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Y CIENCIA DE LOS ALIMENTOS**

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Valorization of plant biomass by emerging technologies: olive and moringa leaves

PhD Thesis

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Burgos, 2021

Valorization of plant biomass by emerging technologies: olive and moringa leaves

Valorización de la biomasa vegetal por tecnologías emergentes:

hojas de olivo y moringa

Memoria que para optar al grado de doctor por la Universidad de Burgos en el programa de Avances en Ciencia y Biotecnología Alimentarias presenta:

Mansoore Kashaninejad

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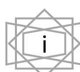

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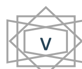

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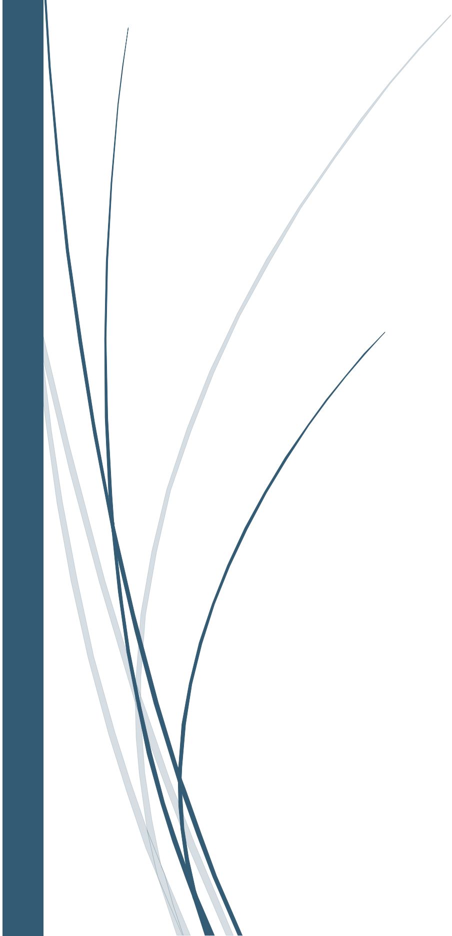
Summary

Valorization of plant biomass by emerging technologies: olive and moringa leaves



Summary of the PhD Thesis

Olive (*Olea europea L.*) leaves have been used for food (herbal teas), pharmacological, cosmetic and medicinal applications from ancient times. Olive trees can live hundreds of years and olive leaves have been historically used as a folk remedy for combating fevers and other diseases, such as malaria. The low incidence of cardiovascular diseases and cancer in Mediterranean countries has drawn the attention of investigators, and the Mediterranean diet consisting of olives and olive products has been thought to be one reason of this. Olive leaves, as one of the "olive by-products", can be found in large amounts in olive oil industries. Similar to other parts of the olive tree, olive leaves obtained during the harvesting of olive fruits, also contain considerable biophenols. Olive leaves are obtained in large quantities during the cultivation of olive trees and are prominent in the olive oil industry (10% of the total weight of harvested olives) and they also accumulate in large quantities during tree pruning.

Polyphenols contained in olive leaves have played an important role to this end, because they have demonstrated to be responsible for their anti-carcinogenic, anti-inflammatory, and antimicrobial properties. From a food waste recovery perspective, the usage of olive leaves is of great interest as they are a great source of nutritionally and bioactive valuable compounds such as polyphenols, volatile compounds, phytosterols, fatty acids, coloring pigments (carotenoids and chlorophylls), tocopherols, and squalene, which can be used as potential food additives and/or nutraceuticals.

On the other hand, *Moringa oleifera* belonging to the family of Moringaceae is an effective remedy for malnutrition. Moringa is rich in nutrition owing to the presence of a variety of essential phytochemicals present in its leaves, pods, and seeds. In fact, moringa is said to provide 7 times more vitamin C than oranges, 10 times more vitamin A than carrots, 17 times more calcium than milk, 9 times more protein than yogurt, 15 times more potassium than bananas, and 25 times more iron than spinach. *M.oleifera* benefits could also be attributed to their composition of phenolic compounds. The search for new antioxidants and phenolics from herbal sources has garnered great attention in the last decade. In this regard, leaf extracts of *M. oleifera* have been reported to exhibit antioxidant activity both *in vitro* and *in vivo*.

The determination of polyphenols in plants has been of great interest because of the natural antioxidant activity of these compounds. Antioxidants can delay or prevent oxidation of cellular oxidative substrates and hence considered very important additives in food processing. Bioactive compounds inside plant materials can be extracted by various classical extraction techniques. Most of these techniques are based on the extracting power of different solvents in use and the application of heat and/or mixing. The selection of a separation method is based on the identification of a suitable characteristic property, whose variation should be important for the component(s) to be separated.

The general objective of this PhD Thesis is characterization and valorization of olive and moringa leaves in terms of bioactive compounds recovery and purification by using emerging clean technologies. For this purpose, comprehensive characterization of olive and moringa leaves were performed primarily. Subsequently, multiple extraction experiments by using conventional, ultrasound-assisted, and pressurized liquid extraction methods with different levels of operating conditions were implemented. Furthermore, solid-liquid adsorption tests were performed on the optimized extracts. 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, olive leaf extracts were processed with membrane based filtrations in order to evaluate the efficiency of membrane technology on the separation of desired valuable compounds available in the phenolic and sugar profile of the extracts. Pressure-driven membrane processes are based on the use of a permselective membrane to achieve a selective transport of fluids and solutes under a hydrostatic pressure difference applied between the two sides of the membrane.

