.

Furthermore, the packaging materials are called coating material, wall material, capsule, membrane, carrier or shell, which can be made of sugars, gums, proteins, natural and modified polysaccharides, lipids and synthetic polymers [213,214]. Various techniques are used for encapsulation. In general, three steps are involved for the encapsulation of bioactive agents: (i) the formation of the wall around the material to be encapsulated; (ii) ensuring that undesired leakage does not occur; (iii) ensuring that undesired materials are kept out [214,215]. The current encapsulation techniques include spray drying, spray cooling/chilling, extrusion, fluidized bed coating, coacervation, liposome and niosome entrapment, emulsification, inclusion complexation, centrifugal suspension separation, lyophilization, etc. [211,214,216]. The main objective of encapsulation is to protect the core material from adverse environmental conditions, such as undesirable effects of light, moisture, and oxygen, thereby contributing to an increase in the shelf life of the product, and promoting a controlled liberation of the encapsulated compound [217]. Microencapsulation can be applied in food industry for several reasons, which have been summarized by Desai and Park [211] as follows: (i) protection of the core material from degradation by reducing its reactivity to its outside environment; (ii) reduction of the evaporation or transfer rate of the core material to the outside environment; (iii) modification of the physical characteristics of the original material to allow easier handling; (iv) tailoring the release of the core material slowly over time, or at a particular time; (v) to mask an unwanted flavor or taste of the core material; (vi) dilution of the core material when only small amounts are required, while achieving uniform dispersion in the host material; (vii) to help separate the components of the mixture that would otherwise react with one another. Food ingredients of acidulants, flavoring agents, sweeteners, colorants, lipids, vitamins and minerals, enzymes and microorganisms, can be encapsulated using different technologies [211,218].

7.1. Encapsulation of polyphenols

Low bioavailability and instability of polyphenols in digestion and absorption process greatly limits their health benefits. In fact, only a small proportion of them taken orally are absorbed, because of insufficient gastric residence time, low permeability, and water solubility [219]. In addition, they are sensitive to physical and chemical conditions, such as light, heat and oxidation [220]. The delivery of these compounds therefore requires protection mechanisms that can maintain their chemical integrity and deliver them to the physiological target [221]. A wide range of technologies have been developed to encapsulate polyphenols, including spray drying, coacervation, emulsions, liposomes, niosomes, micelles, nanoparticles, freeze-drying, cocrystallization and yeast encapsulation [218,220]. Each of them has its own specific strengths and weaknesses in encapsulation, protection, delivery, cost, regulatory status, ease of use, biodegradability and biocompatibility. Among these, emulsions are widely considered as one of the most popular encapsulation and delivery systems for a wide range of lipophilic, hydrophilic and amphiphilic bioactive molecules [222] due to their high-efficiency encapsulation, chemical stability maintenance of encapsulated molecules [223] and controlled release [224]. Furthermore, some emulsion-encapsulated polyphenols presented even higher biological activities compared with pure free molecules [225,226].

7.2. Nanoemulsions

Nanoemulsions usually have better stability to particle aggregation and gravitational separation [227]. Nanoemulsions contain particles that only scatter light waves weakly, and so they are suitable for incorporation into products that need to be optically clear or only slightly turbid, such as fortified soft drinks and waters, and some kinds of soups, sauces, and dips [228–230].

A conventional emulsion, also sometimes known as an emulsion or macroemulsion, typically has particles with mean radii between 100 nm and 100 µm. This type of colloidal dispersion is thermodynamically unstable, i.e., the free energy of the separated oil and water phases is lower than that of the emulsion itself.

Consequently, conventional emulsions always have a tendency to breakdown over time. Conventional emulsions tend to be optically turbid or opaque because their droplet size are similar to the wavelength of incident light ($r \approx \lambda$) and so they scatter light strongly (provided the refractive index contrast between the oil and water phases is not very close to zero). A nanoemulsion can be considered to be a conventional emulsion that contains very small particles, i.e., mean radii between about 10 to 100 nm [227,229]. The relatively small particle size compared to the wavelength of incident light ($r \ll \lambda$) means that nanoemulsions tend to be transparent or only slightly turbid (similar to microemulsions). The very small particle size also means that they have much better stability to gravitational separation and aggregation than conventional emulsions [227,230]. However, these systems are still thermodynamically unstable, and so, will tend to breakdown over time [231].

A system consisting of oil droplets dispersed within a water phase is referred to as an oil-in-water (O/W) nanoemulsion, whereas a system that consists of water droplets dispersed in an oil phase is referred to as a water-in-oil (W/O) nanoemulsion. The liquid that makes up the droplets is usually referred to as the dispersed phase, whereas the liquid that makes up the surrounding liquid is referred to as the continuous phase.

Nanoemulsions often have better stability to gravitational separation, flocculation, and coalescence than conventional emulsions, but are less stable to Ostwald ripening. A major concern of food scientists working with this kind of systems is therefore to create nanoemulsions with sufficiently small particle size and then to ensure that they have a sufficiently long kinetic stability for commercial applications. The kinetic stability of nanoemulsions can be improved by controlling their microstructure (e.g., size distribution), or by incorporating substances known as stabilizers, such as emulsifiers, texture modifiers, weighting agents, or ripening retarders [231]**.**

8. Biorefinery concept for the valorization of olive mill wastes

The use of different residues derived from olive tree cultivation and olive oil extraction, including waste streams, such as wastewater, olive leaves, or olive stones, in a single facility gives rise to the concept of multifeedstock and multiproduct biorefinery. This modular and integrated biorefinery could transform heterogeneous feedstocks (high- and low-moisture residues) into a number of valuable products (food additives, bioproducts, biomaterials, biofuels, and bioenergy). At the same time, the biorefinery can play an interesting environmental role, since the processes developed at this facility represent an alternative to the classical disposal methods of the residues of the olive oil industry. A wide range of final products have been identified as possible outputs for biorefinery, depending on the feedstock considered.

Residues generated in olive oil industry can be categorized as either low/medium- or high-moisture materials. The conversion of low/medium biomasses (in general, lignocellulose) can be directed to heat and power production by thermochemical processes, or be upgraded into added-value products by biochemical/chemical routes (Fig. I3). Biochemical processes generally involve fractionation and hydrolysis of polymers (carbohydrates and lignin) into oligosaccharides or monomers that can further be converted into a wide variety of products. The conversion of wet biomass (wastewater streams) generally involves biofertilization and/or anaerobic digestion [7].
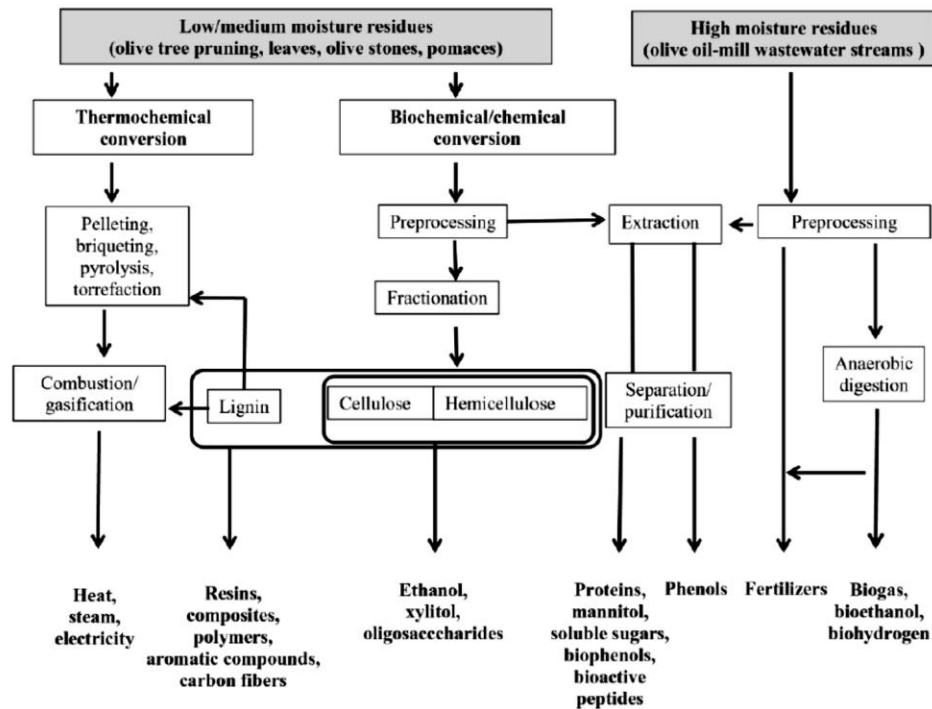
Figure I3. Valorization scheme for different residues generated in the olive oil industry [7].

8.1. Processes for low-medium moisture residues

8.1.1. Direct use as biofuels

The conversion of wastes generated from the olive mill industry into thermal and electrical power is an interesting option in the case of low-moisture residues, as the olive stones or the extracted dry pomace residue produced in the olive pomace oil industry [232]. Currently, the use of olive stones as a biofuel for thermal applications is quite widespread in olive oil productive regions, especially in agroindustries, livestock farms, greenhouses, and domestic heating systems. Extracted dry pomace residue is also used as a fuel in some local industries, like the ceramic industry. However, this residue is less used than olive stones in domestic heating systems because of the higher generation of pollutant emissions. In the pomace olive oil extraction industry, extracted dry pomace residue is usually used

to dry the wet olive pomace whereas olive stones are often employed in the industrial boilers to obtain process steam because it causes less corrosion problems. In recent years, the use of olive stones as a fuel in olive mills boilers instead of extracted dry pomace residue has been progressively increased [7].

8.1.2. Biochemical/chemical conversion routes

Apart from the valorization of low/medium biomasses by thermochemical processes, a variety of valuable products can be obtained by biochemical and chemical conversion technologies based on lignocellulosic nature of these residues. Lignocellulosic-type residues from olive crop and oil mill contain nonstructural minority compounds that could be extracted before the process conversion of structural components (hemicellulose, cellulose and lignin), contributing to the economic viability of a possible biorefinery based on these materials. This is the case of phenolic compounds with antioxidant properties that have a great interest for the food industry as substitutes of synthetic antioxidants. Therefore, a preprocessing strategy, such as an extraction step, could be applied to the residues in order to recover these bioactive components, while improving the yields of the biochemical conversion route of structural components [233,234].

8.1.2.1. Carbohydrate conversion

A key issue for the biorefineries is the cost-effective conversion of cellulose and hemicellulose contained in lignocellulose biomass into fermentable sugars, as a first step in the production of high-added value molecules. Particularly for biofuel (ethanol) production from lignocellulose biomass, process technology generally includes a pretreatment step followed by enzymatic hydrolysis and the subsequent fermentation to ethanol of the sugars released, by fermenting microorganisms. Pretreatment is a critical step for efficient carbohydrates (cellulose and hemicellulose) conversion processes, and it is required to alter the structure of lignocellulose biomass making polysaccharides more accessible to the enzymes that hydrolyze them into fermentable sugars. Moreover, an ideal pretreatment should

result in a complete fractionation of the biomass into its key constituents, cellulose, hemicelluloses, and lignin, which will facilitate a subsequent conversion of main components into fuels and high value products at high yield. Many pretreatment methods have been evaluated for ethanol production [235].

8.1.2.2. Lignin conversion

In most current applications of biochemical and/or chemical based technologies to lignocellulosic materials, lignin component is contained in the final residue after biomass fractionation and carbohydrate hydrolysis, as a complex and disperse compound [236]. A major part of this lignin-rich residue is incinerated to produce process steam and energy, and only a minor part is derived to the production of other valuable products. However, lignin offers a significant opportunity for enhancing the operation of a lignocellulosic biorefinery by the production of more valuable chemicals, such as resins, composites and polymers, aromatic compounds, or carbon fibers. This is viewed as a medium to a long term opportunity depending on the quality and functionality of the lignin that can be obtained [237].

8.2. Processes for high moisture residues

8.2.1. Agricultural uses of extracted OMWW

Reducing the phytotoxicity of OMWW by extracting polyphenols would allow its use as a valuable fertilizer. Its agronomic application to soils is very convenient in Mediterranean countries since water is scarce and soils tend to have a low organic matter content. The land utilization of polyphenols-extracted OMMW represents a cheap source of irrigation water and provides soils with nutrients, mainly potassium and organic matter. High salinity and low pH are the main drawbacks for the application of OMWW to soil, but they can be reduced or eliminated by composting or cocomposting with agricultural residues (e.g., olive pomace or olive tree pruning residues). The agronomic use of composting OMWW does not cause negative

effects on crop productivity and significantly increases organic matter and organic nitrogen in soils [238].

8.2.2. Production of bioenergy and biofuels

OMWW, especially after increasing its biodegradability by the extraction of phenolic compounds, could be a good substrate for fermentative transformations. Although some studies have been performed for bioethanol [239] and biohydrogen [240,241] production, studies have been focused on biomethane production by anaerobic digestion (AD). Among bioprocesses to transform OMWW into biofuels, AD is the best choice since all the macromolecules (lipids, proteins, and carbohydrates) are transformed into biogas. Biogas is a versatile energy carrier that can be used for electricity production, heating purposes, vehicle and jet fuel and replacement of natural gas. In addition, biomethane may be considered as starting compounds for biotechnological production of chemicals.

AD of complex organic substrates, such as OMWW, proceeds through a series of parallel and sequential steps with several groups of microorganism involved. Anaerobic digestion starts with the hydrolysis of high molecular materials and granular organic substrates (lipids, proteins, and carbohydrates) by fermentative bacteria into small molecular materials and soluble organic substrates (fatty acids, amino acids, and glucose), aided by extracellular enzymes (hydrolases), which are excreted by fermentative bacteria. Next, products formed during the hydrolysis are further degraded into volatile fatty acids (e.g., acetate, propionate and butyrate) along with the generation of by-products (e.g., NH_3, CO_2, and H_2S) in a process known as acidogenesis. Finally, the organic substrates produced in the second step are further digested into acetate, H_2, and CO_2 and used by methanogenic archaea for methane production. The anaerobic degradation of OMWW faces some difficulties due to the high content of hardly degradable cellulosic materials and toxic substances, such as phenols, long-chain fatty acids, ethanol, tannins, etc. [242].

In addition to the earlier described technologies to convert olive biomass (including solid or liquid wastes) into bioproducts and biofuels, other interesting applications are nowadays being considered at different development stages, such as animal feed, production of activated carbons to be used as biosorbents, or as ingredients in construction materials [7,243].

9. Clinical applications and therapeutic effects of hydroxytyrosol

The origin of hydroxytyrosol (HT) is the hydrolysis of oleuropein which takes place during the ripening of the olives, and also during the storage and elaboration of table olives [244].

Nowadays, many researchers are looking for new forms of HT to improve the ADME (Absorption, Distribution, Metabolism and Excretion) processes, its stability and its health-biological features. These studies are focused on changing the solubility of HT in order to increase its bioavailability and its plasma half-life [245]. Almost all experiments have been carried out *in vitro* models. Some of these new isolated compounds, their biological activities and their synthesis methods have been reviewed by Bernini et al. [246]:

- *HT esters:* the most important compound of this group is HT acetate which is found in extra virgin olive oil (EVOO). HT acetate has demonstrated a higher antioxidant capacity than HT. In addition, nitro-ester derivatives have shown beneficial effects in Parkinson's disease associated to their antioxidant properties [247].

- *HT alkyl ethers:* a new class of lipophilic HT derived with high cytotoxic activities in A549 lung cancer cells and MRC5 non-malignant lung fibroblasts [248].

- *HT analogues:* with similar structure to HT but different substituents on the aromatic ring and/or a different length of the alcoholic chain.

- *HT thioderivatives:* containing thioacetate, thiol, and disulphide groups.

- *HT derived isochromans:* also presents in EVOO methanolic extracts [249].

9.1. Absorption, distribution, metabolism and excretion (ADME) processes

It is well established that phenolic compounds are absorbed in a dose-dependent manner in the bowel and they suffer an important intestinal/hepatic metabolism [250–252]. Many studies have been performed in animals and humans in order to determine how the ADME processes of HT occur. These studies have shown similar ADME characteristics for HT with other (poly)phenols. In this sense, HT presents different absorption and excretion ways depending on the vehicle employed [253,254].

All (poly)phenols suffer the phase I of metabolism (hydrolysis) in enterocytes and subsequently go through phase II of metabolism, where they are transformed into glucuronide, methylated and sulphate by-products [255]. For all processes described above, 98% of HT can be detected in plasma and urine in glucuronide form, and only 2% as its free form when it is administered in olive oil form [256].

More recent studies were conducted to determine different specific (poly)phenolic metabolites that could explain more concisely the biological activities associated to some components of the Mediterranean diet (MD) as well as to establish some better compliance markers. In this sense, Rubió et al. [257] observed in hypercholesteremic volunteers that after the administration of two different phenolic-enriched EVOO, the most quantifiable and appropriate biomarker for compliance were the sulphate forms of HT, measured both in urine and plasma samples. Finally, a more complex study carried out by Khymenets et al. [258] allowed to corroborate that after the administration of a HT nutraceutical preparation to healthy volunteers, sulphate metabolites of HT and concretely the HT-sulphate-3′ could be selected as the best biomarker of compliance of olive oil ingestion [249].

9.1.1. Absorption process of HT

The absorption of HT mainly occurs by passive transport in the small bowel and the colon with an efficiency that oscillates from 75% up to 100%. The absorption process depends on the vehicle employed, being more effective in the form of olive

oil [254]. These results agrees with Vissers et al. studies [259], which reported an absorption of 70% of HT in patients in whom an ileostomy had been carried out. It is well known that phenolic bioavailability is influenced by different factors such as age, hormonal status [260], or gender, as described by de Bock et al. [261] when leaf extracts were administered to determine the perimenopause activity of HT. In addition, Dominguez-Perles et al. [262] described that gender is a critical feature for the final bioavailability of HT derivatives, persisting this compound for a longer time in the body of female rats after different forms of HT oral administration. Furthermore, other studies have demonstrated that HT achieves its highest urinary levels when it is administered as red wine, probably due to the interaction between ethanol and dopaminergic ways. HT is a dopamine metabolite and the increased levels of HT in urine, when it is administered as red wine, can be consequence of a higher boost to dopamine metabolism [263]. Perez-Mana et al. [264] demonstrated that if HT is administered with ethanol, dopamine metabolism varies to produce HT instead of DOPAC (3,4-dihydroxyphenylacetic acid). The increasing levels of HT in the body can be easily comprehended [249].

9.1.2. Distribution of HT

This compound has a swift absorption process and reaches its maximum plasma concentration around 7 min after the intake [265]. Despite this, other authors have suggested that the highest plasma concentrations of HT are reached between 0.5 and 1 h after its oral administration, being practically undetectable after 4 h. In this sense, when HT is administered in an acetate form, the highest plasma concentration of HT is reached between 0.5 and 2 h after oral administration [262]. Once absorbed, HT will quickly start being part of plasmatic high density lipoproteins, acting as an antioxidant and as a cardiovascular protector [266].

Because of the powerful and quick metabolism of HT, this compound presents a plasma half-life of 1–2 min [267,268]. HT and its metabolites have very good distribution abilities in tissues such as muscle, testis, liver, and brain (HT is able to cross the blood brain barrier) besides it is accumulated in kidney and liver [268].

This widespread distribution is responsible for the health-beneficial properties of HT [249,269].

9.1.3. Metabolism of HT

HT has an intense and rapid metabolism. Firstly, this compound suffers the first step of its metabolism inside enterocytes and subsequently in the liver. In this sense, gut microbiota has been identified as a notably modulator of absorption both of HT and its metabolites. This microflora acts transforming part of HT, HT acetate (HTA), and tyrosol (Tyr) in different catabolites HT-derived, and releasing free-HT from its more complex forms. According to Mosele et al. [270], the metabolism of HT by microbiota occurs firstly by oxidation and this is followed by a transformation into hydroxylated phenylacetic acids [249].

9.1.4. Excretion of HT

Taking into account the structure of HT (Figure I4) and its intense metabolism and transformations, conjugated catabolites are mainly excreted by the kidneys [271]. The time required for the complete elimination from the body, both for HT and its metabolites is approximately 6 h in humans [272,273] and around 4 h in rats [262]. HT is accumulated in kidneys until its excretion [268], where it may perform a nephroprotective role thanks to its antioxidant properties [274]. D'Angelo et al. [268] established that around 5% of total HT is excreted by faeces after 5 h of an injection of HT (1.5 mg/kg–87.5 μCi/kg) [249,268].
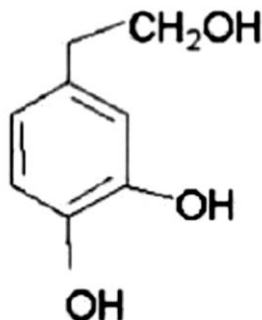
Figure I4. Chemical structure of Hydroxytyrosol [271]

9.2. Toxicity and dose establishment

Nowadays, there is a great interest in using HT as a possible nutraceutical compound against different diseases such as cancer, diabetes or chronical pathologies, although further studies must be performed to understand the possible toxicological effects of HT in human. As consequence of its poor bioavailability, there are no studies that determine if the final blood concentration could have toxic effects after the intake of a high concentration of HT, both as pure extract and as part of enriched foods such as EVOO. In this sense, some authors could determine null toxicity in the first gastrointestinal tract with other (poly)phenols [275]. To date, no toxicity studies have been carried out to stablish if HT could exert any toxicological effect at this level, but taking into account the descriptions of the toxicity studies carried out and also described above, and the high similarity between the behavior of HT and other phenolic compounds, there is a fairly accurate approximation of its null toxicity at all levels, although more studies have to be done to conclude this claim in humans [249].

Regarding to its acute and subchronic toxicity, HT has been studied commonly as olive oil, olive mill wastewater forms, and as extracts in animals and cells. In this sense, there are numerous studies performed in rats with different administration ways to determine the acute toxicity. Some authors have described that the injection (intravenous, subcutaneous or intramuscular) of 2 g/kg in rats provoked piloerection and local redness in the inoculation area disappearing this effects before 48 h [268].

A very interesting study with HIDROX™ (Hydrolysed Aqueous Olive Pulp Extract; OPE), an aqueous olive-pulp extract with a high HT content (around 70%) administered by oral gavage in Sprague Dawley rats and CD1 ICR (BR) mice was performed by Christian et al. [276]. The dose of HT employed was risen to 2 g/kg (equivalent to around 1400 mg/kg of pure HT) to determine the acute toxicity. These authors observed a null mortality and morbidity. The only alterations proven were the presence of diarrheal faeces in rats. This study proposed a LD50 of 2000 mg/kg for HYDROX™, representing a median lethal dose (LD50) of around 1400 mg/kg for pure HT. Besides these results, Christian et al. [276] employed a higher dose in rats (raising 5 g/kg) and they established a LD50 of around 3.5 g/kg of HT. Furthermore, to evaluate subchronic toxicity, other parameters such as body weight, food intake, haematology and histopathology from selected tissues, physical and ophthalmic characteristics were also analyzed. No remarkable changes associated to HT or to the rest of HIDROX's compounds were observed at the end or during the study. Aunon-Calles et al. [277] performed a study to evaluate the toxicological effects of pure hydroxytyrosol. This study reported that the oral administration of HT once a day for 13 weeks at daily doses of 5, 50, and 500 mg/kg did not lead to any micro- and macroscopic alterations or to death. They observed a gain in body weight without interest for toxicological analysis, and higher kidney weights in the group with the highest treatment dose, with no changes in the organ functionality or structure. Therefore, they came to the conclusion that no adverse effects were observed at daily doses of 500 mg/kg. Additionally, Aunon-Calles et al. [278] did not observe any sign of genotoxic and mutagenic effects when HT was employed in *in vitro* models at physiological concentration.

One of the last studies performed in animal models to determine the subchronic toxicity of HT was carried out by Heilman et al. [279]. These authors used an extract called H35 (35% w/w HT) who was administered to female and male Wistar rats by oral gavage for 90 consecutive days, at daily doses of 125, 250 and 500 mg HT/kg. There were no toxicologically significant effects found. These researchers did not observe any adverse effects for 250 mg HT/kg day. Similar conditions were studied by Kirkland et al. [280] using a H40 extract (with a 40% of HT total

content), and no mortality rate was observed, although a decrease in body weight and an increase in relative weight of liver, thymus, kidneys and spleen were found in male rats.

On the other hand, HT presents a pro-apoptotic and antiproliferative effects in different types of tumor cells when treated with pure HT or extract forms. As a result of the pharmacomodulation that have been performed with HT, its selectivity and efficiency on tumor cells has been enhanced by preventing or diminishing their proapoptotic activity on human non-tumor cells [281]. In addition, Anter et al. [282] demonstrated how a simple extraction from olive mill wastewater or using it directly, can exhibit a notable antiproliferative and caspase 3-dependent proapoptotic effects against the human tumor cell line HL60, avoiding its cell toxicity in non-tumor cells.

Taking into account all data described above and he health-beneficial effects in human cardiovascular diseases, the European Food Safety Authority (EFSA) since 2011 approved health claim on olive oil polyphenols and recommended the daily consumption of 5 mg HT and its derivatives (e.g. oleuropein complex and tyrosol) provided by moderate amounts of EVOO (20 g) to maintain a balanced diet. This dose is enough to generate health-beneficial properties by decreasing low density lipoproteins oxidation, increasing high density lipoproteins, maintaining a normal blood pressure, and avoiding the pro-inflammatory processes [249].

9.3. Main activities associated to HT

9.3.1. Antimicrobial effects of HT

Extracts from olive oil and olive tree leaves have been used as soap, solutions or balms for a long time to palliate several pathologies associated with infections caused by microorganisms [283]. It is well known that both olive leaf extract and pure HT have a powerful antimicrobial activity against some microorganisms such *Escherichia coli*, *Candida albicans*, *Clostridium perfringens*, *Streptococcus mutans*, or *Salmonella enterica* [283,284]. Moreover, some studies have demonstrated in *in vitro* models a helpful effect of HT against *Vibrio cholerae*,

Salmonella typhi, *Haemophilus influenza* or *Staphylococcus aureus* even at lower dosages than some antibiotics such as ampicillin [285]. It has also been described an antiparasitic activity by HT against several parasites such as *Lesishmania spp.* [286].

9.3.2. HT as ROS scavenger

One of the most important activities associated to HT is its role as ROS (reactive oxygen species) scavenger. HT and its metabolites have a high capacity to eradicate both intracellular and extracellular production of ROS. At an extracellular level, HT and derivatives have shown a ROS scavenger activity in skin cells by blockading the harmful effects of ROS. This capacity is important when ROS are generated by UV radiation [287] or by peroxynitrite radical [288], both able to break DNA strands and to promote genotoxicity. Intracellularly, HT has also an extraordinary ROS scavenger capacity, particularly with free radical molecules such as superoxide, hydrogen peroxide, and hypochlorous acid, as well as acting as a metal chelator [289–291].

HT can also increase oxidative stress levels in colon cancer cells (DLD1 cells) but not in normal colon epithelial cells (CRL1807 cells). This may help to understand the two main activities of HT: antioxidant and selective antitumor capacity. So there is a certain dichotomy in how HT affects cells, based on how HT runs its action [292–294]. Sun et al. [295] reported that HT treatment increased Akt activity, which later sensitizes cells to get a selective killed by oxidative stress induced apoptosis. Another similar study performed in cells by Luo et al. [296] demonstrated an identical HT action mechanism during ROS formation, only against the human prostate cancer cells (PC-3, DU145) and not in an immortalized non-malignant prostate epithelial cell line (RWPE-1). Moreover, an extra input of extracellular superoxide and hydrogen peroxide facilitated the anti-proliferation effect of HT in prostate cancer cells.

Similar results were obtained by Rosignoli et al. [297], who observed in different types of cancer cell lines (MDA and MCF-7 (breast). LNCap and PC3 (prostate),

SW480 and HCT116 (colon)) how HT performed its antitumor activity by accumulating hydrogen peroxide in the culture medium, acting in this case as a prooxidant-antitumor compound in cancer cells. To date, there is a certain trend, increasingly questioned, that the antioxidants supplements directly generate an antioxidant effect and are therefore beneficial for the body. This fact has been discussed for years and there seems to exist some contradiction in this respect [298].

9.3.3. Role of HT in cardiovascular system

Thanks to its already described antioxidant effect, HT avoids the oxidation of low density lipoprotein (LDL), decreasing the formation of atherosclerotic plaques [290,299,300]. Some HT enriched extracts have also reduced plasmatic levels of total cholesterol and lipids [301,302] and blood pressure [303], decreasing cardiovascular risk factors. This is the main reason why the EFSA recommends a daily ingestion of at least 5 mg of HT (usually in olive oil form).

Several cardiovascular disorders are consequence of drugs administration such as doxorubicin in cancer treatment. In this sense, Granados-Principal et al. [291] showed in 2014 for the first time in a rat *in vivo* model that HT could improve the cardiac disorders generated by a ROS imbalance production and mitochondria damage caused by doxorubicin-based therapy. Therefore, HT could be used as an adjuvant molecule in antitumor therapies that cause an increase in cardiovascular risk by themselves, also facilitating an improvement in the antitumor response and a lower number of adverse effects associated with the treatment. Some nutrigenomics studies have been performed to determine what genes involved in cardiovascular disorders such as atherosclerosis or insulin sensitivity mechanisms could be altered after the administration of OO and HT [249].

9.3.4. How acts HT in metabolic syndrome?

Metabolic syndrome is described as a combination of different abnormalities that includes obesity, dyslipidemia, impaired glucose tolerance, insulin resistance, diabetes, and hypertension [304–306]. It has been demonstrated in middle-aged

overweight men that a phenolic-enriched supplementation with oleuropein and HT for 12 weeks improves the glucose regulation and β-cell secretion capacity of insulin [307]. Furthermore, it is well established that diabetes promotes an increase in lipid peroxidation [308], the alteration of the glutathione redox state, a decrease in plasmatic levels of antioxidants, and a reduction in the antioxidant enzyme activities. All these changes could produce oxidative stress caused by hyperglycemia [309]. Some studies have demonstrated that a HT supplementation given to diabetic rats provoked a significant decrease in intestinal maltase, lactase, sucrose, and lipase activities and an enhancement of the superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities, as well as an increment in reduced glutathione levels, then reducing the oxidative stress status [310].

Further studies showed that HT could normalize obesity, diabetes, dyslipidemia, inflammation, fatty liver, and insulin resistance induced by high fat diet in C57BL/6 mice [311] through down regulating SREBP-1c/FAS pathway, decreasing oxidative stress, weakening mitochondrial anomalies, and suppressing apoptosis. In addition, HT could decrease glucose levels and lipid metabolism present in db/db mice, as a confirmation the relevant effects of HT on metabolic syndrome [311]. In this sense, HT also prevents of high glucose induced generation of ROS and glucose-toxicity in SH-HY-5Y neuroblastoma cells [312]. Altogether, these findings suggest that HT might be an effective agent for the prevention and treatment of diabetes and metabolic syndrome, but more clinical trial should be performed to determine by which mechanisms the HT can act against metabolic syndrome [249].

9.3.5. Anti-inflammatory effects of HT

HT is well established as a powerful phenolic antioxidant with potent anti-platelet, anti-inflammatory, and anti-atherogenic properties [313]. A nutrigenomic study concluded that a high intake of phenol-enriched olive oils can repress the expression of several pro-inflammatory and pro-atherosclerotic genes, promoting a lower inflammatory profile in peripheral blood mononuclear cells [314].

Richard et al. [315] described that HT is the most powerful anti-inflammatory (poly)phenol in aqueous olive extracts. They observed a remarkable inhibition in the production of NO and prostaglandin E2 (PGE2), as well as a decrease in the secretion of cytokines such as IL-1α, IL-1β, IL-6, IL-12, TNF-α, and chemokines (CXCL10/IP-10, CCL2/MPC-1). The iNOS, IL-1α, CXCL10/IP-10, MIP-1β, MMP-9, and PGE2 synthase (PGES) gene expressions were also reduced by HT action. All these findings indicate the great anti-inflammatory capacity of HT and how a daily intake of HT could modulate the inflammatory process and provides a health profit [249].

9.3.6. HT and respiratory diseases

Respiratory diseases are a complex group of different disorders that occur along the whole respiratory system, which comprises from the upper tract down to the pleural cavity, including both muscles and the nervous systems which modulates them. Due to this complexity, respiratory disorders can be derived from multiple causal agents such as pathogens, inflammation, air pollutants or our own individual genetics. Visioli et al. [316] demonstrated that HT and oleuropein can decrease the oxidative stress associated to neutrophils respiratory burst [249].

9.3.7. Neuroprotection associated to HT

It is well known how high levels of oxidative and nitrosative stress can hurt the integrity and functioning of brain tissue, especially in aging. It is remarkable that HT presents a powerful capacity to scavenge radical species [317] and to induce antioxidant enzymes [318] that could improve some neurodegenerative disorders such as Parkinson's disease [319].

In this sense, Schaffer et al. [320] found that HT-rich extracts were able to attenuate the cytotoxic effects of Fe^{2+} and NO in murine dissociated brain cells. Additionally, they also found that the oral administration of 100 mg of HT for 12 days in mice, improved the resistance of dissociated brain cells to oxidative stress, proved by reducing basal and stress-induced lipid peroxidation. Besides, these authors

observed that basal mitochondrial membrane potential was also moderately hyperpolarized, showing for the first time in an ex vivo model that HT could exert a cytoprotection at brain cells level. Other studies in a hypoxia–reoxygenation model in brain slices from rats, showed that HT and HT-acetate had a neuroprotective effect [321,322]. Lactate dehydrogenase efflux in rat brain slices subjected to hypoxia and reoxygenation was significantly reduced under all experimental conditions and by a cytoprotective effect of HT, mediated by a reduction of oxidative and nitrosative stress, also in a hypoxia-reoxygention model [249].

9.3.8.   HT and skin protection

Currently, dermatological conditions are taking great relevance due to the constant exposure to sunlight without protection factors, pollution or factors intrinsic to life itself as aging factors. For a couple of decades, several studies have determined how the Mediterranean Diet (MD), and particularly the olive oil (OO), can reduce the incidence of skin tumors among other health-beneficial aspects [323]. Some authors have employed different olive (poly)phenol-based nutraceuticals to determine that part of the skin protector effects associated to the MD can be attributable to (poly)phenols from MD and particularly from OO. In this sense, Herrera Acosta et al. [324] showed the antipsoriasic-beneficial properties of a (poly)phenol-based cosmeceutical (Alyvium$^®$) in humans. They speculated about the possibility that the positive skin effects of Alyvium$^®$ could be determined as a consequence of its role in the normalization of the redox code, improving the cutaneous manifestation. It is well known that ultraviolet (UV) radiation promotes skin damage by generating ROS, a depletion of endogenous antioxidant system [325] and double-stranded DNA breakage. The increment in oxidative stress produces damage to skin cells, provoking dermatological disorders [326].

9.3.9. HT as antitumor compound

Firstly, it should be noted that ROS are directly linked to tumor formation, mainly due to their key role in the initiation, promotion and progression stages of cancer development. For that reason, the antitumor properties of HT will be mostly associated to its activity as ROS scavenger or its antioxidant system modulation capacity [327,328]. However, in the last decade, a wide number of studies have been performed in order to demonstrate exclusively the antitumor effect of HT.

In human hepatoma cell line (HepG2) acrylamide-induced genotoxicity model, HT can have a chemo modulator effect against genotoxicity by decreasing the ROS production provoked by acrylamide [329]. Thanks to preliminary studies, a dose around 10–17 mg/g of HT has been established to start showing antitumor effects [330]. In addition, numerous studies have tried to explain how HT can act as an antitumor compound and tried to elucidate its antiproliferative action on certain types of tumors, independently of its modulating ability over the antioxidant system [331]. In thyroid cancer cell lines (papillary (TPC-1, FB-2) and follicular (WRO)), HT has also demonstrated its abilities to decrease cell viability and also to activate apoptosis through mitochondria path [332].

On the other hand, there are many studies carried out in several types of healthy and normal cells which have demonstrated non-toxicity and chemo protective effects of HT in non-tumor cell cultures [297,299,333].

To sum up, two different mechanisms of action have been proposed by Granados-Principal et al. (2010) [250] for HT's antitumor effect:

1. Blockade of Cyclin-dependent kinases (CDKs).
2. Blocking messengers involved in cell proliferation, such as ROS [249].

10. Application of recovered bioactive compounds in food products

As it was mentioned, only a small amount of phenolic compounds are transferred into the oily phase, whereas the largest fraction is present in the relevant by-products of the mechanical olive oil extraction process [334]. The well-known antioxidant properties of the main phenolic compounds of OMWW were utilized in different

food applications with significant results: fortification of oils, preservation of fats, and production of functional beverages. On the other hand, the antimicrobial action of the phenols, which nevertheless exists and is widely referred in the literature through *in vitro* studies, requires a greater enhancement in the knowledge also by tests that must be conducted directly on the food. The phenols obtained from the treatment and purification of the OMWW can be directly added to the food products during their processing, or they can be adsorbed on the packaging material (active packaging) in a green and sustainable approach to ensure the food safety and the food quality [335].

In recent years, the number of studies dealing with the reutilization of OMWW recovered compounds in foodstuff has been increased [89,126]. These attempts try to utilize the bioactivities of phenolic compounds and the technological properties of other ones (e.g., gelling properties of pectin). For example, hydroxytyrosol, the most powerful antioxidant compound of OMWW [336–338], has shown advanced antiradical properties compared to vitamin E and C, and thus has been used to prevent oxidation of lipids in fish products [130]. Thereby, it could be used as functional supplement, food preservative in bakery products or life prolonging agents [335]. As mentioned in Section 1.6.4, olive pulp extracts have been approved by FDA in USA with GRAS status (GRN No. 459) for being used as antioxidants in baked goods, beverages, cereals, sauces and dressings, seasonings, snacks, and functional foods at a level up to 3000 mg/kg in the final food [8,339]. The commercial implementation of OMWW to recover polyphenols is nowadays a reality [89,126].

10.1.   Fortification of oil and fat preservation with polyphenols

One of the most significant bioactive properties of phenols is represented by their antioxidant effect, which has been exploited to preserve the stability of some vegetable and animal fats. The introduction of phenolic compounds in foodstuffs could represent an environmentally sustainable alternative to the use of synthetic antioxidants [340,341], such as butylated hydroxyanisole (BHA, E-320), butylated

hydroxytoluene (BHT, E-321), tert-butylhydroquinone (TBHQ, E-319), and propyl gallate (PG, E-310). The latest compounds are subjected to a maximum concentration limit in foodstuffs, due to their potential negative effect on human health [342–346].

Esposto et al. [347] evaluated the effectiveness of phenolic extract (obtained by the purification of phenolic concentrate recovered from fresh OMWW) in controlling the oil stability during a simulated frying process (until 12 h at 180 °C). The phenolic extract was added at different concentrations to a refined olive oil (100, 200, 400, and 1200 mg/kg of polyphenols) and its impact was also compared to a refined olive oil containing BHT and to an EVOO with a high content of polyphenols. This extract recovered from OMWW, mainly consisting of tyrosol, hydroxytyrosol, 3,4-DHPEA-EDA, and verbascoside, demonstrated a better capacity to preserve α-tocopherol content than BHT, reducing their oxidation if used at a concentration of at least 400 mg polyphenols/kg. The other significant effect concerned the reduction of negative volatile compounds in refined olive oil during the frying process, accompanied by a consequent improvement of the organoleptic quality of fried foodstuff. These results demonstrated the possibility of using this phenolic formulation as a bioactive ingredient in oils before frying or cooking processes [335,347].

10.2. Fat replacement with pectin derived from OMWW in meatballs

Olive fruit contains an appreciable amount of fiber with promising functional properties like water holding and cation exchange capacity. The soluble polysaccharides present in OMWW are mainly composed of pectin material [232]. Galanakis et al. [348] proposed a simplified method for the recovery of pectin from thermally concentrated OMWW. Extraction was conducted using acid and solvent extraction, prior the isolation of the alcohol insoluble residue in a precipitation process. The water soluble fraction of the recovered material was able to form gels after a simple isolation and concentration procedure, despite the high ionic concentration, the low pectin content, and the high methylation degree (59%) of

pectic polysaccharides. The method was further improved with regard to the role of endogenous pectin methyl esterase during thermal concentration step [349] and utilized for the recovery of pectin containing material prior its application as fat replacement in meatballs [350]. According to the latter study, olive pectin was able to improve cooking properties of the product, by restricting the oil uptake during deep fat frying, thus giving rise to meatballs with sustained reduced fat content. Besides, this pectin containing extract was successfully clarified from the high ionic concentration using ultrafiltration, with the final purpose of optimizing the functional properties of pectin and improving the taste of the final product [103].

10.3. Antimicrobial applications in foods

Phenols of OMWW can be used as disinfectants in the food and chemical industries [129] since they are known to exhibit antimicrobial action against *E. coli*, *P. aeruginosa*, *S. aureus*, and *B. subtilis* strains. This antimicrobial action is even greater than the respective activities induced by the individual phenolic compounds, indicating the synergistic action of the phenols contained in OMWW [117]. For instance, singular phenolic compounds cannot inhibit the growth of the human pathogens *E. coli*, *K. pneumoniae*, *S.aureus*, and *S. pyogenes* when used at low concentrations, whereas OMWW is effective in the inhibition of Gram-positive and Gram-negative bacteria [351].

10.3.1. Antimicrobial properties of polyphenols in bakery products

The efficacy of commercially available polyphenols recovered from OMWW (in a powder containing 10% polyphenols and 5% hydroxytyrosol) against other natural antioxidants (e.g., ascorbic acid, tocopherols mixture, and α-tocopherol) to inhibit microbial growth of bread and rusks during storage has been investigated [8]. Concentrations were selected in order to assure action against both oxidative deterioration and microbial spoilage. The produced bread and rusks were stored over a period of 20 days and 12 weeks, respectively, and tested periodically to different microbiological assays (e.g., total coliforms, yeasts-moulds, and *Bacillus*

spp.). According to the results, antioxidants were able to induce antimicrobial properties in bakery products and subsequently prolong their shelf life. The optimal concentration of polyphenols was 200 mg/kg flour, as it extended the preservation of both bread and rusk samples. In addition, an emulsification of the powder enhanced the antimicrobial effect of polyphenols [335].

10.3.2. Antimicrobial properties of polyphenols in meat products

The antimicrobial efficacy of a purified OMWW extract to improve the sensory characteristics and hygienic status of hamburgers made of white meat was investigated. Specifically, three doughs were prepared. One of which was the control, the second was prepared with the addition of a purified OMWW extract (0.75 g/kg of meat), and the third with the addition of a purified OMWW extract at a higher concentration (1.50 g/kg of meat). No other ingredients or additives were added. Samples were packaged on a rigid tray, wrapped with PVC film (gas permeable) and stored at 4 °C for up to 11 days. The dough containing added phenolic extract showed a certain delay in microbial growth when compared to the control sample. The effect was more evident in the dough containing 1.5 g/kg compared to that with 0.75 g/kg. In practical terms, if the achievement of a bacterial load equal to 6 log can be considered the critical limit for the marketability of the product, it was noted that the dough with 1.5 g/kg reached 6 log a day later than the control one. Thus, it lengthens the self-life by 24 h, which is noteworthy from the commercial point of view [335].

10.3.3. Antimicrobial and antioxidant applications in feeding

Gerasopoulos et al. [352] used a OMWW microfiltration retentate as an antioxidant ingredient for piglet feed (silage corn, formulated at a corn:OMWW retentate ratio of 24:1 with a final OMWW retentate concentration in feed equal to 4%). The piglets fed for 30 days with OMWW retentate, showed a higher total antioxidant capacity, glutathione and catalase activity both in their blood and tissues, with lower oxidative and stress-induced damage to lipids and proteins than the control group.

With the same feed formula (silage corn:OMWW retentate or permeate 24:1), Gerasopoulos et al. [353] also tested the antioxidant action of the OMWW microfiltration retentate and permeate in broiler chickens, fed for 37 days. The groups of animals fed with the OMWW supplement showed lower values of protein and lipid oxidation and a higher total antioxidant capacity in their plasma and tissues compared to the control group.

11. Application of recovered bioactive compounds in cosmetics

Cosmetic application could be a solution to reuse by-products discarded by several agrifood industries. As it is well known, human skin is a complex organ that regulates body heat and water loss, while preventing the entry of toxic substances and microorganisms [354]. Natural ingredients, phytonutrients, microbial metabolites, dairy derived actives, minerals, and animal protein components are believed to benefit healthy skin ageing [355]. Olive oil has been used on the skin for thousands of years but most of the mechanisms underlying these beneficial effects remain unclear [356]. According to Badiu et al. [356], olive oil has antioxidant properties and contains several essential fatty acids required for the production of phospholipids, being the case of alpha-linolenic acid and gamma-linolenic acid.

Indeed, from a sustainable point of view, this new application could provide, in the near future, a way of recover added value products for olive companies, developing cost-effective processing methods, decreasing the negative impacts of wastes on the environment, and providing other economic advantages for companies [357].

Both chronologic and environment independent processes accelerate skin ageing. Physiological processes such as ageing have been associated with an imbalance between the action of ROS and antioxidants [358]. The cumulative effect of intrinsic age (biological ageing) and photo ageing contributes to a progressive loss of structural integrity and physiological function of the skin. When in excess, reactive oxygen species (ROS) have deleterious effects as lipid oxidation, protein oxidation, DNA strand break, and modulation of gene expression [359]. The

antioxidant activity is an excellent example of a functional benefit that plant extracts can deliver. Cosmetic treatments of the latest generation, developed against wrinkles, rely on antioxidant properties of some ingredients, especially those derived from plants [360]. Antioxidants can mitigate the effects of the skin ageing process by limiting the biochemical consequences of oxidation [354]. Plants are known to contain a variety of natural antioxidants that protect and preserve themselves [361]. The exposure to UV radiation is the initial step to several skin harmful effects as wrinkling, dryness, pigment abnormalities, and in last instance skin cancer [362]. Notwithstanding, a topical application of antioxidant formulas also seems to be an interesting strategy to protect skin against oxidative stress related with several extrinsic agents [357]. Skin exposure to UVA radiation (320–400 nm) can damage DNA molecules and other lipid components. Also UVB radiation (280–320 nm) cause oxidative stress resulting in promutagenic DNA lesions which induce mutations in the epidermal cells [363]. Photoprotection comprises human body mechanisms to minimize the damages suffered when exposed to UV radiation [364]: plants secondary metabolites act, such as UV blockers. Some compounds as phenolic acids and flavonoids can prevent the penetration of the radiation into the skin [357,365].

Once OMW is regarded as a potent source of natural antioxidants, several methodologies have been applied to its recovery. Goula and Lazarides [366] developed an integrated process that can turn OMW and pomegranate wastes into ingredients for further applications. These researchers applied technological processes like fermentation, spray drying, and encapsulation to OMW in order to produce olive paste spread or olive powder and encapsulated phenols. Therefore, OMW powder obtained will be able to be used in a wide variety of food and pharmaceutical applications and in cosmetic products, such as creams, balms, shampoos, or hair conditioners [366].

Due to the sensitivity of natural compounds to the presence of factors like light or oxygen, encapsulation is a promising technology that can be used to improve bioactive compounds stability. Several encapsulation techniques have been studied [367]. In cosmetic formulations it must be assured that the particles obtained are

suitable to skin permeation through the skin barrier, in particular *stratum corneum* [368]. Therefore, it is advisable to exploit encapsulation techniques specific for an application field (in this case, cosmetics), according to what is required: improve compounds stability, control their release, avoid incompatibility of substances, reduce odor of compounds, and assure compounds active function [367].

Hydroxytyrosol, 2-hydroxytyrosol, tyrosol, oleanolic acid, flavonoids, anthocyanins, and tannins are natural antioxidants found in OMW with potential added value. Hydroxytyrosol and tyrosol are the major representative hydrophilic phenols identified, with mean concentrations of 18 and 4 g/L, respectively [369]. Moreover, hydroxytyrosol (HT), the main product of oleuropein degradation, is identified as the olive phenolic compound with the strongest *in vitro* antioxidant properties [370]. It has been demonstrated that HT can have a chemo-protective effect against UVB-induced DNA impairment in a human skin keratinocyte cell line. Hydroxytyrosol (100 μM) reduced intracellular ROS formation and attenuated the expression of p53 and NF-kB in a concentration dependent manner, proposing a positive effect as topical use [363]. Additionally, HT have a good solubility in oil and aqueous media which predicts its successful application in cosmetic products [133,357].

Oleuropein belongs to the secoiridoids group and is an ester of 2-(3, 4-dihydroxyphenyl)-ethanol (hydroxytyrosol). The oleuropein content of olive fruit varies according to the stage of maturation (higher in early stages and declining along maturation). Along with the decline of oleuropein content other oleosides as ligstroside also decline, while other phenolic compounds, such as verbascoside increase [371]. Oleuropein have a potent scavenging effect demonstrated against oxidative substances. It effectively inhibits copper sulfate-induced low-density lipoproteins (LDL) oxidation [372], scavenge nitric oxide and increase the inducible nitric oxide synthase [373], scavenge hypochlorous acid (produced by neutrophil myeloperoxidase) [127], and scavenge oxygen radicals and inhibits lipid peroxidation [374].

Antioxidants are frequently used in sunscreen formulations to complement UV filter photoprotection since they are able to prevent the damage induced by the free

radicals generated by solar irradiation previously mentioned [375]. Nowadays, as UV filters of cosmetics and particularly sunscreens are becoming widespread available, questions have been raised concerning their long-term usage and the resulted skin damage in the presence of UV radiation [376,377]. For instance, synthetic UV filters may penetrate the skin resulting in systemic exposure to potentially harmful xenobiotic and allergic chemicals as well as estrogenic effects [357,377,378].

In this way, antioxidants are becoming a challenge in sunscreens, being typically used to complement UV filter protection. Natural antioxidants found in fruits, vegetables, and relevant bioresources include compounds, such as phenols, ascorbate, and tocopherols [140]. Possessing similar structures, natural phenols can also act with the same mechanism as synthetic sunscreen agents. Subsequently, they have been proposed for their utilization as active compounds in cosmetics [218] and are frequently used in sunscreen formulations to complement UV filter [379]. For instance, quercetin (a typical flavonol) has been reported to enhance photo-stability of two common UVA and UVB filters: methoxydibenzoylmethane and octyl methoxycinnamate, respectively [380]. Hydroxytyrosol has also shown a significant protective ability against UVB-induced DNA damage [363] and against UVA-induced protein damage [381].

Natural or seminatural cosmetics can be used to answer to consumers' current preferences and demands (natural ingredients) and also be a way of preventing skin damage. However, due to the natural instability of antioxidants, these can be easily oxidized before reaching its site of action. Considering the development of new cosmetic formulas, it is important to ensure the stability of bioactive compounds present in the final product. According to some authors and as previously discussed, the encapsulation of polyphenols could ensure some stability [218]. However, it should be highlight that a desirable value of particle size for skin permeation is established as inferior to 500 nm, in order to penetrate the skin epithelium, which is not as easy as expected [357,382].

12. Commercialization of olive mill by-products

12.1. Scale-up and problems

The industrialization of processes dealing with the recovery of compounds from food wastes includes numerous issues such as laboratory research, transfer to pilot plan and full-scale production, protection of intellectual properties, and development of definite applications. These parameters are needed in order to ensure process sustainability, the economic benefit for the involved food industry, and the perpetual establishment of the derived products in the market. In addition, a working scenario focused absolutely on the extraction technologies and not on tailor-made applications research is doomed to fail [126,166].

A scale-up process must be performed without diminishing the functional properties (e.g. antioxidant, viscoelastic, etc.) of the target compounds and, at the same time, a product must be developed that meets the high-quality organoleptic standards of consumers. This is difficult since compounds recovery development challenges the typical scale-up problems, as well as other more complicated procedures related to the particular nature of the recapture procedure [383]. For instance, scale-up of recovery processes meets the same limitations (e.g. mixing and heating time) as any food manufacture procedure. Transition of batch to continuous processes is usually accompanied with extension of mixing and heating time, heavier handling, increased air incorporation, and higher degree of scrutiny [126]. All of these parameters generate numerous interactions and loss of product functionality. Subsequently, process cost is increased, as industrially recovered compounds are used in food formulations in higher concentrations compared with laboratory-recovered compounds.

Furthermore, a more specific problem could be waste collection at the source that often requires additional transportation cost and control of microbial growth. Proper management of the collection process, cooling/freezing of the material, and/or addition of chemical preservatives can provide solutions in a particular case. Another complicated problem is the broad variation of target and non-target compounds from source to source. This fact affects the mass and energy balances

as well as the functionality and the organoleptic character of the final products, especially of more crude extracts. The above problem may be monitored by adding a modification pretreatment step. The latter usually includes a selective mixing of by-product streams at the beginning of the process, taking into account basic parameters (e.g. total antioxidants concentration), or a vacuum concentration of wastewater streams taking into account macroscopic characteristics such as water and solids content [8].

12.2. Commercialized applications of olive mill by-products

A new tendency in the field of food waste recovery concerns the valorization of olive by-products as a source of phenolic compounds [89,232,349,384]. For example, commercial hydroxytyrosol has been recovered from olive mill waste in pure form (99.5 wt. %) using chromatographic columns filled with two resins: nonactivated ionic and XAD-type nonionic [385]. Another popular process is performed using acid treatment of olive mill wastewater, prior to an incubation process that converts oleuropein to hydroxytyrosol. Thereafter, extraction is performed using supercritical fluid extraction and a column operating in the counter-current mode, where a nonselective porous membrane is the barrier interface between the hydroxytyrosol-containing fluid and the dense gas. Ultimately, hydroxytyrosol possesses advanced antiradical properties compared with vitamins E and C, and prevents the oxidation of lipids in fish [386].

Hidrox is a commercially available product from CreAgri (Hayward, USA), granting a GRAS status. According to several scientific studies, Hidrox possesses several beneficial (e.g. anti-inflammatory and antimicrobial) properties. Other commercially available products include: (i) Olnactiv (Glanbia, Milan, Italy), Oleaselect, and Opextan (Indena, Milan, Italy), (ii) Olive Braun Standard 500 (containing 1–2.2 g of hydroxytyrosol and 0.2–0.7 g of tyrosol/kg) from Naturex, (iii) olive polyphenols from Albert Isliker (containing 22–23 g hydroxytyrosol and 6.5–8 g tyrosol/kg), (iv) Prolivols (containing 35% polyphenols, 2% hydroxytyrosol, and 3% tyrosol) from Seppic Inc., and (v) Olive Polyphenols NLT

from Lalilab Inc. (containing 2–6% hydroxytyrosol and 0.7–1.1% tyrosol). Another product is Phenolea Complex, which is a natural hydrophilic extract, obtained directly from aqueous olive pulp and olive mill wastewater. This product is produced without any kind of organic solvent, so the biochemical composition of the final extract reflects the original composition of olive fruit but in a more concentrated formulation. The production process comprises the following steps: (i) collection of olive pulp and mill wastewater after the milling process, (ii) pretreatment of the material, (iii) tangential microfiltration with ceramic membranes, and (iv) vacuum evaporation of the permeate. Olive pulp extracts (in general) have been approved by FDA with GRAS status (GRN No. 459).

Figure I5 illustrates an example for the recovery of valuable compounds from olive mill wastewater, which is adapted to the *5-Stage Universal Recovery Process*. In this case, pretreatment includes two processes (centrifugation and skimming) in order to remove remaining fats from three-phase olive mill wastewater. Thereafter, a vacuum concentration process can remove part of the contained water.

Treatment of concentrated and defatted olive mill wastewater with acids and ethanol can generate two streams: an alcohol insoluble residue rich in dietary fibers and an ethanolic extract rich in polyphenols. Isolation of the latter compounds can be performed using resin adsorption or chromatography. Purification of the water-soluble fraction (mainly pectin) of the residue can be conducted using ultrafiltration [158]. As it was mentioned, pectin derived from olive mill wastewater has been proved to restrict oil uptake of low fat meatballs during deep fat frying [8,103,140,349,350,387].
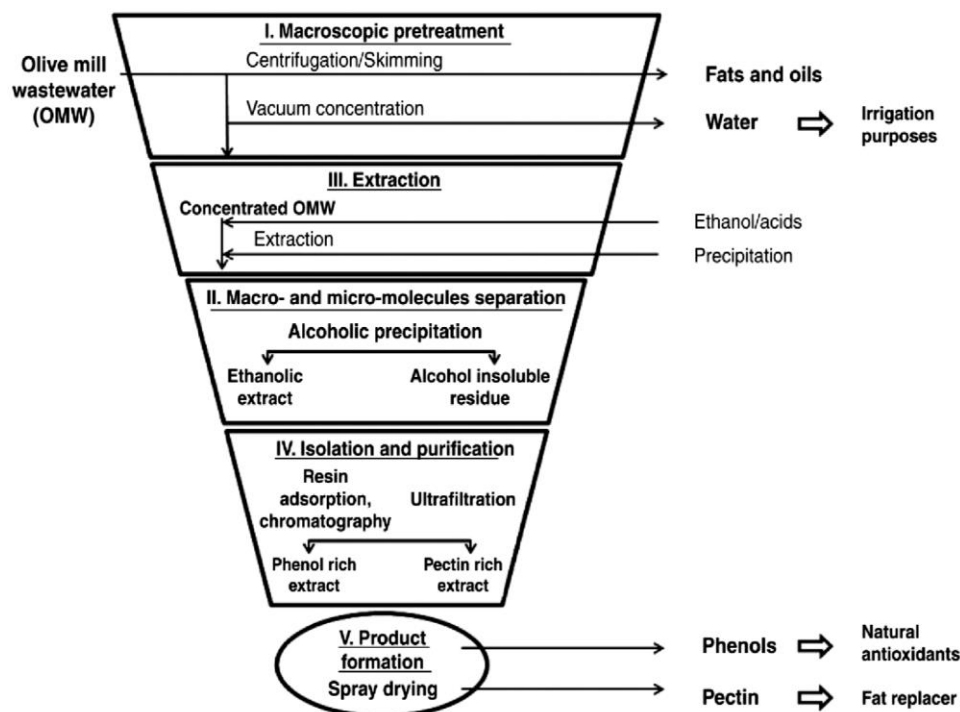
Figure I5. Recovery of valuable compounds from olive mill wastewater (OMWW) and reutilization in different products (adapted from [8])

References

[1] I. E. Kapellakis, K. P. Tsagarakis, and J. C. Crowther, "Olive oil history, production and by-product management", *Rev. Environ. Sci. Bio/Technology*, 7 (1), 1–26, 2008.

[2] A. C. Caputo, F. Scacchia, and P. M. Pelagagge, "Disposal of by-products in olive oil industry: waste-to-energy solutions", *Appl. Therm. Eng.*, 23 (2), 197–214, 2003.

[3] N. Azbar, A. Bayram, A. Filibeli, A. Muezzinoglu, F. Sengul, A. Ozer, "A review of waste management options in olive oil production", *Crit. Rev. Environ. Sci. Technol.*, 34 (3), 209–247, 2004.

[4] S. Souilem, A. El-Abbassi, H. Kiai, A. Hafidi, S. Sayadi, C. M. Galanakis, "Chapter 1 - Olive oil production sector: environmental effects and sustainability challenges", C. M. B. T.-O. M. W. Galanakis, Ed. Academic Press, 2017, pp. 1–28.

[5] A. Roig, M. L. Cayuela, M. A. Sánchez-Monedero, "An overview on olive mill wastes and their valorisation methods", *Waste Manag.*, 26 (9), 960–969, 2006.

[6]   M. K. Doula, J. L. Moreno-Ortego, F. Tinivella, V. J. Inglezakis, A. Sarris, K. Komnitsas, "Chapter 2 - Olive mill waste: recent advances for the sustainable development of olive oil industry", C. M. B. T.-O. M. W. Galanakis, Ed. Academic Press, 2017, pp. 29–56.

[7]   M. J. Negro, P. Manzanares, E. Ruiz, E. Castro, and M. Ballesteros, "Chapter 3 - The biorefinery concept for the industrial valorization of residues from olive oil industry", C. M. B. T.-O. M. W. Galanakis, Ed. Academic Press, 2017, pp. 57–78.

[8]   C. M. Galanakis, N. Martinez-Saez, M. D. del Castillo, F. J. Barba, V. S. Mitropoulou, "Chapter 15 - Patented and commercialized applications", C. M. B. T.-F. W. R. Galanakis, Ed. *San Diego: Academic Press*, 2015, pp. 337–360.

[9]   S. Dermeche, M. Nadour, C. Larroche, F. Moulti-Mati, P. Michaud, "Olive mill wastes: Biochemical characterizations and valorization strategies", *Process Biochem.*, 48 (10), 1532–1552, 2013.

[10]   P. S. Rodis, V. T. Karathanos, A. Mantzavinou, "Partitioning of olive Oil antioxidants between oil and water phases", *J. Agric. Food Chem.*, 50 (3), 596–601, Jan. 2002.

[11]   M. Hamza, S. Sayadi, "Valorisation of olive mill wastewater by enhancement of natural hydroxytyrosol recovery", *Int. J. Food Sci. Technol.*, 50 (3), 826–833, Mar. 2015.

[12]   A.-L. Skaltsounis, A. Argyropoulou, N. Aligiannis, N. Xynos, "11 - Recovery of High Added Value Compounds from Olive Tree Products and Olive Processing Byproducts", D. B. T.-O. and O. O. B. C. Boskou, Ed. AOCS Press, 2015, pp. 333–356.

[13]   P. Chiaiese, F. Palomba, F. Tatino, C. Lanzillo, G. Pinto, A. Pollio, E. Filippone, "Engineered tobacco and microalgae secreting the fungal laccase POXA1b reduce phenol content in olive oil mill wastewater", *Enzyme Microb. Technol.*, 49 (6), 540–546, 2011.

[14]   C. Regulation, "No 1513/2001 of 23 July 2001 Amending Regulations No 136/66/EEC and (EC) No 1638/98 as Regards the Extension of the Period of Validity of the Aid Scheme and the Quality Strategy for Olive Oil", *Off. J. Eur. Communities Luxemb.*, vol. 201, pp. 4–7, 2001.

[15]   E. Gimeno, A. Castellote, R. Lamuela-Raventós, M. Torre, M. López-Sabater , "The effects of harvest and extraction methods on the antioxidant content (phenolics, a -tocopherol, and b -carotene) in virgin olive oil", *Food Chemistry*, 78, 207–211, 2002.

[16]   A. Kiritsakis, G. D. Nanos, Z. Polymenopulos, T. Thomai, and E. M. Sfakiotakis, "Effect of fruit storage conditions on olive oil quality", *J. Am. Oil Chem. Soc.*, 75 (6), 721–724, Jun. 1998.

[17]   P. Catalano, F. Pipitone, A. Calafatello, A. Leone, "Productive efficiency of decanters with short and variable dynamic pressure cones", *Biosyst. Eng.*, 86 (4), 459–464, 2003.

[18]   M. C. Demicheli, L. Bontoux, "Novel technologies for olive oil manufacturing and

their incidence on the environment", *Fresenius Environ. Bull.*, 6 (5–6), 240–247, 1997.

[19]   A. G. Vlyssides, M. Loizides, P. K. Karlis, "Integrated strategic approach for reusing olive oil extraction by-products", *J. Clean. Prod.*, 12 (6), 603–611, 2004.

[20]   R. Arjona, A. García, P. Ollero, *Drying of alpeorujo, a waste product of the olive oil mill industry*, vol. 41. 1999.

[21]   C. Bordons, A. Núñez-Reyes, "Model based predictive control of an olive oil mill", *J. Food Eng.*, 84 (1), 1–11, 2008.

[22]   S. Vichi, P. Boynuegri, J. Caixach, A. Romero, "Quality losses in virgin olive oil due to washing and short-term storage before olive milling", *Eur. J. Lipid Sci. Technol.*, 117 (12), 2015–2022, Dec. 2015.

[23]   M. Servili, S. Esposto, A. Taticchi, S. Urbani, I. Di Maio, G. Veneziani, R. Selvaggini, "New approaches to virgin olive oil quality, technology, and by-products valorization", *Eur. J. Lipid Sci. Technol.*, 117 (11), 1882–1892, Nov. 2015.

[24]   B. A. Jean, B. C. Marie, "OLIVE OIL Huile d'olive varieties", 21 (5), 4–9, 2014.

[25]   P. Reboredo-Rodríguez, C. González-Barreiro, B. Cancho-Grande, J. Simal-Gándara, "Improvements in the malaxation process to enhance the aroma quality of extra virgin olive oils", *Food Chem.*, 158, 534–545, 2014.

[26]   B. Jiménez, A. Sánchez-Ortiz, and A. Rivas, "Influence of the malaxation time and olive ripening stage on oil quality and phenolic compounds of virgin olive oils", *Int. J. Food Sci. Technol.*, 49 (11), 2521–2527, Nov. 2014.

[27]   B. Aliakbarian, D. De Faveri, A. Converti, and P. Perego, "Optimisation of olive oil extraction by means of enzyme processing aids using response surface methodology", *Biochem. Eng. J.*, 42 (1), 34–40, 2008.

[28]   M. L. Clodoveo, R. H. Hbaieb, F. Kotti, G. S. Mugnozza, M. Gargouri, "Mechanical strategies to increase nutritional and sensory quality of virgin olive oil by modulating the endogenous enzyme activities", *Compr. Rev. Food Sci. Food Saf.*, 13 (2), 135–154, Mar. 2014.

[29]   M. L. Clodoveo, "Malaxation: Influence on virgin olive oil quality. Past, present and future - An overview", *Trends Food Sci. Technol.*, 25 (1), 13–23, 2012.

[30]   A. G. Vlyssides, G. K. Lamprou, A. Vlysidis, "Chapter 6 - Industrial case studies on the detoxificaton of OMWW using Fenton oxidation process followed by biological processes for energy and compost production", C.M.B.T.-O. M. W. Galanakis, Ed. Academic Press, 2017, pp. 119–138.

[31]   A. G. Vlyssides, M. Loizidou, K. Gimouhopoulos, A. Zorpas, "Olive oil processing wastes production and their characteristics in relation to olive oil extraction methods", *Fresenius Environ. Bull.*, 7 (5), 308–313, 1998.

[32]   A. Baiano, C. Terracone, I. Viggiani, M. A. Del Nobile, "Changes produced in extra-virgin olive oils from cv. Coratina during a prolonged storage treatment", *Czech J. Food Sci.*, 32 (1), 1–9, 2014.

[33]   M. Avraamides, D. Fatta, "Resource consumption and emissions from olive oil production: a life cycle inventory case study in Cyprus", *J. Clean. Prod.*, 16 (7), 809–821, 2008.

[34]   P. Amirante, P. Catalano, R. Amirante, M. L. Clodoveo, G. L. Montel, A. Leone, A. Tamborrino, "Prove sperimentali di estrazione di oli extravergini di oliva da paste snocciolate", *Olivo Olio*, 6 (6), 16–22, 2002.

[35]   C. J. McNamara, C. C. Anastasiou, V. O'Flaherty, R. Mitchell, "Bioremediation of olive mill wastewater", *Int. Biodeterior. Biodegrad.*, 61 (2), 127–134, 2008.

[36]   A. Ben Sassi, A. Boularbah, A. Jaouad, G. Walker, and A. Boussaid, "A comparison of Olive oil Mill Wastewaters (OMW) from three different processes in Morocco", *Process Biochem.*, 41 (1), 74–78, 2006.

[37]   E. De Marco, M. Savarese, A. Paduano, R. Sacchi, "Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters", *Food Chem.*, 104 (2), 858–867, 2007.

[38]   F. Alagna, R. Mariotti, F. Panara, S. Caporali, S. Urbani, G. Veneziani, S. Esposto, A. Taticchi, A. Rosati, R. Rao, G. Perrotta, M. Servili, L. Baldoni., "Olivve phenolic compounds: metbolic and transcriptional profiling during fruit development", *BMC Plant Biol.*,12 (162), 12-162, 2012.

[39]   N. S. Kumar, K. Min, "Phenolic compounds biosorption onto Schizophyllum commune fungus: FTIR analysis, kinetics and adsorption isotherms modeling", *Chem. Eng. J.*, 168 (2), 562–571, 2011.

[40]   N. Allouche, I. Fki, S. Sayadi, "Toward a high yield recovery of antioxidants and purified hydroxytyrosol from olive mill wastewaters", *J. Agric. Food Chem.*, 52 (2), 267–273, Jan. 2004.

[41]   Fiorentino, A. Gentili, M. Isidori, P. Monaco, A. Nardelli, A. Parrella, F. Temussi, "Environmental effects caused by olive mill wastewaters: toxicity comparison of low-molecular-weight phenol components", *J. Agric. Food Chem.*, 51 (4), 1005–1009, Feb. 2003.

[42]   C. I. Piperidou, C. I. Chaidou, C. D. Stalikas, K. Soulti, G. A. Pilidis, C. Balis, "Bioremediation of olive oil mill wastewater: chemical alterations induced by Azotobacter vinelandii.", *J. Agric. Food Chem.*, 48 (5), 1941–1948, May 2000.