Resumen de la Tesis Doctoral

Las hojas de olivo (*Olea europea L.*) se han utilizado para aplicaciones alimentarias (infusiones), farmacológicas, cosméticas y medicinales desde la antigüedad. Los olivos pueden vivir cientos de años y las hojas de olivo se han utilizado históricamente como remedio popular para combatir la fiebre y otras enfermedades, como la malaria. La baja incidencia de enfermedades cardiovasculares y cáncer en los países mediterráneos ha llamado la atención de los investigadores, y se cree que la dieta mediterránea que consiste en aceitunas y productos de la aceituna es una de las razones. Las hojas de olivo, como uno de los "subproductos de la aceituna", se pueden encontrar en grandes cantidades en las industrias del aceite de oliva. Al igual que en otras partes del olivo, las hojas de olivo obtenidas durante la recolección de los frutos del olivo también contienen una cantidad considerable de biofenoles. Las hojas de olivo se obtienen en grandes cantidades durante el cultivo del olivo y son destacadas en la industria del aceite de oliva (10% del peso total de aceitunas recolectadas) y también se acumulan en grandes cantidades durante la poda de árboles.

Los polifenoles contenidos en las hojas de olivo han jugado un papel importante para este fin, porque han demostrado ser responsables de sus propiedades anticancerígenas, antiinflamatorias y antimicrobianas. Desde la perspectiva de la recuperación de residuos alimentarios, el uso de hojas de olivo es de gran interés, ya que son una gran fuente de compuestos valiosos nutricionalmente y bioactivos como polifenoles, compuestos volátiles, fitoesteroles, ácidos grasos, pigmentos colorantes (carotenoides y clorofilas), tocoferoles y escualeno, que se puede utilizar como posibles aditivos alimentarios y/o nutracéuticos.

Por otro lado, *Moringa oleifera* perteneciente a la familia de las Moringaceae es un remedio eficaz para la desnutrición. La moringa es rica en nutrientes debido a la presencia de una variedad de fitoquímicos esenciales presentes en sus hojas, vainas y semillas. De hecho, se dice que la moringa proporciona 7 veces más vitamina C que las naranjas, 10 veces más vitamina A que las zanahorias, 17 veces más calcio que la leche, 9 veces más proteínas que el yogur, 15 veces más potasio que los plátanos y 25 veces más hierro que la espinaca. Los beneficios de M.oleifera también podrían atribuirse a

su composición de compuestos fenólicos. La búsqueda de nuevos antioxidantes y fenólicos de fuentes herbales ha atraído gran atención en la última década. A este respecto, se ha informado que los extractos de hojas de M. oleifera exhiben actividad antioxidante tanto in vitro como in vivo.

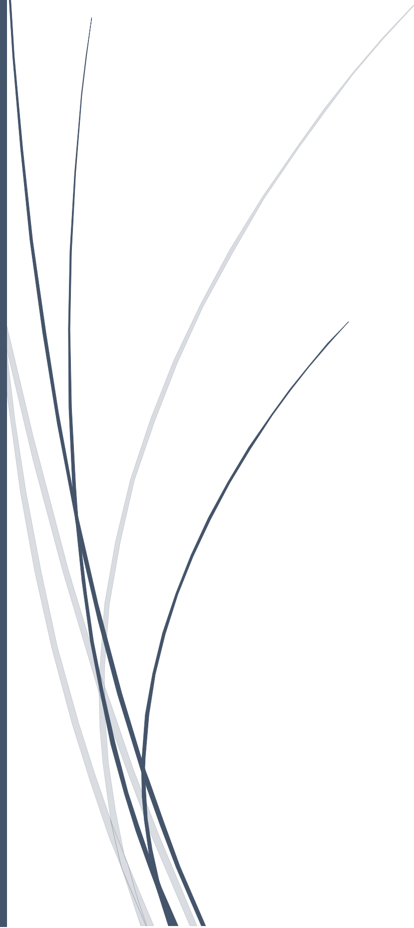
La determinación de polifenoles en plantas ha sido de gran interés debido a la actividad antioxidante natural de estos compuestos. Los antioxidantes pueden retrasar o prevenir la oxidación de sustratos oxidativos celulares y, por lo tanto, se consideran aditivos muy importantes en el procesamiento de alimentos. Los compuestos bioactivos del interior de los materiales vegetales se pueden extraer mediante diversas técnicas de extracción clásicas. La mayoría de estas técnicas se basan en el poder de extracción de diferentes disolventes en uso y la aplicación de calor y/o mezcla. La selección de un método de separación se basa en la identificación de una propiedad característica adecuada, cuya variación debe ser importante para los componentes a separar.

El objetivo general de esta Tesis Doctoral es la caracterización y valorización de hojas de olivo y moringa en términos de recuperación y depuración de compuestos bioactivos mediante el uso de tecnologías limpias emergentes. Para ello, se realizó principalmente una caracterización integral de hojas de olivo y moringa. Posteriormente, se implementaron múltiples experimentos de extracción mediante el uso de métodos convencionales de extracción de líquidos presurizados y asistidos por ultrasonidos con diferentes niveles de condiciones operativas. Además, se realizaron pruebas de adsorción sólido-líquido en los extractos optimizados. Se probaron varios adsorbentes y disolventes en las etapas de adsorción y desorción, con el fin de obtener extractos purificados y concentrados y también para alcanzar el aislamiento selectivo de los compuestos bioactivos deseados.

Además, los extractos de hojas de olivo se procesaron con filtraciones basadas en membranas para evaluar la eficiencia de la tecnología de membranas en la separación de los compuestos valiosos deseados disponibles en el perfil fenólico y de azúcar de los extractos. Los procesos de membrana impulsados por presión se basan en el uso de una membrana permselectiva para lograr un transporte selectivo de fluidos y solutos bajo una diferencia de presión hidrostática aplicada entre los dos lados de la membrana.

Introduction

Valorization of plant biomass by emerging technologies: olive and moringa leaves



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1. Olive trees the oldest and most valuable crops in the Mediterranean basin

Olive trees (Olea europaea L.) are one of the most well-known cultivated species in the Mediterranean countries. Olive tree cultivation has a long history in the Mediterranean countries, and even today consist an important cultural, economic and environmental aspect of the area. The primary and best-known product of the olive tree is olive oil, but there are several by-products derived from olive harvesting or olive oil extraction, which are discarded such as olive leaves, stones, olive pomace, olive mill waste water, etc. Among them, olive leaves are the most important by-product because of their high content in phenolic compounds, which possess beneficial properties to human health. The fruits, leaves, and the oil of the tree have a rich history of nutritional, medicinal, and ceremonial uses. Phenolic compounds are found in all parts of the olive plant, but their nature and concentration varies greatly between the various tissues [1].

From ancient times, olive (Olea europea L.) leaves have been used for food (herbal teas), pharmacological, cosmetic and medicinal applications. Olive trees can live hundreds of years and olive leaves have been historically used as a folk remedy for combating fevers and other diseases, such as malaria. The low incidence of cardiovascular diseases and cancer in Mediterranean countries has drawn the attention of investigators, and the Mediterranean diet consisting of olives and olive products has been thought to be one reason of this [2]. Olive leaf extract has been used in folk medicine for centuries for treating cold and flu [3].

1.1. Olive leaves as a free raw material

By-products derived from olive trees and olive oil extractions are generally known as “olive by-products” [4]. A high number of by-products and residues derived from both olive tree cultivation and the olive processing industry are obtained yearly; most of them have no practical applications. Olive leaves, one of these by-products, can be found in large amounts in olive oil industries [5]. Similar to other parts of the olive tree, olive leaves, an agricultural waste by product obtained during the harvesting of olive fruits, also contain considerable biophenols [6]. Olive leaves are obtained in large

quantities during the cultivation of olive trees and are prominent in the olive oil industry (10% of the total weight of harvested olives) and they also accumulate in large quantities during tree pruning [7]. The overall chemical composition of Olive tree pruning biomass may differ slightly depending on tree age, soil makeup, and climate conditions [8]. Olive leaves are a cheap raw material, which can be used as an advantageous source of high-value-added products [9].

1.2. Different species of olive leaves

Olive trees are grown in warm, dry climates and olive harvest is collected in late fall, although in some varieties and in certain areas, the collection takes place in December. There are over 260 varieties of olive trees, among which 26 are the most representative varieties. These unique varieties are named according to the geographic origin. Usually, olive tree is a long-lived tree that can reach up to 500 years or more. Like other lignocellulosic biomasses, the composition of olive leaves depends on cultivar and to know it is essential for an adequate use [10]. The most widely cultivated olive varieties are Picual, Cornicabra, Hojiblanca, Arbequina, Lenchin, Verdial, Empeltre, Moorish, Gordal or Queen, Manzanilla, and Farga. Picual variety is the world’s largest variety. Picual olive has a very high content of polyphenols, which gives great stability. Picual oil is characterized by fresh flavor and full-bodied, with a degree of bitterness and pungency [11].

1.3. Olive leaves in the olive oil industry

Olive leaves, an agricultural waste obtained during harvesting or processing of olive fruits, are found in large quantities in the olive oil and olive table industries, where they are separated from olives using pneumatic separation systems and create a residue without industrial interest. However, from an economic point of view, as industrial residue from vegetable materials, olive leaves are an excellent source of phytochemicals [12].

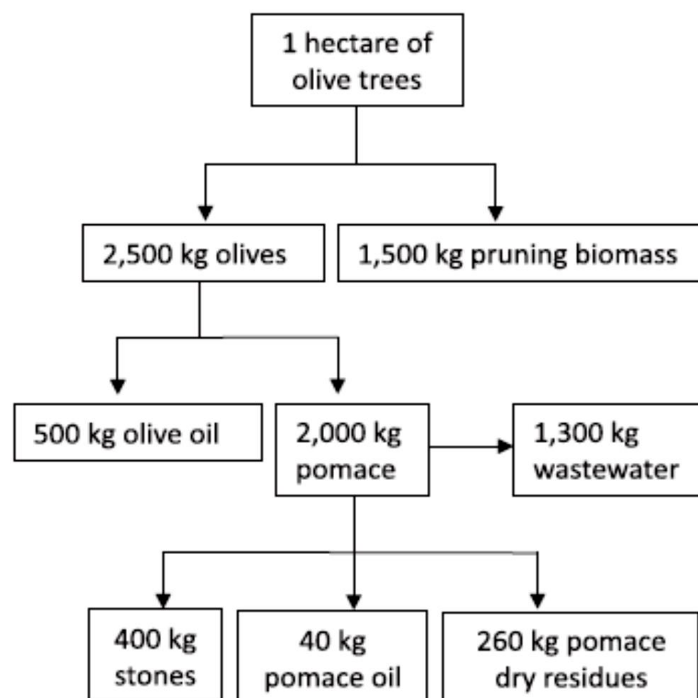


Figure I1. Average mass balance derived from one hectare of olive trees corresponding to Spanish typical cultivation procedure [8].