[43]   J. Fernández-Bolaños, G. Rodríguez, R. Rodríguez, A. Heredia, R. Guillén, A. Jiménez, "Production in large quantities of highly purified hydroxytyrosol from liquid−solid waste of two-phase olive oil processing or 'Alperujo'", *J. Agric. Food Chem.*, 50 (23), 6804–6811, Nov. 2002.

[44]   N. Mulinacci, A. Romani, C. Galardi, P. Pinelli, C. Giaccherini, F. F. Vincieri, "Polyphenolic content in olive oil waste waters and related olive samples", *J. Agric. Food Chem.*, 49 (8), 3509–3514, Aug. 2001.

[45]   L. Lesage-Meessen, D.Navarro, S. Maunier, J. C. Sigoillot, J. Lorquin, M. Delattre, J. L. Simon, M. Asther, M. Labat, , "Simple phenolic content in olive oil residues as a function of extraction systems", *Food Chem.*, 75 (4), 501–507, 2001.

[46]   B. Armandodoriano, B. Francesca, C. Giampaolo, C. Franco, J. Renata, M. Pamela, "Analysis by liquid chromatography-tandem mass spectrometry of biophenolic compounds in olives and vegetation waters, Part I", *J. Sep. Sci.*, 26 (5), 409–416, Apr. 2003.

[47]   C. Romero, M. Brenes, P. García, A. Garrido, "Hydroxytyrosol 4-β-d-glucoside, an important phenolic compound in olive fruits and derived products", *J. Agric. Food Chem.*, 50 (13), 3835–3839, Jun. 2002.

[48]   H. K. Obied, D. R. Bedgood, P. D. Prenzler, K. Robards, "Bioscreening of Australian olive mill waste extracts: Biophenol content, antioxidant, antimicrobial and molluscicidal activities", *Food Chem. Toxicol.*, 45 (7), 1238–1248, 2007.

[49]   A. Albarrán, R. Celis, M. C. Hermosín, A. López-Piñeiro, J. Cornejo, "Behaviour of simazine in soil amended with the final residue of the olive-oil extraction process", *Chemosphere*, 54 (6), 717–724, 2004.

[50]   L. Di Giovacchino, S. Prezioso, "Utilization of olive mill by-products", *Proc. Olivebioteq*, pp. 379–389, 2006.

[51]   T. Lafka, A. E. Lazou, V. J. Sinanoglou, E. S. Lazos, "Phenolic and antioxidant potential of olive oil mill wastes", *Food Chem.*, 125 (1), 92–98, 2011.

[52]   E. Molina-Alcaide, D. Yáñez-Ruiz, "Potential use of olive by-products in ruminant feeding: A review", *Anim. Feed Sci. Technol.*, 147, Nov. 2008.

[53]   J. A. Alburquerque Méndez, J. Gonzálvez, D. García, J. Cegarra Rosique, "Agrochemical characterisation of 'alperujo', a solid by-product of the two-phase centrifugation method for olive oil extraction", *Bioresour. Technol.*, 91 (2), 195–200, 2004.

[54]   E. Garcia-Castello, A. Cassano, A. Criscuoli, C. Conidi, E. Drioli, "Recovery and concentration of polyphenols from olive mill wastewaters by integrated membrane system", *Water Res.*, 44 (13), 3883–3892, 2010.

[55]   I. García García, P. R. Jiménez Peña, J. L. Bonilla Venceslada, A. Martín Martín, M. A. Martín Santos, E. Ramos Gómez, "Removal of phenol compounds from olive mill wastewater using Phanerochaete chrysosporium, Aspergillus niger, Aspergillus terreus and Geotrichum candidum", *Process Biochem.*, 35 (8), 751–758, 2000

[56]   F. Aouidi, H. Gannoun, N. Ben Othman, L. Ayed, M. Hamdi, "Improvement of fermentative decolorization of olive mill wastewater by Lactobacillus paracasei by cheese whey's addition", *Process Biochem.*, 44 (5), 597–601, 2009.

[57]   A. G. Vlyssides, D. L. Bouranis, M. Loizidou, G. Karvouni, "Study of a demonstration plant for the co-composting of olive-oil-processing wastewater and solid residue", *Bioresour. Technol.*, 56 (2–3), 187–193, 1996.

[58]   A. I. M. García, A. Moumen, D. R. Y. Ruiz, E. M. Alcaide, "Chemical composition and nutrients availability for goats and sheep of two-stage olive cake and olive leaves", *Anim. Feed Sci. Technol.*, 107 (1–4), 61–74, 2003.

[59]   R. Ordóñez, P. González, J. V Giráldez, A. García-Ortiz, "Efecto de la enmienda con alperujo sobre los principales nutrientes de un suelo agrícola", *Estúdios la Zo.*

no saturada. R. Muñoz-Carpena, A. Ritter C. Tascón ICIA, 123–126, 1999.

[60]   S. Aktaş, S. Imre, L. Ersoy, "Characterization and lime treatment of olive mill wastewater", *Water Res.*, 35, 2336–2340, Jul. 2001.

[61]   S. Khoufi, F. Aloui, S. Sayadi, "Extraction of antioxidants from olive mill wastewater and electro-coagulation of exhausted fraction to reduce its toxicity on anaerobic digestion", *J. Hazard. Mater.*, 151 (2–3), 531–539, 2008.

[62]   J. A. F. R. De Ursinos, R. Borja-Padilla, "Biomethanization", *Int. Biodeterior. Biodegradation*, 38, no. 3–4, 145–153, 1996.

[63]   J. Cegarra, J. B. Amor, J. Gonzálvez, M. P. Bernal, A. Roig, "Characteristics of a new solid olive-mill by-product ("'alperujo'") and its suitability for composting," in *Proceedings of the International Composting Symposium ICS_99*, 2000, vol. 1, pp. 124–140.

[64]   C. Paredes, J. Cegarra, A. Roig, M. A. Sanchez-Monedero, M. P. Bernal, "Characterization of olive mill wastewater (alpechin) and its sludge for agricultural purposes", *Bioresour. Technol.*, 67 (2), 111–115, 1999.

[65]   T. Yangui, A. Dhouib, A. Rhouma, S. Sayadi, "Potential of hydroxytyrosol-rich composition from olive mill wastewater as a natural disinfectant and its effect on seeds vigour response", *Food Chem.*, 117 (1), 1–8, 2009.

[66]   E. Madejón, E. Galli, U. Tomati, "Composting of wastes produced by low water consuming olive mill technology", *Agrochimica*, XLII (3-4), 1998.

[67]   M. L. Cayuela, "Producción industrial de compost ecológico a partir de residuos de almazara", Universidad de Murcia, 2004.

[68]   I. Sampedro, C. Romero, J. A. Ocampo, M. Brenes, I. García, "Removal of monomeric phenols in dry mill olive residue by saprobic fungi", *J. Agric. Food Chem.*, 52 (14), 4487–4492, Jul. 2004.

[69]   F. Pagnanelli, L. Toro, F. Vegliò, "Olive mill solid residues as heavy metal sorbent material: A preliminary study", *Waste Manag.*, 22 (8), 901–907, 2002.

[70]   B. Aliakbarian, A. A. Casazza, P. Perego, "Valorization of olive oil solid waste using high pressure-high temperature reactor", *Food Chem.*, 128 (3), 704–710, 2011.

[71]   K. Komnitsas, K. Modis, M. Doula, V. Kavvadias, D. Sideri, D. Zaharaki, "Geostatistical estimation of risk for soil and water in the vicinity of olive mill wastewater disposal sites", *Desalin. Water Treat.*, 57 (7), 2982–2995, Feb. 2016.

[72]   S. Ntougias, F. Gaitis, P. Katsaris, S. Skoulika, N. Iliopoulos, G. I. Zervakis, "The effects of olives harvest period and production year on olive mill wastewater properties – Evaluation of Pleurotus strains as bioindicators of the effluent's toxicity", *Chemosphere*, 92 (4), 399–405, 2013.

[73]   I. Karaouzas, N. T. Skoulikidis, U. Giannakou, T. A. Albanis, "Spatial and temporal effects of olive mill wastewaters to stream macroinvertebrates and aquatic ecosystems status", *Water Res.*, 45 (19), 6334–6346, 2011.

[74]   A. Zirehpour, A. Rahimpour, M. Jahanshahi, M. Peyravi, "Mixed matrix membrane application for olive oil wastewater treatment: Process optimization based on Taguchi design method", *J. Environ. Manage.*, 132, 113–120, 2014.

[75]   E. Lagoudianaki, T. Manios, M. Geniatakis, N. Frantzeskaki, V. Manios, "Odor control in evaporation ponds treating olive mill wastewater through the use of Ca(OH)2", *J. Environ. Sci. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng.*, 38 (11), 2537–2547, 2003.

[76]   L. Baeta-Hall, M. Céu Sàágua, M. Lourdes Bartolomeu, A. M. Anselmo, M. Fernanda Rosa, "Bio-degradation of olive oil husks in composting aerated piles", *Bioresour. Technol.*, 96 (1), 69–78, 2005.

[77]   T. Akar, I. Tosun, Z. Kaynak, E. Ozkara, O. Yeni, E. Sahin, S. Tunali Akar, "An attractive agro-industrial by-product in environmental cleanup: Dye biosorption potential of untreated olive pomace", *J. Hazard. Mater.*, 166 (2–3), 1217–1225, 2009.

[78]   P. Galiatsatou, M. Metaxas, D. Arapoglou, "Treatment of olive mill waste water with activated carbons from agricultural by-products", *Waste Management (New York, N.Y.)*, 22, 803–812, 2002.

[79]   K. Al-Malah, M. O. J. Azzam, N. I. Abu-Lail, "Olive mills effluent (OME) wastewater post-treatment using activated clay", *Sep. Purif. Technol.*, 20 (2–3), 225–234, 2000.

[80]   J. Sierra, E. Martí, G. Montserrat, R. Cruaáas, M. A. Garau, "Characterisation and evolution of a soil affected by olive oil mill wastewater disposal", *Sci. Total Environ.*, 279 (1–3), 207–214, 2001.

[81]   M. R. Al-Masri, "In vitro digestible energy of some agricultural residues, as influenced by gamma irradiation and sodium hydroxide", *Appl. Radiat. Isot.*, 50 (2), 295–301, 1999.

[82]   A. G. Vlyssides, D. L. Bouranis, M. Loizidou, G. Karvouni, "Study of a demonstration plant for the co-composting of olive-oil-processing wastewater and solid residue", *Bioresour. Technol.*, 56 (2–3), 187–193, 1996.

[83]   R. Borsani, B. Ferrando, "Ultrafiltration plant for olive vegetation waters by polymeric membrane batteries", *Desalination*, 108 (1–3), 281–286, 1997.

[84]   C. Paredes, J. Cegarra, A. Roig, M. A. Sanchez-Monedero, M. P. Bernal, "Characterization of olive mill wastewater (alpechin) and its sludge for agricultural purposes", *Bioresour. Technol.*, 67 (2), 111–115, 1999.

[85]   A. Saviozzi, R. Levi-Minzi, R. Cardelli, A. Biasci, R. Riffaldi, "Suitability of moist olive pomace as soil amendment", *Water. Air. Soil Pollut.*, 128 (1–2), 13–22, 2001.

[86]   S. Ayoub, K. Al-Absi, S. Al-Shdiefat, D. Al-Majali, D. Hijazean, "Effect of olive mill wastewater land-spreading on soil properties, olive tree performance and oil quality", *Sci. Hortic. (Amsterdam).*, 175, 160–166, 2014.

[87]   C. Di Bene, E. Pellegrino, M. Debolini, N. Silvestri, E. Bonari, "Short- and long-term effects of olive mill wastewater land spreading on soil chemical and biological

properties", *Soil Biol. Biochem.*, 56, 21–30, 2013.

[88]   E. O. Akdemir, A. Ozer, "Application of a statistical technique for olive oil mill wastewater treatment using ultrafiltration process", 62, 222–227, 2008.

[89]   N. Rahmanian, S. M. Jafari, C. M. Galanakis, "Recovery and removal of phenolic compounds from olive mill wastewater", *J. Am. Oil Chem. Soc.*, 91(1), 1–18, Jan. 2014.

[90]   D. P. Zagklis, E. C. Arvaniti, V. G. Papadakis, C. A. Paraskeva, "Sustainability analysis and benchmarking of olive mill wastewater treatment methods", *J. Chem. Technol. Biotechnol.*, 88(5), 742–750, May 2013.

[91]   E. Roselló-Soto, C. M. Galanakis, M. Brnčić, V. Orlien, F. J. Trujillo, R. Mawson, K. Knoerzer, B. K. Tiwari, F. J. Barba, "Clean recovery of antioxidant compounds from plant foods, by-products and algae assisted by ultrasounds processing. Modeling approaches to optimize processing conditions", *Trends Food Sci. Technol.*, 42(2), 134–149, 2015.

[92]   C. M. Galanakis, "Separation of functional macromolecules and micromolecules : From ultrafiltration to the border of nanofiltration", *Trends Food Sci. Technol.*, 42(1), 44–63, 2015.

[93]   C. M. Galanakis, "Food and bioproducts processing short communication emerging technologies for the production of nutraceuticals from agricultural by-products : A viewpoint of opportunities and challenges", *Food Bioprod. Process.*, 91(4), 575–579, 2013.

[94]   M. K. Doula , "Strategies to improve and protect soil quality from the disposal of Olive Oil Mill Wastes in the Mediterranean", *PROSODOL: Results and Achievements of a 4-year demonstration project What to consider, What to do.*, 2012

[95]   J. T. Cookson Jr, "*Bioremediation engineering: Design and application*", McGraw-Hill, Inc., 1995.

[96]   M. K. Doula, K. Elaiopoulos, V. A. Kavvadias, V. Mavraganis, "Use of clinoptilolite to improve and protect soil quality from the disposal of olive oil mills wastes", *J. Hazard. Mater.*, 207–208, 103–110, 2012.

[97]   M. K. Doula, V. A. Kavvadias, K. Elaiopoulos, "Zeolites in soil-remediation processes", *Nat. Zeolites Handb.*, 330–355, 2011.

[98]   P. Paraskeva, E. Diamadopoulos, "Technologies for olive mill wastewater (OMW) treatment: a review", *J. Chem. Technol. Biotechnol.*, 81(9), 1475–1485, Sep. 2006.

[99]   C. M. Galanakis, "Recovery of high components from food wastes : Conventional, emerging technologies and commercialized applications", *Trends Food Sci. Technol.*, 26(2), 68–87, 2012.

[100]   T. Prokopov, Z. Goranova, M. Baeva, A. Slavov, C. M. Galanakis, "Effects of powder from white cabbage outer leaves on sponge cake quality", *International Agrophysis*, 1, 493–500, 2015.

[101]   H. K. Obied, M. S. Allen, D. R. Bedgood, P. D. Prenzler, K. Robards, R. Stockmann, "Bioactivity and analysis of biophenols recovered from olive mill waste", *J. Agric.*

Food Chem., 53(4), 823–837, Feb. 2005.

[102]   O. Bouzida, D. Navarroa, M. Rochec, M. Asthera, M. Haona, M. Delattrea, J. Lorquinb, M. Labatb, M. Asthera, L. Lesage-Meessen, "Fungal enzymes as a powerful tool to release simple phenolic compounds from olive oil by-product", *Process Biochem.*, 40, 1855–1862, 2005.

[103]   C. M. Galanakis, E. Tornberg, V. Gekas, "Clarification of high-added value products from olive mill wastewater", *J. Food Eng.*, 99(2), 190–197, 2010.

[104]   A. Patsioura, C. M. Galanakis, V. Gekas, "Ultrafiltration optimization for the recovery of β-glucan from oat mill waste", *J. Memb. Sci.*, 373(1), 53–63, 2011.

[105]   C. M. Galanakis, G. Fountoulis, V. Gekas, "Nanofiltration of brackish groundwater by using a polypiperazine membrane", *Desalination*, 286, 277–284, 2012.

[106]   P. Ramos, S. Santos, Â. Guerra, O. Guerreiro, L. Felício, E. Jerónimo, A. Silvestre, C. Pascoal, M. Duarte, "Valorization of olive mill residues: Antioxidant and breast cancer antiproliferative activities of hydroxytyrosol-rich extracts derived from olive oil by-products", *Ind. Crop. Prod.*, 46, 359–368, 2013.

[107]   J. Cegarra, C. Paredes, A. Roig, M. P. Bernal, D. García, "Use of olive mill wastewater compost for crop production", *Int. Biodeterior. Biodegradation*, 38(3), 193–203, 1996.

[108]   R. Jarboui, F. Sellami, A. Kharroubi, N. Gharsallah, E. Ammar, "Olive mill wastewater stabilization in open-air ponds: Impact on clay–sandy soil", *Bioresour. Technol.*, 99(16), 7699–7708, 2008.

[109]   A. Ramos-Cormenzana, B. Juárez-Jiménez, M. P. Garcia-Pareja, "Antimicrobial Activity of Olive Mill Waste- Waters (Alpechin) and Biotransformed Olive Oil", *Int. Biodeterior. Biodegradation*, 38(3), 283–290, 1997.

[110]   V. Kavvadias, M. K. Doula, K. Komnitsas, N. Liakopoulou, "Disposal of olive oil mill wastes in evaporation ponds : Effects on soil properties", *J. Hazard. Mater.*, 182(1–3), 144–155, 2010.

[111]   M. H. Alu'datt, T. Rababah, K. Ereifej, I. Alli, "Distribution, antioxidant and characterisation of phenolic compounds in soybeans, flaxseed and olives", *Food Chem.*, 139(1–4), 93–99, 2013.

[112]   D. P. Makris, G. Boskou, N. K. Andrikopoulos, P. Kefalas, "Characterisation of certain major polyphenolic antioxidants in grape (Vitis vinifera cv. Roditis) stems by liquid chromatography-mass spectrometry", *Eur. Food Res. Technol.*, 226 (5), 1075–1079, 2008

[113]   C. M. Galanakis, V. Goulas, S. Tsakona, G. A. Manganaris, V. Gekas, "A knowledge base for the recovery of natural phenols with different solvents", *Int. J. Food Prop.*, 16(2), 382–396, Jan. 2013.

[114]   C. S. Yang, S. Sang, J. D. Lambert, M.-J. Lee, "Bioavailability issues in studying the health effects of plant polyphenolic compounds", *Mol. Nutr. Food Res.*, 52 (SUPPL. 1), S139–S151, 2008.

[115]   A. Karadag, B. Ozcelik, S. Saner, "Review of methods to determine antioxidant

capacities", *Food Anal. Methods*, 2, 41–60, Mar. 2009.

[116] H. Ilyasoglu, B. Ozcelik, V. Van Hoed, R. Verhe, "Cultivar characterization of Aegean olive oils with respect to their volatile compounds", *Sci. Hortic. (Amsterdam).*, 129(2), 279–282, 2011.

[117] H. K. Obied, D. R. Bedgood, P. D. Prenzler, K. Robards, "Bioscreening of Australian olive mill waste extracts: Biophenol content, antioxidant, antimicrobial and molluscicidal activities", *Food Chem. Toxicol.*, 45(7), 1238–1248, 2007.

[118] F. D'Alessandro, C. Marucchini, L. Minuti, C. Zadra, A. Taticchi, "GC/MS-SIM Analysis of phenolic compounds in olive oil waste waters", *Ital. J. Food Sci.*, 17(1), 83–88, 2005.

[119] V. Balice, O. Cera, "Acidic phenolic fraction of the olive vegetation water determined by a gas chromatographic method", *Grasas Aceites*, 35(5), 178–180, 1984.

[120] M. DellaGreca, L. Previtera, F. Temussi, A. Zarrelli, "Low-molecular-weight components of olive oil mill waste-waters", *Phytochem. Anal. An Int. J. Plant Chem. Biochem. Tech.*, 15(3), 184–188, 2004.

[121] G. Knupp, G. Rücker, A. Ramos-Cormenzana, S. Garrido Hoyos, M. Neugebauer, T. Ossenkop, "Problems of identifying phenolic compounds during the microbial degradation of olive mill wastewater", *Int. Biodeterior. Biodegradation*, 38(3), 277–282, 1996.

[122] B. Bayram, B. Ozcelik, G. Schultheiss, J. Frank, G. Rimbach, "A validated method for the determination of selected phenolics in olive oil using high-performance liquid chromatography with coulometric electrochemical detection and a fused-core column", *Food Chem.*, 138(2), 1663–1669, 2013.

[123] F. Boukhoubza, A. Jail, F. Korchi, L. Loukili Idrissi, H. Hannache, J. C. Duarte, L. Hassani, A. Nejmeddine, "Application of lime and calcium hypochlorite in the dephenolisation and discolouration of olive mill wastewater", *J. Environ. Manage.*, 91(1), 124–132, 2009.

[124] H. K. Obied, D. R. Bedgood, P. D. Prenzler, K. Robards, "Chemical screening of olive biophenol extracts by hyphenated liquid chromatography", *Anal. Chim. Acta*, 603(2), 176–189, 2007.

[125] F. Visioli, A. Romani, N. Mulinacci, S. Zarini, D. Conte, F. Vincieri, C. Galli, "Antioxidant and other biological activities of olive mill waste waters", *J. Agric. Food Chem.*, 47(8), 3397–3401, Aug. 1999.

[126] C. M. Galanakis, "Recovery of high added-value components from food wastes: Conventional, emerging technologies and commercialized applications", *Trends Food Sci. Technol.*, 26(2), 68–87, 2012.

[127] F. Visioli, C. Galli, G. Galli, D. Caruso, "Biological activities and metabolic fate of olive oil phenols", *Eur. J. Lipid Sci. Technol.*, 104(9–10), 677–684, 2002.

[128] S. McDonald, P. D. Prenzler, M. Antolovich, K. Robards, "Phenolic content and antioxidant activity of olive extracts", *Food Chem.*, 73(1), 73–84, 2001.

[129]   T. Yangui, S. Sayadi, A. Rhouma, A. Dhouib, "Potential use of hydroxytyrosol-rich extract from olive mill wastewater as a biological fungicide against Botrytis cinerea in tomato", *J. Pest Sci. (2004).*, 83(4), 437–445, 2010.

[130]   B. J. Fernández-bolaños, G. Rodríguez, R. Rodríguez, R. Guillén, A. Jiménez, "Potential use of olive by-products Extraction of interesting organic compounds from olive oil waste", *Grasas y Aceites*, 57(1), 95–106, 2006.

[131]   F. Visioli, D. Caruso, E. Plasmati, R. Patelli, N. Mulinacci, A. Romani, G. Galli, C. Galli, "Hydroxytyrosol, as a component of olive mill waste water, is dose-dependently absorbed and increases the antioxidant capacity of rat plasma", *Free Radic. Res.*, 34(3), 301–305, Jan. 2001.

[132]   C. L. Leger, N. Kadiri-Hassani, B. Descomps, "Decreased superoxide anion production in cultured human promonocyte cells (THP-1) due to polyphenol mixtures from olive oil processing wastewaters", *J. Agric. Food Chem.*, 48(10), 5061–5067, 2000.

[133]   O. Bouzid, D. Navarro, M. Roche, M. Asther, M. Haon, M. Delattre, J. Lorquin, M. Labat, M. Asther, L. Lesage-Meessen, "Fungal enzymes as a powerful tool to release simple phenolic compounds from olive oil by-product", *Process Biochem.*, 40(5), 1855–1862, 2005.

[134]   M.-I. Covas, K. de la Torre, M. Farré-Albaladejo, J. Kaikkonen, M. Fitó, C. López-Sabater, M. Pujadas-Bastardes, J. Joglar, T. Weinbrenner, R. Lamuela-Raventós, R. de la Torre, "Postprandial LDL phenolic content and LDL oxidation are modulated by olive oil phenolic compounds in humans", *Free Radic. Biol. Med.*, 40(4), 608–616, 2006.

[135]   E. De Marco, M. Savarese, A. Paduano, R. Sacchi, "Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters", *Food Chem.*, 104(2), 858–867, 2007.

[136]   E. Panel, A. Nda, "Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL-cholesterol concentrations (ID 1639)", vol. 9(1924), pp. 1–25, 2011.

[137]   T. K. Sunil Kumar, P. A. Sherena, "An efficient process for the preparation of lycopene containing oleoresin and lycopene crystals for human consumption", EP2757904B1, (July, 30, 2014)

[138]   C. M. Galanakis, K. Kotsiou, "Chapter 10- Recovery of bioactive compounds from olive mill waste", C. M. B. T.-O. M. W. Galanakis, Ed. *Academic Press*, 2017, pp. 205–229.

[139]   C. M. Galanakis, "Recovery of high added-value components from food wastes: Conventional, emerging technologies and commercialized applications", *Trends Food Sci. Technol.*, 26(2), 68–87, 2012.

[140]   C. M. Galanakis, E. Tornberg, V. Gekas, "Recovery and preservation of phenols from olive waste in ethanolic extracts", *J. Chem. Technol. Biotechnol.*, 85(8), 1148–1155, Aug. 2010.

[141] A. Zafra, M. J. B. Juárez, R. Blanc, A. Navalón, J. González, J. L. Vílchez, "Determination of polyphenolic compounds in wastewater olive oil by gas chromatography-mass spectrometry", *Talanta*, 70(1), SPEC. ISS., 213–218, 2006.

[142] E. Roselló-Soto, C. M. Galanakis, M. Brnčić, V. Orlien, F. J. Trujillo, R. Mawson, K. Knoerzer, B. K. Tiwari, F. J. Barba, "Clean recovery of antioxidant compounds from plant foods, by-products and algae assisted by ultrasounds processing. Modeling approaches to optimize processing conditions", *Trends Food Sci. Technol.*, 42(2), 134–149, 2015.

[143] R. Japón-Luján, J. Ruiz-Jiménez, M. D. Luque de Castro, "Discrimination and Classification of Olive Tree Varieties and Cultivation Zones by Biophenol Contents", *J. Agric. Food Chem.*, 54(26), 9706–9712, Dec. 2006.

[144] K. G. Zinoviadou, C. M. Galanakis, M. Brnčić, N. Grimi, N. Boussetta, M. J. Mota, J. A. Saraiva, A. Patras, B. Tiwari, F. J. Barba, "Fruit juice sonication: Implications on food safety and physicochemical and nutritional properties", *Food Res. Int.*, 77, 743–752, 2015.

[145] W. W. Heng, L. W. Xiong, R. N. Ramanan, T. L. Hong, K. W. Kong, C. M. Galanakis, K. N. Prasad, "Two level factorial design for the optimization of phenolics and flavonoids recovery from palm kernel by-product", *Ind. Crop. Prod.*, 63, 238–248, 2015.

[146] F. Le Floch, M. T. Tena, A. Ríos, M. Valcárcel, "Supercritical fluid extraction of phenol compounds from olive leaves", *Talanta*, 46(5), 1123–1130, 1998.

[147] C. M. Galanakis, "Emerging technologies for the production of nutraceuticals from agricultural by-products: A viewpoint of opportunities and challenges", *Food Bioprod. Process.*, 91(4), 575–579, 2013.

[148] C. M. Galanakis, A. Schieber, "Editorial", *Food Res. Int.*, 65(PC), 299–300, 2014.

[149] E. Roselló-Soto, F. Barba, O. Parniakov, C. M. Galanakis, N. Lebovka, N. Grimi, E. Vorobiev, "High voltage electrical discharges, pulsed electric field, and ultrasound assisted extraction of protein and phenolic compounds from olive kernel", *Food Bioprocess Technol.*, 8(4), 885–894, 2015.

[150] A. Scoma, L. Bertin, G. Zanaroli, S. Fraraccio, F. Fava, "A physicochemical-biotechnological approach for an integrated valorization of olive mill wastewater", *Bioresour. Technol.*, 102(22), 10273–10279, 2011.

[151] C. M. Galanakis, "Separation of functional macromolecules and micromolecules: From ultrafiltration to the border of nanofiltration", *Trends Food Sci. Technol.*, 42(1), 44–63, 2015.

[152] C. A. Paraskeva, V. G. Papadakis, E. Tsarouchi, D. G. Kanellopoulou, P. G. Koutsoukos, "Membrane processing for olive mill wastewater fractionation", *Desalination*, 213(1–3), 218–229, 2007.

[153] C. A. Paraskeva, V. G. Papadakis, D. G. Kanellopoulou, P. G. Koutsoukos, K. C. Angelopoulos, "Membrane filtration of olive mill wastewater and exploitation of its fractions", *Water Environ. Res.*, 79(4), 421–429, 2007.

[154] T. Coskun, E. Debik, N. M. Demir, "Treatment of olive mill wastewaters by nanofiltration and reverse osmosis membranes", *Desalination*, 259(1–3), 65–70, 2010.

[155] A. De Leonardis, A. Aretini, G. Alfano, V. MacCiola, G. Ranalli, "Isolation of a hydroxytyrosol-rich extract from olive leaves (Olea Europaea L.) and evaluation of its antioxidant properties and bioactivity", *Eur. Food Res. Technol.*, 226(4), 653–659, 2008.

[156] C. Russo, "A new membrane process for the selective fractionation and total recovery of polyphenols, water and organic substances from vegetation waters (VW)", *J. Memb. Sci.*, 288(1), 239–246, 2007.

[157] C. M. Galanakis, "The universal recovery strategy", in *Food waste recovery*, Elsevier, 2015, 59–81.

[158] C. M. Galanakis, "Separation of functional macromolecules and micromolecules: From ultrafiltration to the border of nanofiltration", *Trends Food Sci. Technol.*, 42(1), 44–63, 2015.

[159] F. Sannino, A. De Martino, R. Capasso, I. El Hadrami, "Valorisation of organic matter in olive mill wastewaters: Recovery of highly pure hydroxytyrosol", *J. Geochemical Explor.*, 129, 34–39, 2013.

[160] L. Lesage-Meessen, D. Navarro, S. Maunier, J. C. Sigoillot, J. Lorquin, M. Delattre, J. L. Simon, M. Asther, M. Labat, "Simple phenolic content in olive oil residues as a function of extraction systems", *Food Chem.*, 75(4), 501–507, 2001.

[161] A. De Leonardis, V. Macciola, G. Lembo, A. Aretini, A. Nag, "Studies on oxidative stabilisation of lard by natural antioxidants recovered from olive-oil mill wastewater", *Food Chem.*, 100(3), 998–1004, 2007.

[162] S. Tsakona, C. M. Galanakis, V. Gekas, "Hydro-ethanolic mixtures for the recovery of phenols from Mediterranean plant materials", *Food Bioprocess Technol.*, 5(4), 1384–1393, 2012.

[163] C. M. Galanakis, V. Goulas, S. Tsakona, G. A. Manganaris, V. Gekas, "A knowledge base for the recovery of natural phenols with different solvents", *Int. J. Food Prop.*, 16(2), 382–396, 2013.

[164] L. Wang, C. L. Weller, "Recent advances in extraction of nutraceuticals from plants", *Trends Food Sci. Technol.*, 17(6), 300–312, 2006.

[165] S. Takaç, A. Karakaya, "Recovery of phenolic antioxidants from olive mill wastewater", *Recent Patents Chem. Eng.*, 2(3), 230–237, 2009.

[166] C. M. Galanakis, "Emerging technologies for the production of nutraceuticals from agricultural by-products: A viewpoint of opportunities and challenges", *Food Bioprod. Process.*, 91(4), 575–579, 2013.

[167] D. Deng, H. Deng, L. Zhang, Y. Su, "Determination of ephedrine and pseudoephedrine by field-amplified sample injection capillary electrophoresis", *J. Chromatogr. Sci.*, 52(4), 357–362, Apr. 2013.

[168] F. J. Barba, C. M. Galanakis, M. J. Esteve, A. Frigola, E. Vorobiev, "Potential use

of pulsed electric technologies and ultrasounds to improve the recovery of high-added value compounds from blackberries", *J. Food Eng.*, 167, 38–44, 2015.

[169]   W. H. Wong, W. X. Lee, R. N. Ramanan, L. H. Tee, K. W. Kong, C. M. Galanakis, J. Sun, K. N. Prasad, "Two level half factorial design for the extraction of phenolics, flavonoids and antioxidants recovery from palm kernel by-product", *Ind. Crops Prod.*, 63, 238–248, 2015.

[170]   K. G. Zinoviadou, C. M. Galanakis, M. Brnčić, N. Grimi, N. Boussetta, M. J. Mota, J. A. Saraiva, A. Patras, B. Tiwari, F. J. Barba, "Fruit juice sonication: Implications on food safety and physicochemical and nutritional properties", *Food Res. Int.*, 77, 743–752, 2015.

[171]   J. O'Sullivan, B. Murray, C. Flynn, I. Norton, "The effect of ultrasound treatment on the structural, physical and emulsifying properties of animal and vegetable proteins", *Food Hydrocoll.*, 53, 141–154, 2016.

[172]   S. Şahin, R. Şamlı, "Optimization of olive leaf extract obtained by ultrasound-assisted extraction with response surface methodology", *Ultrason. Sonochem.*, 20(1), 595–602, 2013.

[173]   N. Boussetta, E. Vorobiev, "Extraction of valuable biocompounds assisted by high voltage electrical discharges: A review" *Comptes Rendus Chim.*, 17(3), 197–203, 2014.

[174]   E. Vorobiev, N. Lebovka, "Enhanced extraction from solid foods and biosuspensions by pulsed electrical energy", *Food Eng. Rev.*, 2(2), 95–108, 2010.

[175]   I. Sabbah, T. Marsook, S. Basheer, "The effect of pretreatment on anaerobic activity of olive mill wastewater using batch and continuous systems", *Process Biochem.*, 39(12), 1947–1951, 2004.

[176]   M. Achak, A. Hafidi, N. Ouazzani, S. Sayadi, L. Mandi, "Low cost biosorbent 'banana peel' for the removal of phenolic compounds from olive mill wastewater: Kinetic and equilibrium studies", *J. Hazard. Mater.*, 166(1), 117–125, 2009.

[177]   S. J. Doktycz, K. S. Suslick, "Interparticle collisions driven by ultrasound", *Science*, 247(4946), 1067–1069, Mar. 1990.

[178]   M. C. Herrera, M. D. Luque De Castro, "Ultrasound-assisted extraction of phenolic compounds from strawberries prior to liquid chromatographic separation and photodiode array ultraviolet detection", *J. Chromatogr. A*, 1100(1), 1–7, 2005.

[179]   T. J. Mason, L. Paniwnyk, J. P. Lorimer, "The uses of ultrasound in food technology", *Ultrason. Sonochem.*, 3(3), 1996.

[180]   J. Azmir, I. Zaidul, M. Rahman, K. Sharif, A. Mohamed, F. Sahena, M. Jahurul, K. Ghafoor, N. Norulaini, A. Omar, "Techniques for extraction of bioactive compounds from plant materials: A review", *J. Food Eng.*, 117(4), 426–436, 2013.

[181]   O. A. Mudimu, M. Peters, F. Brauner, G. Braun, "Overview of membrane processes for the recovery of polyphenols from olive mill wastewater olive mill wastewater", *Am. J. Environ. Sci.*, 8(3), 195–201, 2012.

[182]   C. M. Galanakis, S. Chasiotis, G. Botsaris, V. Gekas, "Separation and recovery of

proteins and sugars from Halloumi cheese whey", *Food Res. Int.*, 65(PC), 477–483, 2014.

[183]   C. M. Galanakis, E. Markouli, V. Gekas, "Recovery and fractionation of different phenolic classes from winery sludge using ultrafiltration", *Sep. Purif. Technol.*, 107, 245–251, 2013.

[184]   E. Turano, S. Curcio, M. G. De Paola, V. Calabrò, G. Iorio, "An integrated centrifugation – ultrafiltration system in the treatment of olive mill wastewater", 209, 519–531, 2002.

[185]   M. Pizzichini, Process for recovering the components of olive mill wastewater with membrane technologies, WO 2005/123603 A1, (June, 16, 2004)

[186]   L. Villanova, L. Villanova, G. Fasiello, A. Merendino, Process for the recovery of tyrosol and hydroxytyrosol from oil mill wastewaters and catalytic oxidation method in order to convert tyrosol in hydroxytyrosol, US20060070953A1, (April, 6, 2006)

[187]   H. I. Abdel-Shafy, G. Schories, M. S. Mohamed-Mansour, V. Bordei, "Integrated membranes for the recovery and concentration of antioxidant from olive mill wastewater", *Desalin. Water Treat.*, 56(2), 305–314, Oct. 2015.

[188]   D. R. Kammerer, Z. S. Saleh, R. Carle, R. A. Stanley, "Adsorptive recovery of phenolic compounds from apple juice", *Eur. Food Res. Technol.*, 224(5), 605–613, 2007.

[189]   J. Kammerer, D. R. Kammerer, U. Jensen, R. Carle, "Interaction of apple polyphenols in a multi-compound system upon adsorption onto a food-grade resin", *J. Food Eng.*, 96(4), 544–554, 2010.

[190]   M. L. Soto, A. Moure, H. Domínguez, J. C. Parajó, "Recovery, concentration and purification of phenolic compounds by adsorption: A review", *J. Food Eng.*, 105(1), 1–27, 2011.

[191]   M. Niaounakis, C. P. Halvadakis, "Olive processing waste management: literature review and patent survey", *Elsevier*, 2006.

[192]   J. Fernández-Bolaños, A. Heredia, G. Rodrıguez, R. Rodrıguez, R. Guillén, A. Jiménez, "Method for obtaining purified hydroxytyrosol from products and by-products derived from the olive tree, World Intellectual Property Organization", WO/2002/064537, 2002.

[193]   J. G. Fernandez-Bolanos, O. Lopez, J. F.-B., G. Rodriguez-Gutierrez, "Hydroxytyrosol and Derivatives: Isolation, Synthesis, and Biological Properties", *Current Organic Chemistry*, 12(6). 442–463, 2008.

[194]   Z. Kaleh, S. U. Geißen, "Selective isolation of valuable biophenols from olive mill wastewater", *J. Environ. Chem. Eng.*, 4(1), 373–384, 2016.

[195]   J. Toth, "Adsorption: Theory, Modeling and Analysis. Surfactant Sci. Series", vol. 107, Dekker, New York, 2002.

[196]   D. Do Duong, "Adsorption Analysis: Equilibria And Kinetics (With CD Containing

Computer Matlab Programs)", *World Scientific*, vol. 2, 1998.

[197]   D. M. Ruthven, "Principles of adsorption and adsorption processes", *John Wiley & Sons*, 1984.

[198]   B. Crittenden, W. J. Thomas, "Adsorption technology and design", *Elsevier*, 1998.

[199]   M. Suzuki, "Adsorption engineering", *Kodansha*, 1990.

[200]   A. Dąbrowski, P. Podkościelny, Z. Hubicki, and M. Barczak, "Adsorption of phenolic compounds by activated carbon - A critical review", *Chemosphere*, 58(8), 1049–1070, 2005.

[201]   M. Ahmaruzzaman, "Adsorption of phenolic compounds on low-cost adsorbents: A review", *Adv. Colloid Interface Sci.*, 143(1–2), 48–67, 2008.

[202]   G. Berčič, A. Pintar, J. Levec, "Desorption of phenol from activated carbon by hot water regeneration. Desorption isotherms", *Ind. Eng. Chem. Res.*, 35(12), 4619–4625, 1996.

[203]   D. R. Kammerer, R. Carle, R. A. Stanley, Z. S. Saleh, "Pilot-scale resin adsorption as a means to recover and fractionate apple polyphenols", *J. Agric. Food Chem.*, 58(11), 6787–6796, 2010.

[204]   M. Carmona, A. De Lucas, J. L. Valverde, B. Velasco, J. F. Rodriguez, "Combined adsorption and ion exchange equilibrium of phenol on Amberlite IRA-420", *Chem. Eng. J.*, 117(2), 155–160, 2006.

[205]   M. Caetano, C. Valderrama, A. Farran, and J. L. Cortina, "Phenol removal from aqueous solution by adsorption and ion exchange mechanisms onto polymeric resins", *J. Colloid Interface Sci.*, 338(2), 402–409, 2009.

[206]   K. Mohanty, M. Jha, B. C. Meikap, and M. N. Biswas, "Preparation and characterization of activated carbons from Terminalia arjuna nut with zinc chloride activation for the removal of phenol from wastewater", *Ind. Eng. Chem. Res.*, 44(11), 4128–4138, 2005.

[207]   J. F. Garcia-Araya, F. J. Beltran, P. Alvarez, F. J. Masa, "Activated carbon adsorption of some phenolic compounds present in agroindustrial wastewater", *Adsorption*, 9(2), 107–115, 2003.

[208]   V. Caqueret, S. Bostyn, B. Cagnon, H. Fauduet, "Purification of sugar beet vinasse– adsorption of polyphenolic and dark colored compounds on different commercial activated carbons", *Bioresour. Technol.*, 99(13), 5814–5821, 2008.

[209]   M. Ugurlu, A. Gurses, M. Yalcin, C. Dogar, "Removal of phenolic and lignin compounds from bleached kraft mill effluent by fly ash and sepiolite", *Adsorption*, 11(1), 87–97, 2005.

[210]   European Medicines Agency, "Summary of Information Supporting the Generally recognized as safe (GRAS) status of hydroxytyrosol (>99 % pure) for use as an ingredient in selected foods," https://www.fda.gov/food/generally-recognized-safe-gras/gras-notice-inventory, no. 726, 2017.

[211]   K. G. H. Desai, H. Jin Park, "Recent developments in microencapsulation of food

ingredients", *Dry. Technol.*, 23(7), 1361–1394, 2005.

[212] P. Vilstrup, *Microencapsulation of food ingredients*. Woodhead Publishing Limited, 2001.

[213] M. R. Mozafari, "Bioactive entrapment and targeting using nanocarrier technologies: an introduction", in *Nanocarrier technologies*, Springer, 2006, 1–16.

[214] B. F. GIBBS, "a kol. Encapsulation in the food industry: a review", *Int. J. Food Sci. Nutr.*, 385–390.

[215] M. R. Mozafari, K. Khosravi-Darani, G. G. Borazan, J. Cui, A. Pardakhty, S. Yurdugul, "Encapsulation of food ingredients using nanoliposome technology", *Int. J. Food Prop.*, 11(4), 833–844, 2008.

[216] M. A. Augustin, Y. Hemar, "Nano-and micro-structured assemblies for encapsulation of food ingredients", *Chem. Soc. Rev.*, 38(4), 902–912, 2009.

[217] F. Shahidi, X. Han, "Encapsulation of food ingredients", *Crit. Rev. Food Sci. Nutr.*, vol. 33(6), pp. 501–547, 1993.

[218] Z. Fang, B. Bhandari, "Encapsulation of polyphenols - A review", *Trends Food Sci. Technol.*, 21(10), 510–523, 2010.

[219] R. E. C. Wildman, R. S. Bruno, *Handbook of nutraceuticals and functional foods*. CRC press, 2019.

[220] A. Munin, F. Edwards-Lévy, "Encapsulation of natural polyphenolic compounds; a review", *Pharmaceutics*, 3(4), 793–829, 2011.

[221] L. Chen, G. E. Remondetto, M. Subirade, "Food protein-based materials as nutraceutical delivery systems", *Trends Food Sci. Technol.*, 17(5), 272–283, 2006.

[222] D. J. McClements, "Emulsion design to improve the delivery of functional lipophilic components", *Annu. Rev. Food Sci. Technol.*, 1, 241–269, 2010.

[223] U. Klinkesorn, P. Sophanodora, P. Chinachoti, D. J. McClements, E. A. Decker, "Increasing the oxidative stability of liquid and dried tuna oil-in-water emulsions with electrostatic layer-by-layer deposition technology", *J. Agric. Food Chem.*, 53(11), 4561–4566, 2005.

[224] L. Mao, Y. H. Roos, D. J. O'Callaghan, S. Miao, "Volatile release from whey protein isolate–pectin multilayer stabilized emulsions: effect of pH, salt, and artificial salivas", *J. Agric. Food Chem.*, 61(26), 6231–6239, 2013.

[225] X. Wang, Y. Jiang, Y.-W. Wang, M.-T. Huang, C.-T. Ho, Q. Huang, "Enhancing anti-inflammation activity of curcumin through O/W nanoemulsions", *Food Chem.*, 108(2), 419–424, 2008.

[226] W. Lu, A. L. Kelly, S. Miao, "Emulsion-based encapsulation and delivery systems for polyphenols", *Trends Food Sci. Technol.*, 47, 1–9, 2016.

[227] T. Tadros, P. Izquierdo, J. Esquena, C. Solans, "Formation and stability of nano-emulsions", *Adv. Colloid Interface Sci.*, 108, 303–318, 2004.

[228] K. P. Velikov, E. Pelan, "Colloidal delivery systems for micronutrients and

nutraceuticals", *Soft Matter*, 4(10), 1964–1980, 2008.

[229] T. G. Mason, J. N. Wilking, K. Meleson, C. B. Chang, S. M. Graves, "Nanoemulsions: formation, structure, and physical properties", *J. Phys. Condens. Matter*, 18(41), p. R635, 2006.

[230] T. J. Wooster, M. Golding, P. Sanguansri, "Impact of Oil Type on Nanoemulsion Formation and Ostwald Ripening Stability", *Langmuir*, 24(22), 12758–12765, Nov. 2008.

[231] D. J. McClements, J. Rao, "Food-Grade nanoemulsions: Formulation, fabrication, properties, performance, biological fate, and potential Toxicity", *Crit. Rev. Food Sci. Nutr.*, 51(4), 285–330, 2011.

[232] C. M. Galanakis, "Olive fruit dietary fiber: Components, recovery and applications", *Trends Food Sci. Technol.*, 22(4), 175–184, 2011.

[233] J. M. Romero-García, A. Sanchez, G. Rendón-Acosta, J. C. Martínez-Patiño, E. Ruiz, G. Magaña, E. Castro, "Ethanol production from glucose and xylose obtained from steam exploded water-extracted olive tree pruning using phosphoric acid as catalyst", *Bioresour. Technol.*, 153, 101–107, 2014.

[234] I.Ballesteros, M.Ballesteros, C.Cara, F.Sáez, E.Castro, P.Manzanares, M.J.Negro, J.M.Oliva, "Effect of water extraction on sugars recovery from steam exploded olive tree pruning", *Bioresour. Technol.*, 102(11), 6611–6616, 2011.

[235] P. Alvira, E. Tomás-Pejó, M. Ballesteros, M. J. Negro, "Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review", *Bioresour. Technol.*, 101(13), 4851–4861, 2010.

[236] A. Vishtal, A. Kraslawski, "Challenges in industrial applications of technical lignins", *BioResources*, 6(3), 3547–3568, 2011.

[237] E. de Jong, A. Higson, P. Walsh, M. Wellisch, "Task 42 Biobased Chemicals - Value Added Products from Biorefineries", *A Rep. Prep. IEA Bioenergy-Task*, p. 36, 2011.

[238] P. Martin-Olmedo, F. Cabrera, R. Lopez, J. M. Murillo, "Residual effect of composted olive oil mill sludge on plant growth", *Fresenius Environ. Bull.*, 4(4), 221–226, 1995.

[239] M. I. Massadeh, N. Modallal, "Ethanol Production from Olive Mill Wastewater (OMW) Pretreated with Pleurotus sajor-caju", *Energy & Fuels*, 22(1), 150–154, Jan. 2008.

[240] E. Eroğlu, U. Gündüz, M. Yücel, L. Türker, I. Eroğlu, "Photobiological hydrogen production by using olive mill wastewater as a sole substrate source", *Int. J. Hydrogen Energy*, 29(2), 163–171, 2004.

[241] E. Eroğlu, I. Eroğlu, U. Gündüz, M. Yücel, "Treatment of olive mill wastewater by different physicochemical methods and utilization of their liquid effluents for biological hydrogen production", *Biomass and Bioenergy*, 33(4), 701–705, 2009.

[242] A. Gunay, D. Karadag, "Recent developments in the anaerobic digestion of olive

mill effluents", *Process Biochem.*, 50(11), 1893–1903, 2015.

[243] F. Paiva-Martins, T. Ribeirinha, A. Silva, R. Gonçalves, V. Pinheiro, J. L. Mourão, D. Outor-Monteiro, "Effects of the dietary incorporation of olive leaves on growth performance, digestibility, blood parameters and meat quality of growing pigs", *J. Sci. Food Agric.*, 94(14), 3023–3029, Nov. 2014.

[244] S. Charoenprasert, A. Mitchell, "Factors influencing phenolic compounds in table olives (Olea europaea)", *J. Agric. Food Chem.*, 60(29), 7081–7095, 2012.

[245] R. Mateos, G. Pereira-Caro, S. Saha, R. Cert, M. Redondo-Horcajo, L. Bravo, P. A. Kroon, "Acetylation of hydroxytyrosol enhances its transport across differentiated Caco-2 cell monolayers", *Food Chem.*, 125(3), 865–872, 2011.

[246] R. Bernini, M. S. Gilardini Montani, N. Merendino, A. Romani, F. Velotti, "Hydroxytyrosol-derived compounds: A basis for the creation of new pharmacological agents for cancer prevention and therapy", *J. Med. Chem.*, 58(23), 9089–9107, Dec. 2015.

[247] M. Trujillo, E. Gallardo, A. Madrona, L. Bravo, B. Sarriá, J. A. González-Correa, R. Mateos, J. L. Espartero, "Synthesis and Antioxidant Activity of Nitrohydroxytyrosol and Its Acyl Derivatives", *J. Agric. Food Chem.*, 62(42), 10297–10303, Oct. 2014.

[248] J. Manuel, M. L. Orta, S. Mateos, J. L. Espartero, M. Lo, "Selective cytotoxic activity of new lipophilic hydroxytyrosol alkyl ether derivatives", *J. Agric. Food Chem.*, 61(21), 5046–5053, May 2013.

[249] M. Robles-almazan, M. Pulido-moran, J. Moreno-fernandez, C. Ramirez-tortosa, C. Rodriguez-garcia, J. Quiles, "Hydroxytyrosol: Bioavailability, toxicity, and clinical applications", *Food Res. Int.*, 105, 654–667, 2018.

[250] S. Granados-principal, J. L. Quiles, C. L. Ramirez-tortosa, P. Sanchez-rovira, and M. C. Ramirez-tortosa, "Hydroxytyrosol : from laboratory investigations to future clinical trials", *Nutrition Reviews,* 68(4), 191–206, 2010.

[251] F. Visioli, C. Galli, S. Grande, K. Colonnelli, C. Patelli, and G. Galli, "Nutrient metabolism- research communication hydroxytyrosol xxcretion differs between rats and humans and depends on the vehicle of administration", *American Society for Nutritional Sciences*, 2612–2615, 2003.

[252] L. Rubió, R. Valls, A. Macià, A. Pedret, M. Giralt, M. P. Romero, R. Torre, M. I. Covas, R. Solà, M. J. Motilva, "Impact of olive oil phenolic concentration on human plasmatic phenolic metabolites", *Food Chem.,* 135, 2922–2929, 2012.

[253] M. González-santiago, J. Fonollá, E. Lopez-huertas, "Human absorption of a supplement containing purified hydroxytyrosol, a natural antioxidant from olive oil, and evidence for its transient association with low-density lipoproteins", *Pharmacol. Res.*, 61, 364–370, 2010.

[254] K. L. Tuck, M. P. Freeman, P. J. Hayball, G. L. Stretch, I. Stupans, "The in vivo fate of hydroxytyrosol and tyrosol, antioxidant phenolic constituents of olive oil, after intravenous and oral dosing of labeled compounds to rats", *J. Nutr.*, 131(7),

1993–1996, 2001.

[255] M. Suárez, R. M. Valls, M. P. Romero, A. Macia, S. Fernández, M. Giralt, R. SM. J. Motilva, "Bioavailability of phenols from a phenol-enriched olive oil", *Br. J. Nutr.*, 106(11), 1691–1701, 2011.

[256] E. Miro-casas, M. Covas, M. Farre, "Hydroxytyrosol disposition in humans", *Clinical Chemistry*, 952, 945–952, 2003.

[257] L. Rubió, M. Farràs, R. D. La, A. Macià, M. Romero, R. M. Valls, R. Solà, M. Farré, M. Motilva, Metabolite pro fi ling of olive oil and thyme phenols after a sustained intake of two phenol-enriched olive oils by humans: Identification of compliance markers. *FRIN*, *65*, 59–68, 2014

[258] O. Khymenets, M. C. Crespo, O. Dangles, "Human hydroxytyrosol's absorption and excretion from a nutraceutical", *J. Funct. Foods*, 23, 278–282, 2016.

[259] M. N. Vissers, P. L. Zock, A. J. C. Roodenburg, R. Leenen, M. B. Katan, "Olive Oil Phenols Are Absorbed in Humans", *J. Nutr.*, 132(3), 409–417, 2002.

[260] S. Roowi, W. Mullen, C. A. Edwards, A. Crozier, "Yoghurt impacts on the excretion of phenolic acids derived from colonic breakdown of orange juice flavanones in humans", *Mol. Nutr. Food Res.*, 53(S1), S68–S75, 2009.

[261] M. de Bock, E. B. Thorstensen, J. G. B. Derraik, H. V. Henderson, P. L. Hofman, W. S. Cutfield, "Human absorption and metabolism of oleuropein and hydroxytyrosol ingested as olive (Olea europaea L.) leaf extract", *Mol. Nutr. Food Res.*, 57(11), 2079–2085, 2013.

[262] R. Domínguez-Perles, D. Auñón, F. Ferreres, A. Gil-Izquierdo, "Gender differences in plasma and urine metabolites from Sprague–Dawley rats after oral administration of normal and high doses of hydroxytyrosol, hydroxytyrosol acetate, and DOPAC", *Eur. J. Nutr.*, 56(1), 215–224, 2017.

[263] R. de la Torre, M. I. Covas, M. A. Pujadas, M. Fitó, M. Farré, "Is dopamine behind the health benefits of red wine?", *Eur. J. Nutr.*, 45(5), 307–310, 2006.

[264] C. Pérez-Mañá, M. Farré, M. Pujadas, C. Mustata, E. Menoyo, A. Pastor, K. Langohr, R. De La Torre , "Ethanol induces hydroxytyrosol formation in humans", *Pharmacol. Res.*, 95–96, 27–33, 2015.

[265] C. Bai, X. Yan, M. Takenaka, K. Sekiya, T. Nagata, "Determination of synthetic hydroxytyrosol in rat plasma by GC-MS", *J. Agric. Food Chem.*, 46(10), 3998–4001, 1998.

[266] C. Fernández-Ávila, R. Montes, A. I. Castellote, A. M. Chisaguano, M. Fitó, M. I. Covas, D. Muñoz-Aguallo, K. Nyyssönen, H. J. Zunft, M. C. López-Sabater, "Fast determination of virgin olive oil phenolic metabolites in human high-density lipoproteins", *Biomed. Chromatogr.*, 29(7), 1035–1041, 2015.

[267] S. Granados-Principal, N. El-Azem, R. Pamplona, C. Ramirez-Tortosa, M. Pulido-Moran, L. Vera-Ramirez, J. L. Quiles, P. Sanchez-Rovira, A. Naudí, M. Portero Otin, P. Perez-Lopez, M. Ramirez-Tortosa , "Hydroxytyrosol ameliorates oxidative

stress and mitochondrial dysfunction in doxorubicin-induced cardiotoxicity in rats with breast cancer", *Biochem. Pharmacol.*, 90(1), 25–33, 2014.

[268]   S. D'Angelo, C. Manna, V. Migliardi, O. Mazzoni, P. Morrica, G. Capasso, G. Pontoni, P. Galletti, V. Zappia, "Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil", *Drug Metab. Dispos.*, 29(11), 1492–1498, 2001.

[269]   A. Serra, L. Rubió, X. Borràs, A. Macià, M. P. Romero, M. J. Motilva, "Distribution of olive oil phenolic compounds in rat tissues after administration of a phenolic extract from olive cake", *Mol. Nutr. Food Res.*, 56(3), 486–496, 2012.

[270]   J. I. Mosele, S. Martín-Peláez, A. Macià, M. Farràs, R. Valls, Ú. Catalán, M. Motilva, "Faecal microbial metabolism of olive oil phenolic compounds: In vitro and in vivo approaches", *Mol. Nutr. Food Res.*, 58(9), 1809–1819, 2014.

[271]   F. Visioli, C. Galli, S. Grande, K. Colonnelli, C. Patelli, G. Galli, "Nutrient metabolism-research communication hydroxytyrosol excretion differs between rats and humans and depends on the vehicle of administration", *J. Nutr.*, 133(8), 2612–2615, 2003.

[272]   M. Suárez, R. M. Valls, M. Romero, A. Macia, S. Fernández, M. Giralt, R. Sola, M. Motilva, "Bioavailability of phenols from a phenol-enriched olive oil", *Br. J. Nutr.*, 106(11), 1691–1701, 2011.

[273]   J. Rodríguez-Morató, A. Boronat, A. Kotronoulas, M. Pujadas, A. Pastor, E. Olesti, C. Perez-Mana, O. Khymenets, M. Fito, M. Farre, "Metabolic disposition and biological significance of simple phenols of dietary origin: hydroxytyrosol and tyrosol", *Drug Metab. Rev.*, 48(2), 218–236, 2016.

[274]   N. A. Chashmi, S. Emadi, H. Khastar, "Protective effects of hydroxytyrosol on gentamicin induced nephrotoxicity in mice", *Biochem. Biophys. Res. Commun.*, 482(4), 1427–1429, 2017.

[275]   H. Babich, F. Visioli, "In vitro cytotoxicity to human cells in culture of some phenolics from olive oil", *Farmaco*, 58(5), 403–407, 2003.

[276]   M. S. Christian, V. A. Sharper, A. M. Hoberman, J. E. Seng, L. Fu, D. Covell, R. M. Diener, C. M. Bitler, R. Crea, "The toxicity profile of hydrolyzed aqueous olive pulp extract", *Drug Chem. Toxicol.*, 27(4), 309–330, 2004.

[277]   D. Auñon-Calles, L. Canut, F. Visioli, "Toxicological evaluation of pure hydroxytyrosol", *Food Chem. Toxicol.*, 55, 498–504, 2013.

[278]   D. Auñon-Calles, E. Giordano, S. Bohnenberger, F. Visioli, "Hydroxytyrosol is not genotoxic in vitro", *Pharmacol. Res.*, 74, 87–93, 2013.

[279]   J. Heilman, N. Anyangwe, N. Tran, J. Edwards, P. Beilstein, J. López, "Toxicological evaluation of an olive extract, H35: Subchronic toxicity in the rat", *Food Chem. Toxicol.*, 84, 18–28, 2015.

[280]   D. Kirkland, J. Edwards, T. Woehrle, P. Beilstein, "Investigations into the genotoxic potential of olive extracts", *Mutat. Res.- Genet. Toxicol. Environ. Mutagen.*, 777(1), 17–28, 2015.

[281] S. Burattini, S. Salucci, V. Baldassarri, A. Accorsi, E. Piatti, A. Madrona, J. L. Espartero, M. Candiracci, G. Zappia, E. Falcieri , "Anti-apoptotic activity of hydroxytyrosol and hydroxytyrosyl laurate", *Food Chem. Toxicol.*, 55, 248–256, 2013.

[282] J. Anter, I. Tasset, S. Demyda-Peyrás, I. Ranchal, M. Moreno-Millán, M. Romero-Jimenez, J. Muntané, M. Dolores L. de Castro, A. Muñoz-Serrano, Á. Alonso-Moraga, "Evaluation of potential antigenotoxic, cytotoxic and proapoptotic effects of the olive oil by-product 'alperujo', hydroxytyrosol, tyrosol and verbascoside", *Mutat. Res.-Genet. Toxicol. Environ. Mutagen.*, 772, 25–33, 2014.

[283] E. Medina, A. de Castro, C. Romero, M. Brenes, "Comparison of the concentrations of phenolic compounds in olive oils and other plant oils: correlation with antimicrobial activity", *J. Agric. Food Chem.*, 54(14), 4954–4961, Jul. 2006.

[284] N. Zoric, I, Horvat, N. Kopjar, A. Vucemilovic, D. Kremer, S. Tomic, I. Kosalec, "Hydroxytyrosol expresses antifungal activity in vitro", *Curr. Drug Targets*, 14(9), 992–998, 2013.

[285] G. Bisignano, A. Tomaino, R. Cascio, G. Crisafi, "On the in-vitro antimicrobial activity of oleuropein and hydroxytyrosol", *J. Pharm. Pharmacol,* 971–974, 1999.

[286] J. D. Kyriazis, N. Aligiannis, P. Polychronopoulos, A. Skaltsounis, E. Dotsika, "Phytomedicine Leishmanicidal activity assessment of olive tree extracts", *Eur. J. Integr. Med.*, 20(3–4), 275–281, 2013.

[287] E. Zwane, A. Parker, T. Kudanga, L. M. Davids, S. G. Burton, "Novel, biocatalytically produced hydroxytyrosol dimer protects against ultraviolet-induced cell death in human immortalized keratinocytes", *J. Agric. Food Chem.*, 60, 11509–17, 2012.

[288] M. Deiana, O. Aruoma, P. Maria de Lourdes, J. Spencer, H. Kaur, B. Halliwell, R. Aeschbach, S. Banni, M. A. Dessi, F. Corongiu , "Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol", *Free Radic. Biol. Med.*, 26(5–6), 762–769, 1999.

[289] C. Manna, F. Della Ragione, V. Cucciolla, A. Borriello, S. D'Angelo, P. Galletti, V. Zappia, "Biological effects of hydroxytyrosol, a polyphenol from olive oil endowed with antioxidant activity", in *Advances in Nutrition and Cancer 2*, Springer, 1999, 115–130.

[290] S. Granados-Principal, J. Quiles, C. Ramirez-Tortosa, J. Ochoa-Herrera, P. Perez-Lopez, M. Pulido-Moran, M. Ramirez-Tortosa, "Squalene ameliorates atherosclerotic lesions through the reduction of CD 36 scavenger receptor expression in macrophages", *Mol. Nutr. Food Res.*, 56(5), 733–740, 2012.

[291] S. Granados-Principal, N. El-Azem, R. Pamplona, C. Ramirez-Tortosa, M. Pulido-Moran, L. Vera-Ramirez, J. Quiles, P. Sanchez-Rovira, A. Naudí, M. Portero-Otin, P. Perez-Lopez, M. Ramirez-Tortosa, "Hydroxytyrosol ameliorates oxidative stress and mitochondrial dysfunction in doxorubicin-induced cardiotoxicity in rats with breast cancer", *Biochem. Pharmacol.*, 90, 25–33, 2014.

[292] V. Nogueira, Y. Park, C. Chen, P. Xu, M. Chen, I. Tonic, T. Unterman, N. Hay, "Akt determines replicative senescence and oxidative or oncogenic premature senescence and sensitizes cells to oxidative apoptosis", *Cancer Cell*, 14(6), 458–470, 2008.

[293] A. Maalej, Z. Bouallagui, F. Hadrich, H. Isoda, and S. Sayadi, "ScienceDirect Assessment of Olea europaea L . fruit extracts : Phytochemical characterization and anticancer pathway investigation", *Biomed. Pharmacother.*, 90, 179–186, 2017.

[294] A. Atzeri, R. Lucas, A. Incani, P. Peñalver, A. Zafra-Gómez, M. Melis, R. Pizzala, J. C. Morales, M. Deiana, "Hydroxytyrosol and tyrosol sulfate metabolites protect against the oxidized cholesterol pro-oxidant effect in Caco-2 human enterocyte-like cells", *Food Funct.*, 7(1), 337–346, 2016.

[295] L. Sun, C. Luo, J. Liu, "Hydroxytyrosol induces apoptosis in human colon cancer cells through ROS generation", *Food Funct.*, 5(8), 1909–1914, 2014.

[296] C. Luo, Y. Li, H. Wang, Y. Cui, Z. Feng, H. Li, Y. Li, Y. Wang, K. Wurtz, P. Weber, "Hydroxytyrosol promotes superoxide production and defects in autophagy leading to anti-proliferation and apoptosis on human prostate cancer cells", *Curr. Cancer Drug Targets*, 13(6), 625–639, 2013.

[297] P. Rosignoli, R. Fuccelli, M. V. Sepporta, R. Fabiani, "In vitro chemo-preventive activities of hydroxytyrosol: The main phenolic compound present in extra-virgin olive oil", *Food Funct.*, 7(1), 301–307, 2016.

[298] G. Bjelakovic, D. Nikolova, C. Gluud, "Antioxidant supplements and mortality", *Curr. Opin. Clin. Nutr. Metab. Care*, 17(1), 40–44, 2014.

[299] F. Warleta, C. S. Quesada, M. Campos, Y. Allouche, G. Beltrán, J. J. Gaforio, "Hydroxytyrosol protects against oxidative DNA damage in human breast cells", *Nutrients*, 3(10), 839–857, 2011.

[300] M. Gonz, E. Mart, "One-month administration of hydroxytyrosol, a phenolic antioxidant present in olive oil, to hyperlipemic rabbits improves blood lipid profile, antioxidant status and reduces atherosclerosis development", *Atherosclerosis*, 188, 35–42, 2006.

[301] H. Poudyal, F. Campbell, L. Brown, "Olive leaf extract attenuates cardiac, hepatic, and metabolic changes in high carbohydrate-, high fat-fed rats", *J. Nutr.*, 140(5), 946–953, 2010.

[302] H. Jemai, M. Bouaziz, I. Fki, A. El, S. Sayadi, "Chemico-biological interactions hypolipidimic and antioxidant activities of oleuropein and its hydrolysis derivative-rich extracts from Chemlali olive leaves", *Chemico-Biological Interactions*, 176, 88–98, 2008.

[303] M. T. Khayyal, M. A. El-Ghazaly, D. M. Abdallah, N. N. Nassar, S. N. Okpanyi, M.-H. Kreuter, "Blood pressure lowering effect of an olive leaf extract (Olea europaed) in L-NAME induced hypertension in rats", *Arzneimittelforschung*, 52(11), 797–802, 2002.

[304] Ú. Catalán, L. Rubió, M. López de las Hazas, P. Herrero, P. Nadal, N. Canela, A. Pedret, M. Motilva, R. Solà, "Hydroxytyrosol and its complex forms (secoiridoids)

modulate aorta and heart proteome in healthy rats: Potential cardio-protective effects", *Mol. Nutr. Food Res.*, 60(10), 2114–2129, 2016.

[305]   P. Dandona, A. Aljada, A. Chaudhuri, P. Mohanty, R. Garg, "Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation", *Circulation*, 111(11), 1448–1454, 2005.

[306]   M. A. Cornier, D. Dabelea, T. Hernandez, R. Lindstrom, A. Steig, N. Stob, R. Van Pelt, H. Wang, R. Eckel , "The metabolic syndrome", *Endocr. Rev.*, 29(7), 777–822, 2008.

[307]   M. de Bock, J. Derraik, C. Brennan, J. Biggs, P. Morgan, S. Hodgkinson, P. Hofman, W. Cutfield, "Olive (Olea europaea L.) leaf polyphenols improve insulin sensitivity in middle-aged overweight men: a randomized, placebo-controlled, crossover trial", *PLoS One*, 8(3), p. e57622, 2013.

[308]   A. Gumieniczek, "Effects of pioglitazone on hyperglycemia-induced alterations in antioxidative system in tissues of alloxan-treated diabetic animals", *Exp. Toxicol. Pathol.*, 56(4–5), 321–326, 2005.

[309]   J. Chaudhry, N. N. Ghosh, K. Roy, R. Chandra, "Antihyperglycemic effect of a new thiazolidinedione analogue and its role in ameliorating oxidative stress in alloxan-induced diabetic rats", *Life Sci.*, 80(12), 1135–1142, 2007.

[310]   K. Hamden, N. Allouche, B. Jouadi, S. El-Fazaa, N. Gharbi, S. Carreau, M. Damak, A. Elfeki, "Inhibitory action of purified hydroxytyrosol from stored olive mill waste on intestinal disaccharidases and lipase activities and pancreatic toxicity in diabetic rats", *Food Sci. Biotechnol.*, 19(2), 439–447, 2010.

[311]   K. Cao, J. Xu, X. Zou, Y. Li, C. Chen, A. Zheng, H. Li, H. Li, I. Szeto, Y. Shi, J. Long, J. Liu, Z. Feng, "Free Radical Biology and Medicine Hydroxytyrosol prevents diet-induced metabolic syndrome and attenuates mitochondrial abnormalities in obese mice", *Free Radic. Biol. Med.*, 67, 396–407, 2014.

[312]   A. Zheng, H. Li, J. Xu, K. Cao, H. Li, W. Pu, Z. Yang, Y. Peng, J. Long, J. Liu, "Hydroxytyrosol improves mitochondrial function and reduces oxidative stress in the brain of db/db mice: role of AMP-activated protein kinase activation", *Br. J. Nutr.*, 113(11), 1667–1676, 2015.

[313]   S. Granados-Principal, J. L. Quiles, C. L. Ramirez-Tortosa, P. Sanchez-Rovira, M. C. Ramirez-Tortosa, "Hydroxytyrosol: from laboratory investigations to future clinical trials", *Nutr. Rev.*, 68(4), 191–206, 2010.