Olive leaves can represent up to 5% of the total biomass processed in the olive oil industry. Olive leaves are usually eliminated by either burning or grinding and scattering them on fields, which causes CO_2 emissions and the consequent risk of fire or propagation of diseases [13]. The amount of olive leaves accumulated annually along these industries may exceed 1 million ton. Harvested olives are accompanied by approximately 6% of leaves and annual world production of olives exceeds 18 million tons [14]. Olive leaves in the olive oil industry can be used in a circular economy platform for the recovery of active molecules from olive leaf and processing by-products as shown in Figure I1.

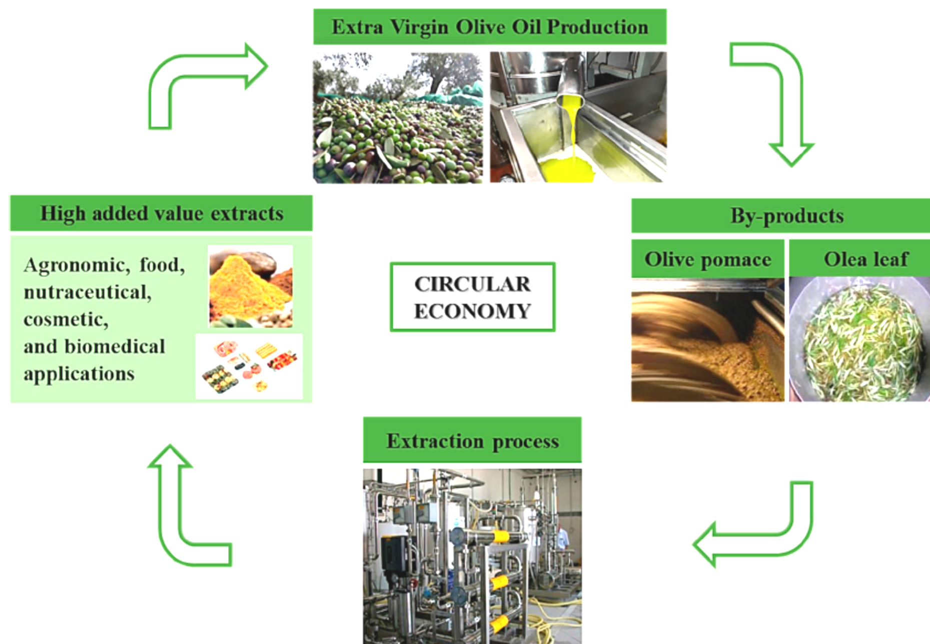
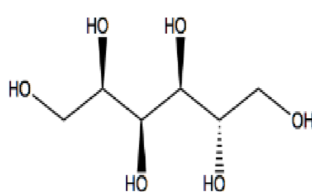


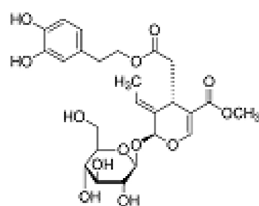
Figure I2. Circular economy platform for the recovery of active molecules from olive leaf and processing by-products [15].

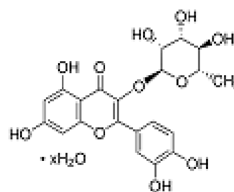
2. Bioactive profile of olive leaves

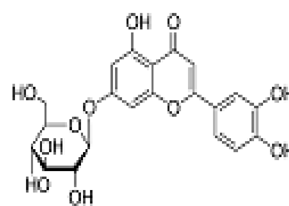
Olive leaves (OL) are gaining recently particular attention as an ample source of antioxidants and bioactive substances such as phenolic derivatives and flavonoids. There has always been an interest in extracting and separating bioactive components from natural plant resources, because natural active compounds have greater safety to human health (Figure I2). Olive leaves and pulp extracts are complex mixtures containing hundreds of different compounds, and their composition can change as a function of the cultivar [16], geographic origin and maturity index [17]. The OL composition varies depending on its origin, proportion of branches, storage conditions, climatic conditions, moisture content, and degree of contamination with soil and oils [4]. Olive leaves have always aroused an important interest, especially for folk medicine. Polyphenols contained in olive leaves have played an important role to this end, because they have demonstrated to be responsible for their anti-carcinogenic, anti-inflammatory, and antimicrobial proprieties. The leaves of olive trees have not yet been

exploited industrially and they have been used only as animal feed or simply burning. From a food waste recovery perspective, the usage of olive leaves is of great interest as they are a great source of nutritionally and bioactive valuable compounds such as polyphenols, volatile compounds, phytosterols, fatty acids, coloring pigments (carotenoids and chlorophylls), tocopherols and squalene, which can be used as potential food additives and/or nutraceuticals [18]. The major active components in the olive leaf are oleuropein and its derivatives, such as hydroxytyrosol and tyrosol, as well as caffeic acid, p-coumaric acid, vanillic acid, vanillin, luteolin, diosmetin, rutin, luteolin-7-glucoside, apigenin-7-glucoside, and diosmetin-7-glucoside (Figure I3) [19].









Mannitol ***Oleuropein*** ***Verbascoside*** ***Luteolin-7-O-glucoside***

Figure I3. Molecular structure of the most abundant bioactive compounds in olive leaves[19].

2.1. Phenolic compounds

Olive leaves have common phenolics with other plants, but they also contain phenolics belonging to the Secoiridoids family (exclusive to the Oleaceae family). Chemical, agronomical and medicinal researches have contributed together to highlight the interest in the use of olive leaves as a potential source of phenolic compounds for the production of functional food and nutraceuticals [5]. Phenolic composition of olive leaves includes secoiridoids and flavonoids such as oleuropein, verbascoside, luteolin-7-O-glucoside, apigenin-7-O-glucoside and quercetin [20]. The phenolic content makes the olive leaf extracts to show great potential as natural antioxidant. As it can be observed form Table I1, oleuropein is the main compound responsible for antioxidant properties of hydroalcoholic extracts (produced with ethanol or methanol at lab scale but also commercial extracts) [21].

Table I1. Phenolic groups present in olive leaf extracts (OLE) [21].

Group	Compound name	Content in olive leaf extract (%)
Oleuropeosides	Oleuropein	24.54
	Verbascoside	11.11
Flavones	Luteolin-7-glucoside	1.38
	Apigenin-7-glucoside	1.37
	Diosmetin-7-glucoside	0.54
	Luteolin	0.21
	Diosmetin	0.05
Flavonols	Rutin	0.05
Flavan-3-ols	Catechin	0.04
Substituted phenols	Tyrosol	0.71
	Hydroxytyrosol	1.46
	Vanillin	0.05
	Vanillic acid	0.63
	Caffeic acid	0.34

2.1.1. Oleuropein

In the last few years, oleuropein has attracted much attention as the main active components with cardioprotective and neuroprotective effects [22]. However, this interest in oleuropein has contributed to the identification and characterization of other biological activities of this compound with potential functional, pharmacological, biomedical, nutraceutical and cosmeceutical interest [23].

Oleuropein is a phenolic secoiridoid glycoside that consists of a polyphenol, namely 4-(2-hydroxyethyl) benzene-1,2-diol, commonly known as hydroxytyrosol, a secoiridoid called elenolic acid and a glucose molecule. It is one of the most abundant bioactive components contained in the leaves of the olive tree (*Olea europaea*). The content of oleuropein varies depending on the variety, organ, olive product, climate, ripeness of the olives at harvesting, and the processing system employed. Oleuropein occurs mainly in the Oleaceae family where it was considered as a chemotaxonomical marker for the infra-generic classification [24].

A lot of studies have reported the amazing physiological and pharmacological properties of oleuropein. Accordingly, *in vitro* and *in vivo* studies have documented the antioxidant, antimicrobial, antifungal, anti-tumoural, hypolipidaemic, and especially hypotensive, anticancer and cardioprotective properties of oleuropein [25–28].

One of the more prominent properties of oleuropein is its strong antioxidant activity, particularly as a free radical scavenger [29]. The proof of such feature has been established by using *in vitro* and *in vivo* tests. In these assays, the ability of oleuropein and oleuropein-rich extracts to scavenge the 2,2-diphenyl-1-picryhydrazyl (DPPH) radical was found to be higher than the synthetic antioxidant BHT (butylated hydroxytoluene) [30–33]. Such effect was primarily attributed to their donating electron ability [23,34].

2.1.2. Luteolin-7-O-Glucoside

Qualitative and quantitative compositional analysis using high-performance liquid chromatography (HPLC) also revealed that luteolin-7-O-glucoside, a flavonoid, is another major polyphenolic compound present in olive leaf extracts [35]. This phytocompound can be a potent drug against colon carcinogenesis [36], that also

demonstrated gastroprotective effects when studied on different ulcer models using rats [37].

2.1.3. Hydroxytyrosol

Hydroxytyrosol is an oleuropein derivative from olive leaves, and it has been associated with low incidence of diverse types of cancer (Figure I4). Testing hydroxytyrosol in the human cell lines showing that it is able to inhibit proliferation, and induce cell cycle arrest and apoptosis in order to control cancer. Anti-cancer effects of hydroxytyrosol in human hepato cellular carcinoma (HCC) cell, it can also significantly inhibit the tumor growth, angiogenesis. Hydroxytyrosol can be a promising candidate agent for the treatment of some cancers [38]. It is a strong antioxidant, an easily oxidizing compound and is synthesized very difficultly and expensively, hence, studies focused on obtaining it from the natural sources and hydroxytyrosol production from an oleuropein source has been proposed very recently [7].

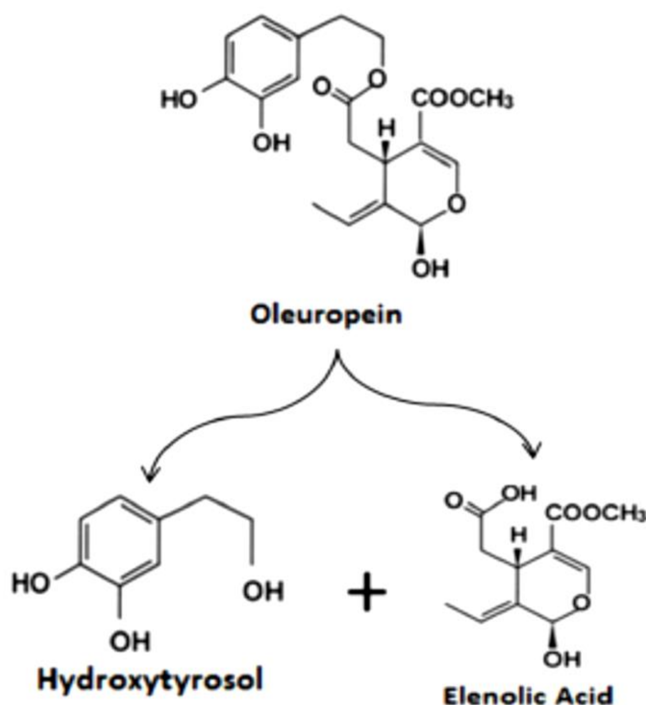


Figure I4. Hydroxytyrosol as a product of oleuropein hydrolysis [39].