[314]   A. Camargo, J. Ruano, J. Fernandez, L. Parnell, A. Jimenez, M. Santos-Gonzalez, C. Marin, P. Perez-Martinez, M. Uceda, J. Lopez-Miranda, "Gene expression changes in mononuclear cells in patients with metabolic syndrome after acute intake of phenol-rich virgin olive oil", *BMC Genomics*, 11(1), 253, 2010.

[315]   N. Richard, S. Arnold, U. Hoeller, C. Kilpert, K. Wertz, J. Schwager, "Hydroxytyrosol is the major anti-inflammatory compound in aqueous olive extracts and impairs cytokine and chemokine production in macrophages", *Planta Med.*, 77(17), 1890–1897, 2011.

[316]   F. Visioli, G. Bellomo, C. Galli, "Free radical-scavenging properties of olive oil

polyphenols", *Biochem. Biophys. Res. Commun.*, 247(1), 60–64, 1998.

[317]   L. Goya, R. Mateos, L. Bravo, "Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells: Protection against oxidative stress induced by tert-butylhydroperoxide", *Eur. J. Nutr.*, 46(2), 70–78, 2007.

[318]   M. A. Martin, S. Ramos, A. Granado-Serrano, I. Rodriguez-Ramiro, M. Trujillo, L. Bravo, L. Goya, "Hydroxytyrosol induces antioxidant/detoxificant enzymes and Nrf2 translocation via extracellular regulated kinases and phosphatidylinositol-3-kinase/ protein kinase B pathways in HepG2 cells", *Mol. Nutr. Food Res.*, 54(7), 956–966, 2010.

[319]   D. T. Dextera, P. Jenner, "Parkinson disease: From pathology to molecular disease mechanisms", *Free Radic. Biol. Med.*, 62, 132–144, 2013.

[320]   S. Schaffer, M. Podstawa, F. Visioli, P. Bogani, W. E. Müller, G. P. Eckert, "Hydroxytyrosol-rich olive mill wastewater extract protects brain cells in vitro and ex vivo", *J. Agric. Food Chem.*, 55(13), 5043–5049, 2007.

[321]   J. A. González-Correa, M. D. Navas, J. A. Lopez-Villodres, M. Trujillo, J. L. Espartero, J. P. De La Cruz, "Neuroprotective effect of hydroxytyrosol and hydroxytyrosol acetate in rat brain slices subjected to hypoxia-reoxygenation", *Neurosci. Lett.*, 446(2–3), 143–146, 2008.

[322]   J. Muñoz-Marín, J. De La Cruz, A. Guerrero, I. López-Leiva, J. López-Villodres, J. Reyes, J. Espartero, A. Madrona, M. Labajos, J. González-Correa , "Cytoprotective effect of hydroxytyrosyl alkyl ether derivatives after oral administration to rats in a model of glucose-oxygen deprivation in brain slices", *J. Agric. Food Chem.*, 60(31), 7659–7664, 2012.

[323]   R. W. Owen, A. Giacosa, W. Hull, R. Haubner, G. Würtele, B. Spiegelhalder, H. Bartsch , "Olive-oil consumption and health: the possible role of antioxidants", *Lancet Oncol.*, 1(2), 107–112, 2000.

[324]   E. Herrera Acosta, J. Alonso Suárez Pérez, J. Aguilera Arjona, F. Visioli, "An olive polyphenol-based nutraceutical improves cutaneous manifestations of psoriasis in humans", *PharmaNutrition*, 4(4), 151–153, 2016.

[325]   G. Park, H. G. Kim, S.-P. Hong, S. Y. Kim, M. S. Oh, "Walnuts (seeds of Juglandis sinensis L.) protect human epidermal keratinocytes against UVB-induced mitochondria-mediated apoptosis through upregulation of ROS elimination pathways", *Skin Pharmacol. Physiol.*, 27(3), 132–140, 2014.

[326]   M. S. Cooke, M. D. Evans, M. Dizdaroglu, J. Lunec, "Oxidative DNA damage: mechanisms, mutation, and disease", *FASEB J.*, 17(10), 1195–1214, 2003.

[327]   E. Lopez-Huertas, J. Fonolla, "Hydroxytyrosol supplementation increases vitamin C levels in vivo. A human volunteer trial", *Redox Biol.*, 11, 384–389, 2017.

[328]   R. Fabiani, "Anti-cancer properties of olive oil secoiridoid phenols: A systematic review of: In vivo studies", *Food Funct.*, 7(10), 4145–4159, 2016.

[329]   X. Zhang, K. A. Merkler, M. P. McLean, "Characterization of regulatory intronic and exonic sequences involved in alternative splicing of scavenger receptor class B

gene", *Biochem. Biophys. Res. Commun.*, 372(1), 173–178, 2008.

[330]   R. Quirantes-Piné, G. Zurek, E. Barrajón-Catalán, C. Bäßmann, V. Micol, A. Segura-Carretero, A. Fernández-Gutiérrez, "A metabolite-profiling approach to assess the uptake and metabolism of phenolic compounds from olive leaves in SKBR3 cells by HPLC-ESI-QTOF-MS", *J. Pharm. Biomed. Anal.*, 72, 121–126, 2013.

[331]   E. Terzuoli, A. Giachetti, M. Ziche, S. Donnini, "Hydroxytyrosol, a product from olive oil, reduces colon cancer growth by enhancing epidermal growth factor receptor degradation", *Mol. Nutr. Food Res.*, 60(3), 519–529, 2016.

[332]   G. Toteda, S. Lupinacci, D. Vizza, R. Bonofiglio, E. Perri, M. Bonofiglio, D. Lofaro, A. La Russa, F. Leone, P. Gigliotti, R.A. Cifarelli, A. Perri, "High doses of hydroxytyrosol induce apoptosis in papillary and follicular thyroid cancer cells", *J. Endocrinol. Invest.*, 40(2), 153–162, 2017.

[333]   R. Fabiani, A. De Bartolomeo, P. Rosignoli, M. Servili, G. F. Montedoro, G. Morozzi, "Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis", *Eur. J. Cancer Prev.*, 11(4), 351–358, 2002.

[334]   M. Servili, S. Esposto, G. Veneziani, S. Urbani, A. Taticchi, I. Di Maio, R. Selvaggini, B. Sordini, G. Montedoro, "Improvement of bioactive phenol content in virgin olive oil with an olive-vegetation water concentrate produced by membrane treatment", *Food Chem.*, 124(4), 1308–1315, 2011.

[335]   G. Veneziani, E. Novelli, S. Esposto, A. Taticchi, M. Servili, "Chapter 11 - Applications of recovered bioactive compounds in food products", C. M. B. T.-O. M. W. Galanakis, *Ed. Academic Press*, 2017, pp. 231–253.

[336]   A. Carrasco-Pancorbo, L. Cerretani, A. Bendini, A. Segura-Carretero, M. Del Carlo, T. Gallina-Toschi, G. Lercker, D. Compagnone, A. Fernández-Gutiérrez, "Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil", *J. Agric. Food Chem.*, 53(23), 8918–8925, Nov. 2005.

[337]   M. Baldioli, M. Servili, G. Perretti, G. F. Montedoro, "Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil", *JAOCS, J. Am. Oil Chem. Soc.*, 73(11), 1589–1593, 1996.

[338]   D. Angelino, L. Gennari, M. Blasa, R. Selvaggini, S. Urbani, S. Esposto, M. Servili, P. Ninfali, "Chemical and cellular antioxidant activity of phytochemicals purified from olive mill waste waters", *J. Agric. Food Chem.*, 59(5), 2011–2018, Mar. 2011.

[339]   FDA, "Expedited Programs for Serious Conditions – Drugs and Biologics", *Guid. Ind.*, vol. 2020, no. 0910, 2020.

[340]   T. I. Lafka, A. E. Lazou, V. J. Sinanoglou, E. S. Lazos, "Phenolic and antioxidant potential of olive oil mill wastes", *Food Chem.*, 125(1), 92–98, 2011.

[341]   I. Fki, N. Allouche, S. Sayadi, "The use of polyphenolic extract, purified hydroxytyrosol and 3,4-dihydroxyphenyl acetic acid from olive mill wastewater for the stabilization of refined oils: A potential alternative to synthetic antioxidants", *Food Chem.*, 93(2), 197–204, 2005.

[342] A. N. S. Efsa, "Scientific Opinion on the re-evaluation of butylated hydroxytoluene BHT (E 321) as a food additive. EFSA J 10: 2588", 2012.

[343] M. Araújo, F. B. Pimentel, R. C. Alves, M. B. P. Oliveira, "Phenolic compounds from olive mill wastes: Health effects, analytical approach and application as food antioxidants", *Trends Food Sci. Technol.*, 45(2), 200–211, 2015.

[344] F. Aguilar, R. Crebelli, B. Dusemund, P. Galtier, D. Gott, U. Gundert-Remy, J. Konig, C. Lambre, J.-C. Leblanc, A. Mortensen, "Scientific opinion on the re-evaluation of propyl gallate (E 310) as a food additive", *EFSA J.*, 12(4), 2014.

[345] (ANS), EFSA Panel on Food Additives and Nutrient Sources added to Food, "Statement on the refined exposure assessment of tertiary-butyl hydroquinone (E 319)", *EFSA J.*, 14(1), p. 4363, 2016.

[346] (ANS), EFSA Panel on Food Additives and Nutrient Sources added to Food, "Scientific Opinion on the re-evaluation of butylated hydroxyanisole–BHA (E 320) as a food additive", *EFSA J.*, 9(10), p. 2392, 2011.

[347] S. Esposto, A. Taticchi, I. Di Maio, S. Urbani, G. Veneziani, R. Selvaggini, B. Sordini, M. Servili, "Effect of an olive phenolic extract on the quality of vegetable oils during frying", *Food Chem.*, 176, 184–192, 2015.

[348] C. M. Galanakis, E. Tornberg, V. Gekas, "A study of the recovery of the dietary fibres from olive mill wastewater and the gelling ability of the soluble fibre fraction", *LWT - Food Sci. Technol.*, 43(7), 1009–1017, 2010.

[349] C. M. Galanakis, E. Tornberg, V. Gekas, "The effect of heat processing on the functional properties of pectin contained in olive mill wastewater", *LWT - Food Sci. Technol.*, 43(7), 1001–1008, 2010.

[350] C. M. Galanakis, E. Tornberg, V. Gekas, "Dietary fiber suspensions from olive mill wastewater as potential fat replacements in meatballs", *LWT - Food Sci. Technol.*, 43(7), 1018–1025, 2010.

[351] A. Tafesh, N. Najami, J. Jadoun, F. Halahlih, H. Riepl, H. Azaizeh, "Synergistic antibacterial effects of polyphenolic compounds from olive mill wastewater", *Evidence-Based Complement. Altern. Med.*, vol. 2011, 2011.

[352] K. Gerasopoulos, D. Stagos, S. Kokkas, K. Petrotos, D. Kantas, P. Goulas, D. Kouretas, "Feed supplemented with byproducts from olive oil mill wastewater processing increases antioxidant capacity in broiler chickens", *Food Chem. Toxicol.*, 82, 42–49, 2015.

[353] K. Gerasopoulos, D. Stagos, K. Petrotos, S. Kokkas, D. Kantas, P. Goulas, D. Kouretas, "Feed supplemented with polyphenolic byproduct from olive mill wastewater processing improves the redox status in blood and tissues of piglets", *Food Chem. Toxicol.*, 86, 319–327, 2015.

[354] F. Rodrigues, F. B. Pimentel, M. B. P. Oliveira, "Olive by-products : Challenge application in cosmetic industry", *Ind. Crop. Prod.*, 70, 116–124, 2015.

[355] L. Prakash, M. Majeed, "Natural ingredients for anti-ageing skin care", *Househ. Pers. Care TODAY*, 2, 44–46, 2009.

[356] D. Badiu, R. Luque, R. Rajendram, "Chapter 123 - Effect of Olive Oil on the Skin", V. R. Preedy ,R. R. B. T.-O, O. O. in H. and D. P. Watson, *Eds. San Diego: Academic Press*, 2010, pp. 1125–1132.

[357] F. Rodrigues, M. A. da M. Nunes, M. B. P. P. Oliveira, "Chapter 12 - Applications of recovered bioactive compounds in cosmetics and health care products", C. M. B. T.-O. M. W. Galanakis, *Ed. Academic Press*, 2017, pp. 255–274.

[358] S. Bulotta, M. Celano, S. M. Lepore, T. Montalcini, A. Pujia, D. Russo, "Beneficial effects of the olive oil phenolic components oleuropein and hydroxytyrosol: focus on protection against cardiovascular and metabolic diseases", *J. Transl. Med.*, 12(1), p. 219, 2014.

[359] C. Esteve, M. L. Marina, and M. C. García, "Novel strategy for the revalorization of olive (Olea europaea) residues based on the extraction of bioactive peptides", *FOOD Chem.*, 167, 272–280, 2015.

[360] C. Rona, F. Vailati, E. Berardesca, "The cosmetic treatment of wrinkles", *J. Cosmet. Dermatol.*, 3(1), 26–34, Jan. 2004.

[361] C. K. Angerhofer, D. Maes, P. U. Giacomoni, "Chapter 10 - The Use of Natural Compounds and Botanicals in the Development of Anti-Aging Skin Care Products", in *Personal Care & Cosmetic Technology*, N. B. T.-S. A. H. Dayan, Ed. Norwich, NY: William Andrew Publishing, 2009, pp. 205–263.

[362] J. A. Nichols, S. K. Katiyar, "Skin photoprotection by natural polyphenols: anti-inflammatory, antioxidant and DNA repair mechanisms", *Arch. Dermatol. Res.*, 302(2), 71–83, 2010.

[363] W. Guo, Y. An, L. Jiang, C. Geng, L. Zhong, "The protective effects of hydroxytyrosol against UVB-induced DNA damage in HaCaT cells", *Phyther. Res.*, 24(3), 352–359, Mar. 2010.

[364] J. Caballero, R. D'Andria, M. Fernández, A. Garrido, L. Rallo, N. Arcas, R. Fernandez Escobar, J. López-Miranda, M. Msallem, M. Parras, R. Zanoli, "Present and future of the Mediterranean olive sector", *Options Méditerranéennes: Série A. Séminaires Méditerranéens*, vol. 106. Zaragoza: CIHEAM / IOC, p. 207, 2013.

[365] N. Saewan, A. Jimtaisong, "Natural products as photoprotection", *J. Cosmet. Dermatol.*, 14(1), 47–63, Mar. 2015.

[366] A. M. Goula, H. N. Lazarides, "Integrated processes can turn industrial food waste into valuable food by-products and/or ingredients: The cases of olive mill and pomegranate wastes", *J. Food Eng.*, 167, 45–50, 2015.

[367] M. L. Soto, E. Falqué, H. Domínguez, "Relevance of natural phenolics from grape and derivative products in the formulation of cosmetics", *Cosmetics*, 2(3), 259–276, 2015.

[368] A. V Rawlings, P. J. Matts, "Stratum Corneum Moisturization at the Molecular Level: An Update in Relation to the Dry Skin Cycle", *J. Invest. Dermatol.*, 124(6), 1099–1110, 2005.

[369] F. N. Bazoti, E. Gikas, A. L. Skaltsounis, A. Tsarbopoulos, "Development of a

liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI MS/MS) method for the quantification of bioactive substances present in olive oil mill wastewaters", *Anal. Chim. Acta*, 573–574, 258–266, 2006.

[370] C. Romero, M. Brenes, K. Yousfi, P. García, A. García, A. Garrido, "Effect of cultivar and processing method on the contents of polyphenols in table olives", *J. Agric. Food Chem.*, 52(3), 479–484, Feb. 2004.

[371] S. H. Omar, "Oleuropein in olive and its pharmacological effects", *Sci. Pharm.*, 78(2), 133–154, 2010.

[372] F. Visioli, P. Bogani, C. Galli, "8 - Healthful Properties of Olive Oil Minor Components", D. B. T.-O. O. (Second E. Boskou, Ed. AOCS Press, 2006, pp. 173–190.

[373] R. De La Puerta, M. E. M. Domínguez, V. Ruíz-Gutíerrez, J. A. Flavill, J. R. S. Hoult, "Effects of virgin olive oil phenolics on scavenging of reactive nitrogen species and upon nitrergic neurotransmission", *Life Sci.*, 69(10), 1213–1222, 2001.

[374] A. Umeno, M. Takashima, K. Murotomi, Y. Nakajima, T. Koike, T. Matsuo, Y. Yoshida, "Radical-scavenging activity and antioxidative effects of olive leaf components oleuropein and hydroxytyrosol in comparison with homovanillic alcohol", *J. Oleo Sci.*, 64(7), 793–800, 2015.

[375] R. K. Chaudhuri, "Role of antioxidants in sun care products", in *Sunscreens: Regulations and Commercial Development*, 2005, 603–638.

[376] F. P. Gasparro, M. Mitchnick, J. F. Nash, "A review of sunscreen safety and efficacy", *Photochem. Photobiol.*, 68(3), 243–256, 1998.

[377] G. J. Nohynek, H. Schaefer, "Benefit and risk of organic ultraviolet filters", *Regul. Toxicol. Pharmacol.*, 33(3), 285–299, 2001.

[378] F. Boehm, H. Meffert, S. Tribius, "Hazards of sunscreens-Cytotoxic properties of micronized titandioxide", *Biol. Eff. Light*, 446–451, 1995.

[379] E. Damiani, L. Rosati, R. Castagna, P. Carloni, L. Greci, "Changes in ultraviolet absorbance and hence in protective efficacy against lipid peroxidation of organic sunscreens after UVA irradiation", *J. Photochem. Photobiol. B Biol.*, 82(3), 204–213, 2006.

[380] S. Scalia, M. Mezzena, "Photostabilization effect of quercetin on the UV filter combination, butyl methoxydibenzoylmethane–octyl methoxycinnamate", *Photochem. Photobiol.*, 86(2), 273–278, Mar. 2010.

[381] S. D'Angelo, D. Ingrosso, V. Migliardi, A. Sorrentino, G. Donnarumma, A. Baroni, L. Masella, M. Tufano, M. Zappia, P. Galletti, "Hydroxytyrosol, a natural antioxidant from olive oil, prevents protein damage induced by long-wave ultraviolet radiation in melanoma cells", *Free Radic. Biol. Med.*, 38(7), 908–919, 2005.

[382] A. K. Kohli, H. O. Alpar, "Potential use of nanoparticles for transcutaneous vaccine delivery: effect of particle size and charge", *Int. J. Pharm.*, 275(1), 13–17, 2004.

[383] C. M. Galanakis, A. Schieber, "Editorial", *Food Res. Int.*, vol. 65, no. PC, pp. 299–

300, 2014.

[384] E. Roselló-Soto, F. Barba, O. Parniakov, C. M. Galanakis, N. Lebovka, N. Grimi, E. Vorobiev, "High Voltage Electrical Discharges, Pulsed Electric Field, and Ultrasound Assisted Extraction of Protein and Phenolic Compounds from Olive Kernel", *Food Bioprocess Technol.*, 8, 885-894, Dec. 2014.

[385] A. Heredia, R. Gutierrez, R. R. Arcos, A. J. Araujo, and R. G. Bejarano, "(12) Patent Application Publication (10) Pub. No.: US 2004/0102657 A1", vol. 1, no. 19, 2004.

[386] J. Fernández-Bolaños, G. Rodríguez, R. Rodríguez, R. Guillén, A. Jiménez, "Extraction of interesting organic compounds from olive oil waste", *Grasas y Aceites*, 57(1), 95–106, 2006.

[387] C. M. Galanakis, E. Tornberg, V. Gekas, "A study of the recovery of the dietary fibres from olive mill wastewater and the gelling ability of the soluble fibre fraction", *LWT - Food Sci. Technol.*, 43(7), 1009–1017, 2010.

OBJECTIVES

VALORIZATION OF RESIDUES GENERATED IN THE PRODUCTION OF OLIVE OIL THROUGH CLEAN TECHNOLOGIES

The general objective of this PhD Thesis is the valorization of olive mill wastes (OMW) in terms of olive pomace oil extraction and bioactive compounds recovery using clean and emerging technologies, with subsequent encapsulation of the recovered biocompounds in single and double nanoemulsions for their future use in food, cosmetic and pharmaceutical applications.

In order to achieve this main goal, the following specific objectives have been also established:

- To evaluate the olive pomace oil extraction from olive mill solid residues, obtained from three-phase and two-phase centrifugation systems, by conventional solvent extraction and ultrasound-assisted extraction methods using response surface methodology in order to optimize the oil yield recovery.

- To determine the fatty acid profile of the extracted olive pomace oils by gas chromatography.

- To evaluate the technical feasibility of ultrasound-assisted extraction methods for the recovery of phenolic compounds present in the defatted olive mill solid residues, optimizing the operating conditions by means of response surface methodology to obtain phenolic extracts with high antioxidant activity (AA), total phenolics content (TPC) and total flavonoids content (TFC).

- To purify and concentrate the obtained phenolic extracts by adsorption-desorption processes using polymeric resins and activated charcoals, optimizing the process in terms of total phenolics recovery and also selective isolation of hydroxytyrosol, tyrosol and oleuropein, the main components of the phenolic fraction present in olive mill waste.

- To characterize the olive mill wastewater (OMWW) obtained from three-phase centrifugation system in terms of total phenolics content, antioxidant activity, zeta potential, particle size and stability, for its further pretreatment by acidification and centrifugation in order to increase its hydroxytyrosol content.

- To study the use of integrated membrane processes, using several ultrafiltration, microfiltration and nanofiltration membranes, for the treatment

of the acidified and centrifuged OMWW to obtain a retentate with high hydroxytyrosol content and a final permeate stream with low chemical oxygen demand.

- To optimize the composition and operating conditions for the preparation of stable water-in-oil (W/O) and water-in-oil-in-water (W/O/W) nanoemulsions loaded with phenolic inner aqueous phase using high energy methods.

RESULTS

VALORIZATION OF RESIDUES GENERATED IN THE PRODUCTION OF OLIVE OIL THROUGH CLEAN TECHNOLOGIES

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VALORIZATION OF RESIDUES GENERATED IN THE PRODUCTION OF OLIVE OIL THROUGH CLEAN TECHNOLOGIES

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En este primer trabajo se estudia la extracción de aceite de orujo de oliva mediante diferentes métodos. El orujo es el residuo sólido generado en la almazara tras la extracción del aceite de oliva virgen, obteniendo aproximadamente 35–40 kg de orujo por cada 100 kg de aceitunas. Está compuesto por restos de agua, huesos, pulpa y piel de la aceituna, y contiene una cantidad residual de aceite que hace que sea un subproducto valorizable de interés comercial. La recuperación del aceite de orujo de oliva se lleva a cabo habitualmente mediante extracción con disolventes, generalmente con hexano. El objetivo de este trabajo ha sido optimizar la extracción del aceite de orujo de oliva en términos de rendimiento de recuperación de aceite (masa de aceite extraído respecto a la masa de orujo utilizada) aplicando la metodología de superficie de respuesta (RSM), considerando el tiempo de extracción, la temperatura y la relación sólido/disolvente como principales variables de diseño de experimentos. Se estudiaron tres métodos de extracción de aceite utilizando hexano como disolvente: extracción Soxhlet convencional, extracción asistida por ultrasonidos y extracción con disolvente en un baño de agua con agitación. Los mejores resultados se obtuvieron mediante extracción asistida por ultrasonidos, con un rendimiento de extracción de aceite del 15,97% en las condiciones óptimas de tiempo (60 min), temperatura (50 °C) y relación sólido/disolvente (1% p/v), siendo un 22,28% y un 9,46% superior al rendimiento obtenido mediante extracción Sohxlet y extracción con hexano en un baño de agua, respectivamente. Estos resultados indican que la extracción asistida con ultrasonidos es la mejor de las tres opciones estudiadas en términos de menor temperatura necesaria para llevar a cabo la extracción y mayor rendimiento de recuperación de aceite. El perfil de ácidos grasos de los aceites de orujo de oliva extraídos se determinó mediante cromatografía de gases, mostrando que la mayoría de los ácidos grasos (89,2–92,7%) presentes en la composición del aceite de orujo de oliva son ácido oleico, ácido linoleico y ácido palmítico, cumpliendo ampliamente los rangos aceptables publicados por la Organización Mundial de la Salud (OMS).

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1.1. Introduction

A great amount of residues are generated as a result of agro-industrial activities and biomass valorization has become imperative [1–3]. Olive pomace is one of the most abundant by-product of olive oil production process and a very important biomass source for the Mediterranean countries. Its main constituents are water, oil, olive peel, and kernels [4–6]. During olive oil production process approximately 35–40 kg of olive pomace are released per 100 kg of olives.

Olive pomace can reach up to 30% of olive oil manufacturing, depending on the milling process. However, a large quantity of olive mill solid residue remains without actual application because only small amounts are used as natural fertilizers, combustible biomass and additives in animal feeding [7,8]. Although being lignocellulosic material, olive pomace contains 5–10% protein as well as 4–15% of oil and its oil content varies depending on the olive cultivation region and the employed extraction method [5,9]. The commercial value of the olive pomace depends on its oil content, where the composition is similar to, but not identical with, that of typical olive oil [10,11]. Extraction of oil from olive pomace is commonly carried out by solvent extraction, mostly by hexane. However, this has some disadvantages in industry, such as the use of an organic solvent, inefficient solvent removal and high energy requirements. Furthermore, the olive pomace oil obtained by this process requires an extra refining step which is expensive and usually causes thermal degradation of valuable compounds but has to be applied in order to comply with the food regulations [4,10,12].

Currently, the growing interest in olive pomace oil is due to its bioactive minor constituents generally included in the unsaponifiable matter (USM), which contains significant amounts of sterols, fatty alcohols, tocopherols, triterpene alcohols and squalene. Most of these compounds possess a wide range of interesting properties such as bioactive, nutritional and characteristic compositional properties. They are additionally suggested for their functional properties, e.g. anti-inflammatory, antibacterial, antifungal, anti-ulcerative and anti-tumoral activities [13]. It should be noted that olive pomace oil has a higher USM content than olive oil [11,14] and is also richer in individual

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sterols and polyunsaturated fatty acids such as linoleic acid [15]. Nowadays, there is great interest in the recovery of these valuable compounds and further in their use as additives in new products for food, nutraceutical and pharmaceutical industrial sectors. Therefore, according to the modern trend for searching new ingredients from natural sources the valorization of olive pomace is appeared to be particularly attractive [16].

For that purpose conventional solvent extraction processes have commonly been applied, whereas alternative extraction techniques were also developed to deliver effective and selective oil extraction minimizing the loss of minor compounds. Ultrasound-assisted extraction (UAE) has been recognized as a promising technique that uses acoustic energy and solvents to extract target compounds from various plant materials [17,18]. The main benefit of UAE can be observed in solid plant sample because ultrasound energy facilitates organic and inorganic compounds leaching from plant matrix [19]. Probable mechanism is ultrasound intensification of mass transfer and accelerated access of solvent to cell materials of plant parts. The extraction mechanism by ultrasound involves two main types of physical phenomena, (a) the diffusion across the cell wall and (b) rinsing the contents of cell after breaking the walls [20]. Moisture content of sample, milling degree, particle size and solvent are very important factors for obtaining efficient and effective extraction. Furthermore, temperature, frequency and time of sonication are the governing factors for the action of ultrasound. The advantages of UAE include reduction in extraction time, energy and use of solvent. Ultrasound energy for extraction also facilitates more effective mixing, faster energy transfer, reduced thermal gradients and extraction temperature, reduced equipment size, faster response to process extraction control, quick start-up, increased production and eliminates process steps [21].

Bioactive compounds from plant materials can also be extracted by several conventional extraction techniques, most of them based on the extracting power of different solvents and the application of heat and/or mixing. The Soxhlet extraction has widely been used for extracting valuable bioactive compounds from various natural sources and is used as a model for the comparison of new extraction alternatives [21].

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The aim of this work was to optimize the extraction of olive pomace oil in terms of oil recovery yield by applying the response surface methodology (RSM) and considering the extraction time, temperature and solid/solvent ratio as variables. In this study, in order to optimize the oil recovery yield, three extraction methods were used: ultrasound-assisted extraction, solvent extraction in a shaking water bath and standard Soxhlet extraction. In addition, fatty acid profile of the extracted olive pomace oil was performed by gas chromatography for its characterization.

1.2. Materials and methods

1.2.1. Samples and chemicals

Olive pomace used in this study was kindly provided by Cooperativa de Montán (Castellón, Spain). It was obtained as a by-product by means of a two-phase cold oil mechanical extraction process using olives from olive trees of the variety *Serrana de Espadán*. The olive pomace samples were dried at 100 ºC for 24 h in an oven (model 2600210, JP Selecta, Barcelona, Spain) to remove most of their moisture (about 60% w/w). They were then ground by using a coffee grinder to obtain an average particle diameter of about 1 mm. Isooctane and n-hexane, both HPLC grade, were purchased from VWR International Eurolab (Barcelona, Spain). Methyl-tricosanoate and other standards of fatty acid methyl esters were purchased from Sigma-Aldrich (Darmstadt, Germany).

1.2.2. Olive pomace oil extraction procedure

1.2.2.1. Conventional extraction using standard Soxhlet process

Olive pomace oil was extracted using a B-811 extraction system (BÜCHI Labortechnik AG, Flawil, Switzerland) for Soxhlet process. The ground olive pomace (about 19 g, 4.7 g in each of the 4 extraction chambers) was placed in the Soxhlet apparatus and continuously refluxed with 360 mL (90 mL for each chamber) of n-hexane for 3 h at 70 ºC. After extraction, n-hexane was removed

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by distillation using the same equipment. The obtained oil was weighed and the recovery yield was calculated using the following equation [13]:

$$Yield = \frac{Oil\ extract\ obtained\ (g)}{Amount\ of\ olive\ pomace\ used\ (g)} \times 100 \tag{1}$$

1.2.2.2.   Ultrasound-assisted extraction (UAE)

The ground olive pomace was first added to 40 mL of n-hexane and then mixed vigorously in a vortex mixer (LBX V05 series, 60 W, Labbox Labware S.L., Barcelona, Spain). The mixture was entered to an ultrasonic bath (Selecta Ultrasons-H, 50/60 Hz, 720 W, JP Selecta, Barcelona, Spain) where the extraction was performed depending on the designed operating conditions (solid/solvent ratio: 1–10 w/v %, time: 20–60 min, temperature: 40–60 ºC). After ultrasonic extraction, the extracts were centrifuged at 10000 rpm for 7 min (Eppendorf 5804 centrifuge, Hamburg, Germany) in order to separate the supernatants from solid particles. As final stage, the solvent was distillated and separated from solid residues within 10 min using the B-811 extraction system. The extracted oil was collected and weighed after getting cooled.

1.2.2.3.   Solvent extraction in shaking water bath

Ground olive cake depending on the considered solid/solvent ratio (1–10 w/v %) in the fitted model was added to n-hexane and then mixed vigorously in the vortex mixer. The mixture was entered to a shaking water bath (Grant Instruments OLS200, Cambridge, UK) where extraction took place at required temperatures (40–60 ºC) and times (20–60 min). As in UAE, final extracts were centrifuged at 10000 rpm for 7 min in order to separate the supernatants from solid particles and the solvent was distillated and separated from solid residues within 10 minutes using the B-811 extraction system. The extracted oil was collected and weighed after getting cooled.

1.2.3.   Analysis of fatty acid profile by gas chromatography (GC)

The fatty acid content of the extracted oils was determined by the AOAC 991.39 official method [22], which is based on the breakdown of all glycerides

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and subsequent derivatization to fatty acid methyl esters (FAMEs). These FAMEs were analyzed by using an Agilent gas chromatograph (6890N Network GC system, Agilent, Santa Clara, CA, USA) equipped with a flame ionization detector (FID) and a fused silica capillary column (OmegawaxTM-320, 30 m × 0.32 mm i.d.) The separation was performed with helium (1.8 mL/min) as carrier gas [23,24]. The chromatographic conditions were as follows: initial column temperature of 180 °C for 15 min, heated to 200 ℃ at 1 ℃/min, held at 200 ℃ for 1 min, heated again to 220 ℃ at 5 ℃/min, and finally held at 220 ℃ for 15 min. A split injector (50:1) at 250 ℃ was used and the FID was also heated to 250 ℃. The injection volume was 1 μL. FAMEs where identified by comparison of their retention times with those of chromatographic standards and their quantification was made by using methyl tricosanoate (C23:0; 1 mg/mL dissolved in isooctane) as internal standard.

1.2.4. Statistical analysis

Experimental data were analyzed by simple statistic parameters in order to detect anomalous data and to express results through the average values and the corresponding standard deviation. Data analysis was performed using Statgraphics Centurion 18 software (Statgraphics Technologies, Inc., Warrenton, VA, USA). Response surface methodology (RSM) with a central composite design 2^3 + star model was selected for this study. The effects of three variable factors, each with 3 levels, were studied on the oil extraction yield, this being the response variable (Y). The following factors were studied: time of extraction (A: 20–60 min), solid/solvent ratio (B: 2–10 w/v %), and extraction temperature (C: 40–60 ℃). The following second-degree polynomial equation was used to express the predicted response (Y) as a function of the independent variables (A, B and C):

$$Y = a_0 + a_1A + a_2B + a_3C + a_{11}A^2 + a_{12}AB + a_{13}AC + a_{22}B^2 + a_{23}BC + a_{33}C^2 \tag{2}$$

where a_0, a_i, a_{ii} and a_{ij} are the regression coefficients in the intercept, linear, quadratic and interaction terms, respectively.

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The value of the factors and their effect on the response was determined by analysis of variance (ANOVA) and LSD (lesser significant difference) test. The model was adjusted by means of multiple linear regressions (MLR) and its validity was determined by ANOVA. The level of significance of each coefficient was evaluated through the values of the statistical parameters F and p (probability), with a confidence level of 95%.

1.3. Results and discussion

1.3.1. Statistical data and optimization of extraction model

Primarily, 15 runs were performed by Soxhlet extraction method with the same operating conditions, as mentioned in Section 2.2.2.1. The sample weight was 4.69 ± 0.21 g while the oil recovery yield was $13.06 \pm 0.75\%$.

Based on the model experimental design, 17 runs of olive pomace oil extraction were performed by UAE. In addition, 17 runs of oil extraction by using shaking water bath were performed with the same operating conditions as those in the UAE for comparative evaluation purposes. The experimental model was planned to have the range of 2–10 w/v % solid/solvent (S/S) ratio. However, better oil recovery yield was obtained with lower S/S ratios in preliminary experiments. Therefore, two extra runs with S/S ratio of 1% were added to the model (runs 5 and 6 in Table 1.1) for both extraction methods, with 19 experimental runs for each method (Table 1.1).

According to the obtained results shown in Table 1.1 and the ANOVA shown in Table 1.2, response surface methodology was a suitable model for this type of experimental design. Generally in ANOVA, large F-value and small p-value show a more significant effect on response [25]. ANOVA (Table 1.2) shows the variability in the yield of extraction (response variable) into separate pieces for each of the effects (factors). It then tests the statistical significance of each factor by comparing the mean square against an estimate of the experimental error. The default model is quadratic with 10 coefficients, and it has been fit to the response variable. The coefficient of determination (R^2) indicates that the model as fitted explains 95.44% and 94.95% of the variability in oil recovery

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yield for ultrasound-assisted extraction and solvent extraction in shaking water bath, respectively.

Table 1.3 shows the values of the regression coefficients of Eq. (2) for both extraction methods. In this case, it can be observed that oil recovery yield for UAE was mainly affected by linear terms of S/S ratio (B), time (A), and the interaction between them (AB), followed by the quadratic term of S/S ratio (B^2) and the interaction between time and temperature (AC): all of them have p-values less than 0.05, indicating that they are significantly different from zero at the 95% confidence level (Table 1.3), whereas for the extraction in the shaking water bath only 2 factors, S/S ratio (B) and temperature (C), are statistically significant ($p \leq 0.05$). F-ratio values indicate that, for the range of studied variables, solid/solvent ratio (B) had stronger influence on the oil recovery yield than the other independent variables.

Based on the observed responses from Table 1.1 for the UAE, the most desirable results were obtained for run 6 (time: 60 min, temperature: 50 ºC, solid/solvent ratio: 1 w/v %) which resulted into 15.97% oil recovery yield. These results are very similar to the predicted optimum operating conditions by the model (time: 59.99 min, temperature: 50.16 ºC, S/S ratio: 1.0002 w/v %, with 16.29% oil recovery yield).

Table 1.1. Design of experiments for olive pomace oil recovery.

Run	Operating conditions			Ultrasound-assisted extraction (UAE)			Extraction in shaking water bath		
	Time (min)	S/S ratio (%)	Temperature (°C)	Weight of sample (g)	Extracted oil weight (g)	Extraction yield (%)	Weight of sample (g)	Extracted oil weight (g)	Extraction yield (%)
1	40	2	50	0.8053	0.1194	14.82	0.8039	0.1031	12.82
2	60	6	50	2.4020	0.3161	13.15	2.4010	0.2988	12.44
3	60	10	50	4.0035	0.4421	11.04	4.0045	0.4470	11.16
4	40	10	50	4.0237	0.4540	11.28	4.0020	0.4432	11.07
5	40	1	50	0.4055	0.0623	15.36	0.4069	0.0578	14.20
6	60	1	50	0.4025	0.0643	15.97	0.4025	0.0554	13.76
7	20	6	50	2.4035	0.2801	11.65	2.4055	0.2909	12.09
8	20	2	50	0.8150	0.1083	13.29	0.8031	0.1081	13.46
9	60	2	60	0.8055	0.1256	15.59	0.8078	0.1128	13.96
10	20	10	60	4.0013	0.4488	11.22	4.0098	0.4634	11.56
11	40	6	60	2.4017	0.2952	12.29	2.4069	0.2891	12.01
12	20	2	60	0.8030	0.0928	11.56	0.8072	0.1178	14.59
13	60	10	60	4.0031	0.4538	11.34	4.0033	0.4965	12.40
14	40	6	40	2.4230	0.2757	11.38	2.4112	0.2807	11.64
15	20	2	40	0.8161	0.1069	13.10	0.8013	0.1035	12.92
16	20	10	40	4.0076	0.4539	11.32	4.0114	0.4232	10.55
17	60	10	40	4.0065	0.4430	11.06	4.0090	0.4409	10.99
18	60	2	40	0.8135	0.1158	14.23	0.8019	0.1090	13.59
19	40	6	50	2.4030	0.2902	12.08	2.4074	0.2814	11.68

Table 1.2. Analysis of variance (ANOVA) for olive pomace oil recovery yield.

Source	Ultrasound-assisted extraction (UAE) ($R^2 = 95.44\%$)					Extraction in shaking water bath ($R^2 = 94.95\%$)				
	SS	DOF	MS	F-ratio	p-value	SS	DOF	MS	F-ratio	p-value
A: Time	3.85759	1	3.85759	14.71	0.0040	0.200018	1	0.200018	1.37	0.2718
B: S/S ratio	24.6379	1	24.6379	93.95	0.0000	18.4879	1	18.4879	126.68	0.0000
C: Temperature	0.08281	1	0.08281	0.32	0.5879	2.33289	1	2.33289	15.99	0.0031
A^2	0.0583865	1	0.0583865	0.22	0.6483	0.336209	1	0.336209	2.30	0.1634
AB	4.40351	1	4.40351	16.79	0.0027	0.21445	1	0.21445	1.47	0.2563
AC	1.3448	1	1.3448	5.13	0.0498	0.10125	1	0.10125	0.69	0.4264
B^2	1.75834	1	1.75834	6.70	0.0292	0.401076	1	0.401076	2.75	0.1317
BC	0.0162	1	0.0162	0.06	0.8093	0.01805	1	0.01805	0.12	0.7332
C^2	0.889099	1	0.889099	3.39	0.0987	0.13248	1	0.13248	0.91	0.3656
Total error	2.36021	9	0.262245	---	---	1.31343	9	0.145937	---	---
Total (corr.)	51.7598	18	---	---	---	25.9867	18	---	---	---

SS: Sum of squares; DOF: Degrees of freedom; MS: Mean Square

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Table 1.3. Values of the regression coefficients of the quadratic polynomial Eq. (2).

Regression coefficient	Ultrasound-assisted extraction (UAE)			Extraction in shaking water bath		
	Value	F-ratio	p-value	Value	F-ratio	p-value
a_0	4.86895	---	---	16.8681	---	---
a_1	-0.00141035	14.71	0.0040	-0.0381824	1.37	0.2718
a_2	-0.571198	93.95	0.0000	-0.658411	126.68	0.0000
a_3	0.378509	0.32	0.5879	-0.11318	15.99	0.0031
a_{11}	-0.00032401	0.22	0.6483	0.000777511	2.30	0.1634
a_{12}	-0.00788864	16.79	0.0027	0.00174087	1.47	0.2563
a_{13}	0.00205	5.13	0.0498	-0.0005625	0.69	0.4264
a_{22}	0.0407419	6.70	0.0292	0.0194582	2.75	0.1317
a_{23}	0.001125	0.06	0.8093	0.0011875	0.12	0.7332
a_{33}	-0.00458159	3.39	0.0987	0.00176855	0.91	0.3656

Regarding solvent extraction in shaking water bath, the predicted optimum operating conditions according to the fitted model were 22.71 min, 59.85 ℃ and 1.0055 w/v % S/S ratio, with a predicted oil recovery yield of 14.66%. These predicted values are so close to the obtained results for run 12 in Table 1.1 (time: 20 min, temperature: 60 ℃, S/S ratio: 2 w/v %, 14.59% oil recovery yield), which can be considered as the optimal conditions for oil recovery yield by solvent extraction in shaking water bath.

1.3.2. Effect of extraction variable factors on oil recovery yield

1.3.2.1. Ultrasonic-assisted extraction

In order to study the effect of the independent variables on the oil recovery yield, surface responses of the quadratic polynomial model (Eq. (2)) were generated by varying two of the independent variables within the experimental range while holding the other constant at its optimum value (Figure 1.1). As it was abovementioned, S/S ratio is the most significant factor in this study. According to Figure 1.1a, extraction time is a significant variable for S/S ratios less than 5%: as the extraction time increases, recovery yield increases considerably. Furthermore, for S/S ratios over 5% increasing time causes a decrease in oil recovery yield. However, according to Figure 1.1b, a temperature change between 40–60 ℃ has no significant effect on the oil recovery yield. The concentration gradient, which is the driving force in extraction process, is assumed to be greater when the S/S ratio is reduced, leading to faster diffusion and greater yields [26]. Similar findings have been reported in other extraction related studies [27,28].

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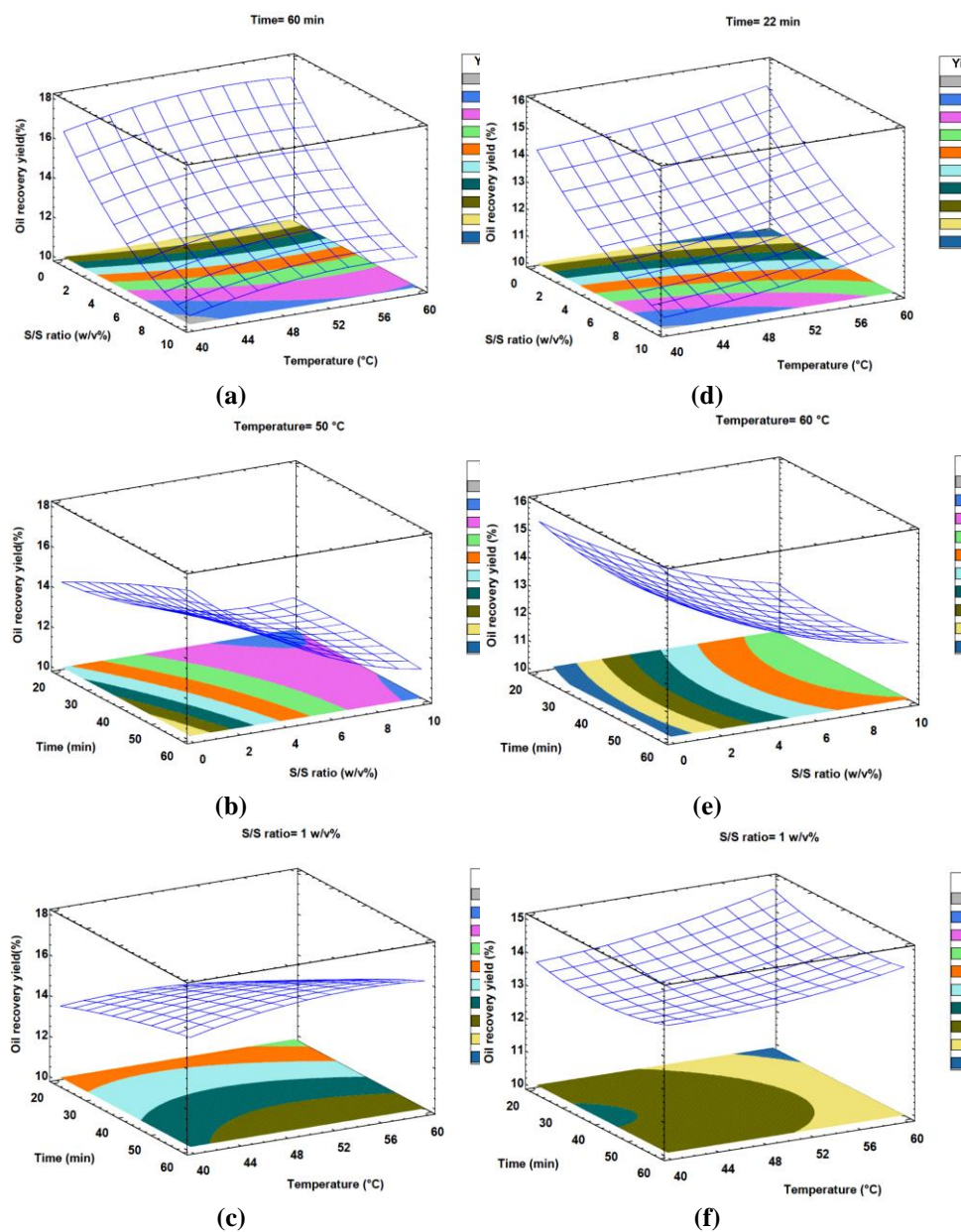
Figure 1.1. Response surface plots of interactions between two input factors, while holding the other constant at its optimum value, on oil recovery yield by ultrasound-assisted extraction (a, b, c) and by solvent extraction in shaking water bath (d, e, f): S/S ratio *vs.* temperature at the optimized time (a, d); S/S ratio *vs.* time at the optimized temperature (b, e); time *vs.* temperature at the optimized S/S ratio (c, f).

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1.3.2.2.   Solvent extraction in shaking water bath

Similar to UAE, the S/S ratio is the most significant factor in solvent extraction in a shaking water bath. It can be seen in Figure 1.1.d. that oil extraction yield increases with increasing temperature and decreasing S/S ratio. As shown in Figure 1.1.e, the change in time from 20 to 60 min hardly affects the oil recovery performance pattern, although the calculated optimum operating time was 22 min. Figure 1.1e indicates that the temperature increase from 40 to 60 ℃ has a increasing effect on oil recovery yield for a S/S over 6% and times lower than 40 min. This is in general agreement with studies of Mani et al. [29] and Minjares-Fuentes et al. [30], who also reported that by increasing the temperature, the extraction yield of oil from the seeds of *Moringa oleifera* and pectin from grape pomace is maximized.

1.3.3.   Optimization of oil recovery methods

As it was aforementioned, multiple runs of standard Soxhlet extraction were performed with similar conditions, obtaining an average oil recovery yield of 13.06 ± 0.75%.

As indicated by the results for ultrasonic-assisted extraction (UAE) and solvent extraction in shaking water bath under similar experimental conditions shown in Table 1.1, it was found that oil recovery yields under optimal conditions were 22.28% and 11.72% higher than that obtained by Soxhlet extraction, respectively. By considering thermal sensitivity of bioactive compounds, especially low molecular weight polyphenols, and their contribution to the antioxidant activity of olive pomace oil, it is obvious that these compounds may have been degraded by Soxhlet method due to the high temperature and long extraction time. In order to assess and compare the efficiency of the three extraction methods, results reveal that UAE is the best option in terms of lower extraction temperature and high oil recovery yield. Experimental results also indicate that oil recovery yield in most of the UAE runs was higher than those for solvent extractions in a shaking water bath. Taking into account that UAE provides lower operating temperature, thus

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less thermal degradation of most of the unstable minor compounds will occur. However, the shorter extraction time and the lower consumption of solvent for extractions performed by shaking water bath lead to energy and economic savings that can be accounted as shaking water bath advantages.

Experimental results obtained in this work are in agreement with those from other studies. Chanioti and Tzia [31,32] proved that S/S ratio is the most significant factor in UAE of olive pomace oil. Also, Stroescu et al. [33] reported that temperature and S/S ratio had the greatest effect on the oil yield from *P. oleracea* seeds, while Goula [27] and Li et al. [28] showed that not only the temperature and S/S ratio but also the particle size significantly affected the oil yield extracted by UAE from pomegranate seeds and *Isatis indigotica* Fort, respectively. Further, Mani et al. [29] observed that particle size had the greatest impact on the oil yield extracted from the seeds of *Moringa oleifera* using n-hexane as solvent, while the combined effects of particle size and extraction temperature were also significant. However, Kostić et al. [34] reported that the interaction between extraction temperature and S/S ratio had significant effect on the hempseed oil recovery yield.

1.3.4.   *Fatty acid profile of extracted olive pomace oil*

GC analysis were performed on olive pomace oil samples obtained under optimal conditions by the three extraction systems tested. Results are depicted in Table 1.4, which shows very similar fatty acid profiles for the three oils. Thus, it can be concluded that the oil extraction method does not have a significant effect on the fatty acid profile of the extracted olive pomace oil. It can also be seen in Table 1.4 that fatty acid composition of recovered olive pomace oils completely fit in the acceptable ranges published by the World Health Organization (WHO), and that main fatty acids (89.2–92.7%) in olive pomace oil are oleic acid (63–65%), linoleic acid (13.3–14.6%) and palmitic acid (12.9–13.1%). These results are in agreement with literature for olive products and by-products [35–37].

External oxidative factors, such as light and oxygen, and intrinsic factors such as antioxidants/prooxidants content can affect the composition of the olive pomace

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oil. The exposure to oxidative factors can lead, for example, to modifications in the fatty acids profile and/or vitamin E content of the remaining oil [38]. Moreover, oil degradation can lead to unpleasant characteristics such as rancidity and discoloration that besides decreasing its economic value, reduces the oil performance as functional ingredient. Furthermore, the degree of the oil instability is also associated with a high content of polyunsaturated fatty acids (PUFA) due to their susceptibility to oxidation [39]. A mixture of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) composes the lipid fraction of the olive pomace. As the olive pomace oil fraction is essentially rich in MUFA, its susceptibility to oxidation is reduced. Additionally, the presence of vitamin E, a potent liposoluble antioxidant, protects long chain fatty acids against oxidative damage [40]. Important olive components that remain in olive pomace, as MUFA, pigments, vitamin E, and phenolic compounds, may act synergistically, making this oil more stable than others [36,41,42]. However, according to Mateos et al. [11], its high MUFA content (67–69.6% of total fatty acids in this work), with demonstrated health benefits, makes olive pomace oil an interesting alternative to reach the FAO (Food and Agriculture Organization) recommendation of consuming 20% of total dietary energy in the form of monounsaturated fatty acids, with the total fat intake representing up to 35% of total caloric value of the diet.

Table 1.4. Fatty acid profile of extracted olive pomace oils.

GC analysis of extracted olive pomace oil			Standard Soxhlet system		Ultrasonic-assisted extraction (UAE)		Extraction in shaking water bath		Fatty acid acceptable ranges for different types of olive oil [43] (% of total fatty acids)		
Type of acid	Formula	Name	Fatty acid concentration Cx (mg/g)	wt. %	Fatty acid concentration Cx (mg/g)	wt. %	Fatty acid concentration Cx (mg/g)	wt. %	Olive pomace oil	Refined olive oil	Virgin olive oil
SFA	C14:0	Myristic acid	0.3±0	0.0±0.05	0.0	0.0	0.2±0	0.0±0.03	0.0–0.05	0.0–0.05	0.0–0.05
SFA	C16:0	Palmitic acid	108.7±9.0	13.1±0.08	120.6±20	13±0.1	119.6±4.0	13.0±0.02	7.5–20	7.5–20	7.5–20
MUFA	C16:1n-7	Palmitoleic acid	8.4±1.0	1.0±0.02	9.3±1.0	1.0±0.03	9.3±0	1.0±0.01	0.3–3.5	0.3–3.5	0.3–3.5
SFA	C17:0	Heptadecanoic acid	1.2±0	0.1±0	1.3±0	0.1±0	1.3±0	0.1±0	0.0–0.3	0.0–0.3	0.0–0.3
PUFA	C16:3n-4	Hexadecatrienoic acid	2.2±0	0.3±0	0.0	0.0	0.0	0.0			
MUFA	C17:1	Heptadecenoic acid	0.0	0.0	2.3±0	0.2±0	2.3±0	0.2±0	0.0–0.3	0.0–0.3	0.0–0.3
SFA	C18:0	Stearic acid	19.9±2.0	2.4±0	23.5±4.0	2.5±0.01	23.2±1.0	2.5±0.01	0.5–5	0.5–5	0.5–5
MUFA	C18:1n-9	Oleic acid	524.0 ±42.0	63.0±0.39	604.6±107	64.9+0.22	598.3±18.0	64.9±0.16	55–83	55–83	55–83
MUFA	C18:1n-7	Vaccenic acid	23.1±2.0	2.8±0.03	24.8±3.0	2.7±0.15	24.3±1.0	2.6±0.2			
PUFA	C18:2n-6	Linoleic acid	121.5±11.0	14.6±0.07	124.1±22.0	13.3±0.03	122.1±3.0	13.2±0.06	3.5–21	3.5–21	3.5–21
PUFA	C18:3n-3	Alpha linolenic acid	6.9±1.0	0.8±0.02	6.3±1.0	0.7±0.01	6.1±0	0.7±0			
SFA	C20:0	Arachidic acid	3.5±0	0.4±0	4.0±1.0	0.4±0.01	4.0±0	0.4±0	0.0–0.6	0.0–0.6	0.0–0.6
MUFA	C20:1n-9	Gondoic acid	2.9±0	0.3±0.03	3.5±1.0	0.4±0.01	3.6±0	0.4±0.01	0.0–0.4	0.0–0.4	0.0–0.4
SFA	C22:0	Behenic acid	1.8±0	0.2±0.01	1.8±0	0.2±0	1.9±0	0.2±0	0.0–0.3	0.0–0.2	0.0–0.2
PUFA	C22:5n-3	Docosapentaenoic acid	1.7±2.0	0.2±0.28	0.0	0.0	0.0	0.0			
SFA	C24:0	Lignoceric acid	1.2±0	0.1±0.01	1.2±0	0.1±0	1.3±0	0.1±0	0.0–0.2	0.0–0.2	0.0–0.2
PUFA	C22:6n-3	Docosahexaenoic acid	3.2±0	0.4±0.01	0.0	0.0	0.0	0.0			
MUFA	C24:1	Nervonic acid	1.2±0	0.1±0.1	3.6±1	0.4±0.01	1.4±1.0	0.2±0.15			
		Total Fatty Acids	831.7±71.5	100±1	931±162.1	100±0.6	922±34.0	100±1.2			
		Saturated fatty acids (SFA)	136.6±11.6	16.42±0.01	152.5±25.9	16.39±0.08	151.3±5.1	16.41±0.03			
		Monounsaturated fatty acids (MUFA)	559.5±45.1	67.3±0.36	648.1±113.2	69.6±0.04	639.2±15.8	69.3±0.49			
		Polyunsaturated fatty acids (PUFA)	135.6±14.8	16.02±0.38	130.3±23	14.0±0.03	131.7±8.4	14.27±0.45			
		SFA+MUFA+PUFA	831.7±71.5	99.7±0	931.0±162.1	100±0	922.2±29.3	100±0			

1.4. Conclusions

In this study, oil was recovered from olive pomace, an important by-product of olive oil production and a rich source of bioactive materials. Standard Soxhlet extraction with multiple runs was firstly performed, which resulted into average oil extraction yield of 13.06±0.75%. Response surface methodology was then applied to determine the optimal conditions (extraction time, temperature and solid/solvent ratio) for oil recovery yield using two methods: ultrasound-assisted extraction (UAE) and solvent extraction in shaking water bath. The most desirable results for UAE (time: 60 min, temperature: 50 ℃, solid/solvent ratio: 1 w/v %) resulted into 15.97% oil yield extraction, whereas for solvent extraction in shaking water bath (time: 20 min, temperature: 60 ℃, S/S ratio: 2 w/v %) resulted into 14.59% oil recovery yield, being 22.28% and 11.72% higher than that of the Soxhlet extraction, respectively. Results of the comparative study revealed that the ultrasound-assisted extraction is the better option in terms of lower extraction temperature and high oil recovery yield. Fatty acid profiles showed that the majority of olive pomace oil composition (89.2–92.7%) consisted of oleic acid (63–65%), linoleic acid (13.3–14.6%) and palmitic acid (12.9–13.1%). Also, fatty acid composition of all recovered oil types completely fit in the acceptable ranges published by World Health Organization.

References

[1] G. Rigane, M. Bouaziz, S. Sayadi, R. Ben Salem, "Identification and characterization of a new iridoid compound from two-phase Chemlali olive pomace", *Eur. Food Res. Technol.*, 234 (6), 1049–1054, 2012.

[2] D. Senyay-Oncel, H. Ertas, O. Yesil-Celiktas, "Effects of supercritical fluid extraction parameters on unsaturated fatty acid yields of pistacia terebinthus Berries", *J. Am. Oil Chem. Soc.*, 88 (7), 1061–1069, 2011.

[3] M. M. R. de Melo, A. J. D. Silvestre, C. M. Silva, "Supercritical fluid extraction of vegetable matrices: Applications, trends and future perspectives of a convincing green technology", *J. Supercrit. Fluids*, 92, 115–176, 2014.

[4] A. de Lucas, J. Rincón, I. Gracia, "Influence of operating variables on yield and quality parameters of olive husk oil extracted with supercritical carbon dioxide", *J. Am. Oil Chem. Soc.*, 79 (3), 237–243, 2002.

PhD Thesis

Chapter 1: Characterization and optimization of olive pomace oil extraction by using ultrasonic and conventional methods

[5] S. Meziane, H. Kadi, "Kinetics and thermodynamics of oil extraction from olive cake", *J. Am. Oil Chem. Soc.*, 85 (4), 391–396, 2008.

[6] T. Miranda, J. I. Arranz, I. Montero, S. Román, C. V Rojas, S. Nogales, "Characterization and combustion of olive pomace and forest residue pellets", *Fuel Process. Technol.*, 103, 91–96, 2012.

[7] F. Pagnanelli, S. Mainelli, F. Vegliò, L. Toro, "Heavy metal removal by olive pomace: Biosorbent characterisation and equilibrium modelling", *Chem. Eng. Sci.*, 58 (20), 4709–4717, 2003.

[8] E. T. Kostas, G. Durán-Jiménez, B. J. Shepherd, W. Meredith, L. A. Stevens, O. S. A. Williams, G. J. Lye, J. P. Robinson, "Microwave pyrolysis of olive pomace for bio-oil and bio-char production", *Chem. Eng. J.*, 387, 123404, 2020.

[9] S. Ramachandran, S. K. Singh, C. Larroche, C. R. Soccol, A. Pandey, "Oil cakes and their biotechnological applications - A review", *Bioresour. Technol.*, 98 (10), 2000–2009, 2007.

[10] F. Akay, A. Kazan, M. S. Celiktas, O. Yesil-Celiktas, "A holistic engineering approach for utilization of olive pomace", *J. Supercrit. Fluids*, 99, 1–7, 2015.

[11] R. Mateos, B. Sarria, L. Bravo, "Nutritional and other health properties of olive pomace oil", *Crit. Rev. Food Sci. Nutr.*, 60, 3506–3521, 2020.

[12] Y. Sánchez-Vicente, A. Cabañas, J. A. R. Renuncio, C. Pando, "Supercritical fluid extraction of peach (Prunus persica) seed oil using carbon dioxide and ethanol", *J. Supercrit. Fluids*, 49 (2), 167–173, 2009.

[13] M. H. Alu'datt, I. Alli, K. Ereifej, M. Alhamad, A. R. Al-Tawaha, T. Rababah, "Optimisation, characterisation and quantification of phenolic compounds in olive cake", *Food Chem.*, 123 (1), 117–122, 2010.

[14] G. Rodríguez-Gutiérrez, A. Lama-Muñoz, M. V Ruiz-Méndez, F. Rubio-Senent, J. Fernández-Bolaños, "New olive-pomace oil improved by hydrothermal pre-treatments". In: D. Boskou (Ed.) *Olive Oil - Constituents, Quality, Health Properties and Bioconversions*, IntechOpen, 2012, pp. 249–266.

[15] S. Rodrigues, G. A. S. Pinto, F. A. N. Fernandes, "Optimization of ultrasound extraction of phenolic compounds from coconut (Cocos nucifera) shell powder by response surface methodology", *Ultrason. Sonochem.*, 15 (1), 95–100, 2008.

[16] T. B. Ribeiro, A. L. Oliveira, C. Costa, J. Nunes, A. A. Vicente, M. Pintado, "Total and sustainable valorisation of olive pomace using a fractionation approach", *Appl. Sci.*, 10 (19), 6785, 2020.

[17] S. Chanioti, G. Liadakis, C. Tzia, "Solid–liquid extraction". In: T. Varzakas, C. Tzia (Eds.) *Food Engineering Handbook: Food Process Engineering*, CRC Press, Boca Raton, 2015, pp. 253–286.

[18] R. Minjares-Fuentes, A. Femenia, M. C. Garau, J. A. Meza-Velázquez, S. Simal, C. Rosselló, "Ultrasound-assisted extraction of pectins from grape pomace using citric acid: A response surface methodology approach", *Carbohydr. Polym.*, 106, (1), 179–189, 2014.

PhD Thesis

Chapter 1: Characterization and optimization of olive pomace oil extraction by using ultrasonic and conventional methods

[19]   M. C. Herrera, M. D. Luque De Castro, "Ultrasound-assisted extraction of phenolic compounds from strawberries prior to liquid chromatographic separation and photodiode array ultraviolet detection", *J. Chromatogr. A*, 1100 (1), 1–7, 2005.

[20]   T. J. Mason, L. Paniwnyk, J. P. Lorimer, "The uses of ultrasound in food technology", *Ultrason. Sonochem.*, 3, S253–S260, 1996.

[21]   J. Azmir, I. Zaidul, M. Rahman, K. Sharif, A. Mohamed, F. Sahena, M. Jahurul, K. Ghafoor, N. Norulaini, A. Omar, "Techniques for extraction of bioactive compounds from plant materials: A review", *J. Food Eng.*, 117 (4), 426–436, 2013.

[22]   AOAC, "AOAC Official Method 991.39. Fatty acids in encapsulated fish oils and fish oil methyl and ethyl esters," 1995.

[23]   S. Rebolleda, S. Beltrán, M. T. Sanz, M. L. González-SanJosé, "Supercritical fluid extraction of wheat bran oil: Study of extraction yield and oil quality", *Eur. J. Lipid Sci. Technol.*, 116 (3), 319–327, 2014.

[24]   Á. G. Solaesa, M. T. Sanz, M. Falkeborg, S. Beltrán, Z. Guo, "Production and concentration of monoacylglycerols rich in omega-3 polyunsaturated fatty acids by enzymatic glycerolysis and molecular distillation", *Food Chem.*, 190, 960–967, 2016.

[25]   P. Ghorbannezhad, A. Bay, M. Yolmeh, R. Yadollahi, J. Y. Moghadam, "Optimization of coagulation–flocculation process for medium density fiberboard (MDF) wastewater through response surface methodology", *Desalin. Water Treat.*, 57 (56), 26916–26931, 2016.

[26]   S. Şahin, R. Şamli, "Optimization of olive leaf extract obtained by ultrasound-assisted extraction with response surface methodology", *Ultrason. Sonochem.*, 20, (1), 595–602, 2013.

[27]   A. M. Goula, "Ultrasound-assisted extraction of pomegranate seed oil - Kinetic modeling", *J. Food Eng.*, 117 (4), 492–498, 2013.

[28]   T. Li, X. Y. Qu, Q. A. Zhang, Z. Z. Wang, "Ultrasound-assisted extraction and profile characteristics of seed oil from Isatis indigotica Fort", *Ind. Crops Prod.*, 35 (1), 98–104, 2012.

[29]   S. Mani, S. Jaya, R. Vadivambal, "Optimization of solvent extraction of moringa (Moringa Oleifera) seed kernel oil using response surface methodology", *Food Bioprod. Process.*, 85 (4C), 328–335, 2007.

[30]   R. Minjares-Fuentes, A. Femenia, M. C. Garau, J. A. Meza-Velázquez, S. Simal, C. Rosselló, "Ultrasound-assisted extraction of pectins from grape pomace using citric acid: A response surface methodology approach", *Carbohydr. Polym.*, 106 (1), 179–189, 2014.

[31]   S. Chanioti, C. Tzia, "Optimization of ultrasound-assisted extraction of oil from olive pomace using response surface technology: Oil recovery, unsaponifiable matter, total phenol content and antioxidant activity", *LWT - Food Sci. Technol.*, 79, 178–189, 2017.

[32]   S. Chanioti, C. Tzia, "Evaluation of ultrasound assisted and conventional methods

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for production of olive pomace oil enriched in sterols and squalene", *LWT-Food Sci. Technol.*, 99, 209–216, 2019.

[33]   M. Stroescu, A. Stoica-Guzun, S. Ghergu, N. Chira, I. Jipa, "Optimization of fatty acids extraction from Portulaca oleracea seed using response surface methodology", *Ind. Crops Prod.*, 43 (1), 405–411, 2013.

[34]   M. D. Kostić, N. M. Joković, O. S. Stamenković, K. M. Rajković, P. S. Milić, V. B. Veljković, "Optimization of hempseed oil extraction by n-hexane", *Ind. Crops Prod.*, 48, 133–143, 2013.

[35]   E. Uribe, R. Lemus-Mondaca, A. Vega-Gálvez, M. Zamorano, I. Quispe-Fuentes, A. Pasten, K. Di Scala, "Influence of process temperature on drying kinetics, physicochemical properties and antioxidant capacity of the olive-waste cake", *Food Chem.*, 147, 170–176, 2014.

[36]   J. Orsavova, L. Misurcova, J. Ambrozova, R. Vicha, J. Mlcek, "Fatty acids composition of vegetable oils and its contribution to dietary energy intake and dependence of cardiovascular mortality on dietary intake of fatty acids", *Int. J. Mol. Sci.*, 16 (6), 12871–12890, 2015.

[37]   V. Dubois, S. Breton, M. Linder, J. Fanni, M. Parmentier, "Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential", *Eur. J. Lipid Sci. Technol.*, 109 (7), 710–732, 2007.

[38]   J.-M. Yun, J. Surh, "Fatty acid composition as a predictor for the oxidation stability of Korean vegetable oils with or without induced oxidative stress", *Prev. Nutr. Food Sci.*, 17, 158-165, 2012.

[39]   H. Li, Y. Fan, J. Li, L. Tang, J. Hu, Z. Deng, "Evaluating and predicting the oxidative stability of vegetable oils with different fatty acid compositions", *J. Food Sci.*, 78 (4), H633–H641, 2013.

[40]   E. Uribe, R. Lemus-Mondaca, A. Vega-Gálvez, L. López, K. Pereira, J. López, K. Ah-Hen, K. Di Scala, "Quality characterization of waste olive cake during hot air drying: nutritional aspects and antioxidant activity", *Food Bioprocess Technol.*, 6 (5), 1207–1217, 2013.

[41]   A. Allalout, D. Krichène, K. Methenni, A. Taamalli, I. Oueslati, D. Daoud, M. Zarrouk, "Characterization of virgin olive oil from Super Intensive Spanish and Greek varieties grown in northern Tunisia", *Sci. Hortic.*, 120 (1), 77–83, 2009.

[42]   M. Antónia Nunes, A. Costa, S. Bessada, J. Santos, H. Puga, R. Alves, V. Freitas, M. Oliveira, "Olive pomace as a valuable source of bioactive compounds: A study regarding its lipid- and water-soluble components", *Sci. Total Environ.*, 644, 229–236, 2018.

[43]   Codex Alimentarius, "Standard for olive oils and olive pomace oils", p. 9, 2013, doi: 10.1017/CBO9781107415324.004.