2.2. Sugar composition of olive leaves

Mannitol and glucose are the two most abundant soluble carbohydrates in olive leaves. Olive leaf is basically a lignocellulosic material [40]. Lignocellulose is a complex structure composed of carbohydrate polymers (cellulose, hemicellulose) and an aromatic polymer (lignin). These carbohydrate polymers contain different sugar monomers. Cellulose is the most abundant component, consisting of a linear polymer of β-1,4 linked glucose units. Cellulose is closely associated with hemicellulose, which is a branched polymer composed of sugars such as xylose and glucose, among others. Lignin is mainly concentrated in the primary cell-wall and it is mostly formed by phenylpropane units, which makes it a highly complex compound [10,41].

Previous studies have reported that extractives can contain nonstructural sugars. Table. I2 shows the non-structural sugars composition of the aqueous extractives removed of olive leaves based on the research performed by Lama-Muñoz et al. [10], who *studied the* sugars composition of the six main cultivars of olives leaves in Spain. This analysis revealed all cultivars contain mainly glucose (from 6.22 to 9.32%) and mannitol (from 3.26 to 5.23%) with lower concentrations of xylose, galactose, arabinose and xylitol. Again, in general, the contents differ statistically depending on whether deal with commercial or wild cultivars. Mannitol can be used for various applications in the food and pharmaceutical industries [42].
The health quality of food depends mainly on nutrients, but also on foreign substances such as food additives. The presence of foreign substances in the food can be justified, allowed or tolerated only when they are harmless to our health. Epidemic obesity and diabetes encouraged the growth of the artificial sweetener industry.

Table I2. Sugar compositions of aqueous extractives fraction of olive leaves from different cultivars expressed in percentage on a dry weight basis [10].

Glucose (%)	Xylose (%)	Galactose (%)	Arabinose (%)	Mannitol (%)	Xylitol (%)	Total (%)
6.22 – 7.69	0.11 – 0.21	0.75 – 0.98	0.42 – 0.93	3.26 – 5.23	0.22 – 0.36	12.79 – 15.14

There are more and more people who are trying to lose weight or keeping the weight off; therefore, sweeteners can be now found in almost all food products. There are two main types of sweeteners, i.e., nutritive and artificial ones. The latter does not provide calories and will not influence blood glucose; however, some of nutritive sweeteners such as sugar alcohols also characterize with lower blood glucose response and can be metabolized without insulin, being at the same time natural compounds.

Sugar alcohols (polyols or polyhydric alcohols) are low digestible carbohydrates, which are obtained by substituting and aldehyde group with a hydroxyl one [43,44]. Among sugar alcohols can be listed hydrogenated monosaccharides (sorbitol, mannitol), hydrogenated disaccharides (isomalt, maltitol, lactitol) and mixtures of hydrogenated mono-di and/ or oligosaccharides (hydrogenated starch hydrolysates) [43–46].

2.2.1. Mannitol

Mannitol, a natural polyalcohol or sugar alcohol with six carbons and a relative sweetness of 40-50% as compared to sucrose, exists in olive leaves, plane or sycamore, manna ash, marine algae, and fruit and vegetables [47]. D-Mannitol is a sugar alcohol with many applications in food, pharmaceuticals, medicine, and chemistry. Mannitol crystallizes in small white needles with a melting point of 165–170°C. Mannitol has a sweet cool taste owing to its high negative heat of solution (−121 kJ/kg). Mannitol has a low solubility in water of only 18% (w/v) at 25 °C. Especially in alkaline solutions, it is a powerful sequestrant of metallic ions. Mannitol is obtained commercially in large quantities by catalytic hydrogenation from glucose fructose mixtures. Glucose is completely converted in to sorbitol, whereas fructose is converted in to mannitol and sorbitol [48]. This 6-carbon sugar alcohol is an isomer of sorbitol (Figure I3). Mannitol is used as a reserve carbohydrate by some bacteria, fungi, brown seaweeds and some higher plants [49,50].

2.2.1.1. Production

Its production is based on the catalytic hydrogenation of glucose/fructose (1:1) mixture, obtained from invert sugar or starch, at high temperatures and pressure [51–53].

However, this process characterizes with low efficiency yielding only 25 % of mannitol in the obtained mixture and a need of elaborate purification step. Thus, fermentative processes have been researched, especially with the use of heterofermentative lactic acid bacteria, resulting in a complete conversion of d-fructose into d-mannitol in mild conditions [54–57]. Recently, cyanobacteria were found useful for mannitol production, mainly due to the fact that sugars are the primary products of photosynthesis [50].

2.2.1.2. Metabolism

Mannitol is poorly absorbed, and thus, the rise in blood glucose and demand for insulin is much less than would be experienced after sucrose ingestion. It is only partially absorbed (~25 %) from the small intestine and not metabolized. However, in the lower part of the intestinal tract, colonic bacteria can slowly metabolize some of the non-absorbed portion, and thus, consumption of doses exceeding 20 g/day might have a laxative effect [43,52,55]. The fermentation yields organic acids production, which can be utilized by human organism [58].