Chapter 2 - Optimization and comparative evaluation of phenolic extraction from olive mill solid wastes and phenol recovery by adsorption process

VALORIZATION OF RESIDUES GENERATED IN THE PRODUCTION OF OLIVE OIL
THROUGH CLEAN TECHNOLOGIES

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La investigación que se presenta en este capítulo surge de la necesidad de valorizar los residuos sólidos de almazara generados en el capítulo 1, una vez extraído el aceite de orujo de oliva. Estos residuos sólidos se consideran una fuente rica de compuestos fenólicos con una amplia gama de actividades biológicas. El objetivo de este trabajo ha sido optimizar la extracción de los compuestos fenólicos presentes en dos tipos de residuos sólidos de almazara, generados en sistemas de centrifugación de dos fases (alperujo) y tres fases (orujo), después de la extracción del aceite que contienen. Inicialmente se seleccionó el disolvente más adecuado y posteriormente se aplicó la metodología de superficie de respuesta (RSM) con el fin de maximizar el contenido de fenoles totales (TPC), flavonoides totales (TFC) y actividad antioxidante (AA) en los extractos finales. Se estudiaron dos métodos de extracción asistida por ultrasonidos: i) utilizando un baño de ultrasonidos, considerando el tiempo de extracción, la temperatura y la relación sólido/disolvente como variables de diseño de experimentos; ii) utilizando un homogeneizador ultrasónico para aplicar directamente los ultrasonidos a la muestra mediante una sonda, siendo en este caso las variables de diseño el tiempo de extracción, la amplitud de los ultrasonidos y la relación sólido/disolvente. Los mejores resultados se lograron utilizando el homogeneizador ultrasónico. Bajo las condiciones de operación óptimas, se obtuvo un extracto de alperujo con las siguientes características: TPC = 18,40 mg GAE/g, TFC = 2,09 mg QE/g y AA =0,859 mM$_{Trolox}$/g, así como un extracto de orujo con TPC = 14,70 mg GAE/g, TFC = 2,48 mg QE/g y AA =0,924 mM$_{Trolox}$/g. Posteriormente, se aplicó un proceso de adsorción-desorción para la recuperación y el fraccionamiento selectivo de biofenoles valiosos. Se utilizaron como adsorbentes dos carbones activados (NPAC y GAC) y dos resinas poliméricas (Amberlita XAD4 y XAD16), y se utilizaron diferentes disolventes en la etapa de desorción. Los resultados se compararon en términos de rendimiento de adsorción y recuperación por desorción de fenoles totales y compuestos fenólicos individuales, concretamente hidroxitirosol, tirosol y oleuropeína. La adsorción utilizando 100 g/L de la resina XAD16 en un dispositivo de agitación magnética produjo los mejores resultados, con un 74,36% de adsorción de fenoles totales y del 39,25%, 68,79% y 100% de hidroxitirosol, tirosol y oleuropeína, respectivamente. Después de una etapa de desorción con etanol puro se logró un 52,27% de recuperación fenoles totales, con un 14,12%, 11,19% y 32,08% de recuperación de los fenoles individuales hidroxitirosol, tirosol y oleuropeína, respectivamente. El uso de etanol-agua al 50% v/v acidificado a pH 2 en la etapa de desroción mejoró los resultados, obteniendo un 57.65% de recuperación total de fenoles, un 19.27% de recuperación de hidroxitirosol y un 45.73% de recuperación de tirosol, pero la recuperación de oleuropeína fue del 0%.

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2.1. Introduction

Olive oil extraction involves different processes such as olive washing, grinding, beating and the extraction itself, that constitutes the basic stage of the whole process. The amount and physico-chemical properties of the wastes produced will depend on the method used for the extraction. There are two ways of extracting the oil: traditional pressing, used for many centuries with only minor modifications, and centrifugation, that the olive oil industry has taken over in the last decades. Two widely used methods for the centrifugation extraction of olive oil include a two-phase or a three-phase centrifugation system [1]. The by -products of oil extraction are black olive mill wastewater and solid waste, or olive cake [1]. The olive oil industry produces 35 kg olive cake and 440 L oil mill wastewater per 100 kg of treated olive [2]. Thus, olive cake is an inexpensive biomass that is available in large quantities in Mediterranean countries. However, it represents a serious environmental problem for olive oil producing countries [3]. Therefore, many studies have aimed to either reduce the environmental impact of olive cake and/or harness its potential economic value [4].

The three-phase system generates a solid husk, oil, and OMWW while the two phase system releases a wet olive husk and oil. Relative to the two-phase centrifugation, the three phase system utilizes 0.6–1.3 m^3 of additional water during the three phase decantation that eventually increases the amount of OMWW [5].

Olive is rich in phenolic compounds [6] and its bitter taste is associated with these compounds, basically [7]. Presence of water soluble biophenolic compounds (98% of the total phenols in the olive fruit), which are partitioned to water from the olive fruit during the oil extraction, represent the highest polluting capacity [8–10]. The important point is that just about 2% of its phenolic content enters the oil phase during oil extraction while about 45% of olive's phenolic compounds enter olive-waste cake [6]. The negative environmental and socioeconomic impacts of this industrial activity are more than obvious since a large number of processing facilities are located close to sea resorts and places of high tourist interest [11]. A

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suitable use of these olive oil residues could not only improve the economic status of olive oil producers but could also reduce such an environmental problem. Thus, it is of great interest to evaluate the possibility of recovering an extract enriched with phenolic compounds, from a low-cost and widely available by-product, especially in the Mediterranean area [12].

This solid waste is of heterogeneous nature and can be found along with many chemical compounds, such as alkaline (potassium) and alkaline-earth (calcium and magnesium metals), sugars and polyphenols, which come from the vegetation water [13]. Since, only 2% of the phenolic compounds are transferred to the oil and as much as 98% retained in the cake, olive pomace has been considered to be an interesting source of phenolic compounds [14]. Phenolic compounds of olive-waste cake are solid, colorless, water soluble with considerable environmental resistance and they decompose hardy. Therefore, entrance of waste cake from olive oil extraction plants, and in fact the entrance of their phenolic compounds to underground water and soil is hazardous. Consequently, these compounds should be extracted and removed from the olive waste cake [15]. Furthermore, extraction of phenolic compounds are economically important due to their applications in pharmaceutical, cosmetic and food industries.

Olive cake is considered a rich source of phenolic compounds with a wide array of biological activities. However, little information has been reported on the recovery of phenolic compounds from olive cake as a potential source of bioactive compounds for the pharmaceutical and nutraceutical industries [16,17]. The major component of the phenolic fraction present in olive mill wastes is hydroxytyrosol, part of which originates from oleuropein (the most abundant phenol contained in several olive cultivars) through an esterase-mediated cleavage during oil extraction. Hydroxytyrosol is a potent antioxidant, with reported abilities to scavenge oxygen and nitrogen free radicals, to inhibit low density lipoprotein oxidation, platelet aggregation, and endothelial cell activation and to protect against DNA damage [18,19]. Furthermore, hydroxytyrosol reduces the synthesis of prostaglandin E2 blocking the transcription of COX-2 and 5-lipooxygenase,

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thereby decreasing the chronic influence associated with diseases such as cancer [20]. Despite these important properties, hydroxytyrosol is not commercially available in large amounts since all the synthetic or extractive developed routes have resulted to be cumbersome, expensive, or not eco-sustainable [21, 22]. Three aspects of antioxidant activity have been evaluated in olive cakes; antioxidant potency [23, 24] anti-radical activities and radical scavenging activities [3, 24].

The traditional extraction methods are very time-consuming and need large amount of solvents for bioactive compounds. Currently, several modern extraction methods have been developed for extraction of phenolic compounds, such as enzymatic extraction, ultrasonic-assisted extraction (UAE), and supercritical fluid extraction [25]. The UAE is less expensive and much easier compared to some other extraction methods such as microwave-assisted extraction (MAE) and supercritical fluid extraction [26]. To enhance overall yield and selectivity of bioactive components from plant materials, ultrasound [27, 28] , pulsed electric field [29], enzyme digestion [30], microwave heating [31], ohmic heating [32], supercritical fluids [33– 38], and accelerated solvents [31, 39] have been studied as non-conventional methods. At the same time conventional extraction methods, such as Soxhlet is still considered as one of the reference method to compare success of newly developed methodology. Substantial number of scientific reports, book chapters and monograms exist where non-conventional methods were extensively reviewed [40– 43].

Ultrasound is a special type of sound wave beyond human hearing. Usually, in chemistry it is 20 kHz to 100 MHz. Like other waves, it passes through a medium by creating compression and expansion. This process produces a phenomenon called cavitation, which means production, growth and collapse of bubbles. A large amount of energy can produce from the conversion of kinetic energy of motion into heating the contents of the bubble [44]. The extraction mechanism by ultrasound involves two main types of physical phenomena, (a) the diffusion across the cell wall and (b) rinsing the contents of cell after breaking the walls [45]. Moisture content of sample, milling degree, particle size and solvent are very

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important factors for obtaining efficient and effective extraction. Furthermore, temperature, pressure, frequency and time of sonication are the governing factors for the action of ultrasound [25].

On the other hand, adsorption technology is one of the most commonly applied processes for the recovery of polyphenols from plant extracts and is gaining increasing importance in food industry [46]. The application of synthetic resins has several advantages such as relatively low operation costs, simple handling, its long service lives resulting of regeneration and reuse ability [47]. Compared with alternative technologies, adsorption is attractive for its relative simplicity of design, operation and scale up, its potential selectivity to toxic substances. Besides, adsorption avoids toxic solvents and minimizes the transformation of the target sub-stances [48]. Among the disadvantages of adsorption technology are the limited overall purification efficiency [49], the loss of capacity observed after the first regeneration and solvent regeneration which has to be developed and/or adapted [50].

It is well known that adsorption is an effective technique which requires relatively low economic investment, quite high energy saving and high efficiency in removal, recovery and selective separation of some compounds. In the recent years, several studies dealt with the uptake of polyphenols and other bioactive compounds from OMW on different adsorbents [51– 56].

In this chapter, two types of olive mill solid residues obtained from two-phase and three-phase centrifugation systems were used as samples which will be hereinafter called olive cake and olive pomace, respectively. Primary objective was solvent optimization and then extraction operating conditions was optimized for olive cake based on response surface methodology with two ultrasonic assisted extraction methods. Subsequently, the optimal ultrasonic method was also used in order to determine the optimal operating conditions for phenolic extraction from olive pomace.

As the next step, adsorption-desorption process was applied for recovery and selective isolation of valuable biophenols of obtained phenolic loaded extract from

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olive pomace. For this purpose XAD-4 and XAD-16 polymeric resins along with two different types of activated charcoals were used as the adsorbents. Furthermore, obtained results from adsorption-desorption experiments were evaluated and optimized in terms of total phenol recovery and also selective isolation of hydroxytyrosol, tyrosol and oleuropein which are major component of the phenolic fraction present in olive mill wastes.

2.2. Materials and methods

2.2.1. Samples and chemicals

Olive cake used in this study was kindly provided by the cooperative "Pagos de Benaval". It was obtained by means of a two-phase cold oil extraction process using olives from olive trees of the variety "Serrana de Espadán", endemic of the Sierra de Espadán natural park (Castellón, Spain). Olive pomace used in this study was kindly provided by the "Mamalan" Agro Industrial Company, Tarom city (Zanjan), Iran. It is obtained by means of a three phase centrifugal extraction process.

The chemicals used in the experiments of this chapter are Folin–Ciocalteu's reagent, acetic acid and acetonitrile and were purchased form VWR International Eurolab (Barcelona, Spain). In addition, Sodium carbonate, gallic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), quercetin, methanol, ethanol, aluminum chloride, potassium acetate, hydrochloric acid (HCl-37%) and Ammonium acetate (NH_4Ac) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). The polymeric resins XAD4 and XAD16 were provided from Fluka (Germany) which have been reported to yield satisfactory adsorption results for phenolic compounds [50, 55, 57– 61].

Furthermore, powdered activated charcoal (Norit-97876) was provided from Sigma Aldrich (Castle Hill, NSW, Australia) and Granulated Activated Charcoal (CA03461000) was purchased from Scharlau (Barcelona, Spain) which hereinafter

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they will be called NPAC and GAC, respectively. Activated Charcoal has been also used in previous researches [22, 62, 63]. Characteristics of all adsorbents are presented in Table 2.1.

Table 2.1. Characteristics of the adsorbents

Adsorbent	Dry density (g/mL)	Mesh	Mean pore size (Å)	Particle diameter (mm)	Specific surface (m²/g)
XAD-4 polymeric resin	1.02	20-60	40	0.25 – 0.84	725
XAD-16 polymeric resin	1.02	20-60	200	0.25 – 0.84	900
Norit Powdered Activated Charcoal (NPAC)	0.25-0.60	-	-	0.15 – 0.25	1000
Granulated Activated Charcoal (GAC)	2-2.1	-	-	1-3	900-1200

2.2.2. Pretreatment of Olive mill solid waste

Olive cake and olive pomace were dried in 100 °C for 24 hours in an oven (model 2600210, JP Selecta, Barcelona, Spain) to remove most of their moisture (about 55-60% w/w). They were then ground by using a coffee grinder to obtain an average particle diameter of about 1 mm.

For applying defatting process, soxhlet method was performed by BUCHI 811 extraction system (BÜCHI Laboratotiums Tchnik AG, Flawil, Switzerland). The ground olive cake (about 20 g, 5 g in each of the 4 extraction chambers) was placed in the Soxhlet apparatus and continuously refluxed with 400 mL (100 mL for each chamber) of n-hexane for 25 cycles (at 69°C for 3 h). After oil extraction, n-hexane was distilled using the same system. The obtained oil was weighted resulting into the average recovery yield of 12.98-13.06% for olive pomace and olive cake, respectively. Then the pretreated solid samples were kept in a dark place at –20 °C for further use.

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2.2.3. Solvent optimization

In order to obtain the optimal solvent for phenolic extraction, seven extraction runs were performed in ultrasonic bath (Selecta Ultrasons-H, 720W, 50/60 Hz, Barcelona, Spain) with different types of solvent but same operating conditions (Time: 60 min, Temperature: 50 °C, solid:solvent ratio: 10% (w/v)), then the supernatants were centrifuged at 10000 rpm for 10 min and filtered. Table 2.2 shows results of solvent optimization process. All the analytical methods used in this work are completely described in the appendix section of this thesis.

Table 2.2.   optimal solvent selection for phenolic extraction

	Type of solvent	**Total Phenolic Content (mg$_{GAE}$/g)**	**Antioxidant Activity (mM$_{Trolox}$/g)**
1	Methanol - Water (50-50%)	12.56	0.846
2	Methanol (100%)	8.09	0.859
3	Ethanol- Water (50-50%)	16.24	0.794
4	Ethanol- Water (75-25 %)	8.58	0.845
5	Ethanol (100 %)	6.06	0.863
6	Ethanol- Water (25-75 %)	10.88	0.787
7	Water (100 %)	10.20	0.792

2.2.4. Phenolic extraction using an ultrasonic bath

Based on response surface methodology and depending on variable factors of the experimental design (time, temperature and S/S ratio), pretreated olive cake was placed in Falcon centrifuge tubes and mixed with the selected solvent. Then it was entered to an ultrasonic bath (Selecta Ultrasons-H, 720W, 50/60 Hz, JP Selecta, Barcelona, Spain). After sonication, phenolic loaded extracts were centrifuged at 10000 rpm for 10 min (Eppendorf Centrifuge model 5804). The supernatants were filtered through 0.45 μm syringe filters and then stored at –20 °C for further analysis.

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2.2.5.   Phenolic extraction using an ultrasonic homogenizer

Based on RSM and depending on variable factors of the experimental design (sonication time, amplitude and S/S ratio), pretreated olive cake samples were placed in Falcon centrifuge tubes and mixed with the selected solvent. Then, these tubes containing samples were entered to a high intensity ultrasonic homogenizer (Sonics VCX500, 500 W, 20 kHz, Newtown, CT, USA) equipped with a titanium alloy microtip probe of 3 mm diameter, where UAE was performed at 30 ºC with ultrasound pulses every 5 seconds (5 s on and 5 s off). After UAE, phenolic loaded extracts were centrifuged at 10000 rpm for 10 min, and the supernatants were filtered and stored at –20 °C for further analysis.

2.2.6.   Preliminary UAE experiments

In this study, the input factor for Solid/Solvent (S/S) ratio was considered to be 1-20 (w/v%). At First, preliminary extractions with predicted optimal operating conditions were performed with S/S ratios of 1-3 (w/v%). Then, secondary extractions were performed with the S/S ratios of 4-20 (w/v%) based on same methodology with the "central composite design: 2^3 + star" model. As it can be observed from Table 2.3, satisfactory results were obtained with 1-3 (w/v %) S/S ratios, but the use of these low ratios is not economically feasible form an industrial point of view, so these results were not considered in the optimization and comparative evaluation of the proposed methods.

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Table 2.3. Preliminary experimental runs for phenolic extraction

ULTRASONIC BATH						
Run number	Operating conditions			TPC (mg$_{GAE}$/g)	TFC (mg$_{QE}$/g)	DPPH assay (mM$_{Trolox}$/g)
	Time (min)	S/S Ratio (w/v%)	Temperature (°c)			
1	120	3	45	17.43	2.36	1.051
2	120	2	45	22.35	2.67	1.050
3	120	1	45	29.10	2.70	0.741
4	60	3	60	20.30	3.22	1.050
5	60	2	60	20.40	2.42	1.023
6	60	1	60	27.20	1.80	0.780
ULTRASONIC HOMOGENIZER						
Run number	Operating conditions			TPC (mg$_{GAE}$/g)	TFC (mg$_{QE}$/g)	DPPH assay (mM$_{Trolox}$/g)
	Time (min)	S/S Ratio (w/v%)	Amplitude (%)			
1	10	3	20	14.63	1.65	1.048
2	10	2	20	17.45	1.98	1.014
3	10	1	20	26.8	2.00	0.586
4	10	3	40	16.1	2.45	1.048
5	10	2	40	18.85	2.36	1.057
6	10	1	40	24.9	2.73	0.782

TPC: total phenolic content, TFC: Total flavonoid content

2.2.7. *Experimental Design and statistical analysis for phenolic extraction*

Data were analyzed using response surface methodology (RSM) with "central composite design: 2^3 + star" (CCD) model by the application of Statgraphics version 18 software (Statgraphics Technologies, Inc, Warrenton, VA, USA). The effects of three input variable factors were studied on the total phenolic content (Y_1), total flavonoids content (Y_2) and antioxidant activity (Y_3). Variable factors (X_1, X_2, X_3) were different depending on the utilized ultrasonic method for each extraction series.

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The model generated 16 experimental settings with two replicates in central point to optimize the phenolic extraction process. A low degree polynomial equation was used to express predicted responses (Y_1-Y_3) as a function of the independent variables under study (X_1, X_2, X_3). The model equation is as Eq. (1):

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 \tag{1}$$

Where Y represents the response variable, a_0 is a constant, and a_i, a_{ii}, a_{ij} are the linear, quadratic and interactive coefficients, respectively.

The significance of each estimated regression coefficient was assessed through values of the statistic parameters, F and p (probability). Data means were separated by the least significant difference (LSD) at $p \leq 0.05$. Generally in ANOVA, large F-value and small p value show a more significant effect on response [64]. The ANOVA table partitions the variability in total phenolic content (TPC), total flavonoid content (TFC) and Antioxidant Activity (AA) into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error.

2.2.7.1. Statistical data for phenolic extraction from olive mill samples

Phenolic extractions with olive cake were performed within two separate experimental designs but by applying same design model and methodology. For this purpose, considered variable factors for phenolic extraction with ultrasonic bath were Time (X_1: 40-120 minutes), Temperature (X_2: 30-60 °C) and S/S ratio (X_3: 4-20% w/v). The considered variable factor ranges for phenolic extraction from olive cake with ultrasonic homogenizer were Time (X_1: 2-10 minutes), S/S ratio (X_2: 4-20% w/v) and Amplitude (X_3: 20-40 %). The corresponding Central Composite Design (CCD) matrixes are presented in Tables 2.4 and 2.5. It should be mentioned that some extra experimental runs were performed for both designs due to fortifying the optimization process of the phenolic extraction.

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Regarding phenolic extraction with olive pomace, experimental design was only executed with ultrasonic homogenizer in order to be able to perform a comparative evaluation between extraction results of olive mill samples from two different centrifugation systems. Thus, considered variable factors for olive pomace were same as olive cake which were Time (X_1: 2-10 minutes), S/S ratio (X_2: 4-20% w/v) and Amplitude (X_3: 20-40 %). Whereas corresponding Central Composite Design (CCD) matrix is presented in Table 2.6.

2.2.8. Resin pretreatment for adsorption process

The pretreatment process was dedicated to polymeric resins (XAD4 and XAD16). Resins were first washed with distilled water and after getting dried were soaked and stirred in acetone at 150 rpm for 6 hours. This step ensured the removal of organic impurities and any monomers trapped in the resins [55]. Then, they were vacuum filtered and dried at room temperature. Subsequently, resins were rinsed in ethanol with 5 $mL_{ethanol}/g_{resin}$ ratio at 150 rpm for 2 hours. After complete removal of ethanol, distilled water was used to wash the resins thrice. Finally, pretreated resins were dried by an oven at 50 °C to constant weight [50, 55, 59].

2.2.9. Adsorption and Desorption Processes

In the adsorption experiments, 1.5 and 3 grams of pretreated resins or Activated charcoals was mixed with 30 mL of optimal olive extract in 50 mL flask (50 and 100 $g_{adsorbent}/L_{sample}$). All the flasks were shaken in incubator shaker (Model G25, New Brunswick, USA) or magnetic stirrer at a shaking speed of 150 rpm for 3 hours at room temperature. After adsorption runs reached equilibrium, adsorbents were separated by vacuum filtration and filtrates were analyzed.

Primary experiments were performed in order to choose between one of the two activated charcoal types (NPAC and GAC) used in this research. Therefore, adsorption experiment were performed with 50 g_{AC}/L_{sample} content in both magnetic

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stirrer and incubator shaker for 3 hours. After selecting the optimal type of activated charcoal, experiments were continued to determine the optimal device and operating conditions for adsorption process. For this purpose, three adsorbents (XAD4, XAD16 and NPAC) with two input contents of 50 and 100 g $_{adsorbent}$/L $_{sample}$ were used, resulting to six experimental runs performed with each device (incubator shaker and magnetic stirrer) being total of twelve adsorption experiments were executed. The adsorption related results were quantified by using the Eq. (2) and Eq. (3) [65, 66]:

$$\text{Adsorption Content: } Q_e = \frac{(C_0 - C_e)V_i}{W} \tag{2}$$

$$\text{Adsorption rate: } A = \frac{(C_0 - C_e)}{C_0} \times 100\% \tag{3}$$

Where Q_e stands for the equilibrated adsorption content (mg $_{Phenols}$/g $_{Adsorbent}$). C_0 and C_e refer to the initial and equilibrium concentration (mg $_{Phenols}$/mL $_{sample}$), respectively. A is the adsorption rate (%) and V_i is the volume of the initial sample solution (mL) and W is the weight of the adsorbent (g).

After each run adsorbents used were vacuum filtered and filtrate was allowed to determinate the content of total phenolic compounds. In addition to total phenolic content, measurement of Hydroxytyrosol, Tyrosol and Oleuropein contents was also performed on the filtrates obtained from adsorption process.

In desorption process, filtered adsorbents were mixed with 30 mL of ethanol and stirred in a magnetic stirrer at 150 rpm for 2 hours. The volume of used solvent in desorption was same as the sample volume in adsorption process. Ethanol was repeatedly used as desorption solvent in bibliography [22, 55, 56, 59, 65]. After each run, adsorbents were vacuum filtered and phenolic loaded ethanol was allowed to determinate the contents of total phenols, Hydroxytyrosol, Tyrosol and Oleuropein. The desorption capacity was evaluated as follows [65, 66]:

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Table 2.4. Central Composite Design (CCD) matrix of olive cake phenolic extraction by ultrasonic bath

	Variable factors			*Responses*		
Run	Time (min)	S/S Ratio (w/v %)	Temp. (°C)	TPC (mg$_{GAE}$/g)	TFC (mg$_{QE}$/g)	Antioxidant Activity (mM$_{Trolox}$/g)
1	120	4	60	14.52	2.53	0.975
2	80	20	45	7.45	1.44	0.889
3	80	4	45	13.55	2.94	0.970
4	80	12	60	7.05	1.89	0.964
5	80	12	45	7.13	2.08	0.912
6	40	20	45	9.23	2.13	0.925
7	40	4	60	11.9	2.32	0.954
8	80	12	30	7.54	1.79	0.927
9	80	12	45	7.77	1.59	0.927
10	120	20	30	6.32	1.62	0.910
11	120	20	60	7.00	1.55	0.845
12	40	20	60	6.49	1.48	0.959
13	120	12	45	9.88	1.96	0.932
14	40	20	30	6.67	1.52	0.920
15	40	4	30	13.97	2.56	0.934
16	120	4	45	13.72	2.53	0.987
17	40	12	45	6.81	1.67	0.963
18	120	4	30	11.77	2.14	0.967

Desorption rate: $\quad D = \frac{C_D V_D}{(C_0 - C_e)V_i} \times 100\%$ (4)

Total recovery: $\quad R = \frac{C_D V_D}{C_0 V_0} \times 100\%$ (5)

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Where D refers to the desorption rate (%). C_D represents the concentration of sample after desorption (mg/mL). V_D is the volume of the desorption solution (mL). R stands for total recovery (%) which shows the relation of final contents of responses after desorption compared to their content after Ethanol evaporation (Table 3.11). Whereas, C_0 and V_0 represent concentration and volume of the sample after Ethanol evaporation, respectively.

2.2.9.1. Adsorption kinetics

The adsorption kinetic models were studied on NPAC and two selected resins (XAD4, XAD16). The kinetic evaluation was conducted in magnetic stirrer at shaking speed of 150 rpm at room temperature. Then, an aliquots (1.0 mL) of supernatant from the each sample solution were taken at the time point of 30, 60, 90, 120, 150 and 180 minutes and the concentrations of phenolic in the solutions were measured by Folin-Ciocalteu colorimetric method to obtain the kinetic curves. Two kinds of models could be used to evaluate the adsorption process, which were analyzed to understand the adsorption mechanism of phenolic compounds on the adsorbents with time [65, 66].

Pseudo-first-order kinetic equation:

$$\ln (q_e - q_t) = -k_1 t + \ln q_e \qquad (6)$$

Pseudo-second-order kinetic equation:

$$\frac{1}{q_t} = \frac{1}{k_2 q_e^2} \times \frac{1}{t} + \frac{1}{q_e} \qquad (7)$$

The particle diffusion kinetic equation:

$$q_t = k_d . t^{1/2} + C \qquad (8)$$

Where q_e is the adsorption contents at equilibrium (mg $_{Phenols}$ /g $_{Adsorbent}$). q_t stands for concentration of phenolic absorbed at time t (mg $_{Phenols}$ /g $_{Adsorbent}$). k_1 and k_2

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represent the rate constant of two kinds of kinetic models, respectively. k_d refers to the rate constant of particle diffusion kinetics model.

Table 2.5. Central Composite Design (CCD) matrix of olive cake phenolic extraction by ultrasonic homogenizer

Run	Variable factors			Responses		
	Time (min)	S/S Ratio (w/v %)	Amplitude (%)	TPC (mg$_{GAE}$/g)	TFC (mg$_{QE}$/g)	Antioxidant Activity (mM$_{Trolox}$/g)
1	2.0	20.0	30.0	7.78	2.50	0.855
2	10.0	20.0	20.0	9.42	2.48	0.683
3	10.0	20.0	40.0	7.36	2.80	0.717
4	10.0	12.0	30.0	9.11	2.06	0.798
5	10.0	4.0	20.0	17.25	1.71	0.857
6	6.0	12.0	20.0	10.65	1.65	0.820
7	2.0	20.0	20.0	6.90	2.30	0.751
8	2.0	20.0	40.0	7.79	2.40	0.846
9	6.0	12.0	30.0	13.42	2.27	0.813
10	6.0	20.0	30.0	7.89	2.40	0.763
11	6.0	12.0	30.0	12.5	2.54	0.810
12	2.0	4.0	40.0	15.6	2.29	0.854
13	10.0	4.0	40.0	16.92	1.68	0.875
14	2.0	4.0	20.0	14.72	1.62	0.859
15	6.0	4.0	30.0	16.45	1.70	0.854
16	6.0	12.0	40.0	10.93	2.11	0.848
17	10.0	4.0	30.0	18.4	2.09	0.859
18	2.0	12.0	30.0	8.06	1.73	0.730
19	6.0	4.0	40	14.62	2.43	0.860
20	2.0	20.0	40	7.47	1.61	0.768
21	6.0	12.0	40	11.73	2.35	0.767

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Table 2.6. Central Composite Design (CCD) matrix of olive pomace phenolic extraction by ultrasonic homogenizer

run	*Variable factors*			*Responses*		
	Time (min)	S/S Ratio (w/v %)	Amplitude (%)	TPC (mg$_{GAE}$/g)	TFC (mg$_{QE}$/g)	Antioxidant Activity (mM$_{Trolox}$/g)
1	10	4	40	14.70	2.48	0.924
2	2	4	20	12.17	1.87	0.936
3	6	12	20	7.04	1.35	0.87
4	2	20	20	6.45	1.43	0.685
5	10	4	20	10.72	1.50	0.883
6	6	12	30	7.77	1.92	0.859
7	10	12	30	7.61	1.77	0.866
8	6	4	30	13.30	1.99	0.92
9	6	12	30	9.16	1.71	0.774
10	2	4	40	13.35	2.06	0.948
11	6	20	30	7.29	1.73	0.537
12	2	20	40	6.92	1.53	0.697
13	10	20	40	8.55	1.72	0.759
14	2	12	30	7.99	1.37	0.865
15	6	12	40	11.48	2.63	0.82
16	10	20	20	5.49	1.25	0.749

Table 2.7. Analyses of variance of the regression coefficients for olive cake phenolic extraction in ultrasonic bath (Time=X_1, Temperature=X_2, S/S ratio=X_3) of the quadratic Eq. (1) for responses (TPC, TFC and AA)

Source	*Total Phenolic Content*			*Total Flavonoids Content*			*Antioxidant Activity*		
	Regression coefficients	F-Ratio	P-Value	Regression coefficients	F-Ratio	P-Value	Regression coefficients	F-Ratio	P-Value
a_0	14.2001	-	-	1.74564	-	-	0.839395	-	-
a_1	-0.0888409	0.32	0.5889	-0.00118326	0.06	0.8178	0.000956354	1.98	0.1973
a_2	0.313441	0.04	0.8463	0.0684196	0.02	0.8795	0.00135441	0.38	0.5523
a_3	-1.30946	88.01	0.0000	-0.166211	28.66	0.0007	0.0083437	25.88	0.0009
a_{11}	0.000281791	0.49	0.5042	-0.0000327004	0.10	0.7626	0.00000487342	0.44	0.5261
a_{12}	0.00118333	3.40	0.1026	0.000125	0.56	0.4747	-0.0000241667	4.25	0.0730
a_{13}	-0.000417194	0.14	0.7210	0.000024789	0.01	0.9346	-0.0000711036	11.94	0.0086
a_{22}	-0.00445842	3.09	0.1168	-0.000824848	1.57	0.2456	0.0000168214	0.13	0.7257
a_{23}	-0.0001875	0.00	0.9549	-0.000270833	0.11	0.7535	-0.00005625	0.92	0.3651
a_{33}	0.0407166	16.33	0.0037	0.00504186	3.72	0.0900	-0.000159415	0.75	0.4111

a_0 is a constant, ai, aii and aij are the linear, quadratic and interactive coefficient

Table 2.8. Analyses of variance of the regression coefficients for olive cake phenolic extraction in ultrasonic homogenizer (Time=X_1, S/S ratio=X_2, Amplitude=X_3) of the quadratic Eq. (1) for responses (TPC, TFC and AA)

Source	Total Phenolic Content			Total Flavonoids Content			Antioxidant Activity		
	Regression coefficients	F-Ratio	P-Value	Regression coefficients	F-Ratio	P-Value	Regression coefficients	F-Ratio	P-Value
a_0	13.4766	-	-	1.39535	-	-	0.763588	-	-
a_1	1.46572	3.99	0.0710	-0.0143101	1.58	0.2348	0.00228592	6.13	0.0308
a_2	-1.04075	107.68	0.0000	0.0134154	81.31	0.0000	-0.00440057	36.72	0.0001
a_3	0.157599	0.17	0.6838	0.0732842	0.14	0.7134	0.00774806	0.89	0.3653
a_{11}	-0.0616793	1.75	0.2130	-0.00014832	0.00	0.9768	0.000080595	0.01	0.9399
a_{12}	-0.0124037	0.78	0.3960	-0.000304824	0.04	0.8430	-0.000789156	6.29	0.0291
a_{13}	-0.0125346	1.11	0.3145	0.0011088	0.76	0.4025	0.0000215375	0.01	0.9370
a_{22}	0.0250736	4.37	0.0606	-0.00238453	3.45	0.0903	-0.000024764	0.01	0.9282
a_{23}	0.00018425	0.00	0.9748	-0.0000536607	0.01	0.9316	0.000102901	0.65	0.4380
a_{33}	-0.0016933	0.06	0.8072	-0.00129423	3.18	0.1019	-0.000137546	0.82	0.3841

a_0 is a constant, a_i, a_{ii} and a_{ij} are the linear, quadratic and interactive coefficients

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Table 2.9. Analyses of variance of the regression coefficients for olive pomace phenolic extraction in ultrasonic homogenizer (Time=X_1, S/S ratio=X_2, Amplitude=X_3) of the quadratic Eq. (1) for responses (TPC, TFC and AA)

Source	Total Phenolic Content			Total Flavonoids Content			Antioxidant Activity		
	Regression coefficients	F-Ratio	P-Value	Regression coefficients	F-Ratio	P-Value	Regression coefficients	F-Ratio	P-Value
a_0	17.6842	-	-	2.28613	-	-	1.29098	-	-
a_1	0.157999	0.01	0.9376	0.106996	0.31	0.5989	-0.0527064	0.08	0.7888
a_2	-0.899188	160.91	0.0000	-0.00650216	7.31	0.0354	0.0123156	44.02	0.0006
a_3	-0.259476	31.79	0.0013	-0.0704034	13.28	0.0108	-0.0209026	0.02	0.8932
a_{11}	-0.0578879	4.17	0.0872	-0.0168642	2.79	0.1456	0.00349246	2.58	0.1590
a_{12}	0.00300781	0.14	0.7243	-0.00015625	0.00	0.9587	0.000792969	1.62	0.2505
a_{13}	0.0168438	6.70	0.0413	0.003625	2.45	0.1686	0.000084375	0.03	0.8712
a_{22}	0.0245124	11.96	0.0135	0.000315194	0.02	0.9046	-0.00126751	5.45	0.0583
a_{23}	-0.00254687	0.61	0.4636	-0.0009375	0.66	0.4492	-0.0000484375	0.04	0.8524
a_{33}	0.00533793	1.39	0.2838	0.00150172	0.87	0.3881	0.000353793	1.04	0.3480

2.3. Results

The principle of ultrasonic assisted extraction is to disrupt plant cell walls and increase mass transfer of intracellular components into the extraction solvent [67]. UAE enhanced the ability of water to extract compounds from the pomace without extracting any additional compounds. This increase can be attributed to the ability of Ultrasound to impact the microstructure of plant materials; since ultrasonic cavitation creates shear forces that disrupt cell walls, which enabled the extraction solvent to penetrate the pomace tissue and extract the phenolic compounds. Similar results have been reported previously [68– 71].

Ultrasonic bath and ultrasonic processor both employ the use of high frequency sound waves to achieve the desired results. However, a major difference between these two equipment lies in the way in which energy is transferred. An ultrasonic bath spreads energy diffusely over a large volume. It's mechanism for energy transfer is cavitation, whereby bubbles form and collapse. Whereas, in ultrasonic processor, the particles directly surrounding the probe get blasted with massive amounts of energy. Bubbles form and collapse in the surrounding solution, creating shear and shock waves. In terms of economical aspect, it is important to note that ultrasonic baths are far less expensive than ultrasonic processors. Furthermore, the probes in ultrasonic processors need to be replaced over time, whereas there are no such consumables in ultrasonic bath. According to the obtained results from both ultrasonic assisted methods, in terms of total phenolic content, ultrasonic homogenizer proved to obtain higher results. Nevertheless, regarding total flavonoids content and antioxidant activity, better results were obtained from ultrasonic bath.

2.3.1. Response surface analysis

The responses (TPC, TFC and AA) corresponding to the CCD matrix for three performed experimental designs are given in Tables 2.4, 2.5 and 2.6. Three response variable and three experimental factors were specified in the CCD design for each series of phenolic extraction. The selected design originally had 16 runs,

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with one sample to be taken during each run. The default model is quadratic with 10 coefficients. Statistical models have been fit to the response variable. Also of interest is the R-squared statistic, which shows the percentage of variation in the response that has been explained by the fitted model. Smaller value of R-squared indicates that dependent variables were less relevant to explaining the variation of behavior [72, 73]. The test is performed by comparing the variability of the current model residuals to the variability between observations at replicate settings of the factors. Significance of different terms of each coefficient was determined using the F-value and p-value. A large F-value and a small p-value would imply a more significant effect on the corresponding response variable [26].

2.3.1.1. Olive cake phenolic extraction by ultrasonic bath

According to data provided in Table 2.4, 18 runs of phenol extraction was executed by using ultrasonic bath. Total phenolic content ranged from 6.32 to 14.52 mg/g, total flavonoid content ranged from 1.44 to 2.56 mg/g and antioxidant activity ranged from 0.845-0.987 mM_{Trolox}/g. According to the obtained results, response surface methodology was a suitable model for this type of experiment design.

As the analysis of variance of the regression coefficients can be seen in Table 3.6, Statistical models have been fit to the response variables. Models with P-values below 0.05, of which there are 3, indicate that the model as fit is statistically significant at the 5.0% significance level. Also of interest is the R-squared statistic, which shows the percentage of variation in the response that has been explained by the fitted model. The R-squared values for TPC, TFC, and AA are from 93.82%, 81.16% and 84.87% respectively.

2.3.1.2. Olive cake phenolic extraction by ultrasonic homogenizer

According to data shown in Table 2.5, phenolic extractions were performed by using ultrasonic homogenizer. As responses, TPC ranged from 6.90 to 18.4 mg/g, TFC ranged from 1.61 to 2.80 mg/g and AA ranged from 0.683-0.875 mM_{Trolox}/g.

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According to the obtained results, response surface model was a suitable model for this type of experiment design.

Analysis of variance of the regression coefficients can be seen in Table 2.8. The significance of different terms of each coefficient was determined using the F-value and p-value. A large F-value and a small p-value would imply a more significant effect on the corresponding response variable [26]. Statistical models have been fit to the response variables. Models with P-values below 0.05, of which there are 3, indicate that the model as fit is statistically significant at the 5.0% significance level. Also of interest is the R-squared statistic, which shows the percentage of variation in the response that has been explained by the fitted model. The R-squared values for TPC, TFC, and AA are from 92.67%, 90.95% and 81.00%, respectively. Temperature was not considered as a controllable factor in this experimental design, however it varied in maximum between 30-60 °C for each run.

2.3.1.3. Olive pomace phenolic extraction by ultrasonic homogenizer

According to the model fitting, the R-Squared statistic indicates that the model as fitted explains 97.32%, 81.84% and 89.78% of the variability in TPC, TFC and AA, respectively. The adjusted R-squared statistic, which is more suitable for comparing models with different numbers of independent variables, is 93.30%, 54.60% and 74.46% for TPC, TFC and AA, respectively. The ANOVA measurement partitions the variability in responses into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, in the coefficients of the quadratic polynomial model (Eq.1), 4, 2 and 1 effects have P-values less than 0.05 for TPC, TFC and AA, respectively. It indicates that they are significantly different from zero at the 95.0% confidence level (Table 2.9). F-values indicate that, for the range of studied variables, S/S ratio had stronger influence on TPC and AA. While regarding TFC, amplitude had the strongest influence.

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2.3.2.   Optimization of phenolic extraction

2.3.2.1. Optimization of olive cake phenolic extraction by ultrasonic bath

Based on the obtained responses shown in Table 2.4, the most desirable results were obtained from run1 (time: 120 minutes, temperature: 60°C, S/S ratio 1:25) which resulted into extracting 14.52 mg_{GAE}/g of total phenolic compounds, 2.53 mg_{QE}/g of total flavonoids content and 0.975 mM_{Trolox}/g of antioxidant activity. Furthermore, the predicted optimal responses executed by response surface methodology with 88.97% of desirability at the optimal point (time: 120.0 minutes, temperature: 50.11 °C, S/S ratio: 4% (w/v)) will obtain 14.399 mg/g, 2.615 mg/g and 0.974 mM_{Trolox}/g for TPC, TFC and Antioxidant Activity, respectively. By comparing obtained results from run 1 in Table 2.4 with the predicted optimized response, it can be seen that the only advantage of predicted model is the slight excess in TFC which can be ignored. Therefore, it can be referred as the optimal condition for extraction of phenolic compounds from olive cake in ultrasonic bath.

2.3.2.2. Optimization of olive cake phenolic extraction in ultrasonic homogenizer

Based on the achieved responses shown in Table 2.5, the most desirable results were obtained from run 17 (time: 10 minutes, amplitude: 30%, S/S ratio: 4% (w/v)) which resulted into extracting 18.40 mg/g of phenolic compounds, 2.09 mg/g of flavonoid content and antioxidant activity of 0.859 mM_{Trolox}/g. Furthermore, in order to evaluate the possibility of obtaining more satisfactory results, extrapolated fitted model was performed which showed that by expanding the operational ranges of extraction with 96.2% desirability (time: 12.48 minutes, amplitude: 33%, S/S ratio: 1.71% (w/v)), predicted responses will be 18.40 mg/g, 2.66 mg/g, 0.900 mM Trolox/g for TPC, TFC and Antioxidant Activity, respectively. it can be concluded that predicted responses from extrapolated model do not differ significantly with optimal experimental results. Therefore, by considering economic advantage of obtained results from run 17 (Table 2.5), it can be referred

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as the optimal condition for extraction of phenolic compounds from olive cake in ultrasonic homogenizer.

2.3.2.3. Optimization of olive pomace phenolic extraction by ultrasonic homogenizer

Numerical optimization was carried out through Design Expert software, using desirability function method. The optimal conditions for the phenolic extraction process used in this work would be those leading to highest TPC, TFC and AA. The combined optimum ingredient levels for all responses with optimum desirability (94.98%) were predicted to be achieved by executing the following operating condition: time= 9.8 minutes, amplitude= 40% and S/S ratio: 4% (w/v). the predicted responses for the optimal input factors are 14.95 mg/g, 2.54 mg/g and 0.92 mM$_{Trolox}$/g for TPC, TFC and AA, respectively.

Based on the observed responses from Table 2.6, most desirable results were obtained from run 1 (time: 10 minutes, amplitude: 40%, S/S ratio: 4% (w/v)) whereas the responses were 14.70 mg/g of TPC, 2.48 mg/g of TFC and antioxidant activity of 0.924 mM$_{Trolox}$/g. Comparison of the predicted and experimental results prove that the results are completely in accordance with the responses predicted by design software. The research performed by Lavecchia and Zuorro (2016) [74] indicate that olive pomace from a three-phase oil production process is a rich source of flavonoids and that they can be recovered by a simple extraction procedure using aqueous ethanol as solvent.

2.3.3. Interactions of the input factors with responses (TPC, TFC, AA)

2.3.3.1. Interactions between responses and phenolic extraction with ultrasonic bath

Figure 2.1.a. shows the interactive effects between time, temperature total phenolic content (TPC) while the S/S ratio was kept constant at optimized point. TPC was increased significantly by decreasing S/S ratio which can be considered as the most

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significant variable factor. The achieved results range in this series of experiments for TPC is between 6.32- 14.52 mg/g, although TPC decreasing rate slows down as the ratio is 12-20 %. This study also revealed that increasing temperature and time separately make an increase in the amount of TPC, but it does not have a significant effect on it. For the times in the range of 40-80 minutes, TPC increases slightly while the temperature is 30-45°C and has a peak at 45°C, but it decreases slowly between 45-60°C. For the time range of 80-120 minutes, TPC increases slightly while the temperature is between 30-45°C, but it remains constant between 45-60 °C.

Figure 2.1.b. shows the interactive effects between time, temperature on total flavonoids content (TFC) while the S/S ratio was kept constant at optimized point. TFC was increased significantly by decreasing S/S ratio which can be considered as the most significant variable factor for this response. On the other hand, temperature in the degrees between 40-50 °C is the best range for extracting highest possible TFC. Besides that, increasing time has only a slight effect on TFC only in low temperatures which causes a slight decrease in total flavonoids content.

As it is displayed Figure 2.1.c., S/S ratio has the highest effect between the variable factors and antioxidant activity (AA). There is a significant interaction between time ranges 60-120 min and the S/S ratio. Somehow, as the S/S ratio increases while time is 60-120 min, antioxidant activity increases considerably.

In can be concluded from 3D response surface plot that increasing time has different effects. It causes and increase in AA while the S/S ratio is 0-4% (w/v). On the contrary, increasing time causes a significant decrease in AA while S/S ratio is 4-20% (w/v). In addition, increasing temperature affects the Antioxidant Activity amount mostly when time in 40-80 min.

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(a)

(b)

(c)

(d)

(e)

(f)
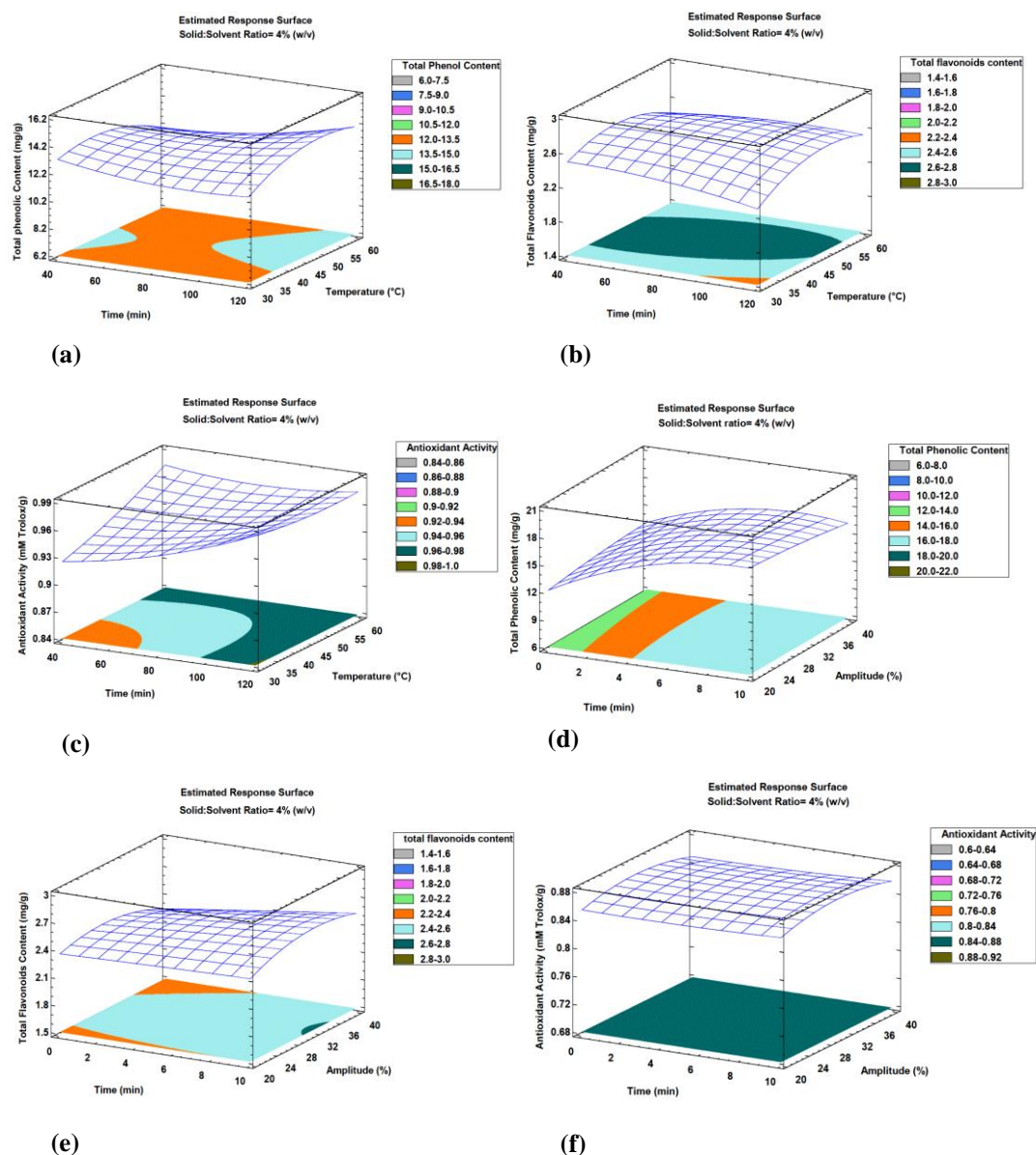
Figure 2.1. Response surface plots of interactions between input factors (X_1-X_3) for each response (Y_1-Y_3) in ultrasonic assisted phenolic extraction from olive cake

(a,b,c): Interaction of Time (X_1) and Temperature (X_2) at constant S:S ratio (X_3) with TPC (Y_1), TFC (Y_2) and AA (Y_3) in ultrasonic bath

(d,e,f): Interaction of Time (X_1) and Temperature (X_3) at constant S:S ratio (X_2) with TPC (Y_1), TFC (Y_2) and AA (Y_3) in ultrasonic homogenizer

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2.3.3.2. Interactions between responses and phenolic extraction with ultrasonic homogenizer

3.3.2.1. Olive cake

Figure 2.1.d. shows the interactive effects between time, amplitude on TPC while the S/S ratio was kept constant at optimized point. TPC was increased significantly by decreasing S/S ratio which can considered as the most significant variable factor. By analyzing the results, it can be concluded that increasing time regardless of the effect of amplitude, causes a slight growth of total phenolic content while S:S ratio is 4-12%. This increasing could be attributed to increase kinetic energy of solvent and its penetrating power [75]. The most significant effect of amplitude appears when S/S ratio is 1-4%, in which increasing amplitude decreases TPC, nevertheless changing amplitude has no significant interaction with other variables.

Figure 2.1.e. shows the interactive effects between time, amplitude on TFC while the S:S ratio was kept constant at optimized point. S/S ratio has the highest interaction with TFC comparing to other input variables. The only considerable interaction between time and amplitude regardless of S/S ratio is when time increases and amplitude is changed from 30% to 40% , this makes TFC reach higher points. On the other hand, changing only amplitude does not have any significant effect on TFC. According to the statistical prediction, the maximum level of TFC is achievable in the S/S ratio of 4% (w/v).

According to Figure 2.1.f., as the S/S ratio increases, antioxidant activity (AA) decreases significantly. Therefore similar last parts, S/S ratio is again the most significant variable. On the other hand, it can be concluded from 3D response surface plot that regardless of amplitude, increasing time is related to decreasing of antioxidant activity while S/S ratio is 12-20%. Nonetheless, for ratios less than 8%, time has no significant effect on AA. In addition, results prove that changing

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amplitude, only cause a slight decrease in AA while S/S ratio is 12-20%, although it cannot be considered as a significant factor.

(a)   (b)

(c)
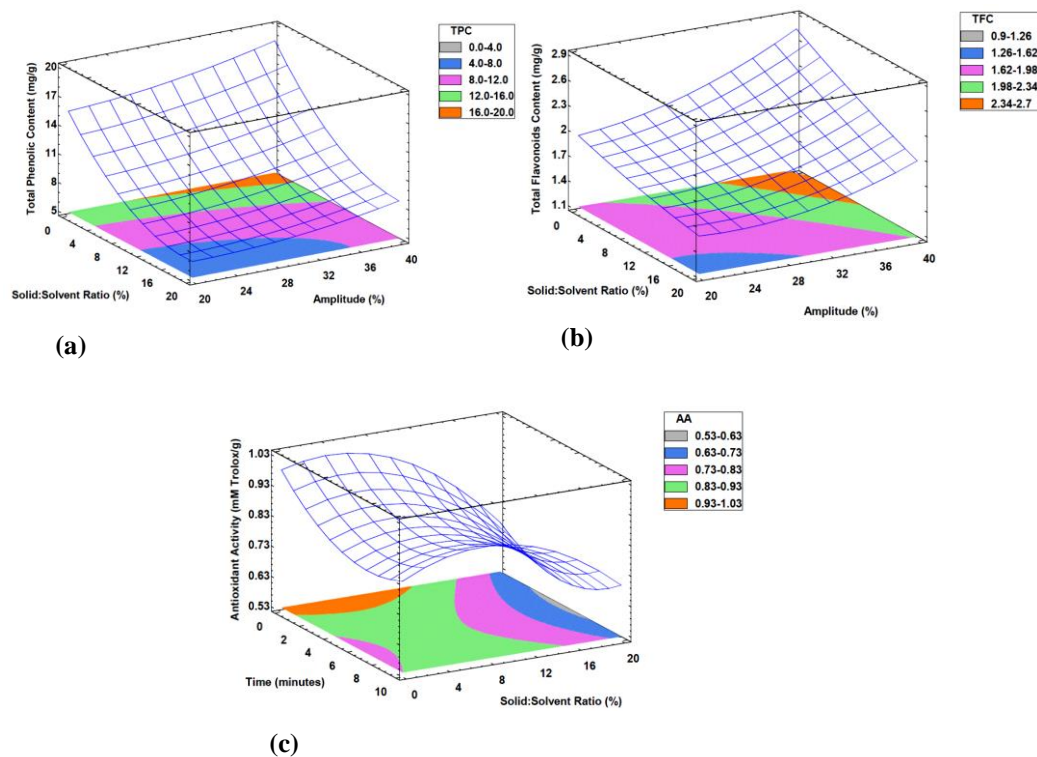
Figure 2.2. Response surface plots of interactions between input factors (X_1, X_2, X_3) and responses (Y_1-Y_3) in ultrasonic assisted phenolic extraction from olive pomace

(a): Interaction of Solid:Solvent ratio (X_2) and Amplitude (X_3) with TPC (Y_1) at central point of Time (X_1); **(b):** Interaction of Solid:Solvent ratio (X_2) and Amplitude (X_3) with TFC (Y_2) at central point of Time (X_1); **(c):** Interaction of Time (X_1) and Solid:Solvent ratio (X_2) with AA (Y_3) at central point of Amplitude (X_3)

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3.3.2.2. Olive pomace

In order to study the effect of the independent variables on responses, surface response plots of the quadratic polynomial model were generated by varying two of significant input variables within the experimental range while holding the third one constant at the central point. Figure 2.2.a. was generated by varying S/S ratio (X_2) and Amplitude (X_3) and their effect on TPC (Y_1) while holding Time (X_1) in central point. It shows that, regardless of amplitude intensity, an increase of S/S ratio causes a continuous decrease in TPC which is not favorable. The interaction of S/S ratio (X_2) and Amplitude (X_3) and their effect on TFC (Y_2) while holding Time (X_1) constant in central point can be observed in figure 2.2.b. This figure shows that high ratios (X_2) and low Amplitude (X_3) are not favorable for obtaining optimal TFC content. Somehow, higher TFC content can be achieved as the S/S ratio decreases and amplitude increases. Figure 2.3.c. shows the interaction between Time (X_1) and S/S ratio (X_2) and their effect on AA (Y_3) while holding Amplitude (X_3) constant at the central point. It can be observed that vary S/S ratio has a considerably more significant effect on AA. Whereas at lower S/S ratios, increasing time causes a slight decrease in AA. While in higher S/S ratios no significant effect occurs for AA by increasing time.

2.3.4.   Adsorption-Desorption batch experiments

The main phenolic compounds and derivatives identified in the olive samples of this study are distributed as follows: Protocatechuic acid, Hydroxytyrosol, Tyrosol, Vanillic acid, p-coumaric acid, Catechol and oleuropein. They are mostly distributed as phenolic alcohols (hydroxytyrosol and tyrosol), lignans (D(+)-erythro-1-(4-hydroxy-3- methoxy)-phenyl-1,2,3-propantriol and pinoresinol), secoiridoids and derivatives (oleuropein derivative, oleoside, oleoside riboside, aldehydic form of decarboxymethyl elenolic acid, elenolic acid derivative, oleoside digluside, verbascoside derivative, caffeoyl-6′-secologanoside) groups. These compounds have been previously described in olive pomace [76– 81].

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Among the major olive pomace phenolic compounds, hydroxytyrosol has gained particular interest over the years. This molecule presents strong antioxidant activity, anti-inflammatory and antimicrobial properties. Therefore, there is a high interest in this compound by cosmetics and pharmaceutical industries. The beneficial role of hydroxytyrosol in cardio and neurovascular diseases, as well as its anti-tumor effect, has also been demonstrated [82]. Moreover, its effect on food quality (chemical, nutritional and sensory properties) has also been explored [81, 83].

Several medicinal and pharmaceutical scientists have demonstrated that olive phenols, especially hydroxytyrosol (3,4-di-hydroxyphenyl-ethanol), were effective in preventing and curing some important diseases [84] . Nevertheless, hydroxytyrosol is not commercially available in large quantities like other food additives. Considering that efficiency of pH adjustment for olive mill wastes can be proved in increasing the concentration of Hydroxytyrosol up to 400% in the feed adding acid to the vegetation water in an amount effective to produce a pH between 2-4, and incubating the acidified vegetation water for a period of at least two months until at least 75% of the oleuropein originally present in the vegetation water will be converted to hydroxytyrosol [85]. On the other hand, adsorption of phenolics is strongly dependent on the ionic strength and/or pH value. At acidic pH, the uptake of phenolics by different sorbents is enhanced because phenols are undissociated and dispersion interactions predominate [48], [50]. Considering the previous conclusions in bibliography, optimal phenolic loaded extract obtained from olive pomace was acidified with HCl (37%) and pH was adjusted to 2.8 and was used as the sample in adsorption experiments.

2.3.4.1. Efficiency of materials and devices in phenol adsorption rates

As it is already mentioned in section 2.9 of this chapter, preliminary adsorption experiments was performed to determine optimal type of activated charcoal. It can be observed from Figure 2.3. that with 50 g_{AC}/L_{sample} content of NPAC and GAC, considerably higher adsorption rate can be achieved by using NPAC regardless of

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the device used for adsorption process. Therefore, NPAC was selected as the preferred type of activated charcoal in the next experiments. In order to determine optimal device and operating condition for performing adsorption process, experiments were continued with contents 50 and 100 g $_{adsorbent}$/L$_{sample}$ as the next step. Therefore, total of 12 adsorption experiments were performed with incubator shaker and magnetic stirrer, being six adsorption experiments performed with each device. After each run, adsorbents used were filtered and filtrate was allowed to determinate the content of total phenolic compounds. Obtained results from Figure 3.4(a,b), prove that there is no significant difference in operating device for adsorption regarding NPAC. Somehow, all obtained results show phenol adsorption rate of more than 90% for NPAC. Situation was totally different for polymeric resins. Comparing data provided in of Figure 2.4 proves that, phenol adsorption rate of both resins increase as the resin content increases from 50 to 100 g $_{resin}$/L$_{sample}$. However, adsorption rates obtained by incubator shaker are much less for both resins compared to the ones with same operating condition obtained by magnetic stirrer. Therefore, it can be concluded the efficiency and advantage of using magnetic stirrer for adsorption processes by the application of polymeric resins.
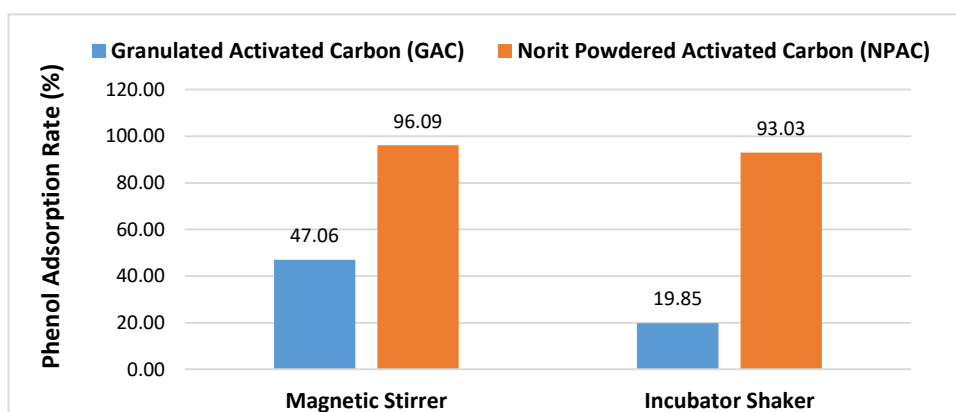
Figure 2.3.   Comparison between NPAC and GAC in 50 g/L adsorption

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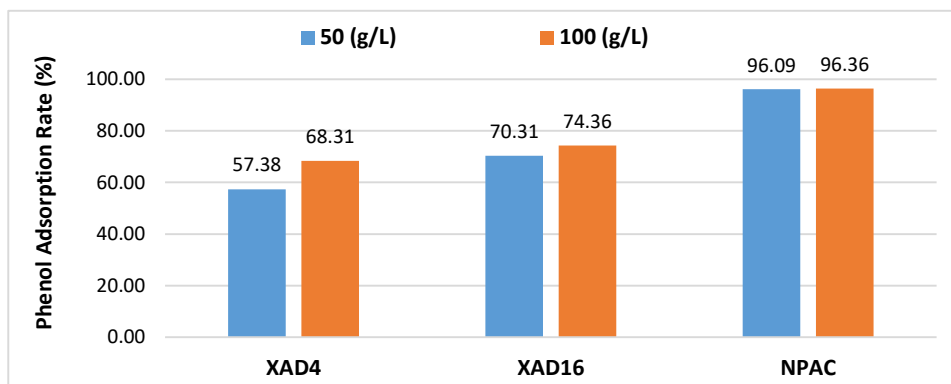
(a)
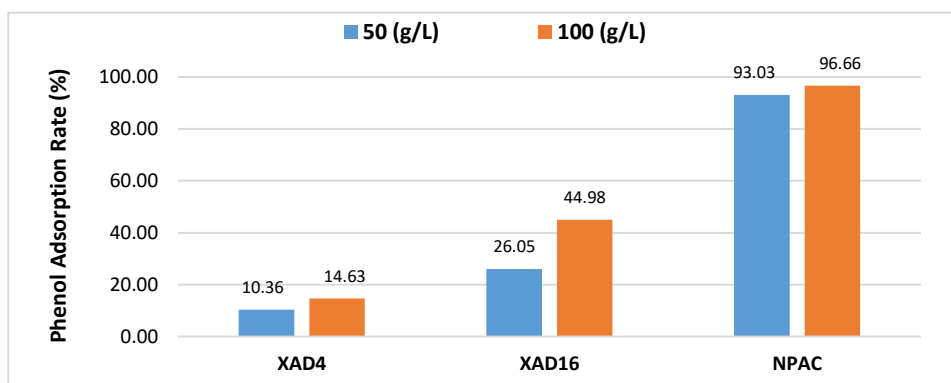
(b)

Figure 2.4. Comparison of the operating devices for adsorption efficiency

(a): Adsorption with magnetic stirrer; **(b):** Adsorption with incubator shaker

2.3.4.2. Adsorption kinetics evaluation

The adsorption kinetic curves of XAD4, XAD16 and NPAC with contents of 50 and 100 g/L for each adsorbent were shown in Figure 2.5. In order to elucidate the adsorption behaviors and mechanisms of all resins, pseudo-first-order, pseudo-second-order and particle diffusion kinetics models [66, 86] were chosen to evaluate the adsorption processes. The pseudo-first-order model is generally applicable over the initial stage of an adsorption process, while the pseudo-second-order model assumes that the rate-limiting step is chemisorption and predicts the

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behavior over the whole range of adsorption [66, 87]. All these equations, including derived parameters such as correlation coefficient and dynamic parameters, were summarized in Table 2.10. It was observed that equilibrium adsorption was achieved before 30 minutes for the experiments with 100 g / L of NPAC and XAD16, so that the kinetics of these experiments could not be evaluated and they are not included in Table 2.10 and Figure 2.5. As shown in Table 2.10, the best linear correlation coefficients correspond to a pseudo-second-order kinetic model for phenolic adsorption of NPAC and particle diffusion kinetic models for both polymer resins. Similar results can be fined elsewhere [66, 88].

2.3.4.3. Phenol selectivity evaluation of adsorption and desorption experiments

As it is shown in Figure 2.6, a wide experimental comparison of adsorption and desorption rates is performed between the three adsorbents (XAD4, XAD16 and NPAC) with 50 and 100 g/L content for each one. Adsorbents were quantitatively compared in terms of total phenols and selectivity for isolation of individual phenolic compounds like hydroxytyrosol, tyrosol and oleuropein.