2.2.1.3. Properties and applications

Mannitol is about 50 % as sweet as sucrose and has a desirable cooling effect, which is efficient in masking bitter tastes [43,52,59]. It can be mixed with other ingredients and sweeteners what can result in a synergistic effect of better sweetness and tasting. This sugar alcohol characterizes with a pleasant taste, stability, even in high temperatures, and a high melting point (165–169 °C). Thus, it is commonly used in pharmaceuticals and nutritional tablets as well as in food industry, i.e., in chocolate-flavored coating agents for ice cream and confections or "breath-freshening" and "sugar-free" products. Mannitol can be also used as a feedstock for bioethanol production [60]. As mannitol is non-hygroscopic, it is used as a bulking agent for sugar free coatings and a dusting powder for chewing gum to prevent the gum from sticking to manufacturing equipment and wrappers [52,61,62].

It can be also applied as a potent osmotic diuretic and is a well-known antioxidant [51,52,59,63–65]. Moreover, it acts as a scavenger of hydroxyl radicals and is claimed

to protect against the development of colon cancer [52,66]. Mannitol is also presumed to be health promoting, and thus, its addition to foods can result in extra nutritional value [55]. When mannitol is inhaled, it helps in mucus and cough clearance in asthmatics and other hypersecretory diseases [67,68]. Mannitol accompanied with hydration during endovascular aortic aneurysm repair might improve renal function [69].

Besides being non-cariogenic, it is also characterized with a low caloric value. As mannitol is not metabolized by humans, it does not induce hyperglycemia and has glycemic and insulinemic indexes of 0 [43,51,55,70].

Similar to other polyols, mannitol is also resistant to oral bacteria which prevents from the increase in the acidity of the mouth after ingestion. Thus, according to the US FDA and European Commission, products containing mannitol can have a health claim on the labeling stating "does not promote tooth decay" [71]. FDA as well as JECFA have approved usage of mannitol as a food additive, which is regarded as safe [46].

3. Emerging techniques for bioactive compounds extraction

The use of bioactive compounds in different commercial sectors such as pharmaceutical, food and chemical industries signifies the need of the most appropriate and standard method to extract these active components from plant materials [72]. In order to obtain olive leaves composition, comprehensive review of different extraction techniques is unavoidable. In the last years, different extraction techniques have been established in order to extract bioactive compounds from olive leaves. All these techniques have some common objectives, (a) to extract targeted bioactive compounds from complex plant sample, (b) to increase selectivity of analytical methods (c) to increase sensitivity of bioassay by increasing the concentration of targeted compounds, (d) to convert the bioactive compounds into a more suitable form for detection and separation, and (e) to provide a strong and reproducible method that is independent of variations in the sample matrix [73].

3.1. Conventional extraction techniques

Bioactive compounds inside plant materials can be extracted by various classical extraction techniques. Most of these techniques are based on the extracting power of different solvents in use and the application of heat and/or mixing. In order to obtain bioactive compounds from plants, the existing classical techniques are: (1) Soxhlet extraction, (2) Maceration and (3) Hydrodistillation [72]. Extraction efficiency of any conventional method mainly depends on the choice of solvents [74]. The polarity of the targeted compound is the most important factor for solvent choice. Molecular affinity between solvent and solute, mass transfer, use of co-solvent, environmental safety, human toxicity and financial feasibility should also consider in selection of solvent for bioactive compound extraction [72].

The major challenges of conventional extraction are longer extraction time, requirement of costly and high purity solvent, evaporation of the huge amount of solvent, low extraction selectivity and thermal decomposition of thermo labile compounds [75]. Soxhlet and solid-liquid extraction by direct contact between the raw material and the solvent, with or without agitation, are common technologies used for the recovery of bioactive compounds. Although these processes provide a fast and reproducible extraction but large quantity of organic solvent is used [76].

3.2. Non-conventional extraction techniques

The major challenges of conventional extraction are longer extraction time, requirement of costly and high purity solvent, evaporation of the huge amount of solvent, low extraction selectivity and thermal decomposition of thermo labile compounds [75]. To overcome these limitations of conventional extraction methods, new and promising extraction techniques are introduced. These techniques are referred as nonconventional extraction techniques. Some of the most promising techniques are ultrasound assisted extraction, enzyme-assisted extraction, microwave-assisted extraction, pulsed electric field assisted extraction, supercritical fluid extraction and pressurized liquid extraction [72].

3.2.1. Ultrasound-assisted extraction (UAE)

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 [77]. Ultrasound technology can benefit the improvement of the extraction efficiency of special compounds. It can be easily implemented to speed up the extraction process causing an expansion in the pores of the cell wall. In that way, the diffusion process of the particular compounds is improved and the mass transfer is enhanced [72].

3.2.2. Pressurized liquid extraction (PLE)

Pressurized liquid extraction (PLE) is shown as an innovative green technology for the effective extraction of the various phytochemicals from food by-products [78]. It is mainly due to its low organic solvent consumption thus considering as a promising innovative extraction technology for recovering polyphenols from olive leaves [79]. The most important PLE parameters are: selection of the appropriate solvents, extraction temperatures, number of extraction cycles, and the length of static extraction times [78].