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(a)

(b)

(c)
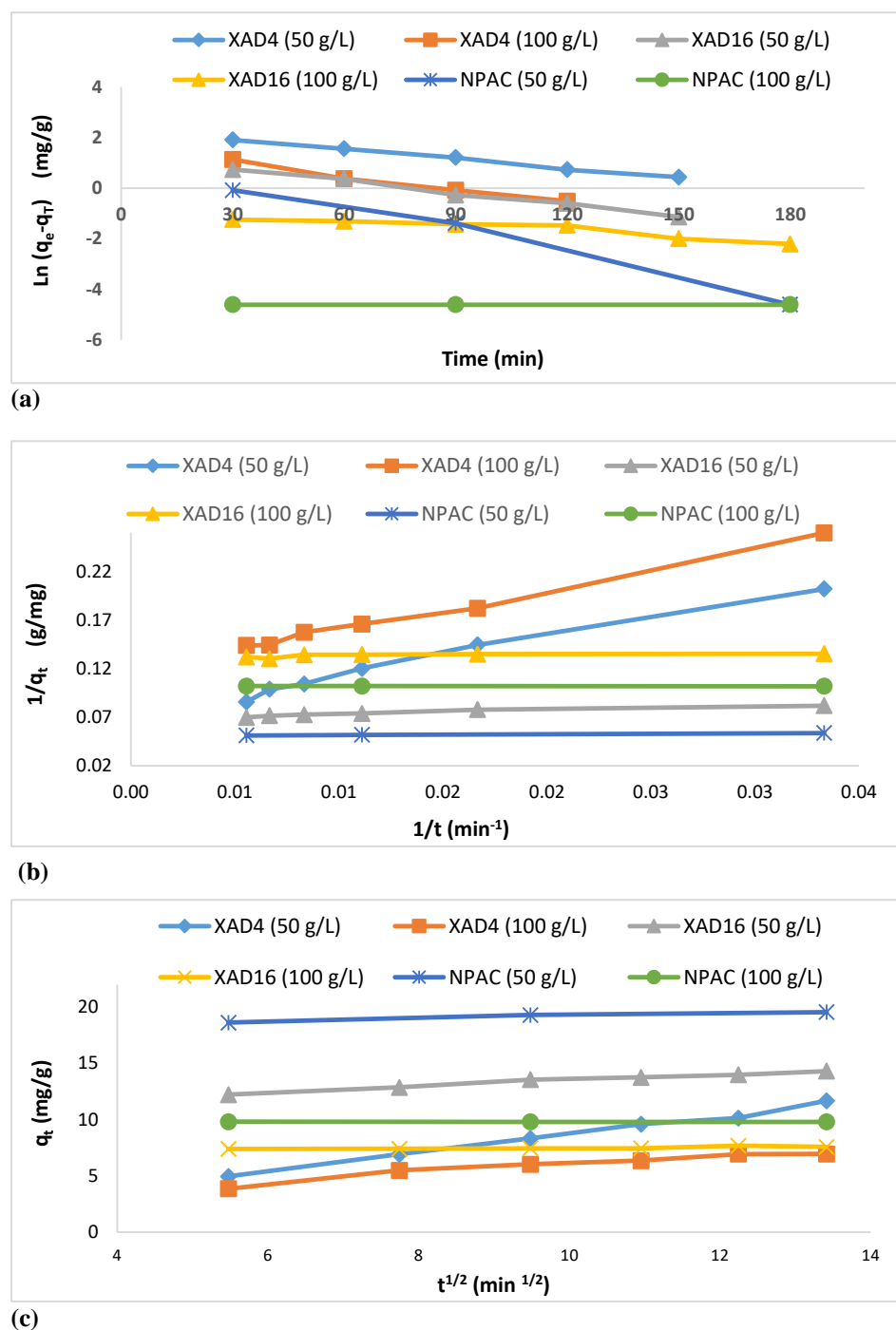
Figure 2.5. Adsorption kinetic curves based on different models. (a): pseudo-first-order kinetic equation; (b): pseudo-second-order kinetic equation; (c): particle diffusion kinetic equation

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Table 2.10. Analysis of the adsorption process based on kinetic models

Adsorbent	Adsorbent content	q_e exp. (mg /g)	Dynamic equation	Dynamic parameters	Correlation coefficient (R^2)
XAD4	50 (g/L)	11.68	$ln\,(q_e\text{-}q_t)= -k_1t + lnq_e$	q_e= 9.966 , K_1 = 0.0125	0.9955
			$\frac{1}{q_t} = \frac{1}{k_2 q_e^2} \times \frac{1}{t} + \frac{1}{q_e}$	q_e= 14.085 , K_2= 0.0018	0.9853
			$q_t = k_d.t^{1/2} + C$	k_d = 0.8154 , C= 0.5373	0.9923
	100 (g/L)	6.95	$ln\,(q_e\text{-}q_t)= -k_1t + lnq_e$	q_e= 4.835 , K_1 = 0.018	0.9799
			$\frac{1}{q_t} = \frac{1}{k_2 q_e^2} \times \frac{1}{t} + \frac{1}{q_e}$	q_e= 8.410 , K_2= 0.0050	0.9930
			$q_t = k_d.t^{1/2} + C$	k_d = 0.3802 , C= 2.1743	0.9288
XAD16	50 (g/L)	14.31	$ln\,(q_e\text{-}q_t)= -k_1t + lnq_e$	q_e= 3.442 , K_1 = 0.0157	0.9915
			$\frac{1}{q_t} = \frac{1}{k_2 q_e^2} \times \frac{1}{t} + \frac{1}{q_e}$	q_e= 14.493 , K_2= 0.0119	0.9336
			$q_t = k_d.t^{1/2} + C$	k_d = 0.2603, C= 10.874	0.9802
	100 (g/L)	7.68	$ln\,(q_e\text{-}q_t)= -k_1t + lnq_e$	q_e= 0.394 , K_1 = 0.0063	0.858
			$\frac{1}{q_t} = \frac{1}{k_2 q_e^2} \times \frac{1}{t} + \frac{1}{q_e}$	q_e= 7.587 , K_2= 0.1354	0.4374
			$q_t = k_d.t^{1/2} + C$	k_d = 0.0305 , C= 7.1889	0.06383
NPAC	50 (g/L)	19.55	$ln\,(q_e\text{-}q_t)= -k_1t + lnq_e$	q_e= 2.812 , K_1 = 0.0306	0.9845
			$\frac{1}{q_t} = \frac{1}{k_2 q_e^2} \times \frac{1}{t} + \frac{1}{q_e}$	q_e= 19.724 , K_2= 0.0294	0.9957
			$q_t = k_d.t^{1/2} + C$	k_d = 0.1161 , C= 18.062	0.9379
	100 (g/L)	9.80	$ln\,(q_e\text{-}q_t)= -k_1t + lnq_e$	q_e= 0.0099 , K_1 = -3E-17	9E-16
			$\frac{1}{q_t} = \frac{1}{k_2 q_e^2} \times \frac{1}{t} + \frac{1}{q_e}$	q_e= 9.794 , K_2= -2.6031	0.9643
			$q_t = k_d.t^{1/2} + C$	k_d = -0.0013 , C= 9.8153	0.755

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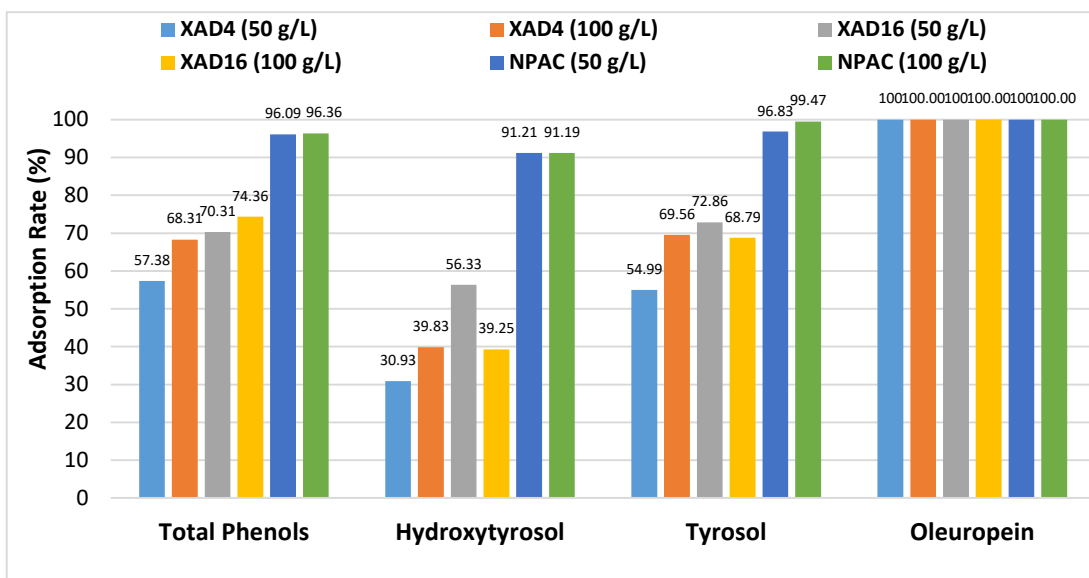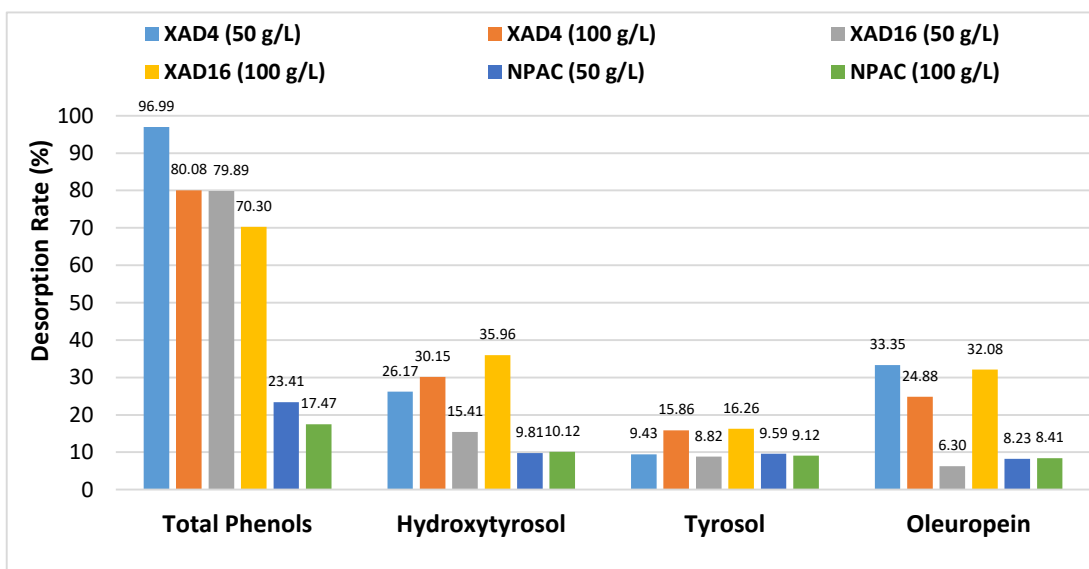
(a): Adsorption rates

(b): Desorption rates

Figure 2.6. Experimental comparison of adsorption and desorption rates between the adsorbents

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In addition, mean values of the performed experiments with the same pattern are presented in Table 2.11 in order to compare with initial content of olive sample. Results in Figure 2.6.a. show that, NPAC had significantly higher adsorption rates for all compounds regardless of the input content (50 or 100 g/L). Whereas, all adsorption rates for NPAC are over 91% which is remarkable. This is in accordance with the study performed by Fava et al. (2017) [22] in which, the prototype operations showed it is possible to recover up to 80% of polyphenols from Olive mill wastewater (OMWW), producing about 6 kg/m3 OMWW of a dry fraction with a purity up to 70% in hydroxytyrosol. Except for NPAC which already proved to be best adsorbent of phenolic compounds in this study, XAD16 with 100 and 50 g/L contents showed high rate of total phenol adsorption with 74.36% and 70.31%, respectively. XAD16 is also been reported to have satisfactory adsorption results in multiple previous studies [57– 59]. Regarding oleuropein isolation, all six series of experiment showed 100% adsorption of oleuropein which can be concluded that all three types of adsorbents have excellent efficiency in selectivity of oleuropein.

Experimental evaluation of desorption rates are presented in Figure 2.6.b. As it is mentioned earlier, Ethanol was used as desorption solvent. In this study, desorption rate correlation of total phenol and individual phenolic compounds was not completely satisfactory. Whereas, XAD4 with 50 g/L content proved highest desorption rate of total phenols with 96.99% but showed 26.17%, 9.43% and 33.35% desorption rates for hydroxytyrosol, tyrosol and oleuropein, respectively. On the other hand, XAD16 with 50 g/L content had almost 10% better desorption rate of total phenols compared to XAD16 with 100 g/L content. However, XAD16 with 100 g/L content showed significantly higher results for desorbing individual phenolic compounds compared to XAD16 with 50 g/L content. Unlike the excellent adsorption results of NPAC, very poor desorption rates were obtained for NPAC (50 or 100 g/L contents) under the tested conditions.

Supplementary desorption kinetic experiments were performed in order to check the equilibrium conditions in the explanation of the results. In these experiments,

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aliquots (1.0 mL) of supernatant of the filtered samples were taken at the time points of 30, 60, 90 and 120 minutes and the TPC in the solutions was measured. Results are depicted in Figure 2.7. It can be observed that for all of experimental series, the majority of phenol recovery occur in the first 30 minutes of desorption process and then they reach the equilibrium. The most important advantage of ethanol as a desorption solvent is its high volatility and ease of use in energy efficient vacuum evaporators. In this way, the final concentration of valuable phenolic compounds will be significantly increased with economic advantages of paramount importance at industrial scale. In this way, Zagklis et al. [89] recovered 74% of the initial amount of hydroxytyrosol by vacuum evaporation of ethanol until reach a volume concentration factor of 5.4.

	XAD4 (50 g/L)	XAD4 (100 g/L)	XAD16 (50 g/L)	XAD16 (100 g/L)	NPAC (50 g/L)	NPAC (100 g/L)
90-120 minutes	1.04	0.64	3.09	0.38	0.16	0.32
60-90 minutes	0.23	0	0.35	0	0.24	0.17
30-60 minutes	1.94	0	0.37	0	0	0
0-30 minutes	52.44	54.06	52.36	51.89	22.1	16.34
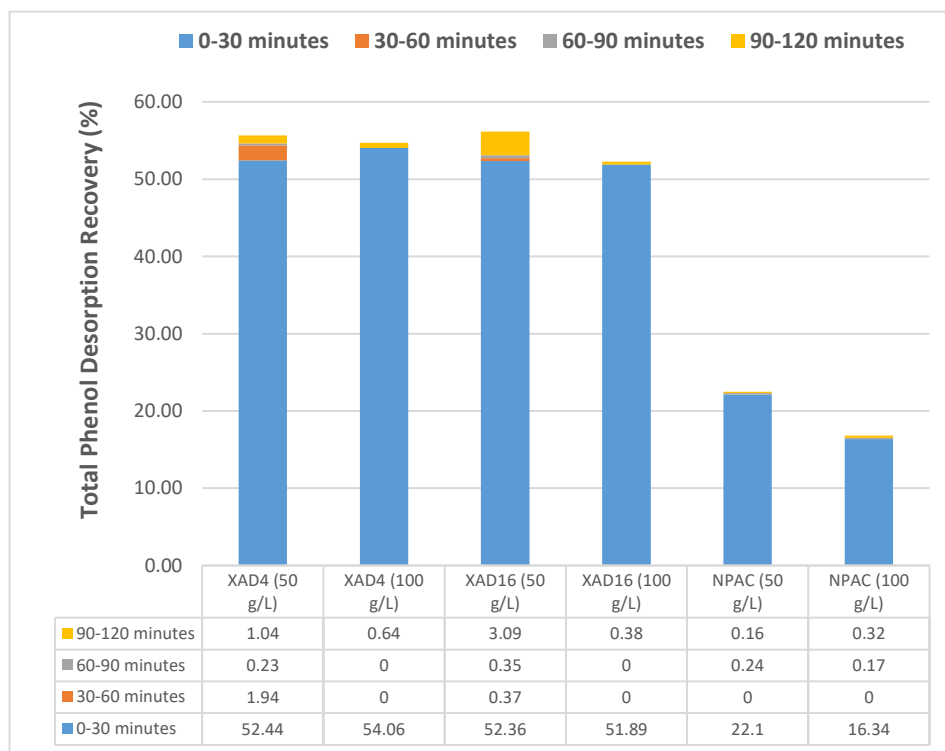
Figure 2.7. Evaluation of total phenol desorption recovery in time periods

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Table 2.11 shows that the total recovery (Eq.5) of TPC with NPAC are 22.50% and 16.83% for contents of 50 and 100 g/L, respectively, which are considerably lower than those of the polymeric resins. According to Table 2.11, despite that XAD4 (50 g/L) showed slightly higher total recovery for TPC and oleuropein, it can be concluded the overall advantage of XAD16 (100 mg/L) over other adsorbents and contents.

Additionally, In order to study the possibility of improvements for recovery results in desorption process, some extra desorption runs was performed after determination of optimal adsorption series which were NPAC (100 g/L) and XAD16 (100 g/L). For this purpose, another four solvents were considered to be used which were acidified Ethanol (pH=2.0), Ethanol-Water (1:1), Ethanol-Water (1:1, acidified to pH=2.3) and Acetic Acid solution (0.5 molar). The corresponding results are provided in Table 2.12. Due to the fact that NPAC showed highest adsorption but unsatisfactory desorption results, it was also selected to be tested in the additional desorption experiments. By comparing the obtained results in tables 2.11 and 2.12, it can be observed the advantage of acidified Ethanol (pH=2.0) in terms of TPC recovery whereas, 64.35% of TPC content was recovered.

Regarding desorption with acetic acid (0.5 Molar) as solvent, although TPC recovery was only 9.11%, but the highest HT recovery was obtained with 24.53% which means that the optimal selective isolation of HT can be achieved with acetic acid (0.5 Molar) as solvent. In overall aspect, optimal results was obtained by acidified ethanol-water solution (1:1) with total recovery of HT (19.27%) and TPC (57.65%) and highest recovery of Tyrosol (45.73%). However, Oleuropein was not identified in the solvent after desorption (Table 2.12). NPAC did not prove any significant improvements in additional desorption experiments, except for total recovery of Tyrosol obtained by acidified ethanol-water solution (21.11%) which was considerably higher than the content obtained by pure ethanol as solvent. By considering the evaporated volume of ethanol from initial olive extract ($V_0=2V_d$), the optimal contents of 654.76±35.36 mg/L for TPC, 20.12±0.35mg/L for HT,

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17.29±2.09 mg/L for Tyrosol and 5.30 ± 2.25 mg/L for Oleuropein was achieved in desorption experiments with 50% volume of the initial olive extract.

As Future trends, more studies should be carried out on the solvent that must be used in the desorption process of NPAC to improve its phenolic recovery, taking advantage of its excellent adsorption capacity. Furthermore, different adsorbents could be investigated in order to improve the selective isolation of Hydroxytyrosol, Tyrosol and Oleuropein.

Table 2.11. Phenolic content in filtrates of adsorption-desorption experiments. Adsorption and desorption rates are calculated by Eqs. 3 and 4, respectively, being C_0 the concentration corresponding to the sample after evaporation of ethanol

Stage	Description	Total Phenols (mg/L)	Rate (%)	Hydroxytyrosol (mg/L)	Rate (%)	Tyrosol (mg/L)	Rate (%)	Oleuropein (mg/L)	Rate (%)
Olive extract	Initial	415.65 ± 9.98	-	14.51 ± 4.17	-	4.58 ± 2.35	-	7.85 ± 0.26	-
	After acidification	497.41 ± 4.16	-	17.99 ± 9.64	-	8.9 ± 4.87	-	9.5 ± 1.28	-
	After ethanol evaporation (C_0)	1017.46 ± 0.54	-	82.03 ± 12.44	-	37.81 ± 9.49	-	16.52 ± 0.94	-
Adsorption	XAD4 (50 g/L)	433.62 ± 15.77	57.38	56.66 ± 0.06	30.93	17.02 ± 0.52	54.99	0	100.00
	XAD4 (100 g/L)	322.46 ± 10.88	68.31	49.36 ± 0.86	39.83	11.51 ± 1.51	69.56	0	100.00
	XAD16 (50 g/L)	302.08 ± 8.16	70.31	35.82 ± 2.0	56.33	10.26 ± 1.89	72.86	0	100.00
	XAD16 (100 g/L)	260.92 ± 2.18	74.36	49.83 ± 0.96	39.25	11.8 ± 0.62	68.79	0	100.00
	NPAC (50 g/L)	39.77 ± 0.54	96.09	7.21 ± 0.13	91.21	1.2 ± 0	96.83	0	100.00
	NPAC (100 g/L)	37.08 ± 1.09	96.36	7.23 ± 0.95	91.19	0.2 ± 0	99.47	0	100.00
Desorption	XAD4 (50 g/L)	566.24 ± 23.29	96.99	6.64 ± 2.40	26.17	1.96 ± 0.03	9.43	5.51 ± 0.01	33.35
	XAD4 (100 g/L)	556.53 ± 19.55	80.08	9.85 ± 2.17	30.15	4.17 ± 2.06	15.84	4.11 ± 0.01	24.88
	XAD16 (50 g/L)	571.53 ± 5.82	79.89	7.12 ± 1.14	15.41	2.43 ± 0.98	8.82	1.04 ± 0.01	6.30
	XAD16 (100 g/L)	531.82 ± 16.22	70.30	11.58 ± 2.41	35.96	4.23 ± 0.06	16.26	5.30 ± 2.25	32.08
	NPAC (50 g/L)	228.88 ± 7.90	23.41	7.34 ± 0.91	9.81	3.51 ± 1.25	9.59	1.36 ± 1.14	8.23
	NPAC (100 g/L)	171.24 ± 2.91	17.47	7.57 ± 3.40	10.12	3.43 ± 0.76	9.12	1.39 ± 0.55	8.41

Table 2.12. Adsorption- desorption experiments with XAD16 and NPAC adsorbents and 100 g/L content using different desorption solvents. Values correspond to the filtrate concentration in desorption stage and recovery calculated by Eq. 5

Solvent Type	Responses	XAD16 (100 g/L)		NPAC (100 g/L)	
		Mean value (mg/L)	Desorption recovery (%)	Mean value (mg/L)	Desorption recovery (%)
Ethanol (100%, pH= natural)	TPC	531.82 ± 16.22	52.27	171.24 ± 2.91	16.83
	HT	11.58 ± 2.41	14.12	7.57 ± 3.40	9.23
	TY	4.23 ± 0.06	11.19	3.43 ± 0.76	9.07
	OLE	5.30 ± 2.25	32.08	1.39 ± 0.55	8.41
Ethanol (100%, pH= 2.0)	TPC	654.76 ± 35.36	64.35	175.35 ± 7.90	17.23
	HT	5.40 ± 1.70	6.58	1.07 ± 0.14	1.30
	TY	3.26 ± 0.11	8.62	3.30 ± 0.33	8.73
	OLE	0.75 ± 0.02	4.54	-	0
Ethanol-Water (1:1, pH=4.1)	TPC	568.38 ± 0.01	55.86	122.87 ± 5.50	12.08
	HT	8.14 ± 0.67	9.92	3.25 ± 0.4	3.96
	TY	2.63 ± 0.2	6.96	4.13 ± 2.21	10.92
	OLE	-	0	-	0
Ethanol-Water (1:1, pH=2.3)	TPC	586.53 ± 19.55	57.65	122.71 ± 2.50	12.06
	HT	15.81 ± 2.02	19.27	3.14 ± 0.36	3.83
	TY	17.29 ± 2.09	45.73	7.98 ± 2.30	21.11
	OLE	-	0	-	
Acetic acid (0.5 M)	TPC	92.68 ± 2.61	9.11	13.23 ± 0.8	1.30
	HT	20.12 ± 0.35	24.53	4.33 ± 0.5	5.28
	TY	1.77 ± 0.51	4.68	-	0
	OLE	-	0	-	0

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2.4. Conclusion

Two types of olive mill solid residues obtained from two-phase and three-phase centrifugation systems (olive cake and pomace) were used as samples in the experiments of this chapter. After determination of optimal solvent being ethanol-water with 1:1 ratio (v/v), main objective was phenolic extraction based on experimental design provided by response surface methodology by application of two ultrasonic assisted extraction methods. Numerical optimization was carried out through Design Expert software, using desirability function method. The effects of three input variable factors were studied on the responses (Y_1, Y_2, Y_3). Variable factors (X_1, X_2, X_3) were different depending on the utilized ultrasonic method for each extraction series.

The optimal operating conditions for phenolic extraction from olive cake in ultrasonic bath was time: 120 minutes, temperature: 60 °C and Solid:Solvent ratio of 4% (w/v) whereas the obtained responses were 14.52 mg_{GAE}/g, 2.53 mg_{QE}/g and 0.975 mM_{Trolox}/g of total phenolic content (TFC), total flavonoid content (TFC) and Antioxidant Activity (AA), respectively. In the next step, phenolic extractions from olive cake were performed by ultrasonic homogenizer. Variable input factors were optimized through experimental runs determined by design software with 90.36% of desirability with following optimal points: time: 10 minutes, amplitude: 30% and solid:solvent ratio 4% (w/v). The obtained responses were 18.40 mg_{GAE}/g, 2.09 mg_{QE}/g and 0.859 mM_{Trolox}/g for TPC, TFC and AA, respectively.

Due to the fact that better results was achieved by ultrasonic homogenizer, it was also used in order to optimize for phenolic extraction of olive pomace with the same methodology. The optimal conditions for the phenolic extraction from olive pomace would be those leading to highest TPC, TFC and AA. The combined optimum ingredient levels for all responses with optimum desirability obtained from time: 10 minutes, amplitude: 40%, S/S ratio: 4%) whereas the responses were 14.70 mg_{GAE}/g of TPC, 2.48 mg_{QE}/g of TFC and 0.924 mM_{Trolox}/g of AA. In terms of economical aspect, it is important to note that ultrasonic baths are far less

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expensive than ultrasonic processors. Nevertheless, it should be considered that ultrasonic homogenizers proved to have better results in phenolic extraction. Subsequently, adsorption-desorption process was applied for recovery of valuable biophenols. For this purpose two activated charcoals (NPAC and GAC) and two polymeric resins (XAD4 and XAD16) using two adsorption devices (magnetic stirrer and incubator shaker) were tested. In these experiments the adsorbent GAC and the incubator shaker were less efficient and were not selected for future experiences. Furthermore, two adsorbent contents of 50 and 100 g adsorbent/L of extract were quantitatively compared in terms of adsorption rate for TPC and individual phenolic compounds like Hydroxytyrosol, Tyrosol and Oleuropein. Kinetic studies of adsorption process attribute a pseudo-second-order kinetic model for NPAC and particle diffusion kinetics for polymeric resins.

NPAC showed the greater (over 91%) adsorption rates for all individual biocompounds, regardless of the input content (50 or 100 g/L), however desorption process for NPAC with pure ethanol was very poor. Whereas, total recovery of TPC with NPAC was considerably lower than those of the polymeric resins. Despite that XAD4 (50 g/L) showed high total recovery for TPC and oleuropein. However, it can be concluded from the results the overall advantage of XAD16 with 100 mg/L content over other adsorbents.

Additionally, In order to study the possibility of improvements for recovery results in desorption process, some extra desorption runs was performed after determination of optimal adsorption series which were NPAC (100 g/L) and XAD16 (100 g/L). For this purpose, another four solvents were considered to be used which were acidified Ethanol (pH=2.0), Ethanol-Water (1:1, pH=natural), Ethanol-Water (1:1, acidified to pH=2.3) and Acetic Acid solution (0.5 molar). By considering the evaporated volume of ethanol from initial olive extract, the optimal contents of 654.76±35.36 mg/L for TPC, 20.12±0.35mg/L for HT, 17.29±2.09 mg/L for Tyrosol and 5.30 ± 2.25 mg/L for Oleuropein was achieved in desorption experiments with 50% volume of the initial olive extract.

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References

[1]   H. K. Obied, M. S. Allen, D. R. Bedgood, P. D. Prenzler, K. Robards, R. Stockmann, "Bioactivity and Analysis of Biophenols Recovered from Olive Mill Waste", *J. Agric. Food Chem.*, 53, 4, 823–837, 2005.

[2]   N. H. Aziz, S. E. Farag, L. A. Mousa, M. A. Abo-Zaid, "Comparative antibacterial and antifungal effects of some phenolic compounds", *Microbios*, 93, 374, 43–54, 1998.

[3]   L. Lesage-Meessen, D. Navarro, S. Maunier, J.-C Sigoillot, J. Lorquin, M. Delattre, J.-L Simon, M. Asther, M. Labat, "Simple phenolic content in olive oil residues as a function of extraction systems", *Food Chem.*, 75, 4, 501–507, 2001.

[4]   T. Manios, "The composting potential of different organic solid wastes: Experience from the island of Crete", *Environ. Int.*, 29, 8, 1079–1089, 2004.

[5]   A. Y. Gebreyohannes, R. Mazzei, L. Giorno, "Trends and current practices of olive mill wastewater treatment: Application of integrated membrane process and its future perspective", *Sep. Purif. Technol.*, 162, 45–60, 2016.

[6]   E. De Marco, M. Savarese, A. Paduano, R. Sacchi, "Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters", *Food Chem.*, 104, 2, 858–867, 2007.

[7]   F. Alagna, R. Mariotti, F. Panara, S. Caporaly, S. Urbani, G. Veneziani, S. Esposto, A. Taticchi, A. Rosati, R. Rao, G. Perrotta, M. Servili, L. Baldoni, "Olivve phenolic compounds: metbolic and transcriptional profiling during fruit development", *BMC Plant Biol.*, 12, 162, 2012.

[8]   R. Borsani, B. Ferrando, "Ultrafiltration plant for olive vegetation waters by polymeric membrane batteries", *Desalination*, 108, 1–3, 281–286, 1997.

[9]   S. Khoufi, M. Hamza, S. Sayadi, "Enzymatic hydrolysis of olive wastewater for hydroxytyrosol enrichment", *Bioresour. Technol.*, 102, 19, 9050–9058, 2011.

[10]   A. Y. Gebreyohannes, E. Curcio, T. Poerio, R. Mazzei, G. Di Profio, E. Drioli, L. Giorno, "Treatment of Olive Mill Wastewater by Forward Osmosis", *Sep. Purif. Technol.*,147, 292–302, 2015.

[11]   E. Tsagaraki, H. Lazarides, "Fouling analysis and performance of tubular ultrafiltration on pretreated olive mill waste water", *Food Bioprocess Technol.*, 1–9, 2010.

[12]   B. Aliakbarian, A. A. Casazza, P. Perego, "Valorization of olive oil solid waste using high pressure-high temperature reactor", *Food Chem.*, 128, 3, 704–710, 2011.

[13]   P. Sánchez Moral, M. V. Ruiz Méndez, "Production of pomace olive oil", *Grasas y Aceites*, 57, 1, 47–55, 2006.

[14]   M. Suarez, M.-P. Romero, T. Ramo, A. Macia, M.-J. Motilva, "Methods for preparing phenolic extracts from olive cake for potential application as food antioxidants", *J. Agric. Food Chem.*, 57, 4, 1463–1472, 2009.

PhD Thesis

Chapter 2: Optimization and comparative evaluation of phenolic extraction from olive mill solid wastes and phenol recovery by adsorption process

[15]   N. S. Kumar, K. Min, "Phenolic compounds biosorption onto Schizophyllum commune fungus: FTIR analysis, kinetics and adsorption isotherms modeling", *Chem. Eng. J.*, 168, 2, 562–571, 2011.

[16]   P. S. Rodis, V. T. Karathanos, A. Mantzavinou, "Partitioning of Olive Oil Antioxidants between Oil and Water Phases", *J. Agric. Food Chem.*, 50, 3, 596–601, 2002.

[17]   M. H. Alu'datt, I. Alli, K. Ereifej, M. Alhamad, A. R. Al-Tawaha, T. Rababah, "Optimisation, characterisation and quantification of phenolic compounds in olive cake", *Food Chem.*, 123, 1, 117–122, 2010.

[18]   T. Hu, X.-W. He, J.-G. Jiang, X.-L. Xu, "Hydroxytyrosol and Its Potential Therapeutic Effects", *J. Agric. Food Chem.*, 62, 7, 1449–1455, 2014.

[19]   X. Zhang, J. Cao, L. Jiang, C. Geng, L. Zhong, "Protective effect of hydroxytyrosol against acrylamide-induced cytotoxicity and DNA damage in HepG2 cells", *Mutat. Res. - Fundam. Mol. Mech. Mutagen.*, 664, 1–2, 64–68, 2009.

[20]   D. G. Cornwell, J. Ma, "Nutritional Benefit of Olive Oil: The Biological Effects of Hydroxytyrosol and Its Arylating Quinone Adducts", *J. Agric. Food Chem.*, 56, 19, 8774–8786, 2008.

[21]   J. C. Espín, C. Soler-Rivas, E. Cantos, F. A. Tomás-Barberán, H. J. Wichers, "Synthesis of the Antioxidant Hydroxytyrosol Using Tyrosinase as Biocatalyst", *J. Agric. Food Chem.*, 49, 3, 1187–1193, 2001.

[22]   G. Fava, M. Domenica Di Mauro, M. Spampinato, D. Biondi, G. Gambera, G. Centonze, R. Maggiore, N. D'Antona, "Hydroxytyrosol Recovery From Olive Mill Wastewater: Process Optimization and Development of a Pilot Plant", *Clean - Soil, Air, Water*, 45, 4, 1–10, 2017.

[23]   A. Ranalli, L. Lucera, S. Contento, "Antioxidizing Potency of Phenol Compounds in Olive Oil Mill Wastewater", *J. Agric. Food Chem.*, 51, 26, 7636–7641, 2003.

[24]   F. Shahidi, M. Naczk, *Phenolics in food and nutraceuticals*. 2006.

[25]   J. Azmir, I. S.M. Zaidul, M. M. Rahman, K. M. Sharif, A. Mohamed, F. Sahena, M. H.A. Jahurul, K. Ghafoor, N. A.N. Norulaini, A. K.M. Omar, "Techniques for extraction of bioactive compounds from plant materials: A review", *J. Food Eng.*, 117, 4, 426–436, 2013.

[26]   M. Yolmeh, M. B. Habibi Najafi, R. Farhoosh, "Optimisation of ultrasound-assisted extraction of natural pigment from annatto seeds by response surface methodology (RSM)", *Food Chem.*, 155, 319–324, 2014.

[27]   M. Vinatoru, M. Toma, O. Radu, P. I. Filip, D. Lazurca, T. J. Mason, "The use of ultrasound for the extraction of bioactive principles from plant materials", *Ultrason. Sonochem.*, 4, 2, 135–139, 1997.

[28]   G. Kashif, H. U. I. Teng, C. Y. Hee, "Optimization of ultrasonic-assisted extraction of total anthocyanins from grape peel using response surface methodology", *J. Food Biochem.*, 35, 3, 735–746, 2011.

PhD Thesis

Chapter 2: Optimization and comparative evaluation of phenolic extraction from olive mill solid wastes and phenol recovery by adsorption process

[29]   S. Toepfl, A. Mathys, V. Heinz, D. Knorr, "Review: Potential of High Hydrostatic Pressure and Pulsed Electric Fields for Energy Efficient and Environmentally Friendly Food Processing", *Food Rev. Int.*, 22, 4, 405–423, 2006.

[30]   R. Gaur, A. Sharma, S. K. Khare, M. N. Gupta, "A novel process for extraction of edible oils. Enzyme assisted three phase partitioning (EATPP)", *Bioresour. Technol.*, 98, 3, 696–699, 2007.

[31]   B. Kaufmann, P. Christen, "Recent extraction techniques for natural products: microwave-assisted extraction and pressurized solvent extraction", *Phytochem. Anal.*, 13, 105–113, 2002.

[32]   N. R. Lakkakula, M. Lima, T. Walker, "Rice bran stabilization and rice bran oil extraction using ohmic heating", *Bioresour. Technol.*, 92, 2, 157–161, 2004.

[33]   R. Marr, T. Gamse, "Use of supercritical fluids for different processes including new developments-a review", *Chem. Eng. Process. Process Intensif.*, 39, 1, 19–28, 2000.

[34]   Q. Lang, C. M. Wai, "Supercritical fluid extraction in herbal and natural product studies - a practical review", *Talanta*, 53, 4, 771–82, 2001.

[35]   M. A. Meireles, *Supercritical extraction from solid: Process design data (2001-2003)*, 7, 2003.

[36]   L. Wang, C. L. Weller, V. L. Schlegel, T. P. Carr, S. L. Cuppett, "Supercritical CO2extraction of lipids from grain sorghum dried distillers grains with solubles", *Bioresour. Technol.*, 99, 5,1373–1382, 2008.

[37]   K. Ghafoor, F. Y. Al-Juhaimi, Y. H. Choi, "Supercritical fluid extraction of phenolic compounds and antioxidants from grape (Vitis labrusca B.) seeds", *Plant Foods Hum. Nutr.*, 67, 4, 407–414, 2012.

[38]   K. Ghafoor, J. Park, Y. H. Choi, "Optimization of supercritical fluid extraction of bioactive compounds from grape (Vitis labrusca B.) peel by using response surface methodology", *Innov. Food Sci. Emerg. Technol.*, 11, 3, 485–490, 2010.

[39]   R. M. Smith, "Extractions with superheated water," *J. Chromatogr. A*, 975, 1, 31–46, 2002.

[40]   S. W., "W. G. Jennings und A. Rapp: Sample Preparation for Gas Chromatographic Analysis. 104 Seiten, 37 Abb., 5 Tab. Dr. Alfred Hüthig Verlag, Heidelberg, Basel, New York 1983. Preis: 46,- DM", *Food / Nahrung*, 27, 10, 1027, 1983.

[41]   M. Szumski, B. Buszewski, *State of the Art in Miniaturized Separation Techniques*, 32. 2002.

[42]   R. M. Smith, "Before the injection-modern methods of sample preparation for separation techniques," *J. Chromatogr. A*, 1000, 1, 3–27, 2003.

[43]   L. Wang, C. L. Weller, "Recent advances in extraction of nutraceuticals from plants", *Trends Food Sci. Technol.*, 17, 6, 300–312, 2006.

[44]   M. C. Herrera, M. D. Luque De Castro, "Ultrasound-assisted extraction of phenolic compounds from strawberries prior to liquid chromatographic separation and

PhD Thesis

Chapter 2: Optimization and comparative evaluation of phenolic extraction from olive mill solid wastes and phenol recovery by adsorption process

photodiode array ultraviolet detection", *J. Chromatogr. A*, 1100, 1, 1–7, 2005.

[45]   T. J. Mason, L. Paniwnyk, J. P. Lorimer, "The uses of ultrasound in food technology", *Ultrason. Sonochem.*, 3, 3, 1996.

[46]   D. R. Kammerer, Z. S. Saleh, R. Carle, R. A. Stanley, "Adsorptive recovery of phenolic compounds from apple juice", *Eur. Food Res. Technol.*, 224, 5, 605–613, 2007.

[47]   J. Kammerer, D. R. Kammerer, U. Jensen, R. Carle, "Interaction of apple polyphenols in a multi-compound system upon adsorption onto a food-grade resin", *J. Food Eng.*, 96, 4, 544–554, 2010.

[48]   M. L. Soto, A. Moure, H. Domínguez, J. C. Parajó, "Recovery, concentration and purification of phenolic compounds by adsorption: A review", *J. Food Eng.*, 105, 1, 1–27, 2011.

[49]   M. Niaounakis, C. P. Halvadakis, *Olive processing waste management: literature review and patent survey*. Elsevier, 2006.

[50]   Z. Kaleh, S. U. Geißen, "Selective isolation of valuable biophenols from olive mill wastewater", *J. Environ. Chem. Eng.*, 4, 1, 373–384, 2016.

[51]   C. A. Santi, S. Cortes, L. P. D'Acqui, E. Sparvoli, B. Pushparaj, "Reduction of organic pollutants in Olive Mill Wastewater by using different mineral substrates as adsorbents", *Bioresour. Technol.*, 99, 6, 1945–1951, 2008.

[52]   A. De Martino, M. Iorio, P. D. Prenzler, D. Ryan, H. K. Obied, M. Arienzo, "Adsorption of phenols from olive oil waste waters on layered double hydroxide, hydroxyaluminium iron co-precipitate and hydroxyaluminium iron montmorillonite complex", *Appl. Clay Sci.*, 80, 154–161, 2013.

[53]   A. Yangui, M. Abderrabba, A. Sayari, "Amine-modified mesoporous silica for quantitative adsorption and release of hydroxytyrosol and other phenolic compounds from olive mill wastewater", *J. Taiwan Inst. Chem. Eng.*, 70, 111–118, 2017.

[54]   A. Ena, C. Pintucci, P. Carlozzi, "The recovery of polyphenols from olive mill waste using two adsorbing vegetable matrices", *J. Biotechnol.*, 157, 4, 573–577, 2012.

[55]   D. P. Zagklis, A. I. Vavouraki, M. E. Kornaros, C. A. Paraskeva, "Purification of olive mill wastewater phenols through membrane filtration and resin adsorption/desorption", *J. Hazard. Mater.*, 285, 69–76, 2015.

[56]   A. Yangui, J. R. Njimou, A. Cicci, M. Bravi, M. Abderrabba, A. Chianese, "Competitive adsorption, selectivity and separation of valuable hydroxytyrosol and toxic phenol from olive mill wastewater", *J. Environ. Chem. Eng.*, 5, 4, 3581–3589, 2017.

[57]   D. Frascari, G. Rubertelli, F. Arous, A. Ragini, L. Bresciani, A. Arzu, D. Pinelli, "Valorisation of olive mill wastewater by phenolic compounds adsorption: Development and application of a procedure for adsorbent selection", *Chem. Eng. J.*, 360, 2018, 124–138, 2019.

PhD Thesis

Chapter 2: Optimization and comparative evaluation of phenolic extraction from olive mill solid wastes and phenol recovery by adsorption process

[58]   D. Pinelli, A. E. Molina Bacca, A. Kaushik, S. Basu, M. Nocentini, L. Bertin, D. Frascari, "Batch and Continuous Flow Adsorption of Phenolic Compounds from Olive Mill Wastewater: A Comparison between Nonionic and Ion Exchange Resins", *Int. J. Chem. Eng.*, 2016, 2016.

[59]   D. P. Zagklis, C. A. Paraskeva, "Isolation of organic compounds with high added values from agro-industrial solid wastes", *J. Environ. Manage.*, 216, 183–191, 2018.

[60]   M. Scordino, A. Di Mauro, A. Passerini, E. Maccarone, "Adsorption of Flavonoids on Resins: Hesperidin", *J. Agric. Food Chem.*, 51, 24, 6998–7004, 2003.

[61]   L. Bertin, F. Ferri, A. Scoma, L. Marchetti, F. Fava, "Recovery of high added value natural polyphenols from actual olive mill wastewater through solid phase extraction", *Chem. Eng. J.*, 171, 1287–1293, 2011.

[62]   M. Ziati, F. Khemmari, O. Cherifi, F. Y. Didouche, "Removal of polyphenols from olive mill wastewater by adsorption on activated carbon prepared from peach stones", *Rev. Roum. Chim.*, 62, 11, 865–874, 2017.

[63]   D. Richard, M. de L. Delgado Núñez, and D. Schweich, "Adsorption of complex phenolic compounds on active charcoal: Adsorption capacity and isotherms," *Chem. Eng. J.*, vol. 148, no. 1, pp. 1–7, 2009.

[64]   P. Ghorbannezhad, A. Bay, M. Yolmeh, R. Yadollahi, J. Y. Moghadam, "Optimization of coagulation–flocculation process for medium density fiberboard (MDF) wastewater through response surface methodology", *Desalin. Water Treat.*, 57, 56, 26916–26931, 2016.

[65]   Z. Wang, C. Wang, J. Yuan, C. Zhang, "Adsorption characteristics of adsorbent resins and antioxidant capacity for enrichment of phenolics from two-phase olive waste", *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, 1040, 38–46, 2017.

[66]   Q. Yang, M. Zhao, and L. Lin, "Adsorption and desorption characteristics of adlay bran free phenolics on macroporous resins," *Food Chem.*, vol. 194, pp. 900–907, 2016.

[67]   B. Yingngam, M. Monschein, A. Brantner, "Ultrasound-assisted extraction of phenolic compounds from Cratoxylum formosum ssp. formosum leaves using central composite design and evaluation of its protective ability against H2O2-induced cell death", *Asian Pac. J. Trop. Med.*, 7, S1, S497–S505, 2014.

[68]   S. Chen, Z. Zeng, N. Hu, B. Bai, H. Wang, Y. Suo, "Simultaneous optimization of the ultrasound-assisted extraction for phenolic compounds content and antioxidant activity of Lycium ruthenicum Murr. fruit using response surface methodology", *Food Chem.*, 242, 2017, 1–8, 2018.

[69]   S. Feng, Z. Luo, B. Tao, C. Chen, "Ultrasonic-assisted extraction and purification of phenolic compounds from sugarcane (Saccharum officinarum L.) rinds", *LWT - Food Sci. Technol.*, 60, 2, 970–976, 2015.

[70]   Y. Tian, Z. Xu, B. Zheng, Y. Martin Lo, "Optimization of ultrasonic-assisted extraction of pomegranate (Punica granatum L.) seed oil", *Ultrason. Sonochem.*, 20, 1, 202–208, 2013.

PhD Thesis

Chapter 2: Optimization and comparative evaluation of phenolic extraction from olive mill solid wastes and phenol recovery by adsorption process

[71]   C. D. Goldsmith, Q. V. Vuong, C. E. Stathopoulos, P. D. Roach, C. J. Scarlett, "Ultrasound increases the aqueous extraction of phenolic compounds with high antioxidant activity from olive pomace", *LWT - Food Sci. Technol.*, 89, 2017, 284–290, 2018.

[72]   T. Mehmood, A. Ahmad, A. Ahmed, Z. Ahmed, "Optimization of olive oil based O/W nanoemulsions prepared through ultrasonic homogenization: A response surface methodology approach", *Food Chem.*, 229, 790–796, 2017.

[73]   R. G. Henika, "Use of response surface methodology in sensory evaluation", *Food Technol*, 36, 11, 96–101, 1982.

[74]   R. Lavecchia, A. Zuorro, "Recovery of flavonoids from three-phase olive pomace by aqueous ethanol extraction", *ARPN J. Eng. Appl. Sci.*, 11, 23, 13802–13809, 2016.

[75]   M. Vinatoru, "An overview of the ultrasonically assisted extraction of bioactive principles from herbs", *Ultrason. Sonochem.*, 8, 3, 303–313, 2001.

[76]   M. DellaGreca, L. Previtera, F. Temussi, A. Zarrelli, "Low-molecular-weight components of olive oil mill waste-waters", *Phytochem. Anal. An Int. J. Plant Chem. Biochem. Tech.*, 15, 3, 184–188, 2004.

[77]   M. Araújo, F. B. Pimentel, R. C. Alves, M. B. P. P. Oliveira, "Phenolic compounds from olive mill wastes: Health effects, analytical approach and application as food antioxidants", *Trends Food Sci. Technol.*, 45, 2, 200–211, 2015.

[78]   W. Yakhlef, R. Arhab, C. Romero, M. Brenes, A. de Castro, E. Medina, "Phenolic composition and antimicrobial activity of Algerian olive products and by-products", *Lwt*, 93, 2017, 323–328, 2018.

[79]   A. Peralbo-Molina, F. Priego-Capote, M. D. Luque de Castro, "Tentative identification of phenolic compounds in olive pomace extracts using liquid chromatography–tandem mass spectrometry with a quadrupole–quadrupole-time-of-flight mass detector", *J. Agric. Food Chem.*, 60, 46, 11542–11550, 2012.

[80]   A. Malapert, E. Reboul, M. Loonis, O. Dangles, V. Tomao, "Direct and rapid profiling of biophenols in olive pomace by UHPLC-DAD-MS", *Food Anal. Methods*, 11, 4, 1001–1010, 2018.

[81]   M. Antónia Nunes, A. S.G. Costa, S. Bessada, J. Santos, H. Puga, R. C. Alves, V. Freitas, M. P.P. Oliveira, "Olive pomace as a valuable source of bioactive compounds: A study regarding its lipid- and water-soluble components", *Sci. Total Environ.*, 644, 229–236, 2018.

[82]   M. Robles-almazan, M. Pulido-moran, J. Moreno-fernandez, C. Ramirez-tortosa, C. Rodriguez-garcia, J. L. Quiles, "Hydroxytyrosol : Bioavailability , toxicity , and clinical applications", *Food Research International*, 105, 654–667, 2018.

[83]   M. A. Nunes, F. B. Pimentel, A. S. G. Costa, R. C. Alves, M. B. P. P. Oliveira, "Olive by-products for functional and food applications : Challenging opportunities to face environmental constraints", *Innov. Food Sci. Emerg. Technol.*, 35, 139–148, 2016.

PhD Thesis

Chapter 2: Optimization and comparative evaluation of phenolic extraction from olive mill solid wastes and phenol recovery by adsorption process

[84]   F. Visioli, G. Bellomo, G. Montedoroc, C. Galli, "Low density lipoprotein oxidation is inhibited in vitro by olive oil constituents", *Atherosclerosis*, 117, 25–32, 1995.

[85]   S. Mateo, "Metodo para obtener hidroxitirosol rico a partir agua de vegetación", 1, 12, 2040–2048, 2002.

[86]   W. Rudzinski, W. Plazinski, "Kinetics of Dyes Adsorption at the Solid−Solution Interfaces: A Theoretical Description Based on the Two-Step Kinetic Model", *Environ. Sci. Technol.*, 42, 7, 2470–2475, 2008.

[87]   C. Duran, D. Ozdes, A. Gundogdu, H. B. Senturk, "Kinetics and Isotherm Analysis of Basic Dyes Adsorption onto Almond Shell (Prunus dulcis) as a Low Cost Adsorbent", *J. Chem. Eng. Data*, 56, 5, 2136–2147, 2011.

[88]   E. Ayranci, N. Hoda, "Adsorption kinetics and isotherms of pesticides onto activated carbon-cloth", *Chemosphere*, 60, 11, 1600–1607, 2005.

[89]   D. P. Zagklis, A. I. Vavouraki, M. E. Kornaros, C. A. Paraskeva, "Purification of olive mill wastewater phenols through membrane filtration and resin adsorption/desorption", *J. Hazard. Mater.*, 285, 69–76, 2015.

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Chapter 3: Study and efficiency evaluation of OMWW pretreatment and membrane based treatment to obtain a final retentate rich in Hydroxytyrosol

En este capítulo se aborda el estudio de la valorización de las aguas residuales de almazara, generadas en el proceso de centrifugación de tres fases (OMWW o alpechín), mediante tecnologías con membranas, que están consideradas como tecnologías limpias y respetuosas con el medio ambiente. Estas aguas residuales son extremadamente contaminantes, pero, a su vez, pueden ser una fuente biológica interesante de compuestos de alto valor agregado, como el hidroxitirosol (HT) u otros compuestos fenólicos antioxidantes. Generalmente son necesarios varios procesos de tratamiento para eliminar los compuestos químicos tóxicos y poder realizar la valorización de los compuestos bioactivos o la reutilización del agua. Debido a su elevado contenido de sólidos en suspensión, es esencial considerar un método de pretratamiento de dichas aguas residuales que sea capaz de prevenir el grave ensuciamiento que estos sólidos producen en las membranas utilizadas habitualmente, lo que redundará en una reducción importante de los costes de capital para procesos de tratamiento a escala industrial.

En primer lugar se evaluó el efecto de diferentes pretratamientos combinados de acidificación, filtración y centrifugación sobre las características del agua residual (concentración de fenoles totales (TPC), concentración de hidroxitirosol, potencial zeta, tamaño y estabilidad de las partículas), seleccionando la acidificación a pH=2 y la centrifugación durante 30 minutos y 4000 rpm como método de pretratamiento que aumentó la concentración de HT en el agua residual hasta cuatro veces debido a la conversión de otros polifenoles en HT.

Posteriormente, se realizó el tratamiento con membranas de las muestras de OMWW pretratadas en las condiciones óptimas indicadas anteriormente. Se utilizaron diferentes tipos de membranas poliméricas planas y cerámicas tubulares de micro, ultra y nanofiltración. Para evaluar la eficiencia y el rendimiento del proceso, se determinó la retención de compuestos fenólicos totales, la eliminación de DQO, el ensuciamiento de la membrana y la densidad de flujo de permeado en estado estacionario en todos los experimentos realizados. Los resultados obtenidos indican que, considerando un factor de concentración en volumen de 2,5, se puede lograr el tratamiento del agua residual en una sola etapa de ultrafiltración utilizando una membrana ST-PES (10 kDa), obteniendo una concentración de HT en la corriente de retenido de $177,43 \pm 18,55$ mg/L, y un 80,06% de reducción de la DQO. Utilizando un proceso integrado con membranas en dos etapas, alimentando el agua residual pretratada a una primera etapa de ultrafiltración (membrana PS, 100 kDa) y luego usando el permeado obtenido como alimentación de la segunda etapa de nanofiltración (membrana NF90, 200 Da), se obtiene un retenido final con una concentración de $364,11 \pm 40,50$ mg/L de HT y un 95,7% de reducción de la DQO, con un permeado final incoloro que podría ser utilizado como agua de riego.

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3.1. Introduction

Olive oil extraction involves different processes such as olive washing, grinding, beating and the extraction itself, that constitutes the basic stage of the whole process. The amount and physico-chemical properties of the wastes produced will depend on the method used for the extraction. There are two ways of extracting the oil: traditional pressing, used for many centuries with only minor modifications, and centrifugation, that the olive oil industry has taken over in the last decades [1]. Vegetation wastewater is one among the numerous end-of-pipe treatment needing food based wastewaters very well known for its significant negative impact on the environment [2]. It is the huge volume of foul smelling acidic dark liquid generated during the extraction of olive oil, and is mainly known as olive mill wastewater (OMWW) [3], which is generated during press, two-phase centrifugal or three-phase centrifugal olive oil extraction [4]. Especially the latter two methods produce a huge amount of wastewater compared to the first one. The press method releases a solid fraction along with an emulsion containing the olive oil that is separated by decantation from the OMWW. The three-phase system generates a solid husk, oil, and OMWW while the two-phase system releases a wet olive husk and oil. Relative to the two-phase centrifugation, the three-phase system utilizes 0.6–1.3 m^3 of additional water during decantation that eventually increases the amount of OMWW [2].

Many OMWW management options have been proposed in recent years. Most of these methods aim to reduce OMWW phytotoxicity in order to reuse it for agricultural purposes, so that it is suitable to be treated in conventional treatment facilities or to recover the biophenolic fraction owing to their interesting pharmacological properties [2,3,5,6].

Polyphenols comprise a large family (about 8000 substances with highly diverse structures) of naturally occurring secondary metabolites of plant-derived foods and are among the principal micronutrients associated with the health beneficial effects of our diet. Molecular weights range from small molecules (<100 Da), such as phenolic acids, to large molecules (>30,000 Da) of highly polymerized

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compounds. The main sources of dietary polyphenols include fruits, vegetables, grains, tea, essential oils, as well as their derived foods, beverages or supplements [7].

Hydroxytyrosol and tyrosol are the main phenolic compounds in extra virgin olive oil [8,9]. Hydrophilic extracts of olive oil contain many phenolic compounds and phenyl-alcohols. Oleuropein, hydroxytyrosol and their derivatives are found in large quantities in olive leaves and olive fruits [8,9].

Membrane separation processes are useful and clean technologies for recovery, fractionation and concentration of phenolic compounds from aqueous and alcoholic processing streams of products, byproducts and wastes from biomass processing [10]. Among them, pressure-driven membrane processes have been successfully employed over the last three decades in food and beverage industries, by treating dairy products, fruit juices, wine, vegetable oils, potable water and agricultural wastewaters. The driving force is the pressure gradient applied between the two sides of a permselective membrane. According to the required transmembrane pressure (TMP) and the pore sizes, pressure driven membrane operations can be divided in: microfiltration (MF, 0.1–10 μm, 0.1–5 bar), ultrafiltration (UF, 1–100 nm, 1–10 bar), nanofiltration (NF, 0.5–10 nm, 10–30 bar) and reverse osmosis (RO, < 0.5 nm, 35–100 bar) [11].

Apart from their many advantages (high selectivity, no phase change required, no organic solvents or harmful materials required, easy operation and low maintenance), membrane processes have also a range of inherent limitations. For example, a membrane system designed to treat OMWW may be limited by the high concentration of suspended solids, feed viscosity or osmotic pressure to reach a target quality. Therefore, the optimal separation process in many cases may be a membrane-based hybrid process. The term hybrid or integrated membrane process refers to integration of two membrane processes or a membrane process with traditional unit operations such as centrifugation. In particular, the membrane–membrane integrated system comprises a sequence of different steps. The integration is supposed to bring performance improvements depending on feed characteristics or desired product quality [12]. Hybridization thus is a strategy

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designed to benefit from the synergy among the different unit operations [13]. For example, hybridization of a UF unit after electro-Fenton oxidation and anaerobic digestion helped to achieve a complete detoxification, decolorization and COD reduction of OMWW [14].

The successive integration for the recovery of biophenols often begins with MF and/or UF. MF is the oldest known membrane process that is used to separate suspended particles with diameters between 0.1 and 10 µm. UF uses finely porous membrane (1–100 nm) to separate water and microsolutes from macromolecules and colloids. Separation in both MF and UF is done mainly based on size exclusion [15]. Hence, they are mostly employed as a pre-treatment step in hybrid system to remove suspended particles and colloids while allowing the polyphenols and other soluble contaminants to pass through. They thus complement other concomitant processes which normally target polyphenols and other soluble components but fail to remove suspended particulate matter [16,17].

NF is a pressure-driven separation process that has ionizable membrane surface charge groups. Hence nano scale separation efficiency depends on steric (sieving) and charges (Donnan) exclusion [15]. It is operated at pressures ranging from 10 to 30 bar enabling low retention of monovalent ions while permitting almost complete rejections of multivalent ions, thus resulting in high solute selectivity. It has growing popularity over last years as an effective yet simplified OMWW treatment technology due to its high solvent permeability, retention of dissolved uncharged polyphenols and other organic molecules with molecular weight greater than 150 Da, and ease of chemical cleaning [18]. This offers benefits in terms of environmental pollution abatement, rejection, recovery and reuse of biophenols, divalent salts, and recovery and reuse of purified water. Although NF is closely related to RO, it uses 21% less energy as compared to RO [2].

Since OMWWs are extremely polluting, several treatment processes are required for removing wastes and toxic chemicals, providing opportunities for water reuse or recycling. Because of their high suspended solids content, a pretreatment method able to abate the severe fouling occurring during the subsequent micro and

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ultrafiltration membrane processes is mandatory. Indeed, the major drawback in the development and large-scale application of membrane processes for olive mill wastewater treatment is the loss of membrane flux due to fouling. Different approaches have been developed and applied as pre-treatment steps based on physical, physical–chemical and biological methods or combination of them [19]. The problem of fouling is also augmented by great variation of OMMW's suspended solid composition during periodic release of huge volume of OMWW in a short time, just after the olive harvest. Therefore, pre-treatment able to standardize OMWW before entering the first membrane process (MF/UF) is necessary. Beside the first membrane stage, in all subsequent membrane operation fouling may not represent a significant effect [2].

It is worth quoting that OMWW are interesting biological sources of high added value compounds, such as hydroxytyrosol or other antioxidant phenolic compounds. In fact, the phenolic compounds are known as important natural antioxidants with nutritional and pharmaceutical properties [20]. Several medicinal and pharmaceutical scientists have demonstrated that olive phenols, especially hydroxytyrosol (3,4-di-hydroxyphenyl-ethanol), were effective in preventing and curing some important diseases [21] . Nevertheless, hydroxytyrosol is not commercially available in large quantities like other food additives. Several methods have been proposed for the production of hydroxytyrosol by means of chemical or enzymatic synthesis [22]. Such protocols are usually slow and expensive, resulting in few numbers of commercially available products containing pure hydroxytyrosol [6].

Bazzarelli et al. [19] deeply investigated the influence of pH variation on the suspension stability as a way to promote aggregates formation, thus improving the effective removal of suspended solids and also reported a comprehensive study of suspension stability as a function of pH using zeta potential and light scattering measurements as a fouling reduction method in membrane processes. These techniques were used to evaluate the electrical charge and the size of suspended solid particles in OMWWs.

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Furthermore, different studies highlighted the importance of pH variation during the pre-treatment process, pH values being mainly adjusted with the aim of preserving polyphenols from undesired oxidation processes. Russo [23] acidified OMWWs with hydrochloric acid and citric acid up to pH 3.5 to prevent phenols oxidation processes. Akdemir and Ozer [24] used two pH values, 2 and 6, both followed by filtration with a cartridge filter. Ochando-Pulido et al. [25] performed pH flocculation process at pH 2.5 followed by two further treatments (photocatalysis and UF) before the nanofiltration step. El-Abbassi et al. [26] studied the effect of pH, ranging from 2 to 12, on the process performance.

The primary objective in this chapter was to achieve a suitable formulation for pretreatment prior to membrane based treatments in order to ultimately obtain a rich composition of bioactive compound. Being able to do so could result into a considerable profit and reduction in the capital costs for industrial scale. Subsequently, the second goal was to obtain high concentration of biophenols, especially hydroxytyrosol, by entering different types of pretreated OMWW as feed in membrane treatment. Integrated membrane treatments within two models of single and double operations were performed including different membrane materials and transmembrane pressures. In order to evaluate the treatment efficiency and performance, phenolic rejection, Chemical Oxygen Demand (COD) removal, membrane fouling and steady state permeate flux were measured for all tests. Ultimately a comparison was made by focusing on the achievement of a treatment design with optimized conditions and possibly lower capital cost at industrial scale.

3.2. Materials and methods

3.2.1. Samples, chemicals and analytical methods

Olive mill wastewater (OMWW) used in this study was obtained by means of a three-phase extraction system and was kindly provided by "Mamalan" Agro

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Industrial Company, Tarom city (Zanjan, Iran). Its physico-chemical composition is provided in Table 3.1.

All chemicals used in this study were analytical grade and provided from VWR International Eurolab (Llinars del Vallès, Spain) and Sigma-Aldrich (Darmstadt, Germany). The chemicals used for each analysis are as follows:

- Folin–Ciocalteu reagent, gallic acid and sodium carbonate were used for Total Phenolic Content (TPC) measurement.

- Potassium dichromate ($K_2Cr_2O_7$), sulfuric acid (H_2SO_4), ferrous ammonium sulfate titrant (FAS) and ferroin were used for Chemical Oxygen Demand (COD) measurement.

- DPPH reagent (1,1-diphenyl-2-picrylhydrazyl) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were used for Antioxidant Activity (AA) analysis (DPPH assay).

- Hydrochloric acid (HCl, 37%) was used for the acidification of OMWW.

- Acetic acid (CH_3COOH), acetonitrile (C_2H_3N) and ammonium acetate (NH_4Ac) were the chemicals used in HPLC chromatography for determining the concentration of individual phenolic compounds.

- Sodium hydroxide (NaOH, 16 g/L) and nitric acid (HNO_3, 60%) in 5 g/L solution were used as alkaline and acid cleaning for ceramic membranes before and after each treatment.

Several types of ceramic and polymeric membranes were used and their characteristics are shown in Table 3.3.

The analytical methods used in this section are total phenolic content (TPC), COD measurement, antioxidant activity by DPPH assay, zeta potential, HPLC chromatography, particle size diameter, particle size distribution and particle stability. Detailed description of each method is provided in the appendix (analytical methods) of this Thesis.

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Table 3.1. Physico-chemical characteristics of Olive Mill Wastewater (OMWW).

Parameter	Unit	Value
Total phenolic content (TPC)	mg/L	1200.15 ± 13.05
Chemical oxygen demand (COD)	mg/L	19418 ± 868
Biochemical oxygen demand (BOD)	mg/L	8600 ± 0.0
Total organic carbon (TOC)	g/L	7.93
Total suspended solids (TSS)	g/L	6.48
pH	---	4.9
Antioxidant activity	mg_{Trolox}/L	185.25 ± 1.21
Turbidity	NTU	8225
SO_4^{2-}	mg/L	170
NO_3^-	mg/L	530

3.2.2. Acidification and centrifugation

pH value of the raw OMWW was measured by means of a glass pH electrode (Crison, Barcelona, Spain) which was 4.9. Afterwards, by considering the work performed by Bazzarelli et al. [19], OMWW was acidified with HCl (37%) in different levels. The purpose of acidification in multiple levels was finding out the optimized point of agglomeration and flocculation of particles in order to destabilize them and reduce the quantity of suspended solids as much as possible. Considering that the effect of centrifugation on the acidified OMWW had not been studied comprehensively in the literature, multiple series of OMWW samples with different pH were centrifuged (Eppendorf centrifuge 5804, Germany) at 4000 rpm for two time levels of 3 and 30 min, in order to find out whether different centrifugation times has any effect on TPC. The operating conditions for centrifugation in this study were the same as those used by Russo [23].

3.2.3. Light scattering methods

Particle size and particle size distribution were measured using a laser light scattering system (Malvern Mastersizer 2000, Malvern Instruments, UK). The mean particle size was expressed as the volume weighted mean diameter (Eq. (1)).

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$$D[4,3] = \frac{\sum D_i^4 n_i}{\sum D_i^3 n_i} \qquad (1)$$

where n_i is the number of particles with a diameter D_i.

The volume weighted mean diameter is most sensitive to the presence of large particles. The width of particle size distribution was expressed as a Span number, calculated by the following equation:

$$Span = \frac{D(0.9) - D(0.1)}{D(0.5)} \qquad (2)$$

where D(0.9) is the particle diameter which in this study signifies the point in the size distribution, up to and including which, 90% of the total number of particles in the sample is contained; The definition for D(0.5), is then the size point below which 50% of the material is contained, this value is also known as the mass median diameter. D(0.1) is that size below which 10% of the particles are contained. The mean particle diameter and Span values were calculated on the basis of five different experimental runs.

In this study, a comprehensive process of light scattering methods was performed on raw and pretreated OMWWs in order to study the effect of processes like acidification, centrifugation and membrane filtration on particle size distribution.

3.2.4. Membrane treatment process

In order to evaluate the extraction of phenolic compounds and the effect of conducted pretreatment, three pretreatment models were considered for OMWW before using it as feed in membrane treatment. In first model (Feed A), only centrifugation was conducted. In second model (Feed B), acidification to pH=1.9 followed by centrifugation was carried out, and third model (Feed C) included centrifugation followed by acidification to pH=1.9. Centrifugation process was performed at 30 minutes and 4000 rpm for all feed types. It may seem that Feed B and Feed C are similar. However, according to light scattering tests which will explained in detail in results, mean particle diameter for Feed B and Feed C were

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considerably different. Therefore, this difference could be the cause of different performances and results in membrane treatment.

One of the main objectives of this section was performing membrane treatment within single stage. The purpose of performing one step membrane treatment was to evaluate the possibility of obtaining a hydroxytyrosol rich retentate by choosing the optimized operating condition and membrane cut-off. One step membrane treatment, if possible, could considerably decrease the capital cost in pilot or industrial scale in comparison with integrated membrane treatments. However, in order to enhance the study, multiple runs of integrated membrane treatments were also performed using MF/UF permeates as feed for the nanofiltration stage.

OMWW treatments were performed using two different membrane modules. The first one was a tubular module supplied by Tami industries (Nyons, France) where permeates were collected in a separate vessel and concentrates were circulated back to the feed tank. The system was equipped with ceramic TiO_2 MF (0.2 µm) or UF (50 kDa) membranes, with a membrane area of 47 cm^2, and a 500 mL capacity feed tank. A positive displacement pump (Masterflex, L/S Easy- Load II, IL, USA) was used to fed the membrane module. Feed temperature was maintained constant by means of a heat plate, and pressure was measured by means of two manometers located at the inlet and outlet of membrane module. Tubular membrane experiments were performed at 40 °C and at a transmembrane pressure (TMP) of 1-2 bar.

Once experiment was finished, membrane was rinsed with distilled water for 10 min, cleaned with an alkaline aqueous solution (16 g/L NaOH) at 50 °C for 30 min, rinsed again with distilled water for 15 min, and cleaned with an acid solution (5mL/L HNO_3 (60%)) at 50 °C for 30 min. Finally, the system was rinsed with distilled water for 15 min until neutrality and water permeate flux was checked.

Flat sheet polymeric membranes shown in Table 3.2 were used for ultrafiltration (UF) and nanofiltration (NF) experiments performed in a stainless steel HP4750 stirred batch cell supplied by Sterlitech Corporation (Kent, WA, USA). Depending on the process, transmembrane pressure of 5-10 bar was provided through a

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nitrogen cylinder to avoid oxidation of the biocompounds. Membrane surface area was 14.6 cm^2 and experiments were performed at room temperature.

Stirred cell modules provide uniform transmembrane pressure and hydrodynamic conditions at all points of the membrane surface, and the effects of operating parameters on process efficiency can be very easily determined using these cells. They are therefore very useful for small-scale process development work. However, advantage of Tubular membrane module include turbulent flow (providing good membrane/solution contact and removing retentate film build-up), relatively easy cleaning, easy handling of suspended solids and viscous fluids and ability to replace or plug a failed tube while the rest of the system runs [27].

The permeate flux was monitored by measuring the permeate volume compiled at a specific time described by Darcy's Equation (Eq. (3)) [28]:

$$J = \frac{1}{A_m} \cdot \frac{dV_p}{dt} \tag{3}$$

where J is the permeate flux, A_m is the effective membrane area, V_P is the permeate volume, and t is the filtration time.

Volume reduction factor is defined as:

$$VRF = \frac{V_F}{V_R} \tag{4}$$

where V_F and V_R are the initial feed volume and the retentate volume ($V_R = V_F - V_P$), respectively. This is an important parameter in concentration operating mode.

Percent rejection (%) of feed components is calculated as:

$$R(\%) = \left(1 - \frac{C_p}{C_F}\right) \times 100 \tag{5}$$

where C_p and C_F are the concentration of total phenols in permeate and feed solutions, respectively [29].

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3.3. Results and discussion

3.3.1. OMWW pretreatment

3.3.1.1. Effect of acidifying and centrifugation on total phenolic content (TPC)

Based on previous studies found in bibliography, centrifugation is able to fully separate suspended solids, and also to improve COD removal and oil recovery. Changes in the chemical environment had a considerable influence on the centrifugation yield. At pH = 2 (acidification by H_2SO_4) the highest oil recovery and a simultaneous high COD decrease (67.8%) were achieved. Furthermore, the sediment obtained from centrifugation at pH = 2 was more cohesive, with the lowest volume (15%) and water content (80%). Centrifugation of OMWW at its natural pH is beneficial for oil mills, because of the significant natural oil recovery (30-50%), and the organic load reduction by 50% at least. However, further COD reduction is not possible by these pretreatment techniques, due to the soluble organics in OMWW, which cannot be removed by these processes [30].

In this study, the effect of centrifugation and filtration was studied in several OMWW samples with different pH values (1.1, 2.9 and 4.9). For this purpose, centrifugation was performed with periods of 3 and 30 minutes. The rotation of centrifuge was kept constant at 4000 rpm. Higher velocities were not used in order to prevent the oxidation of phenolic compounds. As it can be observed from Figure 3.1a, 10-15% TPC decrease was obtained after centrifugation of raw OMWW (pH=4.9), whereas quite different results were obtained for acidified OMWW. Somehow, acidification reduced TPC about 15% and 23% for OMW with pH=2.9 and 1.1, respectively. Nevertheless, it can be observed in Figure 3.1.a that after centrifugation not only TPC did not decrease but also the reduced amount of TPC due to acidification was almost reclaimed for OMWW with pH=1.1. Best results were obtained after 30 min centrifugation time, with several advantages over short centrifugation times in case of phenols preservation and also being able to separate suspended solids in a better form.

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Two filtration steps were also carried out for each level of acidification using syringe filters with different pore size (0.45 µm and 0.1 µm) to evaluate their effect on TPC. As it can be observed in Figure 3.1.b, the highest TPC was obtained for two OMWW treatments with pH=2.9 (3 min centrifugation, 0.1 µm filtration, and 30 min centrifugation, 0.45µm filtration). It can be concluded that the effect of filter pore size is not significant and should not be considered as an important parameter. This analysis proved the efficiency of acidification on preserving phenolic compounds in comparison with using raw OMWW as feed for membrane treatment. Although the possibility of 5-10% of experimental error for every analysis should be considered.

3.3.1.2. Effect of pretreatment on the molecular structure of the phenolic compounds

According to the patent presented by Mateo [31], it is desirable to develop a method which produces an aqueous olive extract with a high percentage of hydroxytyrosol (HT), due to their strong chemoprotective activity. The invention includes a method for producing a hydroxytyrosol-rich composition with the following stages: (a) producing vegetation water from olives, preferably depitted olive meat, (b) adding acid to the vegetation water in an amount effective to produce a pH between 1 and 5, preferably 2-4, and (c) incubating the acidified vegetation water for a period of at least two months, typically 6-12 months until at least 75%, and preferably at least 90% of the oleoeuropein originally present in the vegetation water has been converted to hydroxytyrosol [31].

Table 3.2. Hydroxytyrosol content in raw and pretreated OMWW

Sample name	Hydroxytyrosol content (mg/L)
Raw OMWW	25.30 ± 4.34
centrifuged OMWW	34.76 ± 6.65
Acidified OMWW to pH=2	115.10 ± 6.68
Centrifuged and Acidified to pH=2 OMWW	103.84 ± 4.37

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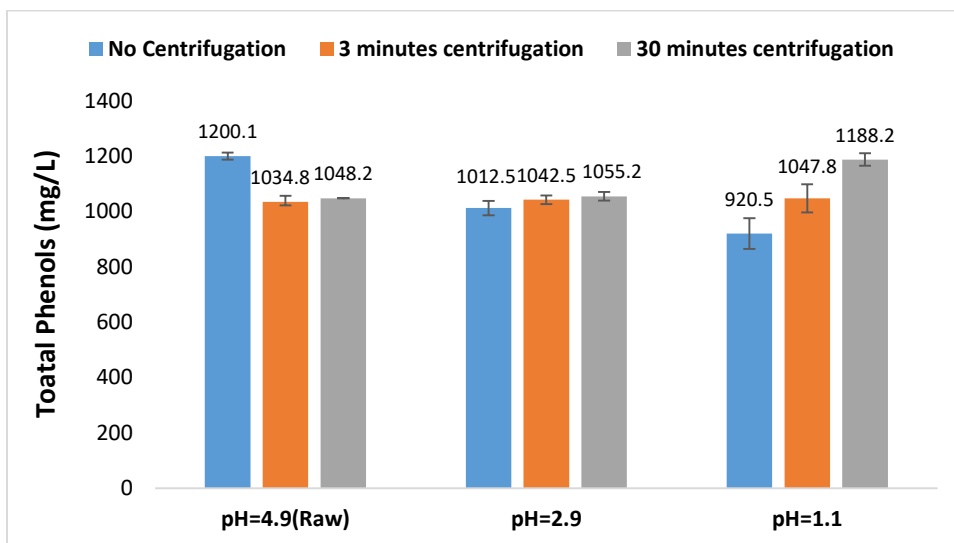
(a)
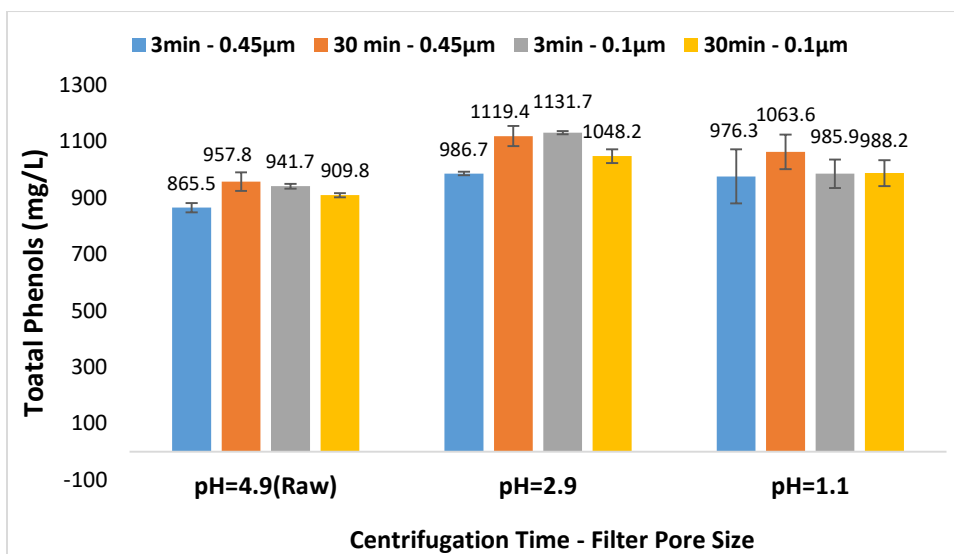
(b)

Figure 3.1. Effect of OMWW pretreatment by centrifugation (a) and centrifugation-filtration (b) on total phenolic content (TPC). Total phenols was changed by TPC in y-axes

Table 3.3. Characteristics of ceramic and polymeric membranes used in this work.

Treatment [1]	NF	NF	NF	UF	UF	UF	UF	MF
Name	NF270	NF90	N0P10	ST-PES	MK-PES	Ceramic	US100	Ceramic
Type	Flat sheet	Flat sheet	Flat sheet	Flat sheet	Flat sheet	Tubular	Flat sheet	Tubular
Manufacturer	Dow Filmtec™	Dow Filmtec™	Microdyn-Nadir	Synder™	Synder™	Tami Industries	Microdyn-Nadir	Tami Industries
Material	Polyamide	Polyamide	Polyethersulfone	Polyethersulfone	Polyethersulfone	Titanium dioxide (TiO_2)	Polysulfone	Titanium dioxide (TiO_2)
Cut-off	200~400 Da	200~400 Da	1000-1200 Da	10 kDa	30 kDa	50 kDa	100 kDa	0.2 μm
pH ranging	2-11	2-11	0-14	2-11	2-11	0-14	1-14	0-14
Water permeability $(L\ m^{-2}\ h^{-1}\ bar^{-1})$	10.5	6.3	7.5	25	25	50	400	170

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[1] MF: Microfiltration; UF: Ultrafiltration; NF: Nanofiltration

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Table 3.4. Results of light scattering methods and total phenolic content analysis of pretreated OMWW.

| | pH | Centrifugation time (min) | TPC (mg/L) | Light scattering methods | | | | | |
| | | | | Nanosizer Nano ZS | | | Mastersizer 2000 | | |
				Zeta potential (mV)	Particle diameter (μm)	PDI	D[4,3] (μm)	Span	D(0.9) (μm)
1	1.5	0	1146.3	+ 0.067	2.087	0.366	4.372	1.138	3.156
2	1.5	30	1072.5	- 0.176	1.198	0.748	7.324	1.364	3.137
3	3.0	0	1066.7	- 4.35	1.888	0.459	4.695	1.140	3.116
4	3.0	30	1032.5	- 4.91	1.418	0.784	7.908	1.434	3.135
5	4.9	0	1200.1	- 26.4	1.967	0.323	4.580	1.201	2.362
6	4.9	30	931.3	- 27.1	1.550	0.862	0.247	1.345	0.120

As it can be seen in Table 3.2, hydroxytyrosol concentration of raw, centrifuged, acidified and acidified+centrifuged OMWWs were measured by application of HPLC chromatography. Results prove that acidification of OMWW has a huge effect on conversion of other phenolic compounds to HT which increased its concentration more than 400%. Somehow, HT concentration in raw OMWW was 25.30 ± 4.34 mg/L whereas after acidification it increased to 115.10 ± 6.68 mg/L. On the other hand, centrifugation did not show any significant effect in changing the concentration of HT and the differences in the quantities could be referred as experimental errors.

3.3.1.3. Effect of pretreatment on zeta potential

Sample stability is an important parameter to minimize or maximize aggregation phenomena, facilitating the permeation or the rejection of particles through filters during waste water treatment. The electrical charges control the interactions between particles and therefore determine the overall behavior of a suspension. The effective particle charge can be modulated altering the media conditions, for example, by varying the pH solution and can be followed by zeta potential measurements. A higher absolute value of zeta potential results in greater electrostatic repulsion forces between particles, reducing aggregation/flocculation phenomena caused by Van der Waals interactions. A zeta potential of ±30 mV is often used as an approximate threshold for stability. Sample particles with zeta potentials values between –30 mV and +30 mV tend to aggregate over time. On the contrary, particles with a zeta potential near to 30 mV (absolute value) should be only marginally stable, with the degree of stability over longer time increasing as the zeta potential increases [19].

Zeta potential analysis in this study was dedicated to OMWW samples with different pH (1.5, 3, 4.9) and a centrifuged model for each pH, which in total made six runs for evaluation. As it can be observed from Table 3.4, as the pH of the OMWW decreases, zeta potential approaches near zero. Zeta potential for OMWW with pH=1.5 is almost zero and for OMWW with pH=3 is between –4.35 to –4.91

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mV while it is –26.4 to –27.1 for raw OMWW. It should be mentioned that centrifugation is not a significant factor in changing the zeta potential value. The obtained results are in accordance with the study performed by Bazarelli et al. [19].

pH range from 1.5 to 2.5 corresponds to the maximum suspension instability, with zeta potential values near the isoelectric point. If particles have low zeta potential values (absolute value) flocculation process occurs. On the contrary, if particles have higher zeta potential values there will be no tendency for particles to aggregate [19]. By analyzing the obtained results from the different effects of OMWW pH level, similar results to those obtained by Bazzarelli et al. [19] were achieved in this study. Thus, in order to prevent any possible damage of membranes due to high level of acidity, pH=2 was selected as the preferred acidification level of OMWW for membrane treatment.

3.3.1.4. Effect of pretreatment on particle stability and size diameter

Experimental results from light scattering analysis shown in Table 3.4 indicates that OMWW acidification increases D(0.9) about 33%. For centrifuged OMWW with pH=1.5, D(0.9)=3.137 μm, while for the raw centrifuged OMWW, D(0.9)=0.120 μm. On the other hand, D[4,3] of OMWWs with pH=1.5 and pH=3 are 4.372 and 4.675 μm, respectively. However, D[4,3] surprisingly increases when centrifugation takes place on OMWW with pH=1.5 and pH=3, reaching to 7.324 and 7.908, respectively. In other words, this analysis indicates that centrifugation of the raw OMWW decreases D(0.9) for about 95%, while centrifugation of acidified OMWW does not have any considerable effect on D(0.9) although it increases D[4,3] for an average of 68%.

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(a)

(b)

(c)
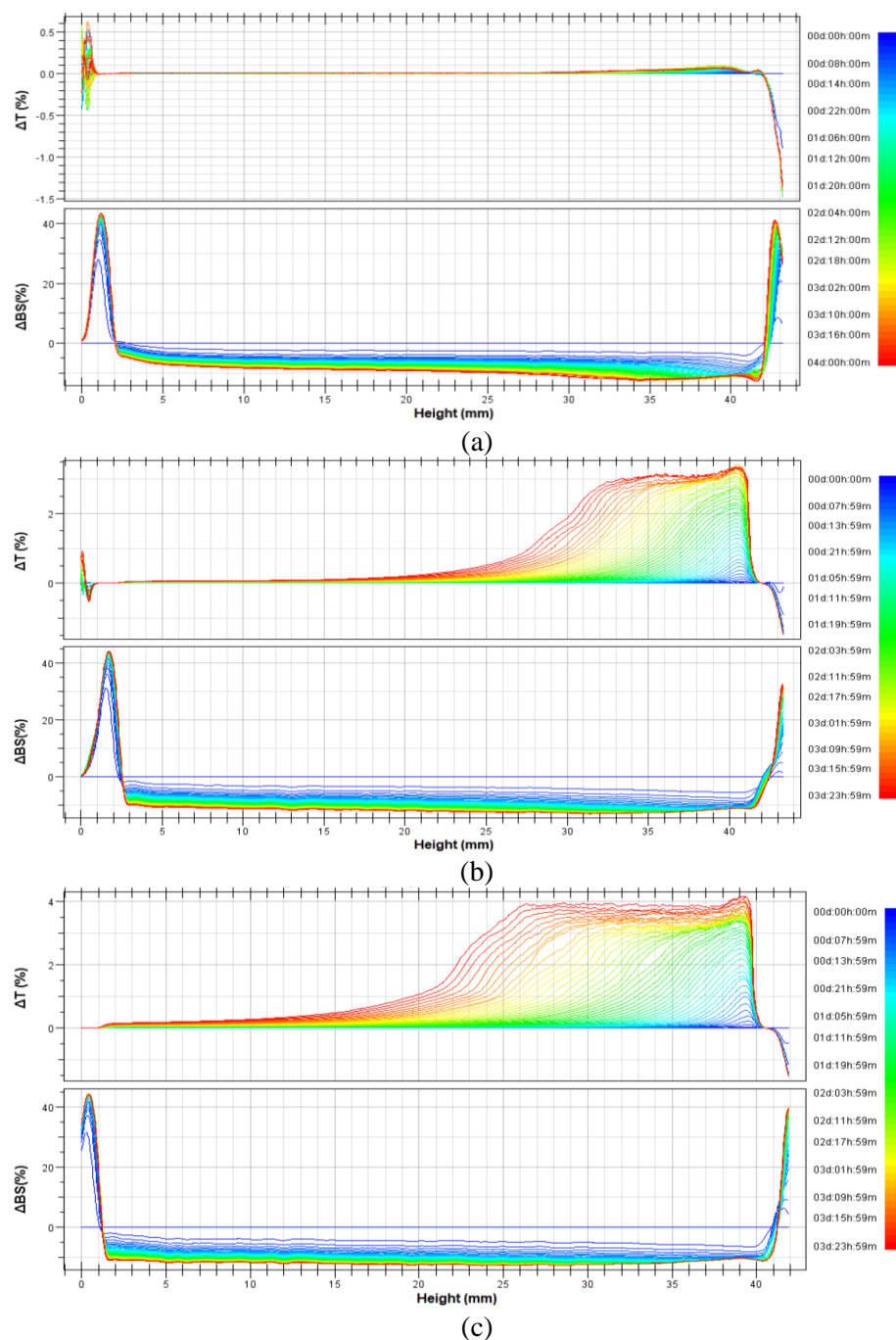
Figure 3.2. Transmission and backscattering profiles of OMWW samples with different pH value: (a) Raw OMWW (pH=4.9); (b) Acidified OMWW (pH=3); (c) Acidified OMWW (pH=1.5).

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It can be understood from the results that performing centrifugation on the acidified OMWW will make the bigger particles getting bigger, increasing their contribution to the volume weighted mean diameter. The opposite situation was found when centrifugation was performed on raw OMWW, where volume weighted mean diameter (D[4,3]) decreased for 95%.

Particle size distribution measurement was also performed by Nano ZS apparatus (Malvern Instruments Ltd., UK). Although, due to the considerable presence of large particles in OMWW samples, results obtained from Nanosizer is not as reliable as the ones obtained by Mastersizer. Nevertheless, in order to be able to compare the analysis from two instruments, size distribution was also measured with Nanosizer.

Data from Table 3.4 also prove that the average particle diameter average is almost the same (about 2 μm) for raw samples regardless of their pH. However, when centrifugation is performed, average particle diameter decreased between 25-40%. On the other hand, polydispersity index (PDI) is considerably higher for centrifuged samples than for raw samples. These high PDI values of centrifuged samples makes their further membrane treatment easier and with less fouling problems.

The stability of three OMWW samples with different pH (1.5, 3, and 4.9) were evaluated using the Turbiscan Lab Expert. The main advantage of this technique is that it can detect changes in the particle size of the sample long before they become visible [32]. Turbiscan Lab measures the light transmitted and backscattered by the sample, which directly depends on the particle mean diameter, at preset intervals (2 hours in this work) over a predetermined period of time (4 days in this study). As it can be seen from Figure 3.2, backscattering and transmission profiles indicate that a considerable flocculation and settling of particles occur for acidified OMWW, but the level of acidification does not make a significant difference in the flocculation intensity. However, sedimentation is

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higher for OMWW with pH=1.5 in comparison with OMWW with pH=3 at the final stage of the evaluation.

3.3.2. Membrane treatment of OMWW

3.3.2.1. Effect of operating conditions on the performance of membrane treatment

Most of the experiments for OMWW treatment by membrane processes found in the literature were carried out for a short period of time: MF from 30 min to 6 h, UF mostly about 3 h, and both NF and RO about 1 h. Within this short operational period, a considerable flux loss was observed; e.g.: MF 30–75%, UF about 50% on average, NF 45–80% and RO about 50% of the initial flux. While fouling due to macromolecules is the main cause at the MF/UF steps, loss in driving force due to increase concentration of small molecules and ions could partially attribute to the flux loss during NF and RO. Therefore, membrane treatment of OMWW is highly dependent on concentration polarization and membrane fouling [33]. Concentration polarization is a reversible phenomenon caused by increased transport resistance in the boundary layer close to the membrane surface. Membrane fouling is an irreversible phenomenon that includes the effects of surface fouling, adsorption, gel layer formation, pore radius reduction, pore blockage, cake formation, and adhesion of particles on the membrane. Permeate flux profiles under fouling typically shows an initial stage sudden drop followed by a smoother but continuous decay [2].

From a macroscopic point of view, membrane fouling can be either due to gel/cake layer formation or through pollutant–membrane interactions. More specifically, the fouling mechanism can be internal pore fouling when the pollutant size is lower than the membrane pore size, pore blocking if pollutant and membrane pore have the same size, or it could be by cake formation on the membrane surface when pollutant size is larger than membrane pore size. Theoretical modeling or prediction based on experimental trends is one way to identify the potential

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membrane fouling mechanism during membrane processing of OMWW. Although theoretical modeling of membrane based filtration is accompanied by several computational challenges, it is useful to predict and monitor membrane productivity, fouling tendency, solute retention and eventually the fouling mechanism [2].

Variation of polyphenols rejection, COD removal, steady state permeate flux and membrane fouling with feed types are shown in Figure 3.3. Ultrafiltration with ST-PES membrane (10 kDa) and nanofiltration with NP010 membrane (1000 Da) were performed using all feed types with same operating conditions. Therefore, their results can be compared in order to analyze the effect of feed types on permeate flux and to determine the optimized feed for single membrane treatment.

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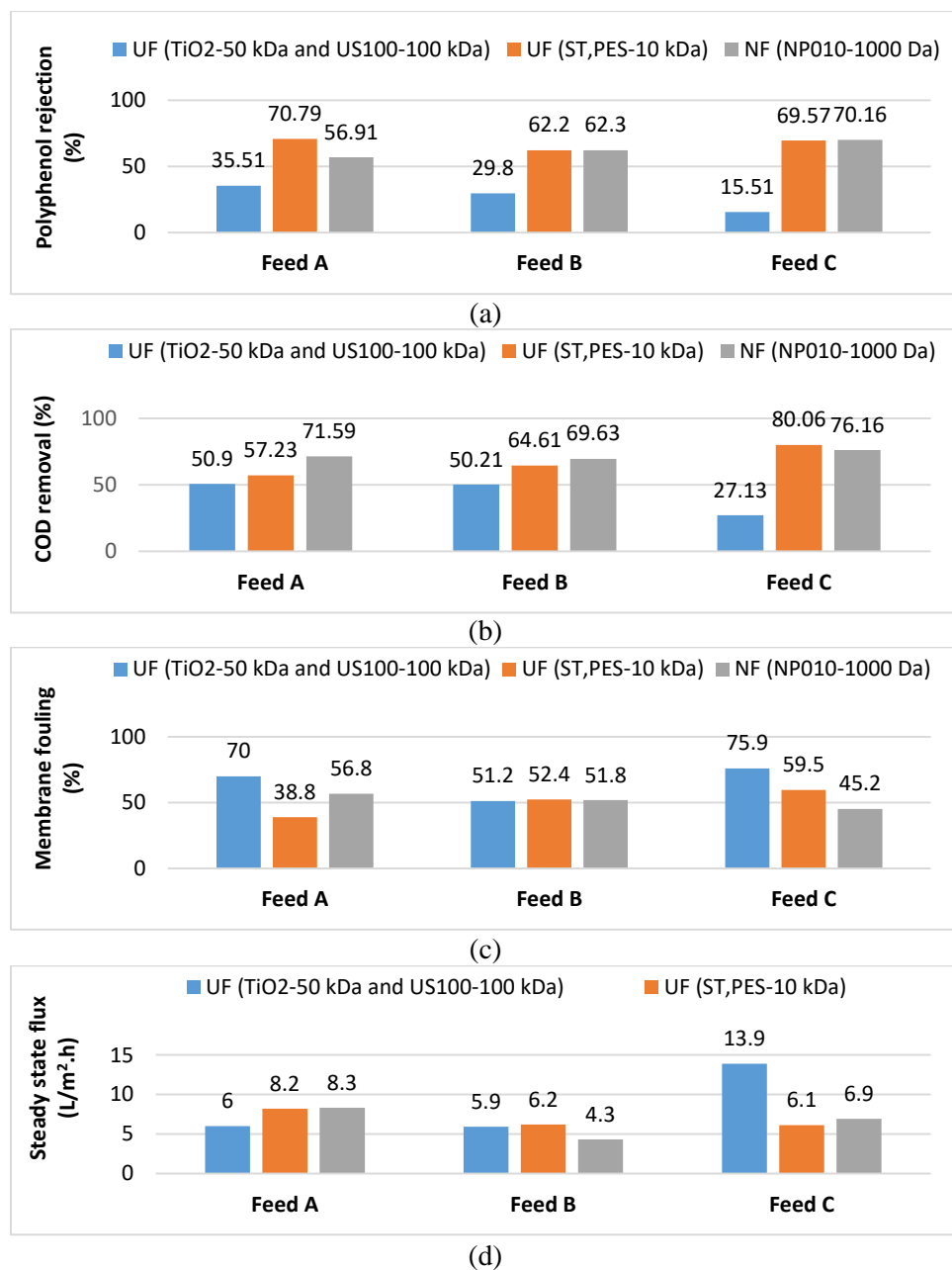
Figure 3.3. Evaluation of feed types on performance of membrane treatment by using membranes with different cut-offs. Operating conditions and feed types are indicated in Table 3.5. (a) Polyphenols rejection; (b) COD removal; (c) Membrane fouling; (d) Steady state permeate flux.

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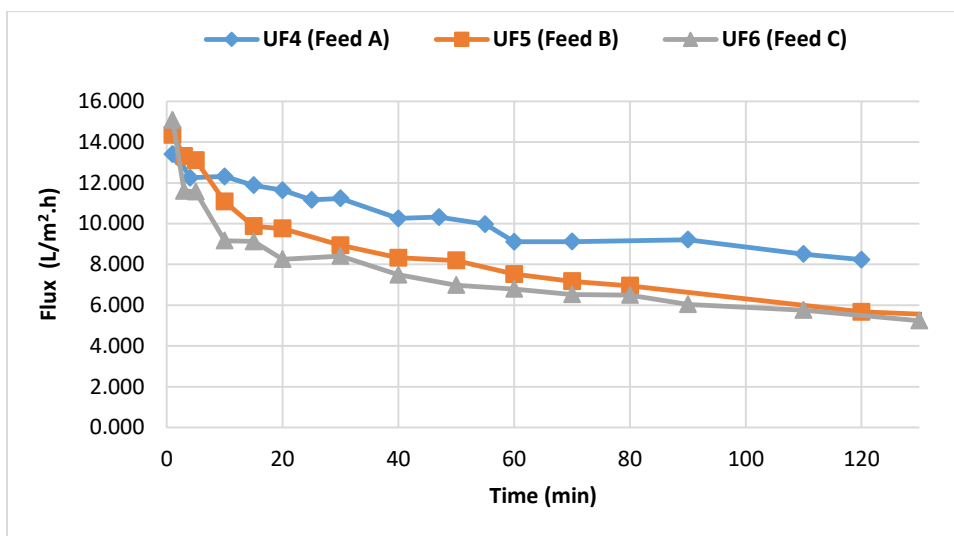
(a)
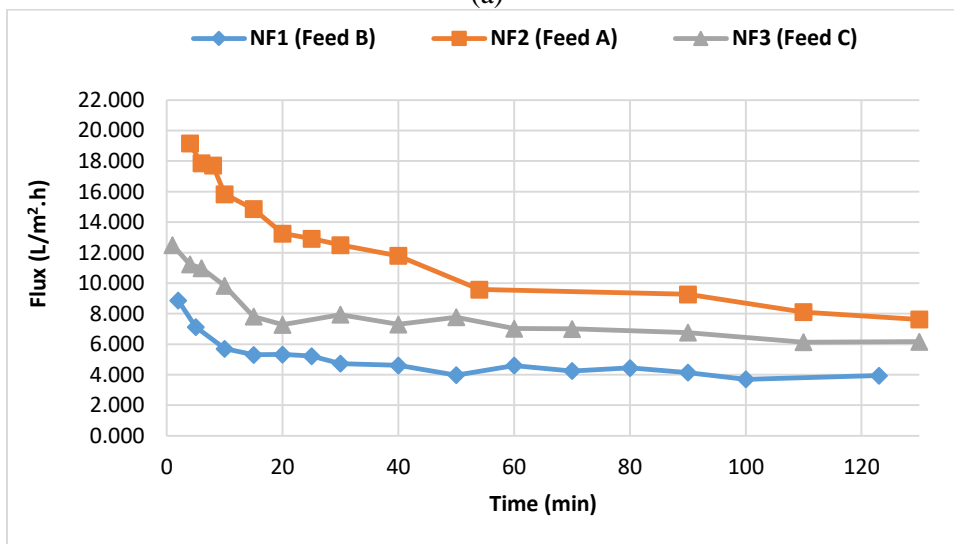
(b)

Figure 3.4. Variation of permeate flux along time for membrane treatment of several OMWWs. Operating conditions and feed types are indicated in Table 3.5. (a) Ultrafiltration (ST-PES membrane, 10 kDa); (b)Nanofiltration (NP010 membrane, 1000 Da).

Table 3.5. Operating conditions and final permeate properties from single and integrated membrane treatments of OMWW.

Code	Feed type[2]	Membrane type / Cut-off	TMP (bar)	Steady state flux (L m^{-2} h^{-1})	Membrane fouling (%)	TPC of permeate mg/L	TPC Rejection (%)	COD of permeate mg/L	COD Removal (%)	Antioxidant activity mg$_{Trolox}$/L	Antioxidant Inhibition (%)	Hydroxytyrosol content in permeate (mg/L)
MF1	Feed B	TiO$_2$ (0.2 µm)	1	9.6	55.6	1086.31 ± 2.18	9.49	11676 ± 931	39.87	188.24 ± 0.0	96.07 ± 0	97.26 ± 8.85 (VRF= 2.5)
MF2	Feed A	TiO$_2$ (0.2 µm)	1	11.7	42.6	1038.23 ± 3.81	13.49	11995 ± 706	38.23	180.125 ± 0.0	91.93 ± 0.0	35.0 ± 2.19 (VRF= 2.5)
UF1	Feed B	TiO$_2$ (50 kDa)	2	5.9	51.2	842.46 ± 17.41	29.80	9668 ± 81	50.21	185.68 ± 1.21	94.77 ± 0.62	95.75 ± 1.32 (VRF= 2.5)
UF2	Feed A	TiO$_2$ (50 kDa)	2	6.0	70.0	774.0 ± 4.35	35.51	9535 ± 134	50.90	179.48 ± 0.30	91.60 ± 0.15	43.94 ± 1.15 (VRF= 1.4)
UF3	Feed A	MK-PES (30 kDa)	8	8.2	55.7	528.23 ± 4.90	55.99	8931 ± 244	54.01	181.41 ± 1.81	92.58 ± 0.93	25.13 ± 0.74 (VRF= 4.4)
UF4	Feed A	ST-PES (10 kDa)	8	8.2	38.8	350.59 ± 5.98	70.79	8305 ± 231	57.23	179.27 ± 4.23	91.49 ± 2.16	77.83 ± 0.43 (VRF=2.2)
UF5	Feed B	ST-PES (10 kDa)	8	6.2	52.4	453.62 ± 12.51	62.20	6873 ± 359	64.61	166.66 ± 3.93	85.06 ± 2.0	89.18 ± 0.93 (VRF=2.0)
UF6	Feed C	ST-PES (10 kDa)	8	6.1	59.5	365.15 ± 7.07	69.57	3872 ± 118	80.06	148.93 ± 0.91	79.57 ± 0.48	131.30 ± 13.73 (VRF=1.85)
UF7	Feed C	PS-US100 (100 kDa)	5	13.9	75.9	1014.0 ± 40.25	15.51	14149 ± 431	27.13	160.89 ± 0.91	93.19 ± 0.53	103.64 ± 1.10 (VRF= 1.89)

[2] *Feed A: centrifuged OMWW / Feed B: pH of OMWW adjusted to 1.9 then centrifuged / Feed C: OMWW first centrifuged then pH was adjusted to 1.9*

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Table 3.5. Continued

Code	Feed type	Membrane type / Cut-off	TMP (bar)	Steady state flux (L m^{-2} h^{-1})	Membrane fouling (%)	TPC of permeate		COD of permeate		Antioxidant activity of the permeate		Hydroxytyrosol content in permeate (mg/L)
						mg/L	Rejection (%)	mg/L	Removal (%)	mg$_{Trolox}$/L	Inhibition (%)	
NF1	Feed B	PM-N0P10 (1000 Da)	10	4.3	51.8	452.46 ± 1.09	62.30	5898 ± 421	69.63	157.69 ± 0.60	84.25 ± 0.32	104.03 ± 1.88 (VRF=1.34)
NF2	Feed A	PM-N0P10 (1000 Da)	10	8.3	56.8	517.08 ± 41.34	56.91	5516 ± 39	71.59	172.86 ± 2.11	92.35 ± 1.13	25.99 ± 0.0 (VRF=2.5)
NF3	Feed C	PM-N0P10 (1000 Da)	10	6.9	45.2	358.10 ± 9.56	70.16	4630 ± 39	76.16	115.17 ± 0.30	61.53 ± 0.16	50.29 ± 3.20 (VRF=1.6)
NF4	MF1 permeate	PM-N0P10 (1000 Da)	10	6.7	52.3	532.85 ± 10.33	50.95	5433 ± 53	53.47	166.02 ± 4.53	88.70 ± 2.42	162.78 ± 8.18 (VRF=2.5)
NF5	UF1 permeate	NF270-PA (270 Da)	10	12.6	11.6	178.23 ± 2.72	78.84	2412 ± 410	75.05	138.03 ± 3.02	73.74 ± 1.61	145.41 ± 0.56 (VRF=2.63)
NF6	UF2 permeate	NF270-PA (270 Da)	10	15.2	63.4	102.46 ± 4.35	86.76	1741 ± 0	81.74	145.08 ± 2.72	77.51 ± 1.45	89.5 ± 3.92 (VRF=3.0)
NF7	UF7 permeate	NF90-PA (200~400 Da)	10	4.5	77.2	56.31 ± 5.44	94.45	828 ± 91	94.15	33.974 ± 0.91	19.68 ± 0.53	234.49 ± 26.08 (VRF=1.61)
NF8	MF2 permeate	NF270-PA (270 Da)	10	9.2	76.8	118.23 ± 3.81	88.61	2729 ± 397	77.25	165.168 ± 2.72	88.24 ± 1.45	116.73± 17.78 (VRF=2.5)

Regarding ultrafiltration, by analyzing experimental runs UF4, UF5 and UF6 from Table 3.5 and observing the variation of permeate flux with time in Figure 3.4.a, it can be concluded that there is no significant difference for maximum permeate flux. However, in the case of steady state flux, UF4 had advantage over feed UF5 and UF6. Also UF4 showed lower membrane fouling comparing to UF5 and UF6. This can be attributed to the fact that by acidification of OMWW, considerable agglomeration and flocculation of particles occurs which can cause the formation of a cake layer on the membrane surface. According to Figure 3.3.a,b and Table 3.5, UF4 and UF6 are more preferable due to higher polyphenols rejection, but UF6 has considerable advantage comparing to UF4 and UF5 regarding COD removal.

Nanofiltration results were slightly different than ultrafiltration ones. NF1, NF2 and NF3 experimental runs were performed with NP010 (1000-1200 Da) membrane. NF3 is preferable comparing to NF1 and NF2 for its higher polyphenols rejection, lower fouling index and higher COD removal. Although, by considering steady state flux from Figure 3.4.b, it can be seen that NF2 has advantage over NF3 and NF1. Regarding antioxidant activity (AA), the aim is to obtain a final permeate with the lowest AA, which indicates a minimum loss of phenolic compounds in permeate. Furthermore, NF3 and UF6 have the lowest AA among single membrane treatments with 115.17 ± 0.30 and 148.93 ± 0.91 mg$_{Trolox}$/L, respectively (Table 3.5).

As it can be seen from Figure 3.5, which shows the results of HPLC analysis on single membrane treatments, efficiency of pH adjustment for OMWW can be proved by increasing the concentration of HT up to 400% in the feed. This is in accordance with Mateo [31] who found out that adding acid to the vegetation water in an amount effective to produce a pH between 2-4, and incubating the acidified vegetation water for a period of at least two months until at least 75% of the oleuropein originally present in the vegetation water will be converted to HT. According to Table 3.5, it should be noted that experiments were performed with

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different VRF. Therefore, in order to being able to compare the efficiency of each process, all concentrations in Figure 3.5 were considered with VRF=2.5.

In order to analyze the results completely, all effective aspects including polyphenols rejection, COD removal, antioxidant activity, membrane fouling and permeate flux should be accounted. Nevertheless, considering that obtaining highest possible concentration of HT was the main goal of current study, observing obtained results from UF4-UF6 and NF1-NF3 retentates proves that UF6 and NF1 are the most suitable runs for HT extraction in UF and NF, respectively. Although UF6 and NF1 may have slight reduction in other parameters but their advantage on HT extraction can overcome the possible shortage in parameters like permeate flux. Thus, it can be concluded that for ultrafiltration operations Feed C (first centrifugation then acidification of OMWW) is the optimized mode of feed type, while for nanofiltration membranes the use of Feed B (first acidification then centrifugation of OMWW) is more suitable.
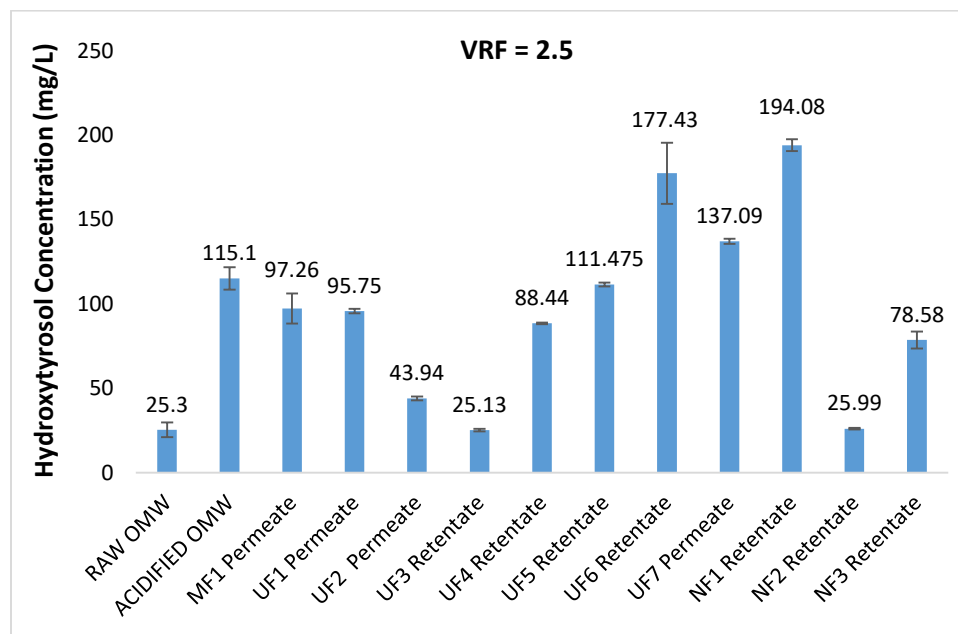
Figure 3.5. Hydroxytyrosol content (mg/L) in final retentate for single membrane treatment operations by considering VRF=2.5.

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3.3.2.2. Efficiency evaluation of membrane treatments

The main process parameters affecting the extent of membrane fouling are feed flow rate, temperature, mode of feed flow and transmembrane pressure. Although change in process parameters can barely affect the fouling behavior, modifying the hydrodynamic conditions, e.g., by increasing the cross-flow velocity, prior to compaction of cake, can hamper growth of the fouling layer on membranes.

Systematic analysis of the effect of TMP, feed flow rate, temperature and membrane molecular weight cut-off showed that all the parameters have significant effect on flux [33]. However, relative comparison of the different parameters indicates TMP had by far the highest impact, followed by temperature, membrane cut-off, and flow rate. Akdemir and Ozer [24] obtained constant flux at higher pressure while using 100 kDa polymeric membrane. The authors achieved an 18% flux enhancement while increasing the TMP from 1 to 3 bar at constant flow rate or increasing the feed flow rate from 100 to 200 L/h at constant TMP. To reduce the required membrane area, it is imperative to increasing the TMP until an acceptable level. However, high pressure may imply irreversible fouling formation and a subsequent fouling induced reduction in the COD and TOC removal rate of MF or UF membrane.

Coskun et al. [16] compared NF performance employing independent centrifugation and UF or their combination as pre-treatments. Centrifugations at 3750 rpm for 30 min or UF using Nadyr 10 kDa membrane at 2 bar resulted in comparable COD removal (30–36%), while their combination gave 56% COD removal efficiency at 70 L m^{-2} h^{-1} UF flux. The NF permeate flux obtained for feed pre-treated by UF was 30% higher than the permeate flux obtained for none pre-treated feed. The study thus established the superiority of using UF as a pre-treatment to increase flux during NF.

Fouling by OMWW is also highly dependent on type and characteristics of the membrane (composition, pore size and thickness). Cassano et al. [34] made a comparative study on the effect of fouling on two polymeric membranes (regenerated cellulose and polyethersulfone (PES) UF membranes). Results showed that regenerated cellulose membranes exhibited lower rejections toward

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phenolic compounds, higher permeate fluxes and lower fouling index compared to PES membranes.

Generally, in order to consider a single membrane treatment functional and effective, it should have highest possible polyphenols rejection, COD removal, steady state flux and preferably as low as possible membrane fouling. Therefore, in current research, the highest polyphenol rejection was obtained from UF4, NF3 and UF6 with 70.79%, 70.16% and 69.57%, respectively. Furthermore, highest COD removal belonged to UF6 and NF3 with 80.06% and 76.16%, respectively (Table 3.5).

As it is already described, HT content in UF6 and NF1 retentates is considerably higher than the other single membrane treatments. In order to compare UF6 and NF1 runs, it should be mentioned that although NF1 has almost 10% more HT content, but by considering further energy consuming to reach higher pressure, lower COD removal and steady state flux of NF1, it can be concluded that choosing UF6 as the selected single membrane treatment is more reasonable. Thus, by using Feed C (centrifuged and acidified OMWW) in stirred cell module equipped with ST-PES Synder membrane with 10 kDa molecular weight cut-off and TMP of 8 bar, it is possible to obtain 177.43 ± 18.55 mg/L of HT in retentate along with 80.06% COD removal from permeate.

Furthermore, several runs of integrated membrane treatments were performed due to necessity of performing nanofiltration as the secondary membrane process in order to reach a satisfactory level of treatment standards and also in order to compare the results with single membrane operation. For this purpose, obtained permeates from MF1, MF2, UF1, UF2 and UF7 runs were used as feed in NF4, NF8, NF5, NF6 and NF7, respectively. By considering overall aspect, UF7 proved to have more satisfactory results in the obtained permeate.

As it can be observed from Table 3.5 and Figure 3.6, low steady state flux and high membrane fouling of NF7 should be considered which can be referred to formation of a cake layer on membrane surface due to larger particle mean diameter. However, NF7 has the highest HT content (Figure 3.7) and also noticeably higher

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COD removal and polyphenols rejection, which makes NF7 as the preferred integrated membrane treatment. Therefore, by entering Feed C (centrifuged and acidified OMWW) in PS membrane with 100kDa molecular weight cut-off and then using the obtained permeate in NF90 membrane as feed, it will result into obtaining the final HT concentration of 364.11 ± 40.50 mg/L in retentate and COD removal from raw OMWW up to 95.7%.

A picture of raw OMWW, pretreated, permeate and retentate samples of the obtained fractionation of OMWW with the selected membrane treatments is shown in Figure 3.8. NF7 permeates are colorless and free of phenolic compounds suggesting a potential use for irrigation or disposal in aquatic streams.
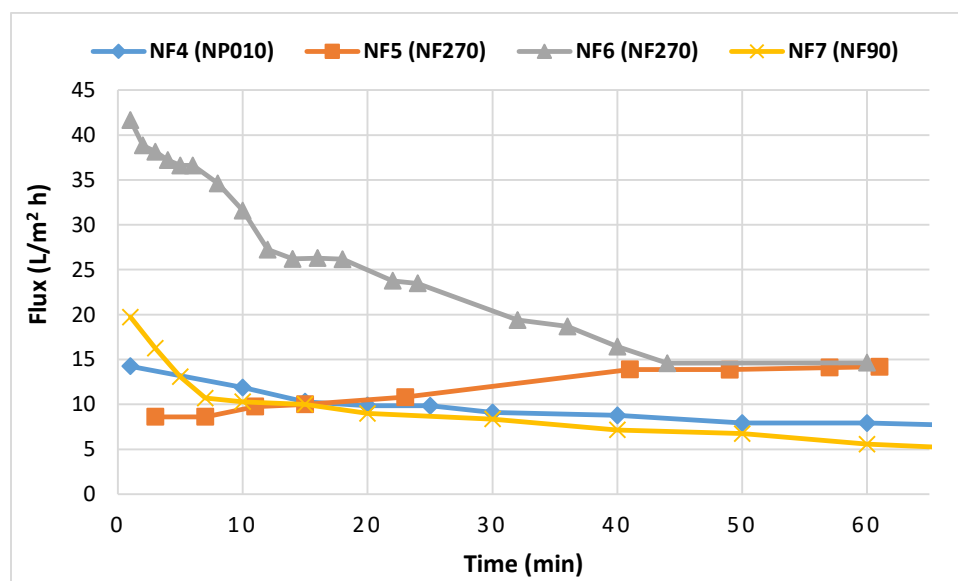
Figure 3.6. Permeate flux variation with time for integrated membrane treatments of OMWW.

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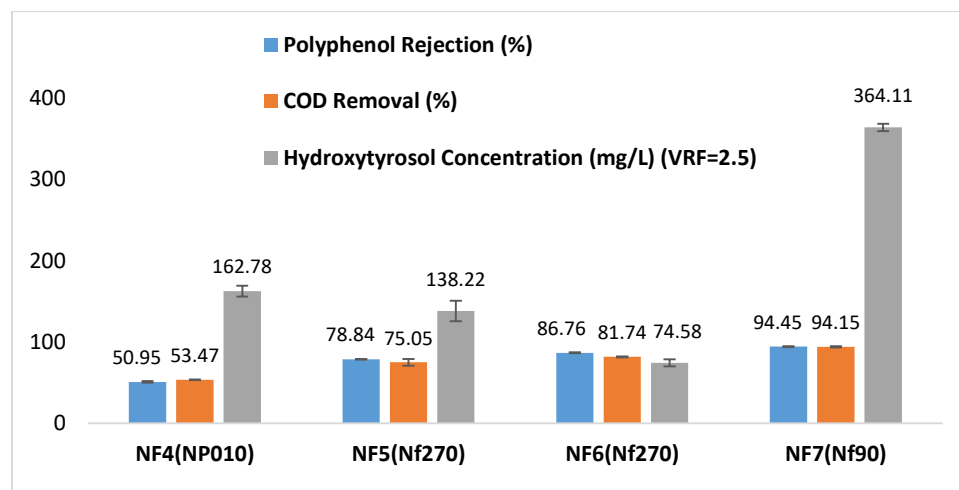
Figure 3.7. Efficiency evaluation of integrated membrane treatments of OMWW by considering all effective parameters.

(a)

(b)
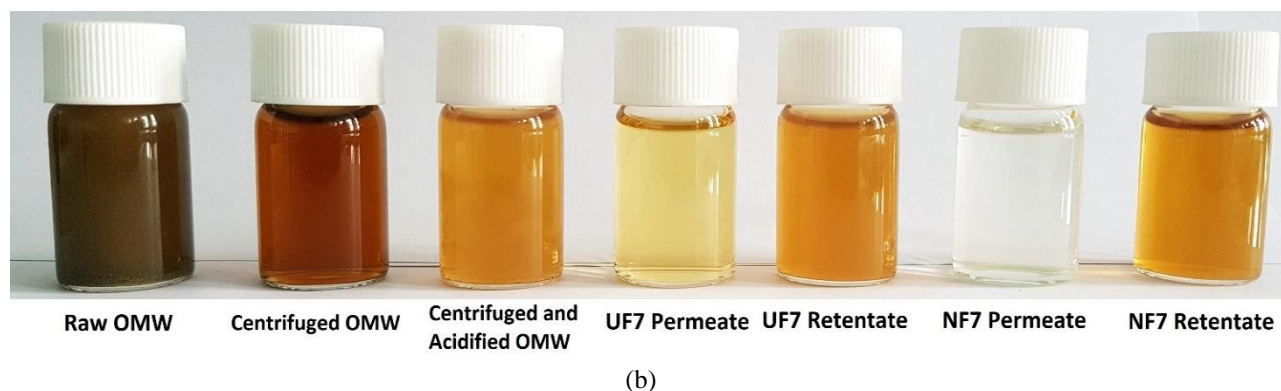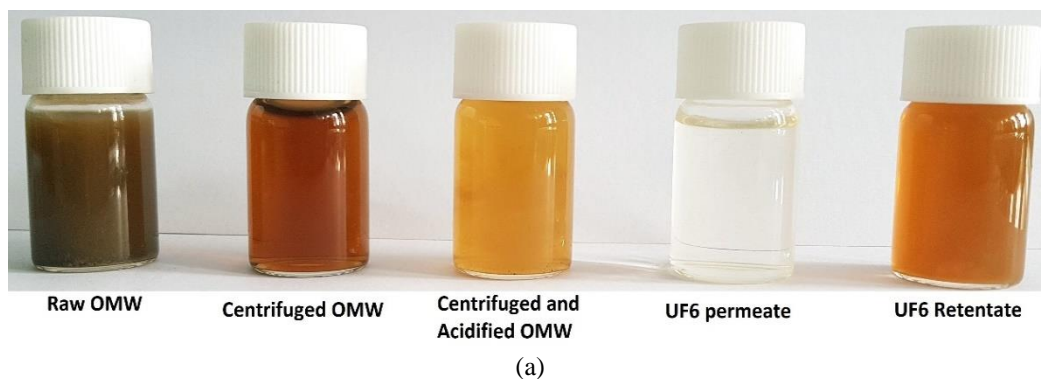
Figure 3.8. Photographs of permeate and retentate samples from selected treatments. (a) Single membrane treatments; (b) Integrated membrane treatments.

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3.4. Conclusions

The primary objective of the research performed in this chapter was to study the effect of OMWW pretreatment (acidification and centrifugation) on performance and probable improvement of further membrane treatment. The second aim was to obtain a final retentate stream enriched with biophenols, especially hydroxytyrosol, by using pretreated OMWW as feed in membrane operations. Results indicated that in the case of raw OMWW, centrifugation decreased total phenolic content (TPC) for 10-15%. Regarding acidified OMWW (adjusted to pH=1.1 and pH=2.9), a slight increase in TPC was observed. Zeta potential approached near zero as the pH of the OMWW decreased: whereas zeta potential is almost zero for acidified OMWW with pH=1.5, it is -4.35 mV for OMWW with pH=3 and -26.4 mV for raw OMWW (pH=4.9). Centrifugation of the raw OMWW decreases D(0.9) for about 95%, while centrifugation of acidified OMWW does not have any considerable effect on D(0.9) although it increases D[4,3] for an average of 68%. According to the obtained results from HPLC analysis (it is not shown, Table 3 is missing), 400% increase in hydroxytyrosol content was occurred because of OMWW acidification. Therefore, it can be concluded that acidification of OMWW has a huge effect on conversion of other polyphenols into Hydroxytyrosol.

Regarding membrane treatment of OMWW, results proved that by considering VRF=2.5 and using the pretreated OMWW as feed in single ultrafiltration process (ST-PES membrane, 10 kDa), 177.43 ± 18.55 mg/L of hydroxytyrosol in retentate along with 80.06% COD removal from permeate can be obtained. Best results were achieved by integrated membrane treatment using pretreated OMWW as feed in ultrafiltration (PS membrane, 100 kDa) stage and then using the obtained UF permeate as feed in nanofiltration (NF90 membrane, 200 Da) stage, with a final hydroxytyrosol content of 364.11 ± 40.50 mg/L in the NF retentate and COD removal up to 95.7%. Furthermore, the final NF permeate obtained with this integrated membrane process using the NF90 membrane is colorless and free of

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phenolic compounds, suggesting its potential use for irrigation or disposal in aquatic streams.

References

[1]   S. Dermeche, M. Nadour, C. Larroche, F. Moulti-Mati, P. Michaud, "Olive mill wastes: Biochemical characterizations and valorization strategies", *Process Biochem.*, 48, 10, 1532–1552, 2013.

[2]   A. Y. Gebreyohannes, R. Mazzei, L. Giorno, "Trends and current practices of olive mill wastewater treatment: Application of integrated membrane process and its future perspective", *Sep. Purif. Technol.*, 162, 45–60, 2016.

[3]   H. Dhaouadi, B. Marrot, "Olive mill wastewater treatment in a membrane bioreactor: Process feasibility and performances", *Chem. Eng. J.*, 145, 2, 225–231, 2008.

[4]   M. Stoller, M. Bravi, "Critical flux analyses on differently pretreated olive vegetation waste water streams: Some case studies", *Desalination*, 250, 2, 578–582, 2010.

[5]   M. Servili, S. Esposto, G. Veneziani, S. Urbani, A. Taticchi, I. Di Maio, R. Selvaggini, B. Sordini, G. Francesco Montedoro, "Improvement of bioactive phenol content in virgin olive oil with an olive-vegetation water concentrate produced by membrane treatment", *Food Chem.*, 124, 4, 1308–1315, 2011.

[6]   A. El-abbassi, M. Khayet, A. Hafidi, "Micellar enhanced ultrafiltration process for the treatment of olive mill wastewater", *Water Res.*, 45, 15, 4522–4530, 2011.

[7]   H. Zhang, R. Tsao, "Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects", *Curr. Opin. Food Sci.*, 8, 33–42, 2016.

[8]   M. Servili, M. Baldioli, R. Selvaggini, E. Miniati, A. Macchioni, G. Montedoro, "High-performance liquid chromatography evaluation of phenols in olive fruit, virgin olive oil, vegetation waters, and pomace and 1D- and 2D-nuclear magnetic resonance characterization", *J. Am. Oil Chem. Soc.*, 76, 7, 873–882, 1999.

[9]   M. Servili, M. Baldioli, R. Selvaggini, A. Macchioni, G. Montedoro, "Phenolic Compounds of Olive Fruit: One- and Two-Dimensional Nuclear Magnetic Resonance Characterization of Nüzhenide and Its Distribution in the Constitutive Parts of Fruit", *J. Agric. Food Chem.*, 47, 1, 12–18, 1999.

[10] E. Conde, B. Díaz Reinoso, M. J. González-Muñoz, A. Moure, H. Domínguez, and J. C. Parajó, "Recovery and concentration of antioxidants from industrial effluents and from processing streams of underutilized vegetal biomass", *Food Public Heal.*, 3, 69–91, 2013.

[11] H. Strathmann, L. Giorno, E. Drioli, "An Introduction to Membrane Science and Technology", *John Wiley & Sons*, 2011.

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Chapter 3: Study and efficiency evaluation of OMWW pretreatment and membrane based treatment to obtain a final retentate rich in Hydroxytyrosol

[12] R. Singh, "Chapter 3 - Hybrid membrane systems – applications and case studies", *Elsevier Science*, 2005, pp. 131–196.

[13] E. Drioli, M. Romano, "Progress and New Perspectives on Integrated Membrane Operations for Sustainable Industrial Growth", *Ind. Eng. Chem. Res.*, 40, 5, 1277–1300, 2001.

[14] S. Khoufi, F. Aloui, S. Sayadi, "Pilot scale hybrid process for olive mill wastewater treatment and reuse", *Chem. Eng. Process. Process Intensif.*, 48, 2, 643–650, 2009.

[15] R. W. Baker, "Membrane technology and applications", *John Wiley & Sons*, 2012.

[16] T. Coskun, E. Debik, N. M. Demir, "Treatment of olive mill wastewaters by nano fi ltration and reverse osmosis membranes", *DES*, 259, 1–3, 65–70, 2010.

[17] A. El-Abbassi, A. Hafidi, M. Khayet, M. C. García-Payo, "Integrated direct contact membrane distillation for olive mill wastewater treatment", *Desalination*, 323, 31–38, 2013.

[18] M. Stoller, "A three year long experience of effective fouling inhibition by threshold flux based optimization methods on a nf membrane module for olive mill wastewater treatment", *Chem. Eng. Trans.*, 32, 3, 37–42, 2013.

[19] F. Bazzarelli, T. Poerio, R. Mazzei, N. D'Agostino, L. Giorno, "Study of OMWWs suspended solids destabilization to improve membrane processes performance", *Sep. Purif. Technol.*, 149, 183–189, 2015.

[20] K. L. Tuck, P. J. Hayball, "Major phenolic compounds in olive oil: metabolism and health effects", *J. Nutr. Biochem.,*13, 636–644, 2002.

[21] F. Visioli, G. Bellomo, G. Montedoroc, C. Galli, "Low density lipoprotein oxidation is inhibited in vitro by olive oil constituents", *Atherosclerosis*, vol. 117, pp. 25–32, 1995.

[22] K. L. Tuck, H.-W. Tan, P. J. Hayball, "Synthesis of Tritium-Labeled Hydroxytyrosol, a Phenolic Compound Found in Olive Oil", *J. Agric. Food Chem.*, 48, 9, 4087–4090, 2000.

[23] C. Russo, "A new membrane process for the selective fractionation and total recovery of polyphenols, water and organic substances from vegetation waters (VW)", *J. Memb. Sci.,* 288, 239–246, 2007.

[24] E. O. Akdemir, A. Ozer, "Investigation of two ultrafiltration membranes for treatment of olive oil mill wastewater", *Desalination*, 249, 2, 660–666, 2009.

[25] J. M. Ochando-Pulido, G. Hodaifa, M. D. Victor-Ortega, A. Martínez-Ferez, "Fouling control by threshold flux measurements in the treatment of different olive mill wastewater streams by membranes-in-series process", *Desalination*, 343, 162–168, 2014.

[26] A. El-Abbassi, H. Kiai, J. Raiti, A. Hafidi, "Application of ultrafiltration for olive processing wastewaters treatment", *J. Clean. Prod.*, 65, 432–438, 2014.

[27] M. Separation, "Chapter 1, מבוי," *Tractates Sabbat 'Eruvin*, 13–47, 2012.

PhD Thesis

Chapter 3: Study and efficiency evaluation of OMWW pretreatment and membrane based treatment to obtain a final retentate rich in Hydroxytyrosol

[28]   M. H. V. Mulder, "Basic principles of membrane technology", *Kluwer Academic Press*, 1991.

[29]   A. Zirehpour, M. Jahanshahi, A. Rahimpour, "Unique membrane process integration for olive oil mill wastewater purification", *Sep. Purif. Technol.*, 96, 124–131, 2012.

[30]   M. Mitrakas, G. Papageorgiou, A. Docoslis, G. Sakellaropoulos, "Evaluation of various pretreatment methods for olive oil mill wastewaters", *Eur. Water Pollut. Control*, 6, 6, 10–16, 1996.

[31]   S. Mateo, "Metodo para obtener hidroxitirosol rico a partir agua de vegetación," vol. 1, no. 12, pp. 2040–2048, 2002.

[32]   Y. Yuan, Y. Gao, J. Zhao, L. Mao, "Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions", *Food Res. Int.*, 41, 1, 61–68, 2008.

[33]   E. Tsagaraki, H. Lazarides, "Fouling analysis and performance of tubular ultrafiltration on pretreated olive mill waste water", *Food Bioprocess Technol.*, 1–9, 2010.

[34]   A. Cassano, C. Conidi, E. Drioli, "Comparison of the performance of UF membranes in olive mill wastewaters treatment", *Water Res.*, 45, 10, 3197–3204, 2011.

Chapter 4: Formulation and fabrication of single and double emulsions loaded with phenolic inner aqueous phase by application of high energy emulsification methods

VALORIZATION OF RESIDUES GENERATED IN THE PRODUCTION OF OLIVE OIL THROUGH CLEAN TECHNOLOGIES

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En este capítulo se estudia la encapsulación de los extractos fenólicos obtenidos bajo las condiciones de operación óptimas en los capítulos 2 y 3. El objetivo de este trabajo fue obtener nanoemulsiones estables con el fin de mejorar la estabilidad y biodisponibilidad de los compuestos fenólicos encapsulados, principalmente del hidroxitirosol, tirosol y oleuropeína.

Esta investigación se desarrolla en dos partes. En primer lugar, se utilizó la metodología de superficie de respuesta (RSM) para determinar la composición y las condiciones de operación óptimas necesarias para preparar emulsiones dobles de agua en aceite en agua (W/O/W) con el fin de encapsular los extractos fenólicos obtenidos en el capítulo 3 a partir del alpechín (OMWW). Se utilizaron componentes de grado alimentario (aceite Miglyol 812 y tensioactivos Span 80 y Tween 80) para la formulación y se probaron tres métodos de emulsificación de alta energía: homogenización a alta velocidad, homogeneización ultrasónica y microfluidización. Los resultados óptimos se alcanzaron mediante microfluidización a 148 MPa durante siete ciclos, obteniendo una nanoemulsión con gotas de $105,3 \pm 3,2$ nm de diámetro medio y un índice de polidispersión (PDI) de $0,233 \pm 0,020$, sin cambios apreciables de tamaño de gota, potencial zeta o estabilidad durante 35 días de almacenamiento. Además, presentó una elevada retención de compuestos fenólicos (68,6%) y actividad antioxidante (89,5%) después de los 35 días de almacenamiento a temperatura ambiente.

En segundo lugar, se procedió a la optimización de la composición y de las condiciones de operación necesarias para la preparación de emulsionas simples de agua en aceite (W/O), con el fin de encapsular los extractos fenólicos obtenidos en el capítulo 2 a partir del orujo una vez extraído el aceite. Se utilizaron también componentes de grado alimentario (aceite Miglyol 812 y tensioactivos Tween 80 y polirricinoleato de poliglicerol (PGPR)) para la formulación y se probaron únicamente dos métodos de emulsificación de alta energía: homogenización a alta velocidad y homogeneización ultrasónica. En este caso se obtuvieron dos formulaciones óptimas: i) obtenida mediante homogenización ultrasónica, con un diámetro medio de gota de $104,9 \pm 6,68$ nm y un PDI de $0,156 \pm 0,085$; ii) obtenida mediante homogenización a alta velocidad, con un diámetro medio de gota de

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109,8 ± 17,81 nm y un PDI de 0,087 ± 0,069, ambas con una buena estabilidad física después de 30 días de almacenamiento. En ambos casos se obtuvieron elevadas retenciones de compuestos fenólicos después de los 30 días de almacenamiento a temperatura ambiente: 83,1% y 87,2%, respectivamente. Todas estas nanoemulsiones con formulación óptima, tanto simples como dobles, son adecuadas para su uso en la industria alimentaria.

Una parte de los resultados obtenidos en este trabajo se han publicado en la revista *Foods* 9 (2020) 1411. https://doi.org/10.3390/foods9101411

Además, la elevada retención de compuestos fenólicos (68,6%) y actividad antioxidante (89,5%) después de esos 35 días de almacenamiento a temperatura ambiente la hace adecuada para su aplicación en la industria alimentaria

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4.1. Introduction

Emulsion technology is generally applied for the encapsulation of bioactives in aqueous solutions, which can either be used directly in the liquid state or can be dried to form powders (e.g., by spray, roller, or freeze drying) after emulsification. Therefore, it is actually a part of encapsulation process. Basically, an emulsion consists of at least two immiscible liquids, usually as oil and water, with one of the liquids being dispersed as small spherical droplets in the other [1, 2]. Typically, the diameters of the droplets in food systems range from 0.1 to 100 mm [3]. Emulsions can be classified according to the spatial organization of the oil and water phases. A system that consists of oil droplets dispersed in an aqueous phase is called an oil-in-water (O/W) emulsion, whereas a system that consists of water droplets dispersed in an oil phase is called a water-in-oil (W/O) emulsion. Furthermore, various types of multiple emulsions can be developed, such as oil-in-water-in-oil (O/W/O) or water-in-oil-in-water (W/O/W) emulsions [4,5]. To obtain a kinetically stable solution, stabilizers such as emulsifiers or texture modifiers are commonly added in the emulsion systems [6].

The selection of an appropriate emulsifier (or combination of emulsifiers) is one of the most important factors to consider for the proper design of a nanoemulsion. An emulsifier is a surface-active molecule that is capable of adsorbing to droplet surfaces, facilitating droplet disruption, and protecting droplets against aggregation [7, 2]. In the food industry, the choice of surfactant is very important due to the fact that it must be able not only to create and stabilize the dispersed phase droplets, but also be biodegradable and nontoxic [8]. In high-energy approaches, the emulsifier facilitates droplet disruption within the homogenizer by lowering the interfacial tension, which favors the production of small droplets. In low-energy approaches, the emulsifier facilitates the spontaneous formation of small droplets due to its ability to produce very low interfacial tensions under certain environmental and solution conditions. The stability of a nanoemulsion to environmental stresses such as pH, ionic strength, heating, cooling, or long-term storage is often predominantly determined by the kind of emulsifier used [9].

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Attractive characteristics of nanoemulsions include small droplet size and kinetic stability [10]which is interesting for practical applications in agricultural, cosmetic, chemicals, food, and pharmaceutical industries. For example, W/O nanoemulsions serve as micro-reactors to control nanoparticles growth [11,12].

The aqueous phase used to prepare a nanoemulsion typically consists primarily of water, but it may also contain a variety of other polar components, including co-solvents (such as simple alcohols and polyols), carbohydrates, proteins, minerals, acids, and bases. The type and concentration of these components determines the physicochemical characteristics of the aqueous phase, which in turn will impact the formation, stability, and physicochemical properties of the emulsion produced [9].

Polyphenols are a structural class of mainly natural, but also synthetic or semisynthetic, organic chemicals, which are characterized by the presence of large multiples of phenol structural units; they widely exist in numerous natural plants and foods, e.g., herbs, fruits and vegetables [13]. Unfortunately, the concentrations of polyphenols that appear effective in vitro are often of an order of magnitude higher than the levels measured in vivo. The effectiveness of nutraceutical products in preventing diseases depends on preserving the bioavailability of the active ingredients [6].

Several studies on encapsulation and delivery of polyphenols by emulsion-based delivery systems have been reviewed [6, 14, 23–28, 15–22]. It is clear that the use of encapsulated polyphenols instead of free molecules improves both the stability and bioavailability of the molecules *in vitro* and *in vivo*. Many emulsion-based encapsulation and delivery systems for polyphenols have been well established, including single, multiple, and nanoemulsions. However, variations in composition and preparation technologies result in the formation of a range of emulsions with novel properties, which may show even greater potentials in delivery of polyphenols. Studies on these emulsions will contribute to the establishment of high-performance delivery systems and extend the application of both polyphenols and emulsions, e.g., using unsaturated fatty acids as the oily

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phase of polyphenol-encapsulated emulsion, which can achieve the delivery of multiple nutrients (unsaturated fatty acids and polyphenols) at the same time. Furthermore, very few preliminary studies have evaluated the *in vivo* absorption, transportation and targeted release of polyphenol-incorporated emulsions, which are essential to their deeper and wider applications. Hence, systematic and intensive investigation of *in vivo* metabolic mechanism and physiological effects of encapsulated polyphenols or any other bioactive nutrients are urgently required [29].

Preparation of an emulsion system directly from two separate liquids is considered as primary homogenization, whereas the reduction of droplet size in existing emulsions is defined as secondary homogenization. The preparation of emulsions always involves the use of one or both of these homogenization processes [30]. Properties of emulsions prepared by homogenization are often largely affected by the homogenization conditions used, including temperature, pressure and cycles [31]. These properties mainly include droplet size, stability and viscosity. Hence, emulsions with desired properties can be achieved by controlling the homogenization conditions for encapsulation and targeted delivery of polyphenols.

Water-in-oil-in-water (W/O/W) double emulsions consist of small water droplets contained within larger oil droplets that are dispersed in an aqueous continuous phase. W/O/W emulsions can also be more clearly defined as $W_1/O/W_2$ emulsions, where W_1 is the inner water phase while W_2 is the outer water phase. In principle, it is possible to design properties of inner water phase and oil phase (e.g., droplet size and droplet size distribution), and interfaces between water and oil, such as surface charge, and environmental response behaviors. Compared with O/W emulsions, W/O/W emulsions are ideal protected encapsulation systems for hydrophilic polyphenols. These compounds can be trapped in the internal water phase, which is isolated from the outer water phase by the oil phase, preventing their diffusion across the water-oil interface into the outer water phase [5, 32]. Furthermore, release of polyphenols entrapped within the inner water phase will be prolonged and can be controlled [33] . Moreover, W/O/W emulsions can be also

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designed to encapsulate both lipophilic and hydrophilic bioactive polyphenols at the same time [34], which will achieve multiple targeted delivery of multiple bioactive compounds in one particular system. Another potential advantage of W/O/W emulsions is that they can be structured to have the same dispersed phase volume and droplet size as conventional O/W emulsions, but with lower fat content, which facilitates the development of functional food products with encapsulated polyphenols, with lower fat content but the same properties as the full-fat products [35]. However, W/O/W emulsions, like conventional O/W emulsions, are also highly susceptible to environmental stresses (thermal processing, freeze and dehydration), which can induce instability, such as conventional flocculation, coalescence, and Ostwald ripening, which potentially will influence the delivery of encapsulated polyphenols. Furthermore, the diffusion of encapsulated hydrophilic polyphenols or water molecules from the inner to the outer aqueous phase or expulsion of whole water droplets from oil droplets, induced by limited solubility of encapsulated compounds in oil phase, can also lead to the instability of W/O/W emulsions [32, 36].

Water-in-oil (W/O) nanoemulsions [37, 38] form a class of emulsions that have nano-sized water droplets dispersed in organic media through the action of surfactants. Due to nanoemulsions with small droplet sizes and narrow distributions, these emulsions may appear transparent or translucent like microemulsion [39]. Small droplet size makes them stable against sedimentation and creaming for a long time, hence increasing overall stability of the emulsions. However, nanoemulsions are thermodynamically unstable and the size of the droplets tends to increase with time [12, 40].

The stability of emulsions to gravitational separation, flocculation, and coalescence is greatly improved when their size is reduced, which may help extend the shelf-life of some emulsion-based products. Nevertheless, it is important to design the system so that Ostwald ripening does not occur since nanoemulsions are highly susceptible to degradation through this mechanism. This can be achieved by preparing them from a carrier oil that has very low water-solubility

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[41, 42], or by controlling the mechanical properties of the interfacial layer to restrict changes in droplet size [43]. In addition, the very small size of the droplets in nanoemulsions may promote the chemical degradation of encapsulated components [44]. Nanoemulsions have very large specific surface areas so that any chemical degradation reaction that occurs at the oil-water interface may be promoted, such as lipid oxidation. In addition, transparent nanoemulsions will allow UV and visible light to penetrate them easily, which may promote any light-sensitive chemical degradation reactions [9].

In this study, response surface methodology (RSM) was applied to detect the optimal composition and operating conditions for the preparation of W/O and W/O/W nanoemulsions loaded with phenolic containing inner aqueous phase which was previously obtained from olive mill solid and liquid residues. The emulsion formulation was first optimized and then operating conditions to obtain a double nanoemulsion using high energy emulsification methods were examined. In addition to droplet size analysis, physical and chemical stability of double emulsions over time for the optimal formulation were also evaluated.

4.2. Materials and methods

4.2.1. Samples and Materials

In this chapter, two types of olive mill residues were used. Primarily, Olive mill wastewater (OMWW) which was obtained by means of a three phase olive oil extraction and centrifugation system and was kindly provided by Mamalan Agro Industrial Company (Zanjan, Iran).

In the preparation of W/O emulsion, olive cake was used which is the solid residue obtained by means of a two-phase cold oil extraction process using olives from olive trees of the variety "Serrana de Espadán", endemic of the Sierra de Espadán natural park (Castellón, Spain), and was kindly provided by Cooperativa de Montán (Castellón, Spain). Furthermore, the following materials were used in the preparation of W/O/W and W/O emulsions:

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Miglyol 812 oil, a mixture of C6-C12 medium chain triglycerides (MCT), was purchased from Cremer Co. (Germany). Folin-Ciocalteu reagent and hydrochloric acid (37%) were purchased from VWR International Eurolab (Spain) and Acros Organics (Belgium), respectively. Sodium carbonate, methanol, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), sorbitan monooleate (Span 80), polyoxyethylene (20) sorbitan monooleate (Tween 80) and Polyglycerol polyricinoleate (PGPR) were purchased from Sigma Aldrich (Germany). Milli-Q water (Millipore, USA) was used in all samples.

4.2.2. Preparation of phenolic rich OMWW for using in W/O/W emulsion

OMWW was first centrifuged (Eppendorf 5804 centrifuge, Germany) for 30 min at 4000 rpm in order to separate the remaining solid particles, obtaining a liquid phase with a pH value of 4.85 (Crison GLP-21 pH-meter, Crison, Spain). Afterwards, based on the method described by Bazzarelli et al. [45], acidification with HCl (37%) was performed by 0.003 (v/w%) addition to reach pH =1.8. After 24 hours, the pretreated OMWW was used in a two-stage membrane treatment process including ultrafiltration (UF) followed by nanofiltration (NF).

Both UF and NF were performed in batch concentration mode using a stainless steel HP4750 high-pressure stirred cell of 300 mL capacity supplied by Sterlitech Corporation (Kent, WA, USA). For this purpose, an UF flat sheet polysulfone membrane (US100, 100 kDa, Microdyn-Nadir, Germany) was used and the permeate obtained from UF was entered to the same stirred cell module equipped with a NF flat sheet polyamide membrane (NF90, 200–400 Da, Dow Filmtec, USA). Transmembrane pressures of 5 bar and 10 bar for UF and NF processes, respectively, were supplied by a nitrogen cylinder. Membrane surface area was 14.6 cm^2 and both UF and NF treatments were performed at room temperature. The retentate solution obtained by NF treatment was used as the inner aqueous phase in the optimal formulation of W/O/W nanoemulsions.

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4.2.3. Preparation of phenolic loaded extract for using in W/O emulsion

Olive cake samples were dried at 100 ºC for 24 h in an oven (model 2600210, JP Selecta, Barcelona, Spain) to remove most of their moisture (about 60% w/w). They were then ground by using a coffee grinder to obtain an average particle diameter of about 1 mm. For applying defatting process, Soxhlet method was performed using a B-811 extraction system (BÜCHI Labortechnik AG, Flawil, Switzerland). Ultrasound-assisted extraction (UAE) of phenolic compounds from ground and defatted olive cake was performed in a high intensity ultrasonic homogenizer (Sonics VCX500, 500 W, 20 kHz, Newtown, CT, USA) at 30 ºC for 10 min effective time with ultrasound pulses every 5 s (5 s on and 5 s off) and 30% amplitude, using aqueous ethanolic solution (50 vol % ethanol) as solvent. After UAE, phenolic loaded extracts were centrifuged at 10000 rpm for 10 min (Eppendorf Centrifuge model 5804). The supernatants were filtered through 0.45 µm Millipore syringe filters and then stored at –20 °C for further use as aqueous phase in W/O emulsion.

4.2.4. Preparation process of water in oil in water emulsion (W/O/W)

W/O emulsions which is the inner composition of the W/O/W emulsion were prepared by mixing Miglyol 812 and Span80 in amounts described by response surface methodology (RSM). Then the aqueous dispersed phase (OMWW) was added drop wise and the mixture was stirred for 10 minutes at 500 rpm. The prepared W/O emulsions were immediately used as the dispersed phase in the preparation of W/O/W double emulsions. Subsequently, W/O/W emulsions were prepared by the drop wise addition of the dispersed phase (W/O emulsion) to a continuous phase formed by Milli-Q water and Tween 80 surfactant.

The primary goal for preparation of double emulsion was optimization of formulation. For this purpose, emulsification process was performed with 26 different compositions based on RSM experimental design (Table 4.1). The emulsification was performed by using a high intensity ultrasonic homogenizer,

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described below, for 6 min effective time, in 5-second pulses (5 s off and 5 s on) and 50% amplitude.

The next objective was emulsification of optimized formulation with different high energy methods in order to achieve the optimal operating conditions. A high speed blender (Miccra D9 equipped with a DS-5/K-1 rotor-stator, ART Prozess- & Labortechnik, Mülheim, Germany), a high intensity ultrasonic homogenizer (Sonics VCX 500, 500 W, 20 kHz, Newtown, CT, USA) with a titanium alloy microtip probe of 3 mm diameter, and a microfluidizer high shear fluid processor (LM20, Microfluidics, MA, USA) were the equipment used for the formation of W/O/W emulsions.

4.2.5. Preparation of water in oil nanoemulsion (W/O)

W/O emulsions were prepared by mixing Miglyol 812 with polyglycerol polyricinoleate (PGPR) and Polysorbate 80 (Tween 80) based on the formulation provided by response surface methodology. The dispersed phase was added dropwise to the continuous phase and the mixture was stirred for 5 min at 500 rpm. The emulsification was performed by using the aforementioned high intensity ultrasonic homogenizer for 10 min effective time, in 5 s pulses (5 s off and 5 s on) and 40% amplitude. Due to limited amount of olive extracts, the experiments generated by RSM were performed using ethanol-water (50 vol %) solution as the aqueous phase. Then, emulsification of optimized formulation was performed by using phenolic olive extract as the aqueous phase and two of the aforementioned high energy emulsification methods: high speed homogenization and high intensity ultrasonic homogenization.

4.2.6. Experimental design

All data were analyzed by a response surface methodology (RSM) using a central composite design (CCD) of type 2^3 + star with two replicates from the central point. Statgraphics Centurion 18 software (Statgraphics Technologies, Inc,

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Warrenton, VA, USA) was used in this study. The value of the factors and their effect on the response was determined by analysis of variance (ANOVA) and LSD (lesser significant difference) test. The model was adjusted by means of multiple linear regressions (MLR) and its validity was determined by ANOVA. The level of significance of each coefficient was evaluated through the values of the statistical parameters F and p (probability) [46], with a confidence level of 95%.

4.2.6.1. W/O/W emulsion experimental design

Regarding W/O/W emulsion runs, the effects of four variable factors, each with 3 levels, were studied on emulsion droplet size being the response (Y). The following factors were studied: aqueous phase content in W/O emulsions (X_1: 4-20% w/w), surfactant content in W/O emulsions (X_2: 4-20% w/w), W/O content in W/O/W emulsions (X_3: 10-40% w/w), and surfactant content in W/O/W emulsions (X_4: 4-10% w/w). The model generated 26 experimental runs (Table 5.1). The following second-degree polynomial Eq. (1) was used to express the predicted response (Y) as a function of the independent variables (X_1, X_2, X_3 and X_4):

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_{11}X_1^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{22}X_2^2 + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{33}X_3^2 + a_{34}X_3X_4 + a_{44}X_4^2 \qquad (1)$$

where Y represents the response variable (droplet size in this case), a_0 is a constant, and a_i, a_{ii} and a_{ij} are the linear, quadratic and interactive coefficients, respectively.

After optimization of the W/O/W formulation, three experimental designs based on RSM were applied in order to determine the optimal emulsification method. Three apparatus were tested: a rotor-stator mixer, an ultrasonic homogenizer and a microfluidizer processor. For this purpose, the CCD model generated 10 experimental runs with two replicates in the central point for each emulsification method. The factors selected were rotation speed (X_1: 11000-29000 rpm) and time (X_2: 5-15 minutes) for the rotor-stator mixer, sonication time (X_1: 5-15 min) and amplitude (X_2: 20-60%) for ultrasonic homogenizer (while temperature (30 °C)

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and pulses (5 s off and 5 s on) were kept constant), and pressure (X_1: 50-150 MPa) and number of cycles (X_2: 1-11) for microfluidizer processor (Table 5.3). The following polynomial equation was used to express predicted responses (Y) as a function of the independent variables (X_1, X_2) under study:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_{11}X_1^2 + a_{22}X_2^2 + a_{12}X_1X_2 \tag{2}$$

where Y represents the response (droplet size in this case), a_0 is a constant, and a_i, a_{ii} and a_{ij} are the linear, quadratic and interactive coefficients, respectively.

4.2.6.2. W/O emulsion experimental design

The effects of three variable factors, each with 3 levels, were studied on the droplet size of the nanoemulsions, this being the response variable (Y). The following factors were studied: aqueous phase concentration (X_1: 2-20% w/w), surfactant concentration (X_2: 2–20% w/w) and HLB number (X_3: 3–11). HLB number was fixed for each experiment, based on pre-calculation of necessary content of each material in the surfactant mixture to be prepared. The model generated 16 experimental runs with two replicates of the central point to optimize the formulation of nanoemulsion. A second-order polynomial equation, Eq. (3), was used to express the predicted response (Y) as a function of the independent variables under study (X_1, X_2 and X_3):

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 +$$
$$a_{13}X_1X_3 + a_{23}X_2X_3 \tag{3}$$

where Y represents the response variable (droplet size in this case), a_0 is a constant, and a_i, a_{ii}, a_{ij} are the linear, quadratic and interactive coefficients, respectively. The significance of each estimated regression coefficient was assessed through values of the statistic parameters, F and p (probability).

After optimizing the W/O formulation, two experimental designs based on RSM were apllied in order to determine the optimal emulsification method by using a

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rotor-stator mixer and a ultrasonic homogenizer. For this purpose, the CCD model generated 10 experimental runs with two replicates of the central point for each emulsification method.

Table 4.1. Experimental matrix of W/O/W emulsion formulations based on central composite design (CCD)

Run	Independent variables				Response variable			
	Aqueous phase content in W/O emulsion (X_1, w/w%)	Surfactant content in W/O emulsion (X_2, w/w%)	W/O content in W/O/W emulsion (X_3, w/w%)	Surfactant content in W/O/W emulsion (X_4, w/w%)	Droplet Size (Y, nm)		PDI	
					mean	ST dev.	mean	ST dev.
1	12	12	10	7	470.4	3.7	0.426	0.043
2	20	12	25	7	415.2	12.8	0.357	0.068
3	20	4	40	4	512.2	14.8	0.561	0.033
4	20	20	10	4	279.7	18.1	0.439	0.015
5	12	4	25	7	344.8	10.1	0.486	0.032
6	20	4	40	10	1767	119.5	0.592	0.071
7	4	20	10	4	388.7	6.3	0.458	0.020
8	20	4	10	10	470.3	4.7	0.437	0.024
9	20	20	40	4	478.3	19.1	0.607	0.076
10	12	12	25	4	316.2	13.0	0.571	0.027
11	4	4	10	10	363.5	11.4	0.329	0.054
12	12	20	25	7	371.8	7.4	0.454	0.009
13	12	12	40	7	768.9	17.3	0.62	0.009
14	20	20	40	10	1828	58.3	0.501	0.015
15	12	12	25	7	585.8	19.1	0.553	0.041
16	20	4	10	4	242.6	5.6	0.283	0.011
17	4	20	10	10	330	10.4	0.273	0.011
18	4	20	40	4	923.6	24.3	0.61	0.045
19	12	12	25	7	857.4	13.3	0.487	0.047
20	4	4	10	4	232.3	2.5	0.365	0.045
21	20	20	10	10	216.3	5.0	0.460	0.032
22	4	4	40	10	1336	246.6	0.907	0.162
23	12	12	25	10	795.7	11.5	0.514	0.017
24	4	20	40	10	362.5	8.4	0.574	0.018
25	4	12	25	7	476.5	4.2	0.467	0.006
26	4	4	40	4	415.5	37.9	0.872	0.190

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The factors selected were rotation speed (X_1: 11000–29000 rpm) and time (X_2: 5–15 min) for the rotor-stator mixer, and sonication time (X_1: 5–15 min) and amplitude (X_2: 20–60%) for ultrasonic homogenizer (while temperature (30 °C) and pulses (5 s off and 5 s on) were kept constant). The low degree polynomial equation (second-order one) used to express predicted responses (Y) as a function of the independent variables under study (X_1, X_2) was the same as Eq. (2).

Table 4.2. Analysis of variance of the regression coefficients of the quadratic model Eq. (1) for the droplet size of W/O/W emulsions

Source	Regression coefficients	F-Ratio	p-Value
a_0	1029.33	-	-
a_1	-46.5462	1.27	0.2841
a_2	75.8451	0.17	0.6882
a_3	-47.5277	19.37	0.0011
a_4	-171.888	9.00	0.0121
a_{11}	-0.407612	0.02	0.8878
a_{12}	0.149121	0.02	0.8973
a_{13}	0.861198	2.05	0.1805
a_{14}	6.08568	4.08	0.0683
a_{22}	-1.77558	0.40	0.5421
a_{23}	-0.179323	0.09	0.7714
a_{24}	-4.8638	2.61	0.1345
a_{33}	0.656502	0.67	0.4309
a_{34}	3.78764	5.56	0.0379
a_{44}	9.33476	0.22	0.6509

4.3. Results and discussion

4.3.1. Determination of W/O/W emulsion formulation

4.3.1.1. Model fitting of formulation

One response variable, the droplet size, PDI and 4 experimental factors which were used on the CCD for the optimization of formulation are shown in Table 4.1. The

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default model is quadratic with 15 coefficients, and it has been fit to the response variable. The R-squared statistic indicates that the model as fitted explains 80.57% of the variability in particle size. The ANOVA measurement partitions the variability in particle size into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, three of the coefficients (a_3, a_4, and a_{34}) of the quadratic polynomial model, Eq. (1), have p-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level (Table 4.2). F values indicate that, for the range of studied variables, W/O content in W/O/W composition (X_3) had stronger influence on the droplet size of the emulsions than the other independent variables. F values also indicate that the interaction with the highest incidence was the one occurring between the W/O concentration in W/O/W (X_3) and surfactant content in W/O/W emulsion (X_4).

4.3.1.2. Response surface analysis

In order to study the effect of the independent variables on the droplet size, surface responses of the quadratic polynomial model were generated by varying two of the independent variables within the experimental range while holding the other two constant at the central points. Figure 4.1.a was generated by varying the surfactant content in W/O (X_2) and surfactant content in W/O/W (X_4) and keeping constant the aqueous phase content in W/O (X_1) and W/O content in W/O/W (X_3) at their central values. It shows that increasing X_4 in the lower levels of X_2 causes an increase in emulsion particle size which is unfavorable, while for higher levels of X_2, no considerable variations in particle size were observed.

The effect of X_1 and X_4 on the particle size of the emulsion at a fixed content of X_2 and X_3 in their central values can be seen in Figure 4.1.b This figure shows that the increase in X_1 and X_4 hardly affects the particle size, except at the higher levels for both factors. The effect of X_1 and X_2 changing on particle size at the central values of X_3 and X_4 is depicted in Figure 4.1.c It shows that changes of X_1 and X_2 hardly affect the particle size. The effects of X_3 and X_4 variations on particle size

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at central values of X_1 and X_2 are shown in Figure 4.1.d A particle size increase is observed as X_3 increases, being this effect greater at higher levels of X_4. Figure 5.1(e) depicts the effect of X_2 and X_3 on particle size at central values of X_1 and X_4. Somehow, varying X_2 hardly modifies the particle size, while increasing X_3 has a significant effect on particle size growth. Figure 4.1.f shows the effect of X_1 and X_3 variation on particle size at central levels of X_2 and X_4. A significant growth in particle size with the increase in X_1 is only observed at the highest levels of X_3 factor. It can be concluded that these figures prove the significance of X_3 and X_4 over other factors on particle size, as well as the interaction between both factors represented in Fig. 4.1.d

4.3.1.3.Optimization of double emulsion formulation

Following Stoke's law, the stability of emulsion would increase as the droplet size decreases [47]. Furthermore, the emulsion with higher resistance and control to creaming should also be homogenously distributed in particle size [48].

Numerical optimization of W/O/W emulsion formulation loaded with phenolic rich OMWW was carried out through design expert software, using desirability function. The W/O/W optimal formulation is expected to be those leading to a stable emulsion with minimum droplet size. Optimum formulation with 98.8% desirability was predicted for 20% (w/w) of aqueous phase content (X_1), 4% (w/w) Span 80 surfactant content (X_2), 10% (w/w) of aqueous phase (W/O) content (X_3), and 4.6% (w/w) of Tween 80 surfactant content (X_4). Because some of these optimal values are in the lower range of those selected in the CCD matrix, data extrapolation was performed. It was carried out through expert design software to expand factor input levels and to evaluate the possibility of achieving a better response beyond the levels considered. Formulation optimization process for main response (minimum particle size) yields 100% desirability for 20.3% (w/w) of aqueous phase content (X_1), 3.7% (w/w) Span 80 surfactant content (X_2), 9.8% (w/w) of aqueous phase (W/O) content (X_3), and 4.1% (w/w) of Tween 80 surfactant content (X_4). The predicted optimal response is very close to that

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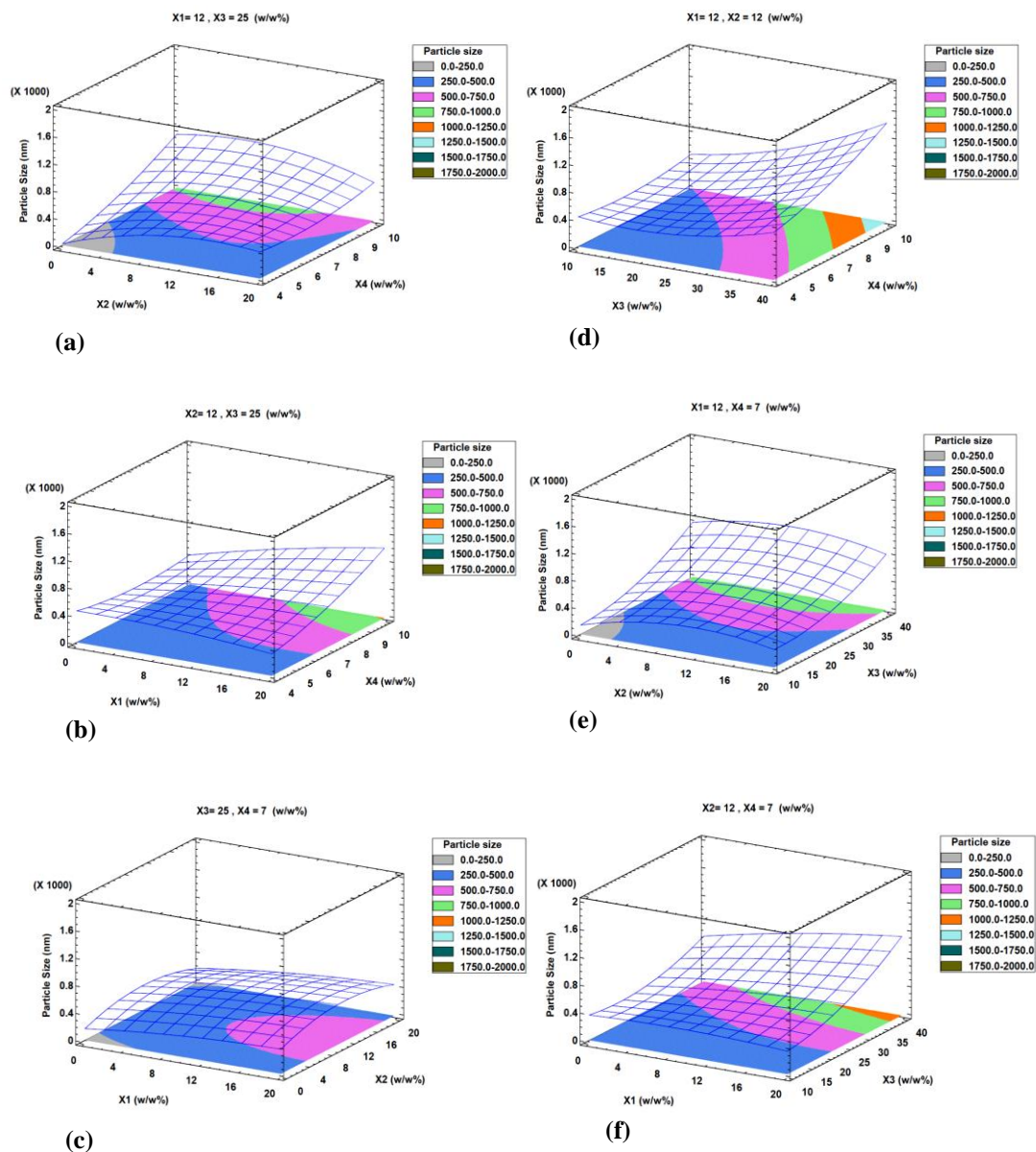
Figure 4.1. Response surface plots of interactions between two input factors, while holding the other two constants at their central points, on particle size of W/O/W emulsions. X_1: Aqueous phase content in W/O emulsion; X_2: Surfactant content in W/O emulsion; X_3: W/O content in W/O/W emulsion; X_4: Surfactant content in W/O/W emulsion.

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4.3.2. Effect of the high energy emulsification methods on W/O/W emulsion droplet size

High-energy approaches are one of the most versatile means of producing food-grade nanoemulsions because they can be used with a wide variety of different oil and emulsifier types. Under suitable homogenization conditions, nanoemulsions can be produced using triacylglycerol oils, flavor oils or essentials oils as oil phase, and proteins, polysaccharides, phospholipids or surfactants as emulsifiers [31, 49, 58–60, 50–57]. Even so, the size of the particles produced depends strongly on the characteristics of the oil and emulsifier used. Low-energy approaches are often more effective at producing small droplet sizes than high-energy approaches, but they are more limited in the types of oils and emulsifiers that can be used [9]. Small droplets are usually obtained when the ratio between dispersed phase viscosity (η_d) to that of the continuous phase (η_c) falls within a certain range ($0.05 < \eta_d/\eta_c < 5$) [61–63]. Finally, it is important to add enough emulsifier to cover all the new droplet surfaces formed during homogenization, and to use an emulsifier which rapidly adsorb at the droplet surfaces to prevent re-coalescence [64]. Usually, there is a linear relationship between the logarithm of the homogenization pressure (P) and the logarithm of the droplet diameter (d): $\log d \propto \log P$. The constant of proportionality depends on the homogenizer: $d \propto P^{-0.6}$ for large homogenizers and low-viscosity fluids, $d \propto P^{-0.75}$ for large homogenizers and high-viscosity fluids, and $d \propto P^{-1.0}$ for small (bench top) homogenizers [2]. To reduce the droplet size to the level required in nanoemulsions it is usually necessary to operate at extremely high pressures and to use multiple passes through the homogenizer. Even then, obtaining droplets of less than 100 nm radius is only possible under certain circumstances (e.g., high emulsifier levels, low interfacial tensions, and appropriate viscosity ratios) [9].

Microfluidizers have traditionally been used in the pharmaceutical industry to produce emulsion based products, but they have also been used in the food and beverage industries to produce flavor emulsions, nutraceutical emulsions, and homogenized milk. The microfluidizer works on the principle of dividing an

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emulsion flowing through one channel into two streams, each stream passing through a separate thin channel, and then directing the two streams to each other to an interaction chamber. Intense disruptive forces are generated within the interaction chamber when the two fast-moving streams of emulsion impinge upon each other, leading to highly efficient droplet disruption [9]. Ultrasonic homogenizers utilize high-intensity ultrasonic waves to create the intense disruptive forces necessary to fracture oil and water phases into very small droplets [65–67]. The energy input is provided using sonicator probes that contain piezoelectric quartz crystals that expand and contract in response to an alternating electrical voltage [68, 69]. Compared to other high-energy methods, ultrasound requires less energy expenditure. One serious downside of this technique is contamination induced by probe. For scale up applicability, commercial homogenizers based on sonication have been developed, in which nanoemulsion is made to flow through a special column capable of producing ultrasonic waves [70]. Rotor-stator mixers (also known as high-speed or high-shear mixers) generally consist of a perforated stator screen mounted outside a rotor. The rotor draws the fluid towards the rotor-stator head axially, accelerates it tangentially and then forces it radially through the stator slots, giving rise to efficient mixing and/or dispersion. RSMs are used for a large and increasing number of chemical engineering processes, especially for high viscosity and high disperse phase volume fraction dispersions, e.g. in pharmaceuticals, cosmetics and food processing [71–73].

After obtaining the optimal W/O/W double emulsion formulation, the next objective of this chapter was achieving the optimal operating conditions for its preparation. For this purpose, the following methods and factors were studied: ultrasonic homogenizer by varying time and amplitude, rotor-stator mixer by varying time and rotor speed, and microfluidizer processor by varying pressure and cycle numbers.

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Table 4.3. Experimental matrix based on central composite design (CCD) for W/O/W emulsion preparation by three high-energy emulsification methods

Run	Rotor-stator mixer					
	Independent variables		*Response variables*			
	Rotation speed (X$_1$, rpm)	Time (X$_2$, min)	Droplet size (Y, nm)		PDI	
			Mean	ST dev.	Mean	ST dev.
1	29000	2	735.6	38.1	0.903	0.028
2	20000	2	468.2	4.0	0.585	0.016
3	11000	2	1255	174.6	0.392	0.147
4	11000	6	580	21.9	0.487	0.032
5	11000	10	352.9	10.5	0.554	0.076
6	29000	6	530.4	10.2	0.484	0.029
7	29000	10	325.5	2.6	0.539	0.007
8	20000	10	301.7	12.1	0.439	0.030
9	20000	6	474.5	9.7	0.457	0.032
10	20000	6	366.4	9.5	0.551	0.019
Run	Ultrasonic homogenizer					
	Independent variables		*Response variables*			
	Time (X$_1$, min)	Amplitude (X$_2$, %)	Droplet Size (Y, nm)		PDI	
			Mean	ST dev.	Mean	ST dev.
11	10	20	338.5	16.4	0.468	0.021
12	6	60	434.6	16.9	0.436	0.010
13	6	20	388.1	2.4	0.468	0.013
14	2	20	622.7	26.6	0.565	0.101
15	6	40	460.4	3.9	0.432	0.014
16	10	40	394.1	3.1	0.368	0.042
17	10	60	375.2	1.4	0.350	0.043
18	2	60	484.5	3.5	0.429	0.009
19	6	40	667.5	6.8	0.410	0.013
20	2	40	482.3	7.9	0.429	0.004

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Table 4.3.	Continued					
	Microfluidizer processor					
	Independent variables		*Response variables*			
Run	Pressure (X₁, MPa)	Cycles (X₂, number)	Droplet size (Y, nm)		PDI	
			Mean	ST dev.	Mean	ST dev.
21	50	1	393.5	7.6	0.387	0.037
22	150	11	120.8	1.9	0.316	0.009
23	150	1	128.8	1.9	0.378	0.007
24	100	6	105.8	1.2	0.468	0.009
25	50	6	195.3	6.9	0.281	0.017
26	100	1	253.4	8.2	0.422	0.005
27	100	11	118.5	1.3	0.454	0.004
28	50	11	234.1	6.3	0.424	0.032
29	100	6	187.3	2.8	0.300	0.003
30	150	6	134.8	4.8	0.387	0.053

Table 4.4. Analysis of variance (ANOVA) of the regression coefficients of the quadratic Eq. (2) for W/O/W emulsion preparation by three high-energy emulsification methods

	Rotor-stator mixer			*Ultrasonic homogenizer*			*Microfluidizer processor*		
Source	*Regression coefficients*	*F-Ratio*	*p-Value*	*Regression coefficients*	*F-Ratio*	*p-Value*	*Regression coefficients*	*F-Ratio*	*p-Value*
a_0	2570.49	-	-	497.885	-	-	616.672	-	-
a_1	-0.139724	2.06	0.2242	-20.3173	3.44	0.1372	-4.52892	13.05	0.0225
a_2	-166.557	12.69	0.0235	7.39658	0.04	0.8426	-48.2018	6.20	0.0675
a_{11}	2.7045E-6	3.90	0.1195	-1.80134	0.17	0.6993	0.0107943	0.69	0.4523
a_{12}	0.00341667	2.11	0.2203	0.546562	0.68	0.4558	0.1514	2.33	0.2014
a_{22}	3.05089	0.19	0.6827	-0.139179	0.64	0.4675	1.91543	2.18	0.2140

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4.3.2.1. Model fitting of emulsification method

Three experimental designs based on RSM were prepared by the application of rotor-stator mixer, ultrasonic homogenizer and microfluidizer processor. For this purpose, the CCD model generated 10 experimental runs with two replicates in central point for each emulsification apparatus. The average droplet size and PDI of the experiments corresponding to the CCD design is given in Table 4.3. The default model is quadratic and statistical models have been fit to the response variables. The R-squared statistic indicates that the fitted model explains 84.23%, 56.13% and 86.18% of the variability in particle size for rotor-stator mixer, ultrasonic homogenizer and microfluidizer processor, respectively. Regarding the rotor-stator mixer, the F values indicate that the time factor (X_2) has a stronger influence on the droplet size of the emulsions than the rotational speed factor (X_1). In the ultrasonic homogenizer, the F values indicate that the time factor (X_1) has a slightly stronger influence on the droplet size of double emulsions than the amplitude (X_2), whereas for microfluidizer processor pressure factor (X_1) has considerable stronger influence on the droplet size of the emulsions than the number of cycles (X_2), as shown in Table 4.4.

ANOVA showed the significance of the coefficients of the quadratic polynomial models (Eq. (2)). Regarding the high speed mixer and microfluidizer, only one coefficient in each model has a p-value less than 0.05, indicating that it is significantly different to zero with 95.0% confidence level. Regarding the ultrasonic homogenizer model, no coefficient has p values less than 0.05 (Table 4.4).
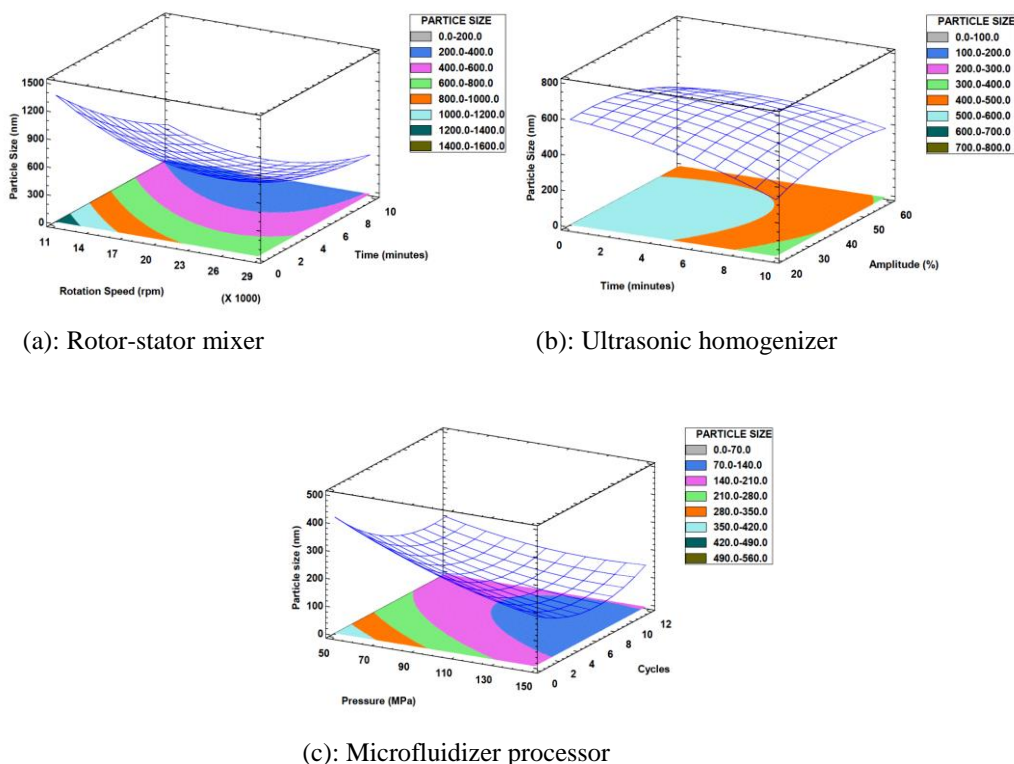
4.3.2.2. Response surface analysis

In order to study the effect of the emulsification methods and operating conditions on the droplet size, surface response plots of the quadratic polynomial model were generated. Figure 4.2.a shows the effects of time and rotation speed in rotor-stator mixer on the emulsion droplet size. This figure shows that regardless of time level,

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20000-23000 rpm is the optimal range for rotation speed. However, increasing time causes a continuous decrease in particle size which is favorable.

The droplet size of nanoemulsions depends on cavitation, turbulence and shear forces, created by ultrasonic homogenizer. Smaller droplets of nanoemulsions were formed with the increase in homogenization time [74]. Moreover, temperature of the nanoemulsions also increases due to increase in homogenization time which leads to decrease in viscosity and interfacial tension. Lower interfacial tension value encourages the interfacial instability. Additionally, due to higher temperature, vapor pressure increases in cavitation region which increase the cavitation intensity and accelerates the breakdown of droplets [54, 75]. Figure 4.2.b shows the interaction of time and amplitude on the achieved particle size in ultrasonic homogenizer; as it can be observed, amplitude hardly affects the particle size except for lower time levels. Furthermore, increasing time is significant on reducing particle size. Somehow, the minimum particle size would be achieved for high time and lower amplitude levels.

In emulsions prepared by microfluidization the mean droplet diameter decreased with increasing homogenization pressure. It is in accordance with the study performed by Bai et al. (2016) [55], in which the mean droplet diameter decreased from around 213 to 150 nm as the homogenization pressure increased from 4 to 14 kbar. The decrease in droplet size with increasing pressure can be attributed to the increase in the magnitude of the disruptive forces generated within the homogenization chamber. Therefore double emulsions containing small droplets with a narrow particle size distribution can be produced by using microfluidizer processor. Figure 4.2.c shows the interaction between pressure and cycles and their effect on particle size in microfluidizer emulsification tests. It can be observed that increasing pressure is significantly effective in decreasing particle size at middle cycle levels. Somehow, the minimum particle size can be achieved at 140-150 MPa pressure levels and 6-7 cycles.

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(a): Rotor-stator mixer

(b): Ultrasonic homogenizer

(c): Microfluidizer processor

Figure 4.2. Response surface plots of interaction between emulsification input factors on particle size

4.3.2.3. Optimization of W/O/W emulsion preparation conditions

The optimal conditions for the emulsification of the phenolic rich OMWW aqueous phase used in this work would be those leading to a stable double emulsion with minimum droplet size. Numerical optimization was performed through design expert software, using desirability function method. Regarding the W/O/W emulsification by rotor-stator mixer, the combined optimum ingredient levels for average particle size with 100% desirability were predicted to be achieved by emulsification at 20000 rpm and 10 min. The predicted response at optimal value was 169.4 nm for particle size and polydispersity index (PDI) of 0.546, while observed results were 301.7 ± 12.1 nm and 0.439 ± 0.030 for particle size and PDI, respectively (Table 4.3). This large difference between predicted and

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observed results is due to imprecision in the average size for heterogeneous populations, as revealed the high PDI values

In the case of optimal double emulsion prepared by ultrasonic homogenizer, response of 316.15 nm for particle size and PDI of 0.453 with 100% desirability was predicted while emulsification for 10 minutes at 20% amplitude was the combined optimum factor levels. The observed results (Table 4.3.), were 331.5 ± 16.5 nm and 0.468 ± 0.021 for particle size and PDI, respectively. The narrow difference between the predicted and observed responses can verify the reliability of the ultrasonic assisted experimental design.

Best results were obtained with microfluidizer processor (Table 4.3.). Numerical optimization performed by the design expert software predicted the optimal factor levels as 148 MPa of pressure and 7 cycles. The predicted response was 96.03 nm and 0.330 for particle size and PDI, respectively, whereas the observed responses for the mentioned factor levels were 105.3 ± 3.2 nm and 0.233 ± 0.020 for particle size and PDI, respectively. The narrow difference between the predicted and observed responses can verify the reliability of the microfluidizer experimental design. Therefore, it can be concluded from the observed responses of optimal points from three emulsification methods that microfluidizer processor can be considered as the optimal method to achieve double emulsions with lower particle size and PDI.

4.3.3. Characterization of optimal W/O/W emulsion

### 4.3.3.1. Stability evaluations during storage

The stability of the optimized double emulsions during their storage was evaluated by two methods. The first one was the comparative evaluation of droplet size (at room temperature and 4 °C) after 1 day and 35 days from emulsification in order to study the possible droplet growth. The second method was the backscattering (BS) evolution throughout time using Turbiscan Lab Expert apparatus to detect possible creaming, sedimentation, coalescence, flocculation or Ostwald ripening

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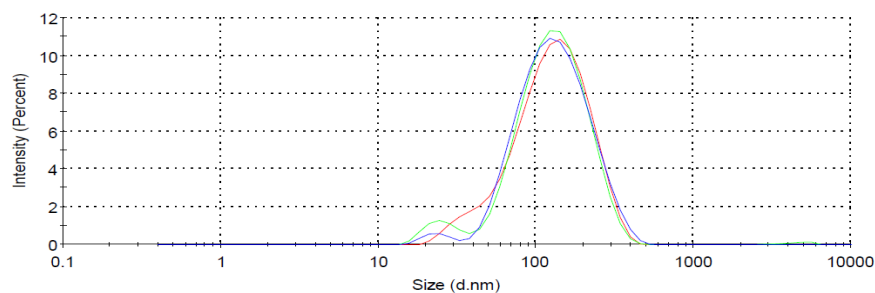
effect. Sedimentation, creaming and flocculation phenomena are unexpected, while those of coalescence and Ostwald ripening are more likely in the behavior of nanoemulsions [52].
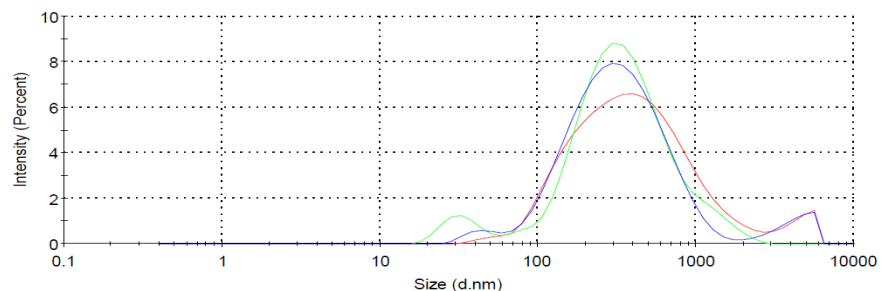
(a): Rotor-stator mixer
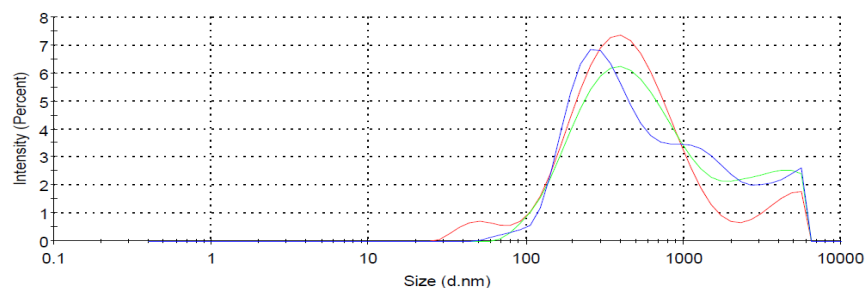
(b): Ultrasonic homogenizer

(c): Microfluidizer processor

Figure 4.3. Particle size distribution of W/O/W emulsions (optimal formulations) prepared by three different devices after 1 day (red line), after 35 days of storage at room temperature (green line) and 35 days of storage at 4 °C (blue line)

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Figure 4.3. show the rate of droplet size changes between 1 day and 35 days of storage at 4 °C and room temperature. As it can be seen from Figure 4.3.a, there is no considerable change in droplet size distribution of rotor-stator optimal W/O/W emulsion during the storage period while the intensity increased regardless of storage temperature condition. According to Figure 4.3.b, which shows the droplet size distribution of emulsion prepared in ultrasonic homogenizer, average droplet size did not vary considerably but PDI was increased during the storage period. It is already mentioned the advantage of optimal W/O/W prepared by microfluidizer processor over other devices due to significantly better average droplet size. Furthermore, it showed the best droplet growth stability (Figure 4.3.c). Neither average droplet size nor PDI was changed after 35 days of storage period at both 4 °C and room temperature. Zeta potential (ζ) was also measured for this sample and the results indicated the stable value of –2.7 mV after 1 day and 35 days of emulsification.

The long-term stability of emulsions is one of the most important factors determining their shelf life in commercial food and beverage applications [76, 77]. There is a direct relationship between the increased emulsion droplet diameter, creaming index and apparent viscosity of emulsion. Creaming is the separation process of bigger size lipid droplets in emulsion. Due to weak steric repulsions, smaller droplets come closer to form bigger size lipid droplets and due to density differences, the bigger lipid droplets floats on the surface of liquid to form a lipid rich cream layer [26, 76]. The coalescence process mainly takes place after double emulsion production and is thus stronger influenced by geometrical parameters, like inner and outer droplet sizes and dispersed phase concentrations, than by process parameters [53].

Backscattering (BS) profiles monitored during 35 days at 25 °C are depicted in Figure 4.4. As it is shown in Figures 4.4.a and 4.4.b, creaming instability occurs in emulsification by rotor-stator mixer and ultrasonic homogenizer. Similar results were obtained by Einhorn-Stoll et al. (2002) [78], who observed a rapid destabilization of emulsions prepared by a single step with the Ultra-Turrax. In

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contrast, the optimal W/O/W emulsion prepared by microfluidizer processor, showed no considerable destabilization during the storage period and the sample was also visually stable, maintaining the same bluish color and semitransparent appearance after 35 days (Figure 4.4.c).

The rotor-stator mixer sample happened to exhibit visual creaming instability after a few hours of storage. In contrast, double emulsion obtained by using ultrasonic homogenizer showed a slightly larger droplet size but visual instability was not observed after the same storage period. However, both chemical analysis and physical appearance indicated slight levels of creaming after few days of storage (Figure 4.4.b). The shifting of the curves in Figures 4.4.b and 4.4.c could be attributed to the presence of air bubbles in cells or being a little shaken during each analysis.

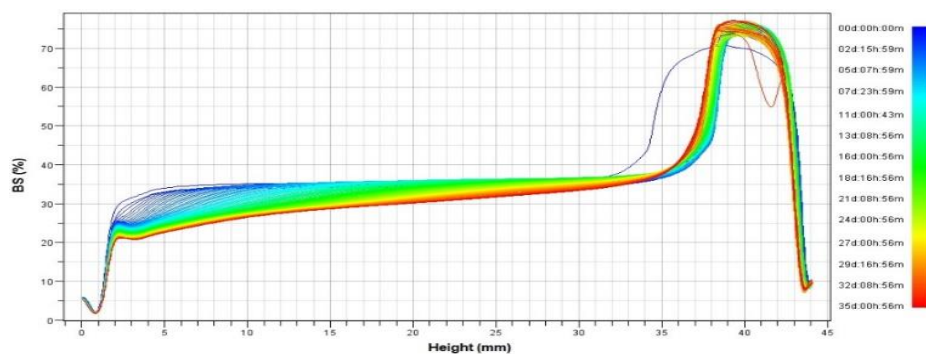### 4.3.3.2. Retention properties of nanoemulsions

As it was aforementioned the OMWW used in this study was acidified to pH = 1.8 prior to membrane treatment in order to increase the content of selective bioactive compounds. Previous studies have determined the impact of pH on physical stability of emulsions. Recently, the effect of pH on curcumin emulsions was investigated by Kharat et al. (2017) [79]. The authors found that more than 85% of curcumin was present after one month of storage at 37 °C when acidic conditions were employed. However, emulsions at pH 7.0, 7.4 and 8.0 contained only 62, 60, and 53% of the initial curcumin, respectively, thus demonstrating low stability. The effect of pH on the stability of emulsions stabilized by pectin-zein complexes has also been studied by Juttulapa et al. (2013) [80]. They found a greater cross-linking polymer network at pH 4 than pH 7, providing thus a smaller droplet size distribution [81].

Chemical stability of W/O/W emulsion was evaluated by measurements of retention levels of total phenolic content (TPC) and antioxidant activity (AA) by DPPH assays, after 35 days of storage at room temperature. TPC and AA

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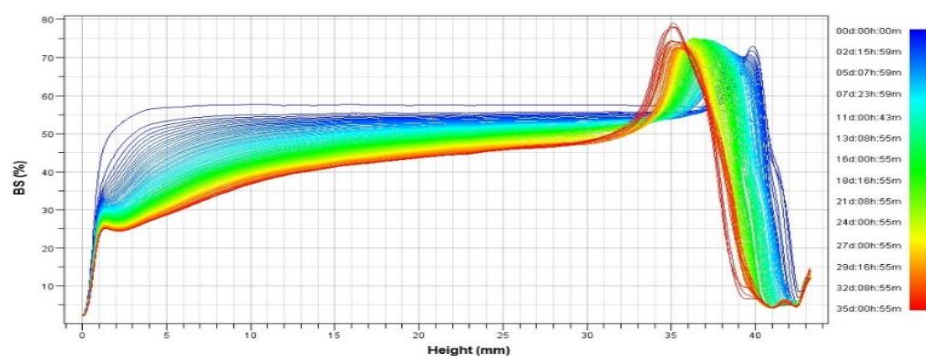
measurements were performed on W/O/W double emulsion using the optimal formulation prepared by microfluidizer processor at 148 MPa and 7 cycles. Polyphenols are very sensitive compounds that can be easily degraded over time; therefore, its encapsulation in suitable double emulsions is an effective solution to prevent the degradation of phenolic compounds at a satisfactory level. The OMWW used in this study (after membrane treatments and before emulsification) had TPC of 1399.8 ± 17.9 mg$_{GAE}$/L and 286.5 ± 0.3 mg$_{Trolox}$/L of AA. After 35 days of storage at room temperature, 68.6% and 89.5% retentions of TPC and AA, respectively, were preserved.
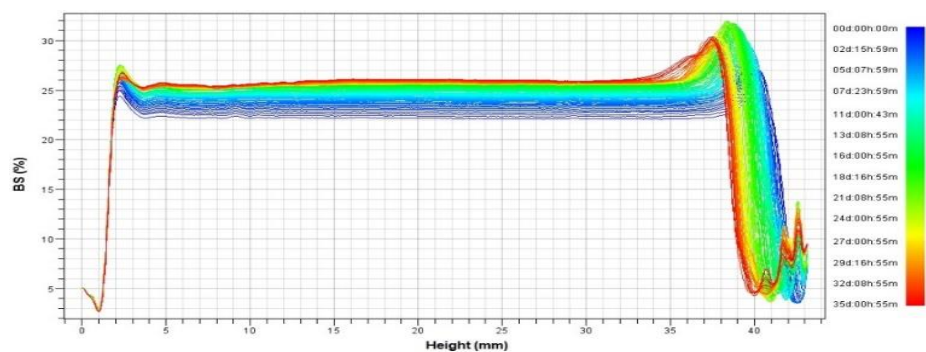
In related studies, Akhtar et al. (2014) [82] reported 72% rutin and anthocyanin flavonoids retention after 10 days of storage in a W/O/W nanoemulsion system, but a polymodal droplet size distribution and inner phase leakage were observed. Gomes et al. (2016) [83] studied the retention of gallic acid in W/O and O/W emulsion systems with soybean oil, obtaining a 15% reduction in gallic acid after 7 days of storage. Mohammadi et al. (2016) [17] reported 22% of phenolic compounds release by preparing double emulsions stabilized only with whey protein concentrate (WPC) containing olive leaf extract after 20 days. Furthermore, in the study performed by Gadkari et al. (2017) [26] it was noted that when the emulsions were stored at temperatures of 277 K, 300 K and 310 K, the green tea polyphenols present in emulsions were degraded by 4.25%, 15.97% and 22.78%, respectively. It can be concluded that the results obtained in the present study are in the usual retention range of phenols encapsulated in emulsions with applications for the food industry.

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(a): Rotor-stator mixer

(b): Ultrasonic homogenizer

(c): Microfluidizer processor

Figure 4.4. Backscattering profiles of W/O/W emulsions (optimal formulations) prepared by three devices through 35 days of storage at 25 ºC

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4.3.4.   Determination of W/O emulsion formulation

4.3.4.1. Model fitting of formulation

The droplet size and polydispersity index (PDI) of the formulation corresponding to the CCD design are shown in Table 4.5. The default model is quadratic with 10 coefficients, and it has been fit to the response variable.

Table 4.5. Experimental matrix for W/O emulsion formulation based on central composite design (CCD).

Run	Independent variables			Response variables	
	Aqueous phase content $(X_1, \%$ w/w)	Surfactant content $(X_2, \%$ w/w)	HLB number (X_3)	Droplet Size $(Y,$ nm) (mean ± SD)	PDI (mean ± SD)
1	2	20	11	3688 ± 484.1	1 ± 0.0
2	20	2	3	313.5 ± 12.7	0.956 ± 0.076
3	2	2	3	143.9 ± 24.6	0.094 ± 0.071
4	20	20	11	692.1 ± 121.0	1 ± 0.0
5	11	20	7	804.2 ± 237.3	1 ± 0.0
6	2	2	11	3856 ± 588.6	1 ± 0.0
7	20	20	3	1152 ± 30.4	0.164 ± 0.148
8	2	11	7	259.3 ± 51.7	0.246 ± 0.210
9	11	11	3	177.4 ± 47.0	0.104 ± 0.009
10	11	11	7	331.8 ± 116.0	0.082 ± 0.054
11	11	11	7	142.3 ± 13.1	0.222 ± 0.094
12	11	2	7	356.1 ± 68.1	0.350 ± 0.278
13	20	2	11	263.2 ± 71.8	1 ± 0.0
14	11	11	11	320.3 ± 64.6	0.666 ± 0.413
15	2	20	3	359.8 ± 14.0	0.055 ± 0.061
16	20	11	7	372.1 ± 136.3	0.416 ± 0.303

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Table 4.6. Analysis of variance (ANOVA) of the regression coefficients of the quadratic model (Eq. (3)) for the droplet size of W/O emulsion.

Source	Regression coefficients	F-ratio	p-value
a_0	-388.136	-	-
a_1	8.48871	169.34	0.0488
a_2	-146.709	17.32	0.1501
a_3	244.208	248.00	0.0404
a_{11}	4.23006	17.24	0.1505
a_{12}	1.88194	10.35	0.1918
a_{13}	-26.217	396.89	0.0319
a_{22}	7.49487	54.11	0.0860
a_{23}	-2.75521	4.38	0.2837
a_{33}	17.2365	11.17	0.1851

The R-squared statistic indicates that the model as fitted explains 90.68% of the variability in Particle size. Three of the coefficients (a_1, a_3, and a_{13}) of the quadratic polynomial model, Eq. (3), have p-values less than 0.05, indicating that they are significantly different from zero at the 95% confidence level (Table 4.6). F-ratio values indicate that, for the range of studied variables, the HLB number (X_3) and aqueous phase concentration (X_1) had stronger influence on the droplet size of the emulsions than surfactant concentration. F-ratio values also indicate that the interaction with the highest incidence was the one occurring between the HLB number and aqueous phase concentration. The significance of HLB number and surfactant content and their influence on particle size was already reported by Pimentel-Moral et al. (2018); Diamante and Lan (2014) ([81, 84]).

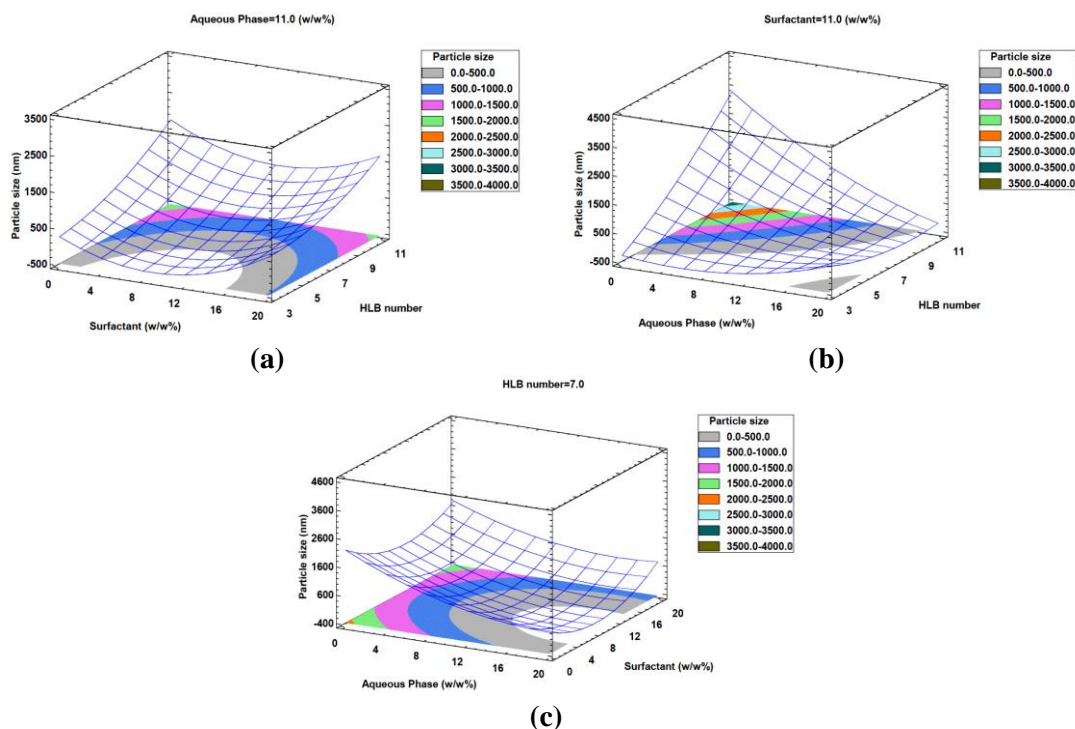### 4.3.4.2. Response surface analysis

In order to study the effect of the independent variables on the droplet size, surface response and contour plots of the quadratic polynomial model were generated by varying two of the independent variables within the experimental range while holding the third one constant at the central point. Figure 4.5.a was generated by varying the HLB number and surfactant content in the emulsion while holding the

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aqueous phase content (11 w/w %). It shows that, regardless of surfactant content, an increase of HLB number between 3 to 11 results in an increase of droplet size of the emulsion. This could be explained by the more efficiency of PGPR than Tween 80 in formation of nanoemulsions with lower particle size. The effects of HLB number and aqueous phase concentration on the droplet size at a fixed surfactant content (11 w/w %) can be observed in Figure 4.5.b. This figure shows that by considering HLB number at higher levels (7-11), an increase in aqueous content will significantly decrease particle size. However, at lower HLB number (3-7), varying aqueous content does not have a significant effect on particle size. Figure 4.5.c shows the effects of surfactant and aqueous phase contents on particle size at a fixed HLB number of 7. It can be observed that varying surfactant content has no considerable effect on particle size at high and low aqueous phase content. However, increasing aqueous phase content has a more significant effect, whereas it will decrease particle size considerably.

4.3.4.3. Optimization of W/O emulsion formulation

According to Stoke's law [48], the particle size of the dispersed phase affects the stability of an emulsion system, which is composed of two immiscible liquids with different densities. In this sense, the emulsion with higher resistance and control to creaming should contain smaller particles with a homogeneous size distribution. Following Stoke's law, the stability of emulsion would increase as the droplet size decreases [47].

Numerical optimization was carried out through Design Expert software, using desirability function method. The optimal conditions for the emulsification of the phenolic rich extract used in this work would be those leading to a stable emulsion with minimum droplet size. The combined optimum ingredient levels for all responses with optimum desirability were predicted to be achieved by combining 11.2% w/w of surfactant content with a HLB number of 3.36 and 7.4% w/w of aqueous phase content.

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Figure 4.5. Response surface plots of interactions between two input factors, while holding the other constant at its central point, on particle size of W/O emulsion. **(a)** Interaction of surfactant content and HLB number; **(b)** Interaction of aqueous phase content and HLB number; **(c)** Interaction of surfactant and aqueous phase contents.

A confirmation of the results using the optimum conditions (81.42% oil, 11.19% surfactant and 7.4% aqueous phase) was carried out by performing three replicates. The average droplet size obtained was 132.91 ± 16.7 nm. The low PDI obtained (0.153 ± 0.134) indicates a narrow droplet size distribution. The droplet size of nanoemulsions depends on cavitation, turbulence and shear forces created by ultrasonic homogenizer. As Anarjan et al. reported [74], smaller nanoemulsion droplets will be formed with the increase in homogenization time. Moreover, temperature of the nanoemulsions also increases due to increase in homogenization time which leads to decrease in viscosity and interfacial tension. Lower interfacial tension value encourages the interfacial instability. Additionally, due to higher temperature, vapor pressure increases in cavitation region which increase the cavitation intensity and accelerates the breakdown of droplets [75].

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4.3.5. *Effects of high energy emulsification methods on W/O emulsion droplet size*

The reason that such intense energies are needed is that the disruptive forces generated by the homogenizer must exceed the restoring forces holding the droplets into spherical shapes [62, 85, 86]. These restorative forces are determined by the Laplace pressure ($P = \gamma / 2r$), which increases with decreasing droplet radius (r) and increasing interfacial tension (γ). Thus, as the droplet radius decreases within a homogenizer, it becomes increasingly difficult to break them up further. The smallest droplet size that can be produced by a particular high-energy device depends on the homogenizer design (e.g., flow and force profiles), homogenizer operating conditions (e.g., energy intensity, duration), environmental conditions (e.g., temperature), sample composition (e.g., oil type, emulsifier type, concentrations), and the physicochemical properties of the component phases (e.g., interfacial tension, viscosity) [41, 67]. Previous studies have shown that the droplet size tends to decrease when the energy intensity or duration increases, the interfacial tension decreases, the emulsifier adsorption rate increases, and the disperse-to-continuous phase viscosity ratio falls within a certain range (0.05 < η_D/η_C < 5) [61–63]. The extent of the η_D/η_C range where small droplets can be produced depends on the nature of the disruptive forces generated by the particular homogenizer used, i.e., simple shear versus extensional flow. High-energy approaches are one of the most versatile means of producing food-grade nanoemulsions because they can be used with a wide variety of different oil and emulsifier types. Provided that the homogenization conditions are suitably optimized, nanoemulsions can be produced using triacylglycerol oils, flavor oils, and essentials oils as the oil phase, and proteins, polysaccharides, phospholipids, and surfactants as emulsifiers. Even so, the size of the particles produced depends strongly on the characteristics of the oil and emulsifier used [9].

The energy needed for the emulsification process can be provided by mechanical agitation (e.g.: stirring, high shear mixing), high-pressure homogenization or high power ultrasound [87], [88]. Although high-pressure homogenization is widely

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used, ultrasonic methods have several advantages, such as lower-cost equipment, smaller footprint and easier cleaning and servicing [87]. In this research W/O nanoemulsions of different droplet sizes were produced in an ultrasonic homogenizer by varying time and amplitude and also in a rotor-stator mixer by varying time and rotational speed of the rotor. Ultrasonic homogenizers utilize high-intensity ultrasonic waves to create the intense disruptive forces necessary to breakup up oil and water phases into very small droplets [65–67]. The energy input is provided using sonicator probes that contain piezoelectric quartz crystals that expand and contract in response to an alternating electrical voltage. Compared to other high energy methods, ultrasonication requires least energy expenditure. One serious downside of this technique is contamination induced by probe. For scale up applicability, commercial homogenizers based on sonication have been developed, in which nanoemulsion is made to flow through a special column capable of producing ultrasonic waves [70]. Rotor-stator mixers (RSMs) (also known as high-speed or high-shear mixers) generally consist of a perforated stator screen mounted outside a rotor. The rotor draws in fluid towards the rotor-stator head axially, accelerates it tangentially and then forces it radially through the stator slots, giving rise to efficient mixing and/or dispersion. RSMs are used for a large and increasing number of chemical engineering processes, especially for high viscosity and high disperse phase volume fraction dispersions, e.g. in pharmaceuticals, cosmetics and food processing [71–73].

4.3.5.1. Model fitting

Two experimental designs based on RSM were prepared by the application of a rotor-stator mixer and an ultrasonic homogenizer. For this purpose, the CCD model generated 10 experimental settings with two replicates of central point for each emulsification method. The average droplet size and PDI of the experiments corresponding to the CCD design is given in Table 4.7.

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Table 4.7. Experimental matrix based in a central composite design (CCD) for W/O nanoemulsion preparation by high-energy emulsification methods.

Run	Rotor-Stator Mixer			
	Independent Variables		Response Variables	
	Rotation speed (X$_1$, rpm)	Time (X$_2$, min)	Droplet size (Y, nm) (mean ± SD)	PDI (mean ± SD)
1	20000	10	105.8 ± 10.3	0.255 ± 0.045
2	20000	5	223.5 ± 26.8	0.435 ± 0.238
3	29000	15	280.7 ± 29.0	0.571 ± 0.093
4	29000	10	133.2 ± 6.65	0.166 ± 0.107
5	20000	10	105 ± 4.7	0.192 ± 0.224
6	11000	15	138.4 ± 2.2	0.187 ± 0.043
7	29000	5	136.4 ± 6.1	0.175 ± 0.016
8	11000	10	148.5 ± 30.0	0.323 ± 0.044
9	11000	5	286.9 ± 15.2	0.556 ± 0.150
10	20000	15	112.6 ± 4.43	0.185 ± 0.020
Run	Ultrasonic Homogenizer			
	Independent Variables		Response Variables	
	Time (X$_1$, min)	Amplitude (X$_2$, %)	Droplet size (Y, nm) (mean ± SD)	PDI (mean ± SD)
11	5	20	71.09 ± 7.35	0.480 ± 0.283
12	15	20	104.9 ± 6.68	0.156 ± 0.085
13	10	20	113.2 ± 6.96	0.136 ± 0.109
14	15	40	114.7 ± 18.14	0.103 ± 0.011
15	15	60	111.5 ± 9.95	0.060 ± 0.035
16	10	60	120.3 ± 12.91	0.074 ± 0.018
17	10	40	175.9 ± 3.46	0.163 ± 0.04
18	10	40	188.1 ± 5.16	0.168 ± 0.044
19	5	40	106.5 ± 6.46	0.192 ± 0.107
20	5	60	228.8 ± 12.7	0.019 ± 0.015

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The default model is quadratic and statistical models have been fit to the response variables. The R-squared statistic indicates that the model as fitted explains 90.93 % and 67.24% of the variability in particle size for rotor-stator mixer and ultrasonic homogenizer, respectively. Regarding rotor-stator mixer, F-ratio value indicate that time (X_2) had stronger effect on the droplet size of the emulsions than rotation speed (X_1). In the case of ultrasonic homogenizer, F-ratio value indicate that amplitude (X_2) had significantly stronger effect on emulsion droplet size than time (X_1) (Table 4.8).

ANOVA showed the significance of the coefficients of the quadratic polynomial model (Eq. (2)). Regarding the rotor-stator mixer, all 5 coefficients have p-values less than 0.05, only one coefficient in each model has a p-value less than 0.05, indicating that it is significantly different to zero with 95% confidence level, indicating that they are significantly different from zero at the 95.0% confidence level (Table 4.8). Moreover, no coefficient has a p-value less than 0.05 for the ultrasonic homogenizer model.
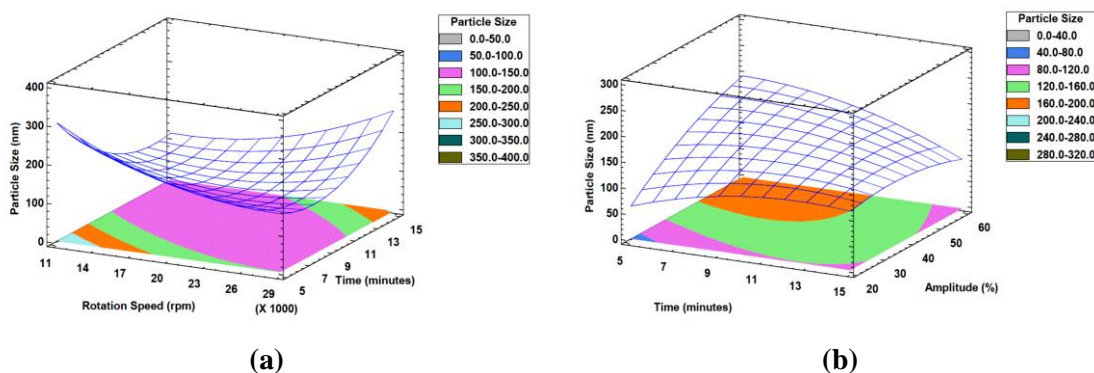
Table 4.8. Analysis of variance (ANOVA) of the regression coefficients of the quadratic model, Eq. (2), for the droplet size of W/O nanoemulsions prepared by high energy emulsification methods

Source	Rotor-Stator Mixer			Ultrasonic Homogenizer		
	Regression coefficients	F-Ratio	p-Value	Regression coefficients	F-Ratio	p-Value
a_0	937.701	-	-	-188.939	-	-
a_1	-0.0362116	287.63	0.0375	31.4482	12.70	0.1742
a_2	-89.7357	6900.01	0.0077	8.68788	65.80	0.0781
a_{11}	4.87742E-7	11380.94	0.0060	-0.942343	17.40	0.1498
a_{12}	0.00162667	66978.00	0.0025	-0.377775	76.71	0.0724
a_{22}	2.66829	32446.77	0.0035	-0.0435214	9.50	0.1997
Lack-of-fit	-	4183.78	0.0112	-	30.08	0.1313

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4.3.5.2. Response surface analysis

In order to study the effect of the emulsification methods and operating conditions on the droplet size, surface response plots of the quadratic polynomial model were generated. Figure 4.6.a shows the effects of time and rotation speed in rotor-stator mixer on the achieved emulsion droplet size. This figure shows that the increase of process time at higher rotation speeds (20000-29000 rpm) causes an increase in particle size, while at lower speeds (11000-20000 rpm) has an opposite effect and decreases the particle size significantly.

The droplet size of nanoemulsions depends on cavitation, turbulence and shear forces created by ultrasonic homogenizer. Smaller droplets of nanoemulsions were formed with the increase in homogenization time [74]. Moreover, temperature of the nanoemulsions also increases due to increase in homogenization time which leads to decrease in viscosity and interfacial tension. Lower interfacial tension value encourages the interfacial instability. Additionally, due to higher temperature, vapor pressure increases in cavitation region which increase the cavitation intensity and accelerates the breakdown of droplets [54, 75]. Figure 4.6.b shows the interaction of time and amplitude on the achieved droplet size in ultrasonic homogenizer. As it can be seen, time variation does not have a noticeable effect on particle size at lower amplitudes. However, as it was abovementioned, amplitude is the most significant factor in ultrasonic assisted experiments. Somehow, for lower time ranges (5-10 minutes), increasing amplitude has a negative effect in this study and increases particle size while at higher time ranges (10-15 minutes) to considerable effect was observed by varying amplitude.

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(a)   **(b)**

Figure 4.6. Response surface plots of interactions between input factors on particle size of W/O nanoemulsion using high energy emulsification methods. **(a)** Effects of time and rotation speed variations on particle size in rotor-stator mixer; **(b)** Effects of time and amplitude variations on particle size in ultrasonic homogenizer.

4.3.5.3. Optimization of W/O nanoemulsion preparation conditions

The optimal conditions for the emulsification of the phenolic rich olive extract used in this work would be those leading to a stable emulsion with minimum droplet size. Numerical optimization was carried out through Design Expert software, using desirability function method. Based on the results shown in Table 4.7, the combined optimum levels for average particle size with 100% desirability regarding nanoemulsions made by rotor-stator mixer were predicted to be achieved by emulsification at 20000 rpm and 10.1 minutes. The predicted response was particle size of 103.01 nm and PDI of 0.211, while observed results for optimal emulsion loaded with phenolic compounds were 109.8 ± 17.81 nm and 0.087 ± 0.069 for particle size and PDI, respectively.

In the case of optimal nanoemulsion prepared by ultrasonic homogenizer, situation were slightly different. Somehow, 63.31 nm of particle size with PDI = 0.422 was predicted to be obtained by emulsification for 5 minutes at 20% amplitude with 100% desirability. However, observed results for the same operating conditions were 71.09 ± 7.35 nm and 0.480 ± 0.283 for average particle size and PDI, respectively (Table 4.7). However, lack of visual stability, droplet size growth after initial days and wide distribution of particle size (PDI) of the mentioned operating conditions were the reason to consider emulsification at 15 minutes and 20%
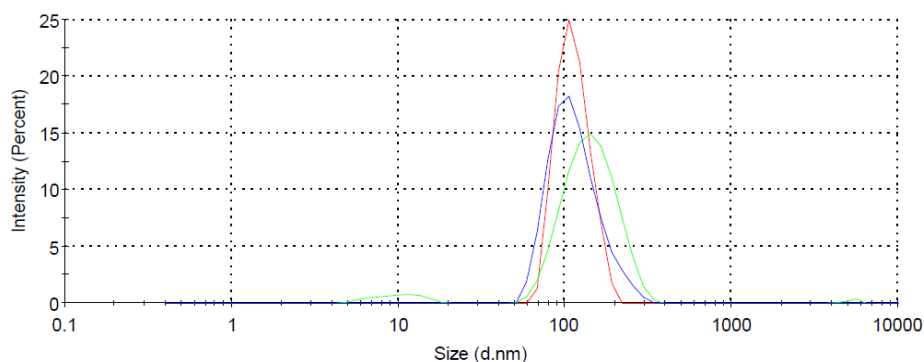
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amplitude as the optimal operating condition with ultrasonic homogenizer. Whereas, by this factor levels, 104.9±6.68 nm and 0.156±0.085 for particle size and PDI was achieved, which has considerably lower PDI with satisfactory particle size diameter.
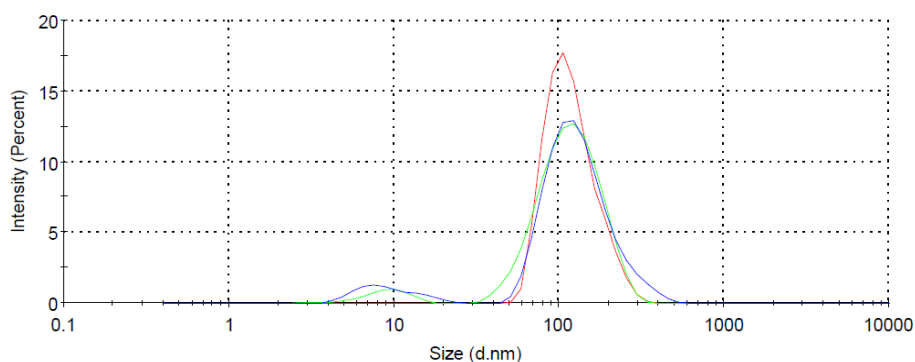
4.3.6. Characterization of optimal W/O nanoemulsion

4.3.6.1.Stability evaluations during storage

In order to evaluate the stability of the optimized nanoemulsions prepared by rotor-stator mixer and ultrasonic homogenizer, two methods were used. The first one was the evaluation of droplet size (at room temperature and 4 °C) after 1 day and 30 days from emulsification in order to study the possible droplet growth rate. The second method was the backscattering (BS) evolution over time using Turbiscan Lab Expert apparatus to detect emulsion instability.

Figure 4.7 shows no significant changes in average droplet size of optimal formulated nanoemulsions, with no variations in visual stability appearance, after 30 days of storage at 4 ºC and room temperature (25 ºC), but polydispersity index (PDI) was increased during the storage period. It can be observed from Figure 4.7.a that lower temperature causes a better effect on stability of optimal nanoemulsion prepared by rotor-stator mixer. Somehow, considerably lower PDI with same particle size was obtained while for optimal nanoemulsion prepared by ultrasonic homogenizer (Figure 4.7.b) no significant difference was observed between storage conditions at both temperatures.

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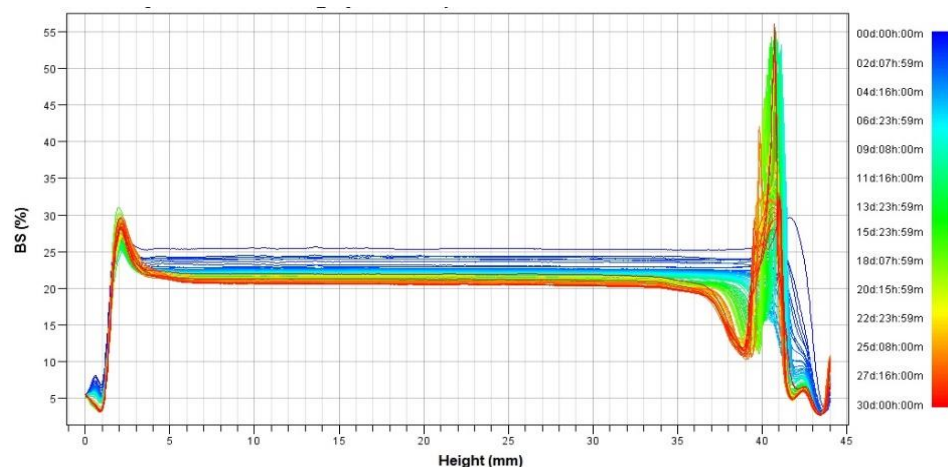
(a): Rotor-stator mixer
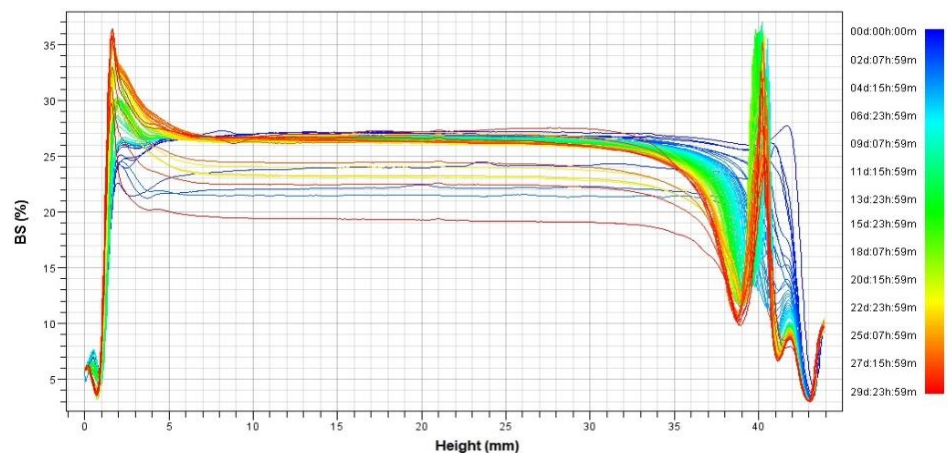

(b): Ultrasonic homogenizer

Fig. 4.7. Particle size distribution of optimal formulated W/O nanoemulsions after 1 day (red line), and after 30 days of storage at room temperature (green line) and at 4 ºC (blue line)

Stability measurements by studying the backscattering (BS) profile and monitoring for 30 days at 20 °C, using the Turbiscan Lab Expert apparatus were also performed. As it is shown in Figure 4.8.a, BS profiles for optimal nanoemulsion prepared by rotor-stator mixer showed sedimentation at the bottom of the sample and also creaming after the first week of storage, indicating a slight emulsion destabilization at the end of the storage period. Regarding the optimal nanoemulsion prepared by ultrasonic homogenizer, the corresponding BS profiles (Figure 4.8.b) showed after 30 days of storage the same levels of sedimentation and creaming as the nanoemulsion prepared by rotor-stator mixer showed after the

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first week of analysis. Besides, it shows slight levels of particle size increase in the last days of storage period. However, optimal nanoemulsions were physically stable and visual appearance did not indicate any destabilization and no phase separation was observed during 30 days of storage at 20 °C.

(a): Rotor-stator mixer

(b): Ultrasonic homogenizer

Figure 4.8. Backscattering profiles of optimal formulated W/O nanoemulsions prepared by two devices through 35 days of storage at 20 ºC

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4.3.6.2. Release properties of W/O nanoemulsion

Chemical stability of W/O nanoemulsion was evaluated by measurements of retention levels of total phenolic content (TPC) after 30 days of storage at room temperature. Polyphenols are very sensitive compounds that can easily be degraded with time and the W/O nanoemulsions were highly effective in preventing phenolic compounds degradation. In the present study, freshly extracted phenolic compounds from olive pomace had a TPC of 17.27±0.87 mg GAE/g olive pomace. After 30 days of storage at room temperature, 87.2% and 83.1% retentions of TPC and AA, respectively, were preserved for the optimal nanoemulsions prepared by rotor-stator mixer and ultrasonic homogenizer, respectively. In the study performed by Rabelo et al. [89], the 2% Açaí berry extract nonencapsulated sample showed highest retention of 94.6% for the phenolic loaded sample after 30 days of storage. Sanchez et al. [90] obtained 90% retention of ACNs in spray-dried cherry juice after 33 days. It can be concluded that the results obtained in the present study are in the usual retention range of phenols encapsulated in emulsions with applications for the food industry.

4.4. Conclusions

Based on studies and reviews regarding encapsulation on polyphenols, it is clear that utilization of encapsulated polyphenols instead of free molecules improves both the stability and bioavailability of the molecules in vitro and in vivo. Response surface methodology was applied in this work to determine both the optimal composition and operating conditions for preparation of W/O and W/O/W emulsions loaded with phenolic loaded inner aqueous phase obtained from olive mill solid and liquid residues.

Regarding preparation of double (W/O/W) emulsion, optimal formulation for primary W/O emulsion was 20.3% (w/w) of phenolic rich aqueous solution as dispersed phase and 3.7% (w/w) of Span 80 in MCT oil as continuous phase. The optimal composition for W/O/W emulsion was 9.8% (w/w) of W/O as dispersed

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phase and 4.1% (w/w) of Tween 80 in Milli-Q water as external phase. Three methods were tested to obtain the optimal emulsification conditions: mechanical homogenization (rotor-stator mixer), ultrasonic homogenization and microfluidization. Optimum results were achieved by microfluidization at 148 MPa and 7 cycles input levels, obtaining a W/O/W nanoemulsion with and average droplet diameter of 105.3±3.2 nm and a polydispersity index of 0.233±0.02. Samples obtained by rotor-stator mixer and ultrasonic homogenizer showed creaming instability after few days of emulsification, while emulsion obtained by microfluidization showed no droplet size growth or changes in stability and zeta potential after 35 days of storage at 25 °C. Furthermore, it showed a satisfactory level of phenolics retention (68.6%) and antioxidant activity (89.5%) after 35 days of storage.

On the other hand, same design methods were used for optimization of water in oil (W/O) nanoemulsion preparation. The optimal formulation was obtained by when 7.4 w/w% of olive cake extract and 11.2 w/w% of a surfactant mixture of PGPR (97%) and Tween 80 (3%) were emulsified in Miglyol 812N (MCT). The optimal emulsification condition with ultrasonic homogenizer was obtained by sonication for 15 minutes at 20% amplitude which resulted in average droplet size of 104.9±6.68 nm and polydispersity index (PDI) of 0.156±0.085. Furthermore, using a rotor-stator mixer for 10.1 minutes at 2000 rpm was the other optimal high energy emulsification method in which average droplet size of 109.8 ± 17.81 nm and PDI of 0.087± 0.069. After 30 days of storage at room temperature, total phenolic content analysis of the optimal nanoemulsions prepared by rotor-stator mixer and ultrasonic homogenizer showed 87.2% and 83.1% retention of the initial phenolic compounds, respectively. in spite of slight levels of sedimentation and creaming for the optimal nanoemulsions prepared by both devices, they showed satisfactory physical stability and no droplet size growth over 30 day storage time at room temperature which make them suitable for application in food or pharmaceutical industries.

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References

[1]   S. Friberg, K. Larsson, J. Sjoblom, "Food emulsions", *CRC Press*, 2003.

[2]   D. J. McClements, "Food Emulsions: Principles, Practice, and Techniques", CRC series in contemporary food science, *Boca Raton: CRC Press*, 2005.

[3]   D. J. McClements, E. A. Decker, Y. Park, J. Weiss, "Structural Design Principles for Delivery of Bioactive Components in Nutraceuticals and Functional Foods", *Crit. Rev. Food Sci. Nutr.*, 49, 6, 577–606, 2009.

[4]   S. Van Der Graaf, C. G. P. H. Schroën, R. M. Boom, "Preparation of double emulsions by membrane emulsification-A review", *J. Memb. Sci.*, 251, 1–2, 7–15, 2005.

[5]   A. Benichou, A. Aserin, N. Garti, "Double emulsions stabilized with hybrids of natural polymers for entrapment and slow release of active matters", *Adv. Colloid Interface Sci.*, 108–109, 29–41, 2004.

[6]   Z. Fang, B. Bhandari, "Encapsulation of polyphenols - A review", *Trends Food Sci. Technol.*, 21, 10, 510–523, 2010.

[7]   I. Kralova, J. Sjöblom, "Surfactants Used in Food Industry: A Review", *J. Dispers. Sci. Technol.*, 30, 9, 1363–1383, 2009.

[8]   R. Llinares, J. Santos, L. A. Trujillo-Cayado, P. Ramírez, J. Muñoz, "Enhancing rosemary oil-in-water microfluidized nanoemulsion properties through formulation optimization by response surface methodology", *Lwt*, 97, 370–375, 2018.

[9]   D. J. McClements, J. Rao, "Food-Grade nanoemulsions: Formulation, fabrication, properties, performance, Biological fate, and Potential Toxicity", *Crit. Rev. Food Sci. Nutr.*, 51, 4, 285–330, 2011.

[10]   S. L. Ee, X. Duan, J. Liew, Q. D. Nguyen, "Droplet size and stability of nano-emulsions produced by the temperature phase inversion method", *Chem. Eng. J.*, 140, 1–3, 626–631, 2008.

[11]   V. Uskoković, M. Drofenik, "Synthesis of materials within reverse micelles", *Surf. Rev. Lett.*, 12, 02, 239–277, 2005.

[12]   L. C. Peng, C. H. Liu, C. C. Kwan, K. F. Huang, "Optimization of water-in-oil nanoemulsions by mixed surfactants", *Colloids Surfaces A Physicochem. Eng. Asp.*, 370, 1–3, 136–142, 2010.

[13]   L. Zhou, R. J. Elias, "Antioxidant and pro-oxidant activity of (-)-epigallocatechin-3-gallate in food emulsions: Influence of pH and phenolic concentration", *Food Chem.*, 138, 2–3, 1503–1509, 2013.

[14]   M. Matos, G. Gutiérrez, J. Coca, C. Pazos, "Preparation of water-in-oil-in-water (W1/O/W2) double emulsions containing trans-resveratrol", *Colloids Surfaces A Physicochem. Eng. Asp.*, 442, 69–79, 2014.

[15]   A. Tolun, Z. Altintas, N. Artik, "Microencapsulation of grape polyphenols using maltodextrin and gum arabic as two alternative coating materials: Development

PhD Thesis

Chapter 4: Formulation and fabrication of single and double emulsions loaded with phenolic inner aqueous phase by application of high energy emulsification methods

and characterization", *J. Biotechnol.*, 239, 23–33, 2016.

[16]   M. Ahmad, P. Mudgil, A. Gani, F. Hamed, F. A. Masoodi, S. Maqsood, "Nano-encapsulation of catechin in starch nanoparticles: Characterization, release behavior and bioactivity retention during simulated in-vitro digestion", *Food Chem.*, 270, 95–104, 2019.

[17]   A. Mohammadi, S. Mahdi, E. Assadpour, A. Faridi, "International Journal of Biological Macromolecules Nano-encapsulation of olive leaf phenolic compounds through WPC – pectin complexes and evaluating their release rate", *Int. J. Biol. Macromol.*, 82, 816–822, 2016.

[18]   J. A. Bhushani, N. K. Kurrey, C. Anandharamakrishnan, "Nanoencapsulation of green tea catechins by electrospraying technique and its effect on controlled release and in-vitro permeability", *J. Food Eng.*, 199, 82–92, 2017.

[19]   A. M. Pisoschi, A. Pop, C. Cimpeanu, V. Turcuş, G. Predoi, F. Iordache, "Nanoencapsulation techniques for compounds and products with antioxidant and antimicrobial activity - A critical view", *Eur. J. Med. Chem.*, 157, 1326–1345, 2018.

[20]   A. Mohammadi, S. M. Jafari, A. F. Esfanjani, S. Akhavan, "Application of nano-encapsulated olive leaf extract in controlling the oxidative stability of soybean oil", *Food Chem.*, 190, 513–519, 2016.

[21]   M. A. Tuyishime, E. Matabaro, A. H. Ali, "Green Tea Polyphenols olyphenols-proteins Nanocomplexes anocomplexes Foaming Behavior , Nanoemulsions anoemulsions and Their Applications pplications", *Ijair*, 5, 1, 149–156, 2016.

[22]   R. Ghanbari, F. Anwar, K. M. Alkharfy, A. H. Gilani, N. Saari, "Valuable nutrients and functional bioactives in different parts of olive (Olea europaea L.)-A review", *Int. J. Mol. Sci.,* 13, 2012.

[23]   T. P. Sari, B. Mann, R. Kumar, R. R.B. Singh, R. Sharma, M. Bhardwaj, S. Athira, "Preparation and characterization of nanoemulsion encapsulating curcumin", *Food Hydrocoll.*, 43, 540–546, 2015.

[24]   C. D. Di Mattia, G. Sacchetti, D. Mastrocola, P. Pittia, "Effect of phenolic antioxidants on the dispersion state and chemical stability of olive oil O/W emulsions", *Food Res. Int.*, 42, 8, 1163–1170, 2009.

[25]   L. F. Ballesteros, M. J. Ramirez, C. E. Orrego, J. A. Teixeira, S. I. Mussatto, "Encapsulation of antioxidant phenolic compounds extracted from spent coffee grounds by freeze-drying and spray-drying using different coating materials", *Food Chem.*, 237, 623–631, 2017.

[26]   P. V. Gadkari, M. G. Shashidhar, M. Balaraman, "Delivery of green tea catechins through Oil-in-Water (O/W) nanoemulsion and assessment of storage stability", *J. Food Eng.*, 199, 65–76, 2017.

[27]   A. De Cristo Soares Alves, R. M. Mainardes, N. M. Khalil, "Nanoencapsulation of gallic acid and evaluation of its cytotoxicity and antioxidant activity", *Mater. Sci. Eng. C*, 60, 126–134, 2016.

PhD Thesis

Chapter 4: Formulation and fabrication of single and double emulsions loaded with phenolic inner aqueous phase by application of high energy emulsification methods

[28]   A. Kumari, S. K. Yadav, Y. B. Pakade, B. Singh, S. C. Yadav, "Development of biodegradable nanoparticles for delivery of quercetin", *Colloids Surfaces B Biointerfaces*, 80, 2, 184–192, 2010.

[29]   W. Lu, A. L. Kelly, S. Miao, "Emulsion-based encapsulation and delivery systems for polyphenols", *Trends Food Sci. Technol.*, 47, 1–9, 2016.

[30]   B. P. Binks, "Modern Aspects of Emulsion Science Royal Society of Chemistry", London, 1999.

[31]   Y. Yuan, Y. Gao, J. Zhao, L. Mao, "Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions", *Food Res. Int.*, 41, 1, 61–68, 2008.

[32]   D. J. Mcclements, "Encapsulation, protection , and release of hydrophilic active components : Potential and limitations of colloidal delivery systems", *Adv. Colloid Interface Sci.*, 219, 27–53, 2015.

[33]   N. Garti, C. Bisperink, "Double emulsions : progress and applications", *Curr. Opin. Colloid Interface Sci.*, 3, 6, 657–667.

[34]   F. Cournarie, M. Savelli, C. Vauthier, J. Grossiord, M. Seiller, "Insulin-loaded W/O/W multiple emulsions: comparison of the performances of systems prepared with medium-chain-triglycerides and fish oil", 58, 477–482, 2004.

[35]   D. J. McClements, E. A. Decker, J. Weiss, "Emulsion-based delivery systems for lipophilic bioactive components", *J. Food Sci.*, 72, 8, R109–R124, 2007.

[36]   N. Garti, "Colloids and Surfaces A: Physicochemical and Engineering Aspects", vol. 124, pp. 233–246, 1997.

[37]   M. Porras, C. Solans, C. González, A. Martínez, A. Guinart, J. M. Gutiérrez, "Studies of formation of W/O nano-emulsions", *Colloids Surfaces A Physicochem. Eng. Asp.*, 249, 1–3, 115–118, 2004.

[38]   E. P. Melo, M. R. Aires-Barros, J. M. S. Cabral, "Reverse micelles and protein biotechnology", 2001.

[39]   L. Wang, J. Dong, J. Chen, J. Eastoe, X. Li, "Design and optimization of a new self-nanoemulsifying drug delivery system", *J. Colloid Interface Sci.*, 330, 2, 443–448, 2009.

[40]   C. M. Pey, A. Maestro, I. Solé, C. González, C. Solans, J. M. Gutiérrez, "Optimization of nano-emulsions prepared by low-energy emulsification methods at constant temperature using a factorial design study", *Colloids Surfaces A Physicochem. Eng. Asp.*, 288, 1–3, 144–150, 2006.

[41]   T. J. Wooster, M. Golding, P. Sanguansri, "Impact of Oil Type on Nanoemulsion Formation and Ostwald Ripening Stability", *Langmuir*, 24, 22, 12758–12765, 2008.

[42]   Y. Li, S. Le Maux, H. Xiao, D. J. McClements, "Emulsion-based delivery systems for tributyrin, a potential colon cancer preventative agent", *J. Agric. Food Chem.*, 57, 19, 9243–9249, 2009.

PhD Thesis

Chapter 4: Formulation and fabrication of single and double emulsions loaded with phenolic inner aqueous phase by application of high energy emulsification methods

[43]   S. Mun, D. J. McClements, "Influence of interfacial characteristics on Ostwald ripening in hydrocarbon oil-in-water emulsions", *Langmuir*, 22, 4, 1551–1554, 2006.

[44]   L. Mao, D. Xu, J. Yang, F. Yuan, Y. Gao, J. Zhao, "Effects of small and large molecule emulsifiers on the characteristics of β-carotene nanoemulsions prepared by high pressure homogenization", *Food Technol. Biotechnol.*, 47, 3, 336–342, 2009.

[45]   F. Bazzarelli, T. Poerio, R. Mazzei, N. D'Agostino, L. Giorno, "Study of OMWWs suspended solids destabilization to improve membrane processes performance", *Sep. Purif. Technol.*, 149, 183–189, 2015.

[46]   P. Ghorbannezhad, A. Bay, M. Yolmeh, R. Yadollahi, J. Y. Moghadam, "Optimization of coagulation–flocculation process for medium density fiberboard (MDF) wastewater through response surface methodology", *Desalin. Water Treat.*, 57, 56, 26916–26931, 2016.

[47]   Y. T. Hu, Y. Ting, J. Y. Hu, S. C. Hsieh, "Techniques and methods to study functional characteristics of emulsion systems", *J. Food Drug Anal.*, 25, 1, 16–26, 2017.

[48]   R. J. Hunter, "Foundations of colloid science", *Oxford university press*, 2001.

[49]   S. M. Jafari, Y. He, B. Bhandari, "Production of sub-micron emulsions by ultrasound and microfluidization techniques", *J. Food Eng.*, 82, 4, 478–488, 2007.

[50]   P. Ganesan, G. Karthivashan, S. Y. Park, J. Kim, D. K. Choi, "Microfluidization trends in the development of nanodelivery systems and applications in chronic disease treatments", *Int. J. Nanomedicine*, 13, 6109–6121, 2018.

[51]   B. Khadem, N. Sheibat-Othman, "Theoretical and Experimental Investigations of Double Emulsion Preparation by Ultrasonication", *Ind. Eng. Chem. Res.*, 58, 19, 8220–8230, 2019.

[52]   H. Kumar, V. Kumar, "Ultrasonication assisted formation and stability of water-in-oil nanoemulsions: Optimization and ternary diagram analysis", *Ultrason. Sonochem.*, 49, 79–88, 2018.

[53]   A. Schuch, J. Wrenger, H. P. Schuchmann, "Production of W/O/W double emulsions. Part II: Influence of emulsification device on release of water by coalescence", *Colloids Surfaces A Physicochem. Eng. Asp.*, 461, 344–351, 2014.

[54]   T. Mehmood, A. Ahmad, A. Ahmed, Z. Ahmed, "Optimization of olive oil based O/W nanoemulsions prepared through ultrasonic homogenization: A response surface methodology approach", *Food Chem.*, 229, 790–796, 2017.

[55]   L. Bai, S. Huan, J. Gu, D. J. McClements, "Fabrication of oil-in-water nanoemulsions by dual-channel microfluidization using natural emulsifiers: Saponins, phospholipids, proteins, and polysaccharides", *Food Hydrocoll.*, 61, 703–711, 2016.

[56]   S. Mahdi Jafari, Y. He, B. Bhandari, "Nano-emulsion production by sonication and microfluidization - A comparison", *Int. J. Food Prop.*, 9, 3, 475–485, 2006.

PhD Thesis

Chapter 4: Formulation and fabrication of single and double emulsions loaded with phenolic inner aqueous phase by application of high energy emulsification methods

[57]   S. Kentish, T. J. Wooster, M. Ashokkumar, S. Balachandran, R. Mawson, L. Simons, "The use of ultrasonics for nanoemulsion preparation", *Innov. Food Sci. Emerg. Technol.*, 9, 2, 170–175, 2008.

[58]   C. Qian, D. J. McClements, "Formation of nanoemulsions stabilized by model food-grade emulsifiers using high-pressure homogenization: Factors affecting particle size", *Food Hydrocoll.*, 25, 5, 1000–1008, 2011.

[59]   M. N. Yukuyama, E. Tomiko Myiake Kato, G. Lima Barros de Araujo, R. Löbenberg, L. Marie Monteiro, F. Rebello Lourenço, N. Araci Bou-Chacra, "Olive oil nanoemulsion preparation using high-pressure homogenization and D-phase emulsification-A design space approach", *J. Drug Deliv. Sci. Technol.*, 49, 622–631, 2019.

[60]   P. H. Li, B. H. Chiang, "Process optimization and stability of d-limonene-in-water nanoemulsions prepared by ultrasonic emulsification using response surface methodology", *Ultrason. Sonochem.*, 19, 1, 192–197, 2012.

[61]   P. Walstra, "Physical chemistry of foods", *CRC Press*, 2002.

[62]   P. Walstra, "Principles of emulsion formation", *Chem. Eng. Sci.*, 48, 2, 333–349, 1993.

[63]   T. Tadros, P. Izquierdo, J. Esquena, C. Solans, "Formation and stability of nano-emulsions", *Adv. Colloid Interface Sci.*, 108, 303–318, 2004.

[64]   S. M. Jafari, E. Assadpoor, Y. He, B. Bhandari, "Re-coalescence of emulsion droplets during high-energy emulsification", *Food Hydrocoll.*, 22, 7, 1191–1202, 2008.

[65]   C.-Y. Lin, L.-W. Chen, "Comparison of fuel properties and emission characteristics of two-and three-phase emulsions prepared by ultrasonically vibrating and mechanically homogenizing emulsification methods", *Fuel*, 87, 10–11, 2154–2161, 2008.

[66]   T. S. H. Leong, T. J. Wooster, S. E. Kentish, M. Ashokkumar, "Minimising oil droplet size using ultrasonic emulsification", *Ultrason. Sonochem.*, 16, 6, 721–727, 2009.

[67]   S. Kentish, T. J. Wooster, M. Ashokkumar, S. Balachandran, R. Mawson, L. Simons, "The use of ultrasonics for nanoemulsion preparation", *Innov. Food Sci. Emerg. Technol.*, 9, 2, 170–175, 2008.

[68]   B. Abismaıl, J. P. Canselier, A. M. Wilhelm, H. Delmas, C. Gourdon, "Emulsification by ultrasound: drop size distribution and stability", *Ultrason. Sonochem.*, 6, 1–2, 75–83, 1999.

[69]   Y.-F. Maa, C. C. Hsu, "Performance of sonication and microfluidization for liquid–liquid emulsification", *Pharm. Dev. Technol.*, 4, 2, 233–240, 1999.

[70]   Y. Singh, J. Gopal Meher, K. Raval, F. Ali Khan, M. Chaurasia, N. K. Jain, M. K. Chourasia, "Nanoemulsion: Concepts, development and applications in drug delivery", *J. Control. Release*, 252, 28–49, 2017.

[71]   H. H. Mortensen, F. Innings, A. Håkansson, "The effect of stator design on

PhD Thesis

Chapter 4: Formulation and fabrication of single and double emulsions loaded with phenolic inner aqueous phase by application of high energy emulsification methods

flowrate and velocity fields in a rotor-stator mixer-An experimental investigation", *Chem. Eng. Res. Des.*, 121, 1, 245–254, 2017.

[72]   V. A. Atiemo-Obeng, R. V Calabrese, "Rotor-stator mixing devices", *Handbook of industrial mixing: Science and practice*. Wiley Online Library, pp. 479–505, 2004.

[73]   J. Zhang, S. Xu, W. Li, "High shear mixers: A review of typical applications and studies on power draw, flow pattern, energy dissipation and transfer properties", *Chem. Eng. Process. Process Intensif.*, 57, 25–41, 2012.

[74]   N. Anarjan, H. Mirhosseini, B. S. Baharin, C. P. Tan, "Effect of processing conditions on physicochemical properties of astaxanthin nanodispersions", *Food Chem.*, 123, 2, 477–483, 2010.

[75]   S. G. Gaikwad, A. B. Pandit, "Ultrasound emulsification: Effect of ultrasonic and physicochemical properties on dispersed phase volume and droplet size", *Ultrason. Sonochem.*, 15, 4, 554–563, 2008.

[76]   D. J. McClements, "Food emulsions: principles, practices, and techniques", *CRC press*, 2015.

[77]   Y. Yang, M. E. Leser, A. A. Sher, D. J. McClements, "Formation and stability of emulsions using a natural small molecule surfactant: Quillaja saponin (Q-Naturale®)", *Food Hydrocoll.*, 30, 2, 589–596, 2013.

[78]   U. Einhorn-Stoll, M. Weiss, H. Kunzek, "Influence of the emulsion components and preparation method on the laboratory-scale preparation of o/w emulsions containing different types of dispersed phases and/or emulsifiers", *Food/Nahrung*, 46, 4, 294–301, 2002.

[79]   M. Kharat, Z. Du, G. Zhang, D. J. McClements, "Physical and Chemical Stability of Curcumin in Aqueous Solutions and Emulsions: Impact of pH, Temperature, and Molecular Environment", *J. Agric. Food Chem.*, 65, 8, 1525–1532, 2017.

[80]   M. Juttulapa, S. Piriyaprasarth, P. Sriamornsak, "Effect of ph on stability of oil-in-water emulsions stabilized by pectin-zein complexes", *Advanced Materials Research*, 747, 127–130, 2013.

[81]   S. Pimentel-Moral, C. Rodríguez-Pérez, A. Segura-Carretero, A. Martínez-Férez, "Development and stability evaluation of water-in-edible oils emulsions formulated with the incorporation of hydrophilic Hibiscus sabdariffa extract", *Food Chem.*, 260, 200–207, 2018.

[82]   M. Akhtar, B. S. Murray, E. I. Afeisume, S. H. Khew, "Encapsulation of flavonoid in multiple emulsion using spinning disc reactor technology", *Food Hydrocoll.*, 34, 62–67, 2014.

[83]   A. Gomes, A. L. R. Costa, F. de Assis Perrechil, R. L. da Cunha, "Role of the phases composition on the incorporation of gallic acid in O/W and W/O emulsions", *J. Food Eng.*, 168, 205–214, 2016.

[84]   L. M. Diamante, T. Lan, "Absolute Viscosities of Vegetable Oils at Different Temperatures and Shear Rate Range of 64.5 to 4835 s^{-1}", *J. Food Process.*, 2014,

PhD Thesis

Chapter 4: Formulation and fabrication of single and double emulsions loaded with phenolic inner aqueous phase by application of high energy emulsification methods

234583, 2014.

[85]   H. Schubert, R. Engel, "Product and formulation engineering of emulsions", *Chem. Eng. Res. Des.*, 82, 9, 1137–1143, 2004.

[86]   H. Schubert, K. Ax, O. Behrend, "Product engineering of dispersed systems", *Trends Food Sci. Technol.*, 14, 1–2, 9–16, 2003.

[87]   A. S. Peshkovsky, S. L. Peshkovsky, S. Bystryak, "Scalable high-power ultrasonic technology for the production of translucent nanoemulsions", *Chem. Eng. Process. Process Intensif.*, 69, 77–82, 2013.

[88]   Q. Huang, H. Yu, Q. Ru, "Bioavailability and delivery of nutraceuticals using nanotechnology", *J. Food Sci.*, 75, 1, 50–57, 2010.

[89]   C. A. S. Rabelo, N. Taarji, N. Khalid, I. Kobayashi, M. Nakajima, M. A. Neves, "Formulation and characterization of water-in-oil nanoemulsions loaded with açaí berry anthocyanins: Insights of degradation kinetics and stability evaluation of anthocyanins and nanoemulsions", *Food Res. Int.*, 106, 542–548, 2018.

[90]   V. Sanchez, R. Baeza, J. Chirife, "Comparison of monomeric anthocyanins and colour stability of fresh, concentrate and freeze-dried encapsulated cherry juice stored at 38 C", *J. Berry Res.*, 5, 4, 243–251, 2015.

CONCLUSIONS

VALORIZATION OF RESIDUES GENERATED IN THE PRODUCTION OF OLIVE OIL THROUGH CLEAN TECHNOLOGIES

Conclusions

The current PhD Thesis collects several theoretical and experimental studies aimed on the valorization of olive mill wastes (OMW) using clean technologies. Considering that each chapter addresses different aspects and has its own conclusions, the general conclusions derived from these works, formulated under an integrative prism aligned with the proposed objectives, are presented below.

- Olive mill wastes generated in olive oil production process are a major environmental issue due to their high toxic organic load, low pH, and high chemical and biological demands. However, these wastes are a considerable source of valuable natural components such as phenolic compounds.

- The recovery of the remaining oil (olive pomace oil) present in olive mill solid residues can be performed by conventional solvent extraction or ultrasound-assisted extraction methods. It has two business aspects that can provide important economic benefits. Taking into account that the majority composition of olive pomace oil is formed by oleic acid (63-65%), linoleic acid (13.3-14.6%) and palmitic acid (12.9-13.1%), it could be used in cosmetic products and also in food products, in which case it must be refined. Furthermore, the solid waste obtained during the production of olive pomace oil can be used for the recovery of phenolic compounds through additional extraction and adsorption-desorption processes. This method provides the selective isolation of desired phenolic compounds like hydroxytyrosol, tyrosol or oleuropein for their further use in food and pharmaceutical applications.

- Ultrasound assisted extraction methods for phenolics recovery from olive mill solid residues were found to be technically feasible and constitutes a great opportunity for valorization of olive mill by-products. Phenolics extraction efficiency depends mainly on solid/solvent ratio.

- The development of an industrial scale-up of the extraction process from olive mill solid residues could yield less polluted environments and less toxicity for plants and soil.

- Although the adsorption process of phenolic compounds from olive extract depends mainly on adsorbent type, the efficiency of the desorption process depends largely on solvent type. As a future work, the effect of temperature and adsorbent/solvent ratio in desorption process should be further studied in order to enhance the desired isolation of phenolic compounds.

- Regarding pretreatment of olive mill wastewater (OMWW) before entering it to membrane-based treatment, the huge effect of acidification (adjustment to pH=2) on conversion of other polyphenols to hydroxytyrosol was observed.

- Membrane treatment process was found to be feasible to produce a purified permeate stream and a phenolic-rich retentate stream from OMWW. The obtained permeate could be used as irrigation water or even as drinking water in the event of being subjected to a reverse osmosis process. Furthermore, the selective recovery of the desired phenolic compounds in the retentate stream could be studied in the future by applying an adsorption-desorption process.

- Extracts obtained from the olive mill by-products treatment can be encapsulated in nanoemulsions to improve the stability and bioavailability of their phenolic molecules. Composition of phenolic-loaded nanoemulsions and operating conditions of the emulsification process were optimized in this work, but further studies *in vitro* and *in vivo* should be carried out to evaluate their stability and bioavailability.

Conclusiones

La presente Tesis Doctoral recoge diversos estudios teóricos y experimentales dirigidos a la valorización de los residuos de almazara (OMW) utilizando tecnologías limpias. Considerando que cada capítulo aborda aspectos diferentes y tiene sus propias conclusiones, a continuación se presentan las conclusiones generales derivadas de estos trabajos, formuladas bajo un prisma integrador alineado con los objetivos propuestos.

- Los residuos de almazara generados en el proceso de producción de aceite de oliva son un problema ambiental importante debido a su alta carga orgánica tóxica, bajo pH y alta demanda química y biológica. Sin embargo, estos residuos son una fuente importante de valiosos componentes naturales, como son los compuestos fenólicos.

- La recuperación del aceite residual (aceite de orujo de oliva) presente en los residuos sólidos de la almazara se puede realizar mediante métodos convencionales de extracción con disolventes o de extracción asistida por ultrasonidos. Esto tiene dos aspectos comerciales que pueden brindar importantes beneficios económicos. Teniendo en cuenta que la composición mayoritaria del aceite de orujo de oliva está formada por ácido oleico (63-65%), ácido linoleico (13,3-14,6%) y ácido palmítico (12,9-13,1%), se podría utilizar en productos cosméticos y también en productos alimentarios, en cuyo caso debe ser refinado. Además, los residuos sólidos obtenidos durante la producción de aceite de orujo de oliva se pueden utilizar para la recuperación de compuestos fenólicos mediante procesos adicionales de extracción y adsorción-desorción. Este método proporciona el aislamiento selectivo de compuestos fenólicos deseados, como hidroxitirosol, tirosol u oleuropeína, para su uso posterior en aplicaciones alimentarias y farmacéuticas.

- Se demostró que los métodos de extracción asistida por ultrasonidos para la recuperación de compuestos fenólicos a partir de los residuos sólidos de la almazara son técnicamente viables y constituyen una gran oportunidad para la

valorización de estos subproductos. La eficiencia de extracción de dichos compuestos fenólicos depende principalmente de la relación sólido/disolvente empleada en el proceso.

- El desarrollo a escala industrial del proceso de extracción de los residuos sólidos de las almazaras podría producir entornos menos contaminados y menor toxicidad para las plantas y el suelo.

- Aunque el proceso de adsorción de compuestos fenólicos del extracto de oliva depende principalmente del tipo de adsorbente, la eficiencia del proceso de desorción depende en gran medida del tipo de disolvente. Como trabajo futuro se propone un estudio más exhaustivo del efecto de la temperatura y la relación adsorbente/disolvente en el proceso de desorción para mejorar la obtención de los compuestos fenólicos deseados.

- En cuanto al pretratamiento de las aguas residuales de almazara (OMWW) antes de someterlas al tratamiento con membranas, se observó el importante efecto de la acidificación (pH = 2) sobre la conversión de otros polifenoles en hidroxitirosol.

- Se demostró la viabilidad técnica del tratamiento con membranas de las aguas residuales (OMWW) para obtener una corriente de permeado con baja carga orgánica y una corriente de retenido rica en compuestos fenólicos. El permeado obtenido podría utilizarse como agua de riego o incluso como agua potable en caso de ser sometido a un proceso de ósmosis inversa. Además, se podría estudiar en un futuro la recuperación selectiva de los compuestos fenólicos deseados en la corriente de retenido mediante la aplicación de un proceso de adsorción-desorción.

- Los extractos obtenidos en el tratamiento de los subproductos de la almazara pueden encapsularse en nanoemulsiones para mejorar la estabilidad y biodisponibilidad de las moléculas fenólicas que contienen. En este trabajo se optimizaron la composición de las nanoemulsiones cargadas con fenoles y las condiciones de operación del proceso de emulsificación, pero se deben realizar más estudios *in vitro* e *in vivo* para evaluar su estabilidad y biodisponibilidad.

Appendix: Analytical Methods

VALORIZATION OF RESIDUES GENERATED IN THE PRODUCTION OF OLIVE OIL THROUGH CLEAN TECHNOLOGIES

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Figures

1. Oil extraction with standard Soxhlet method

Solid-liquid extraction is a basic laboratory operation that consists of separating the components of a solid matrix soluble in an organic solvent. Both phases must come into intimate contact so that the solute or solutes can diffuse from the solid to the liquid phase, producing a separation of the original components of the solid. Two of the most common examples of solid-liquid extraction in food products are extraction of fat with organic solvents and extraction of sugar from beets with hot water. The BUCHI B-811 extraction system (Buchi Laboratotiums Tchnik AG, Flawil, Switzerland) is used for the extraction of liquid solid samples with solvents or solvent mixtures of known composition and the subsequent concentration or drying of the extract. Solvents with a boiling point between 30 ℃ and 150 ℃ can be used. Furthermore, there is the possibility of supplying inert gas for drying thermolabile extracts.

Description

Step1: Extraction

When heating and evaporating the solvent present in the vessels, it ascends to the refrigerant where it condenses and falls on the glass container that contains the sample. The glass tube with the sample, placed in the solvent chamber, is covered with the condensed solvent. When the solvent level reaches the photoelectric cell, the glass valve opens, allowing the passage of the solvent to the lower vessel. This operation is repeated during the preset time and cycles, the number of which defines the duration of the extraction process.

Step 2: washing

At this stage the glass valve remains open, so the evaporating solvent rises to the refrigerant, where it condenses and descends, washing the area where the sample is placed and the solvent chamber as it passes.

Step 3: drying

At this stage the glass valve remains closed so that the solvent does not fall on the solvent cup but is retained in the solvent chamber. It is a stage in which the heating power is lower to avoid heating the extract obtained that remains in the solvent beaker. The "inert gas" option allows minimizing the thermal degradation of the extract during this stage.

In this study, BUCHI B-811 was used for soxhlet process. This extraction system has 4 chambers of extraction which in our study, Ground olive mill solid waste (cake or pomace) about 19 grams in total (4.7 grams in each chamber of extraction) was placed in the Soxhlet apparatus and continuously refluxed with 360 mL (90 ml for each chamber) of n-hexane for 3 hours. After extraction, n-hexane was distilled using the same extraction system. The obtained oil was weighted and the recovery yield was calculated using Eq. (1):

$$\text{Yield} = \frac{\text{Oil extract obtained (g)}}{\text{Amount of olive pomance used (g)}} \times 100 \tag{1}$$

Figure A.1. BUCHI B-811 extraction system

2. *Analysis of fatty acid profile by Gas chromatography (GC-FID)*

GC analysis for the extracted oil from each extraction process was performed. The fatty acid content is determined by the official method of the AOAC [1] which is based on the breakdown of all glycerides and subsequent derivatization to methyl esters of fatty acids. These methyl esters are analyzed by gas chromatography (Agilent 6890N GC system) (Figure A-2) with flame ionization detector (FID) and helium as carrier gas. A capillary column OmegawaxTM-320, 30m x 0.32mm is used. For the identification, standard substances (methyl esters) were used and for the quantification, the methyl-tricosanoate (C23:0) was used as internal standard. For the derivatization the AOAC method is followed, but in the part corresponding to the chromatographic analysis some small modifications have been made.

Separation of compounds was carried out in a column (Omegawax-320, 30m x 0,32mm) using helium as the carrier gas (1.8 mL/min). The chromatographic conditions were as follows: initial temperature: 180 °C; temperature rate: $5°C. \min^{-1}$ up to 220 °C, which was maintained for 15 min; injector temperature: 250 °C; injection: 1μl; transfer-line temperature, 250 °C; split ratio: 1:50. Preparation of all samples for gas chromatography were performed with one replicates.

Reagents

- Isooctane solution of Methyl tricosanoate (C23:0) (1 g/mL), used as internal standard. It was by accurately weighting 25 mg ($\pm$ 0.1 mg) of C23:0 Methyl ester in a 25 mL volumetric flask and diluting with isooctane.
- Boron trifluoride (BF_3) 12% in methanol
- Sodium Hydroxide (NaOH) 0.5 M in Methanol, prepared by dissolving 2.0 g NaOH in 100 mL methanol.
- FAMEs standards, dissolved in isooctane
- Sodium chloride (NaCl) saturated solution, prepared by dissolving 36 g NaCl in 100 mL distilled water.

Figure A.2.   Gas Chromatography equipment (GC-FID)

Sample preparation

Accurately, 1 mL of I.S. Solution was added into a glass tube. Then, 25 mg (± 0.1 mg) of extracted oil from olive mill solid wastes was weighted into the glass tube containing the I.S and 1.5 mL of NaOH-MeOH solution was added. The glass tube was capped and heated 5 minutes in a water bath at 100 °C. Then it was cooled down and 2 mL BF$_3$-MeOH was added. After mixing vigorously, the glass tube was again heated in the water bath at 100 °C for 30 minutes. Subsequently, the mixture was cooled, diluted with 1 mL of isooctane and then mixed in a vortex for 30 seconds. Immediately the mixture was diluted in 5 mL of saturated NaCl solution, mixed and left to stand for phase separation. What phases were separated completely, the upper organic layer was transferred into a glass vial for autosampler injection in the GC column.

Quantification of fatty acids

The amount of each fatty acid in the sample is quantified according to Eq. (2), in which A_x is the area counts for each fatty acid (FA); $A_{I.S.}$ is the area counts of internal standard; $W_{I.S.}$ and W_{sample} are the I.S. and sample weights in mg, respectively; a_x is the slope of the calibration curve of each fatty acid methyl ester (FAME) standard; and $CF_{FAME-FFA}$ is the ratio between molecular weight of each FAME and that of corresponding FFA in each case, which is necessary to express result as mg of fatty acid (FA)/ g oil rather than as FAME.

$$\text{mg }_{FA}/\text{ g }_{oil} = \frac{A_x \cdot W_{I.S.}}{A_{I.S.} \cdot W_{sample} \cdot a_x \cdot CF_{FAME-FFA}} \times 1000 \tag{2}$$

3. Characterization of phenolic contents

Polyphenols account for the antioxidant richness of most of the foods usually consumed by the population. All polyphenols have at least one or more hydroxyl groups attached to an aromatic ring in their structure. Among the polyphenols it is possible to distinguish two major types of compounds: the flavonoids, for which several thousand have been described in the plant kingdom, and whose structure comprises two aromatic rings linked by a heterocycle of three carbon atoms and one of oxygen (C6- C3-C6), and the so-called non-flavonoids (some hundreds) that comprise, mainly, mono-phenolic alcohols, phenolic acids and stilbenes.

They are bioactive compounds with antioxidant capacity that have aroused great interest from a nutritional point of view, for their actions not only in health, but in the prevention of functional and structural changes in various diseases. In recent years, they have been attributed beneficial effects against the development of various diseases (cancer, cardiovascular diseases and neurodegenerative diseases) associated with increased cellular oxidation processes, known as oxidative stress.

3.1 Determination of Total Phenolic Content by Folin-Ciocalateu method

Basis

Total phenolic content (TPC) for each olive mill sample (solid and liquid) was measured using Folin-Ciocalateu standard method [2] with some modifications. The Folin Ciocalteau method is based on an oxidation-reduction reaction between the Folin-Ciocalteu reagent and the polyphenols present in the sample. Reduction of Folin reagent is based on the following pattern: $Mo\ (IV) + e^- \rightarrow Mo\ (V)$. It is reduced to a complex mixture of blue compounds. The absorbance at 750 nm of the blue color formed is measured, since the maximum absorbance is around that wavelength. The absorbance obtained is directly proportional to the concentration of polyphenols in the sample. Although the reagent reduction test is simple, sensitive and precise, it should be noted that said reagent is capable of being reduced not only by all polyphenols, but also by reducers such as ascorbic acid, sodium metabisulfite, iron (II) salts, EDTA, certain amino acids, fructose and glucose, among others. The latter is extremely important since when this method is applied to samples containing one or more of said interferers, without taking corrective measures, a result will be erroneously obtained that represents an over-estimation of the real value of total polyphenols. In these cases, the appropriate controls must be incorporated, using modifications to the original method that allow analytical discrimination between the contribution made to the reduction of said reagent by the polyphenols to be measured and those non-polyphenolic "interfering" components present in a sample.

Reagents

- Folin Ciocalteau: Commercial reagent, mixture of phosphotungstanate and phosphomolybdate (kept at 4°C)
- Olive mill sample (the extract obtained from solid residues or wastewater)
- Sodium Carbonate (Na_2CO_3) 7.5% (w/v) dissolved in distilled water
- Distilled water

Development

A standard calibration curve ($R^2 = 0.99$) was prepared using gallic acid solution (50mg/50ml). 100 µl of olive mill samples containing phenolic compounds was added to a test tube. Then 2.8 ml of distilled water, 2 ml of 7.5% sodium carbonate, 100 µl Folin-Ciocalteu reagent added to the test tube. Contents were mixed and after 60 minutes in the dark at room temperature, the absorbance was measured spectrophotomertrically at 750 nm (Hitachi U-2000 spectrophotometer) (Figure A-3). In this thesis, TPC was expressed as milligrams of Gallic acid equivalents per grams of olive mill solid residues (mg/g) or as mg of Gallic acid per liter of olive mill wastewater (mg/L).

Figure A.3. Spectrophotometry equipment (Hitachi U2000)

3.2 Identification of individual polyphenols by HPLC / DAD

The identification and quantification of individual polyphenols was carried out by reverse phase HPLC at various wavelengths (280 and 330 nm). Analytical-scale HPLC analysis of the olive extracts and fractions were performed with a High

Efficiency Liquid Chromatograph (HPLC-DAD Agilent 1100, CA, USA) equipped with a Kinetex®5 μm Biphenyl 100 Å column of 250 × 4.6 mm (Phenomenex, Inc. CA, U.S.A.). Separation was achieved, using a linear gradient of two solvents: solvent A (ammonium acetate 5 mol/m^3 with 1% (v/v) acetic acid in water) and solvent B (ammonium acetate 5 mol/m^3 with 1% (v/v) acetic acid in acetonitrile). A linear gradient was run at 25 °C with flow rate of 0.8 ml/min from 2% B in 7 min, from 2% to 8% B in 20 min, from 8% to 10% B in 35 min, from 10% to 18% B in 55 min, from 18% to 38% B in 65 min, from 38% to 65% B in 75 min and from 65% to 80% B in 80 min. the injection volume was 10-100μl depending on the sample content. All the tests were performed in duplicate and the results were averaged. The evaluation of each phenolic compound was based on comparison of peak sequence and intensity with standard solutions. Regarding liquid samples (olive mill wastewater), results were expressed as milligrams of compound per liter of OMWW (mg/L), while for dried and defatted olive mill solid wastes (cake and pomace) results were expressed as milligrams of compound per grams of olive mill sample (mg/g).


Figure A.4. HPLC-DAD Agilent 1100 equipment

3.3 Determination of Total Flavonoid Content

The objective of this method is to determine the flavonoid content using a colorimetric method with aluminum chloride. Total Flavonoids Content (TFC) for olive mill solid wastes (residues obtained from two-phase and three-phase oil extraction) extract was measured using method prepared by Chang et al. (2002) [3]. Flavonoids are secondary metabolites of plants that are synthesized from a phenylalanine molecule and 3 from malonyl-coA, forming a cyclized base structure (Figure A-5) thanks to the action of an enzyme isomerase. Later this structure can undergo many modifications and additions of functional groups, so there is a great variety of compounds within this family. The main classes are: flavones, flavonols, flavonones, isoflavones, anthocyanins, and chaconas. Foods generally contain a complex mixture of flavonoids in their composition. Their functions in vegetables are varied, from being responsible for pigmentation to acting as chemical signaling agents and modulators of enzyme activity. Numerous studies support the beneficial biological properties of flavonoids, mainly due to their antioxidant properties.
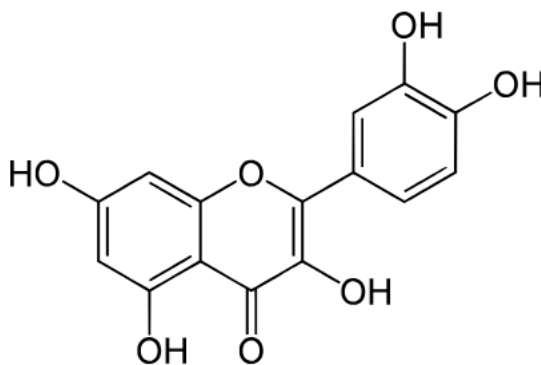
Figure A.5.   Quercetin molecule - Example of flavonoid structure

Principle

415 nm is established as the measurement wavelength, since it is where these colored compounds have an absorption maximum. The measurement is proportional to the amount of flavonoids in the sample. The groups of flavonoids

that mainly establish colored complexes with aluminum chloride are flavones and flavonols.

Reagents

Following materials with analytical grade were used.

- Flavonoid standard (In this study quercetin was used as standard)
- 100% ethanol
- Aluminum chloride 10% (Accurately, 5g of $AlCl_3$ was taken and made up to 50 ml in a volumetric flask by mixing with distilled water under an extractor hood)
- 1M potassium acetate (4.907 g of potassium acetate (CH_3COOK) dilute by mixing with 50 mL of distilled water).
- Distilled water

Preparation of the standard curve

Quercetin standard solutions were made at different concentrations between approximately 10 and 500 µg/mL in ethanol-water solution with 1:1 ratio (v/v) which was the basic solvent for phenolic extraction from olive mill wastes. Subsequently, a standard calibration curve ($R^2 = 0.99$) was prepared using quercetine solution.

Preparation of samples

500 µl of olive extracts (sample) was added to a test tube. Then 2.8 ml of distilled water, 1.5 ml of ethanol 95%, 100 µl of $AlCl_3$ 10% and 100 µl of potassium acetate (CH_3CO_2K) 0.1M added to the test tube. Contents were mixed and after 30 minutes in the dark at room temperature, the absorbance was measured spectrophotomertrically at 415 nm (Hitachi U-2000 spectrophotometer) (Figure A-3). TFC was expressed as milligrams of quercetin equivalents per grams of olive pomace (mg/g).

3.4 Determination of Antioxidant Activity by DPPH assay

Definition

The purpose of this method is to determine the antioxidant capacity of the products obtained according to different extraction methods. Antioxidant Activity (AA) of the olive mill extracts was measured by following DPPH method described by Brand-William et al. (1994); Shen et al. (2010) ([4,5]). An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation reactions can produce free radicals that start chain reactions that damage cells. Antioxidants terminate these reactions by removing intermediates from the free radical and inhibit other oxidation reactions by oxidizing themselves. Therefore, antioxidants are often reducing agents such as thiols or polyphenols.

From a chemical point of view, a free radical is any species (atom, molecule or ion) that contains at least one unpaired electron in its outermost orbital, and which in turn is capable of existing independently (hence the free term). The presence of an "unpaired" electron in the outermost orbital of an atom gives the latter an increased ability to react with other atoms and / or molecules present in its environment, normally lipids, proteins and nucleic acids. The interaction between free radicals and said substrates gives rise to alterations in the structural, and eventually functional, properties of the latter.

This method takes its name from the base radical of the reaction, DPPH (2,2-diphenyyl-1-picrylhydrazyl free radical). The basic reaction mechanism in the DPPH method is electron transfer. In the presence of an antioxidant or radical agent, the absorbance value of the original reagent (DPPH) decreases.

Reagents

- Methanol (analytical grade)
- The solution 50.7 µM of DPPH (dissolving 0.0010 g in 50 ml methanol) was prepared. Then the solution was stored in dark at 4°C for at least 4 hours after preparation. This reagent was then kept covered with aluminum foil and in the dark. The absorbance value of the reagent is approximately 0.7.

Preparation of the sample

As mentioned earlier, solvent used for olive mill solid wastes was ethanol-water solution with 1:1 (v/v) ratio while regarding olive mill liquid wastewater (OMWW), distilled water was considered as solvent.

In order to perform DPPH assay, an amount of 60 µl of sample extract with 2940 µl of DPPH reagent solution were mixed and for preparing the control solution, 60 µl of the solvent with 2940 µl of DPPH reagent solution were mixed. The mixtures were allowed to stand in the dark at room temperature for 60 minutes. The absorbance of the samples were measured at 517 nm using a UV-VIS spectrophotometer model (Hitachi U-2000 spectrophotometer) (Figure A-3). Antioxidant activity was expressed as millimoles of Trolox equivalents per grams of olive pomace (mM_{Trolox}/g) or per liter of OMWW (mM_{Trolox}/L).

Calibration curve

It was made with trolox as standard under the same conditions as the samples (in the same solvent). In the calibration curve, the concentration of trolox as in the form of molarity (250.29 g /mol) is indicated on the X axis and on the Y axis, the difference in absorbance (Ar-Am) was placed. In this study, a standard calibration curve ($R^2 = 0.995$) was prepared using Trolox solution.

Expression of results

For the calculation of antioxidant capacity, the following expression is taken into account:

$$Absorbance\ difference = A_r - (A_m - A_0) \tag{3}$$

Where A_r = absorbance of reagent; A_m= absorbance of the sample; A_0 = absorbance of the blank made with the sample and the solvent used for the extraction. The value of the difference in absorbance is taken to the calibration curve. Also the capability of scavenging the DPPH radical was calculated by using the Eq. (4):

$$DPPH\ scavenging\ effect\ (\%\ inhibition) = \{(A_0 - A_1)/A_0 \times 100\} \tag{4}$$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the olive extract samples and reference. All the tests in this study were performed in duplicate and the results were averaged.

4. *Characterization of olive mill wastewater*

4.1 *Determination of Chemical Oxygen Demand (COD)*

Chemical oxygen demand (COD) is a parameter that could be useful for evaluation of waste biodegradability and also for evaluating the mass or the energetic balance of the process. The COD value provides relevant information about the efficiency of the AD process [6,7]. COD is defined as the amount of oxygen required to oxidize an organic compound to carbon dioxide, ammonia, and water, and it is used as one of the main water quality parameters in wastewater treatment facilities [8].

In this research, Analysis of COD for raw and treated olive mill wastewater (OMWW) samples was performed in accordance of the protocols described in the Standard method for examination of water and wastewater [9]. Briefly, OMWW samples were diluted with ratios 1:50 and 1:100 depending on their prior treatment. Then 5 mL of diluted sample were poured in a glass tube along with 3 mL of $k_2Cr_2O_7$ and 7mL of H_2SO_4. Also a Blank and title sample were prepared by using distilled water instead of OMWW. The mixtures in all glass tubes were sealed and heated at 160 °C for at least two hours except for Title sample. After getting cold and adding ferroin as indicator, contents of each glass tube were titrated with Standard Ferrous Ammonium Sulfate titrant (FAS). COD value was measured by the application based on Eq. (5):

$$COD = f \times \frac{(B-M)}{T} \times D.F \qquad (5)$$

Where f equals to 960 and D.F is dilution factor. Also B, T and M are consumed volume in titration process for blank, title and OMWW samples, respectively. All samples in this COD analysis were prepared with two replicates.

4.2 Light scattering methods

4.2.1 Particle size measurement

Particle size and particle size distribution in this research was determined via laser diffraction (LD) method according to official method proposed by ISO standards [10]. This technique is based on the fact that particles passing through a laser beam are able to scatter light at a certain angle. The scattered angle increases logarithmically as particle size decreases, whereas the intensity of scattered light decreases with the cross-sectional area of particle (Figure A-6).
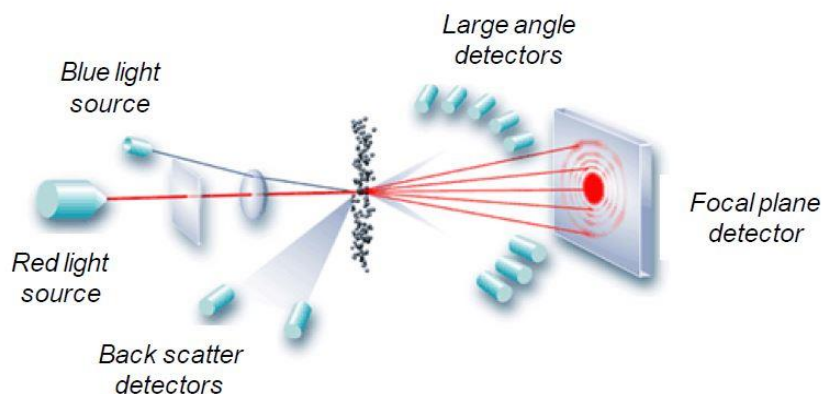
Figure A.6. Particle size analysis by laser diffraction (adopted from www.malvern.com)

Part of particle size measurements of this study was measured using a laser light scattering system (Malvern Mastersizer 2000/2000E, Malvern Instruments) which is shown in Figure A-7. The mean particle size was expressed as the volume weighted mean diameter (D[4,3]) based on Eq. (6).

$$D[4,3] = \frac{\sum D_i^4 n_i}{\sum D_i^3 n_i} \tag{6}$$

The volume weighted mean diameter is most sensitive to the presence of large particles. The width of particle size distribution was expressed as a Span number, calculated by following Eq. (7):

$$\text{Span} = \frac{D(0.9) - D(0.1)}{D(0.5)} \tag{7}$$

where D(0.9) is the particle diameter which in this study signifies the point in the size distribution, up to and including which, 90% of the total number of particles in the sample is contained; The definition for D(0.5), is then the size point below which 50% of the material is contained, this value is also known as the mass median diameter. And the D(0.1) is that size below which 10% of the particles are contained.

The mean particle diameter and span values were calculated on the basis of five different experimental runs. In this study, a comprehensive process of light scattering methods were performed on raw and pretreated samples of olive mill wastewater in order to study the effect of processes like acidification and centrifugation and filtration on particle size distribution.

Figure A.7. Malvern Mastersizer 2000 apparatus with hydra 2000SM dispersion unit

4.2.2   Zeta potential and Nanosizing measurement

A typical dynamic light scattering system comprises of six main components. Firstly, a laser (1) provides a light source to illuminate the sample contained in a cell (2). For dilute concentrations, most of the laser beam passes through the sample, but some is scattered by the particles within the sample at all angles. A detector (3) is used to measure the scattered light. In the Zetasizer Nano series, the detector position will be at either 173° or 90°, depending upon the particular model. The intensity of scattered light must be within a specific range for the detector to successfully measure it. If too much light is detected, then the detector will become saturated. To overcome this, an attenuator (4) is used to reduce the intensity of the laser source and hence reduce the intensity of scattering. For samples that do not scatter much light, such as very small particles or samples of low concentration, the amount of scattered light must be increased. In this situation, the attenuator will allow more laser light through to the sample.

For samples that scatter more light, such as large particles or samples at higher concentration, the intensity of scattered light must be decreased. The appropriate attenuator position is automatically determined by the Nano software and covers a transmission range of 100% to 0.0003% [11].

In current thesis, Droplet size distribution, mean droplet diameter and polydispersity index (PDI) of OMWW samples in nanoscale were measured by dynamic light scattering (DLS), using a Zetasizer Nano ZS apparatus (Malvern Instruments Ltd., UK) (Figure A-8). The apparatus is equipped with a He–Ne laser emitting at 633 nm and with a 4.0mW power source. The instrument uses a backscattering configuration where detection is done at a scattering angle of 173°. Samples were first diluted 1:10 to avoid multiple scattering effects and then 2 ml samples were poured into DTS0012 square disposable polystyrene cuvettes. Measurements were performed at 20°C. The hydrodynamic diameter was calculated, using the Stokes–Einstein equation with the assumption that the particles were monodisperse spheres.

Zeta potential (ζ) was also measured using the mentioned Zetasizer Nano ZS apparatus. The measurement was conducted for each diluted sample at 20 °C, using

DTS1061 disposable folded capillary cells. The Zeta potential (ζ), was calculated in an applied electric field, using the Smoluchowski approximation.

Figure A.8. Malvern Zetasizer Nano ZS apparatus

4.2.3 Physical stability evaluation

Physical stability of olive mill wastewater samples was measured in terms of their droplet growth ratio using a Turbiscan Lab Expert equipment (Formulaction Co., L'Union, France) (Figure A-9) by static multiple light scattering (MLS), sending a light beam from an electroluminescent diode ($\lambda = 880$ nm) through a cylindrical glass cell containing the sample. Since acidified samples tend to aggregate during storage, the droplet size obtained in this work was measured at 4 days at the bottom of the cell containing them. Three different levels of pH (1.5, 3 and 4.9) were prepared.

stability analysis was done for the sample without dilution which was placed in a cylindrical glass cell and two synchronous optical sensors received the light transmitted through the sample (180° from the incident light) and the light backscattered by the droplets in the sample (45° from the incident light). The

optical reading head scans the height of the sample in the cell (about 40 mm), by acquiring transmission and backscattering data every 40 µm. Transmitted and backscattered light were monitored as a function of time and cell height for 4 days at 25 °C.

Figure A.9.   Turbiscan Lab Expert equipment

5.   *Characterization of single (W/O) and double (W/O/W) emulsions*

In current thesis, characterization process of encapsulated phenolic compounds within W/O and W/O/W emulsions was performed by the executing following analysis:

- Droplet size diameter and polydispersity index (PDI) determination
- Zeta potential (ζ) measurement
- Total phenolic content (Folin-Ciocalateu method)
- Antioxidant activity (DPPH assay)
- Physical stability measurement
- Chemical stability measurement

Except for stability measurements, rest of the above mentioned analysis including their related equipment were similar to the processes performed for previous analysis which are completely described earlier in sections 3.1, 3.4 and 4.2.2 of this Appendix.

5.1 Chemical stability measurements

The percentage of phenolic compounds held within the aqueous phase after several days of storage at room temperature was measured following the method proposed by Regan and Mulvihill [12] (30 days for W/O emulsion and 35 days for W/O/W emulsion). Thus, 3 g of optimal emulsions were mixed with 3 g of phosphate buffer solution (pH 7) and centrifuged (Eppendorf 5804 centrifuge) at 4500 rpm for 90 min. Then, the lower phase was collected carefully for total phenolic content (TPC) and antioxidant activity (AA) analysis. The percentage of encapsulated compounds was identified by using the following equation:

$$E\ (\%) = \left(1 - \frac{c_2}{c_1}\right) \times 100 \tag{8}$$

where C_2 is the concentration of phenolic compounds found in the aqueous phase after centrifugation and C_1 is the initial concentration of phenolic compounds in the inner aqueous phase [12, 13].

5.2 Physical stability measurements

Stability of W/O and W/O/W emulsions was measured in terms of their droplet growth ratio. Since emulsions tend to aggregate during storage, the droplet size of the emulsions was measured after 1 day and several days after preparation (30 days for W/O emulsion and 35 days for W/O/W emulsion). Two different storage conditions were evaluated: 4 °C and room temperature in darkness. Furthermore, optical characterization of the optimal double emulsion was done by static multiple light scattering (S-MLS) using a Turbiscan Lab Expert equipment (Formulaction Co., L'Union, France) (Figure A-9). As described earlier, the apparatus send a light beam from an electroluminescent diode ($\lambda = 880$ nm) through a cylindrical glass

cell containing the sample. The emulsion sample (20 mL) without dilution was placed in a cylindrical glass cell and two synchronous optical sensors received the light transmitted through the sample (180° from the incident light) and the light backscattered by the droplets in the sample (45° from the incident light). The optical reading head scans the height of the sample in the cell (about 40 mm), by acquiring transmission and backscattering data every 40 μm. Transmitted and backscattered light were monitored as a function of time and cell height at 25 °C [14,15].

References

[1]   AOAC International, "AOAC Official Method 2012.13: Determination of Labeled Fatty Acids Content in Milk Products and Infant Formula", *AOAC Int.*, 1–9, 2012.

[2]   R. W. Owen, W. Mier, A. Giacosa, W. E. Hull, B. Spiegelhalder, "Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene", *Food Chem. Toxicol.*, vol. 38, no. 8, pp. 647–659, 2000.

[3]   C. C. Chang, M. H. Yang, H. M. Wen, J. C. Chern, "Estimation of Total Flavonoid Content in Propolis by Two Complementary Colorimetric Methods", *J. Food Drug Anal.*, 10, 178–182, 2002.

[4]   W. Brand-Williams, M. E. Cuvelier, C. Berset, "Use of a free radical method to evaluate antioxidant activity", *LWT - Food Sci. Technol.*, 28, 1, 25–30, 1995.

[5]   Q. Shen, B. Zhang, R. Xu, Y. Wang, X. Ding, P. Li, "Antioxidant activity in vitro of the selenium-contained protein from the Se-enriched Bifidobacterium animalis 01", *Anaerobe*, 16, 4, 380–386, 2010.

[6]   L. André, A. Pauss, T. Ribeiro, "A modified method for COD determination of solid waste, using a commercial COD kit and an adapted disposable weighing support", *Bioprocess Biosyst. Eng.*, 40, 3, 473–478, 2017.

[7]   C. M. W. Harnadek, N. G. H. Guilford, E. A. Edwards, "Chemical oxygen demand analysis of anaerobic digester contents", *STEM Fellowsh. J.*, 1, 2, 2–5, 2016.

[8]   G. Cazaudehore, B. Schraauwers, C. Peyrelasse, C. Lagnet, F. Monlau, "Determination of chemical oxygen demand of agricultural wastes by combining acid hydrolysis and commercial COD kit analysis", *J. Environ. Manage.*, 250, 109464, 2019.

[9]   I. V. Carranzo, "Standard Methods for examination of water and wastewater",

APHA, AWWA, WEF, 5, 2, 185–186, 2012.

[10]   ISO 10426-5, "International Standard International Standard", *61010-1 © Iec2001*, vol. 2006, p. 13, 2006.

[11]   Malvern Instruments, "Technical Note: Dynamic Light Scattering", pp. 1–8, 2001.

[12]   J. O. Regan, D. M. Mulvihill, "Water soluble inner aqueous phase markers as indicators of the encapsulation properties of water-in-oil-in-water emulsions stabilized with sodium caseinate", *Food Hydrocoll.*, 23, 8, 2339–2345, 2009.

[13]   A. Mohammadi, S. Mahdi, E. Assadpour, A. Faridi, "International Journal of Biological Macromolecules Nano-encapsulation of olive leaf phenolic compounds through WPC – pectin complexes and evaluating their release rate", *Int. J. Biol. Macromol.*, 82, 816–822, 2016.

[14]   S. Rebolleda, M. T. Sanz, J. M. Benito, S. Beltrán, I. Escudero, M. L. González San-José, "Formulation and characterisation of wheat bran oil-in-water nanoemulsions", *Food Chem.*, 167, 16–23, 2015.

[15]   D. Allende, Á. Cambiella, J. M. Benito, C. Pazos, J. Coca, "Destabilization-enhanced centrifugation of metalworking oil-in-water emulsions: effect of demulsifying agents", *Chem. Eng. Technol. Ind. Chem. Equipment-Process Eng.*, 31, 7, 1007–1014, 2008.