3.2.3. Subcritical water extraction (SWE)

Subcritical water extraction is an operation that explores the solvating power of water at high temperatures (100–374 °C) at pressures high enough to maintain the water in a liquid state. The dielectric constant of water, which is responsible for solubility, reduces under SWE conditions to values similar to organic solvents like ethanol [80]. Due to the campaign for eco-friendly and sustainable processes, extraction techniques like subcritical water extraction are recommended as alternatives to organic solvent extraction [81]. Among the green extraction techniques, subcritical water extraction is

one of the most promising modern extraction techniques for isolation of bioactive compounds from plant materials [82].

3.2.4. Supercritical fluid extraction (SFE)

Nowadays, in order to reduce the use of the organic solvents, water extraction has recently been applied in some food and pharmaceutical applications and it has led to new extraction methods including supercritical fluid extraction (SFE) (a sustainable green technology) [7]. Supercritical solvents are used in material processing, extractions, micronization, chemical reactions, cleaning and drying among other applications. Other important properties of supercritical fluids are their low surface tensions, low viscosities and moderately high diffusion coefficients [83]. SFE has received increasing attention in a variety of fields due to the reasons that supercritical fluids provide high solubility and improved mass transfer rates and the operation being manipulated by changing the temperature or pressure [84].

3.2.4.1. Supercritical carbon dioxide extraction (SC-CO_2)

Supercritical fluid extraction with CO_2 is a very attractive, clean and rapid alternative method to conventional Soxhlet extraction [85] . Carbon dioxide is considered as an ideal solvent for SFE. The critical temperature of CO_2 (31 °C) is close to room temperature, and the low critical pressure (74 bars) offers the possibility to operate at moderate pressures, generally between 100 and 450 bar [86]. Since the solvent is safe, non-toxic, non-flammable, inexpensive, odorless, colorless, tasteless, highly selective, readily available, easily removable, and fast, the extraction parameters can be changed in a wide range of temperature and pressure. Hence, supercritical carbon dioxide extraction (SC-CO_2) is also an effective and green technology for the extraction of bioactive compounds (carotenoids, flavonoids, phenols, etc.) from different fruits and vegetables, owing to the relatively low temperature used and the production of extracts without organic solvents [87].

3.2.5. Microwave assisted extraction (MAE)

The microwave-assisted extraction is also considered as a novel method for extracting soluble products into a fluid from a wide range of materials using microwave energy [88]. Microwaves are electromagnetic fields in the frequency range from 300 MHz to 300 GHz. They are made up of two oscillating fields that are perpendicular such as electric field and magnetic field. The principle of heating using microwave is based upon its direct impacts on polar materials [89]. Several advantages of MAE have been described such as quicker heating for the extraction of bioactive substances from plant materials; reduced thermal gradients; reduced equipment size and increased extract yield [90].

4. Methods for separation and purification of bioactive compounds

The selection of a separation method is based on the identification of a suitable characteristic property, whose variation should be important for the component(s) to be separated. In order to obtain active components at high concentration, the extraction, separation and purification of flavonoids and oleuropein from olive resources has become increasingly appealing. Recently, silica-gel column chromatography, liquid–liquid extraction, solid-phase extraction, high-speed counter-current chromatography and dynamic ultrasound-assisted extraction, among other techniques have been used for the extraction and separation of flavonoids and oleuropein from olive leaves [91]. Furthermore, biopolymers and polymeric absorbents have been used in separating and purifying target compounds from natural plant resources [92].

4.1. Macroporous resin (MAR)

In recent years, macroporous resins (MARs) have been widely used in recovery and separation of plant secondary metabolites, such as flavonoids and polyphenols. Macroporous resin (MAR) is an important polymeric absorbent, which is usually applied in preparative separation and purification of bioactive compounds from natural resources [91]. MARs are durable polar, nonpolar, or slightly hydrophilic polymers with high adsorption capacity. They can selectively adsorb targeted constituents

through electrostatic force, hydrogen bonding interaction, complexation, and size sieving action. Compared to the chemical methods, MARs are applied for some advantages, such as mechanical strength, diverse structures, good performance, low costs, and environmentally friendly [93]. MARs produce good recovery because of their unique adsorption properties and other advantages, including ideal pore structure and availability of various surface functional groups, low operational cost and easy regeneration [94].

4.2. Pressure-driven membrane processes

Pressure-driven membrane processes Pressure-driven membrane processes are based on the use of a permselective membrane to achieve a selective transport of fluids and solutes under a hydrostatic pressure difference applied between the two sides of the membrane. Consequently, the feed solution is converted into a retentate (stream or concentrate) containing all components retained by the membrane and a permeate stream (purified fraction) containing all components that pass through the membrane. These processes are classified according to the pore size of the membranes and the required transmembrane pressure (TMP) in: microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). MF is based on the use of symmetric membranes able to separate particles with diameters of 0.05–10 mm from a solvent or other low molecular weight compounds. UF involves the use of asymmetric membranes with pore size in the skin layer of 2–10 nm, providing high retention of macromolecules and colloids from a solution. UF membranes are typically characterized by their molecular weight cut-off (MWCO), defined as the equivalent molecular weight of the smallest species that Exhibit 90% rejection. For UF membranes, MWCO is between 10^3 and 10^6 Da. Hydrostatic pressures of 2–10 bar are typically used [95]. The separation mechanism in both MF and UF processes is mainly based on a sieving effect and particles are separated according to their dimensions although other factors, such as shape and charge, as well as interactions between the membrane itself and particles being filtered, play a key role in the separation mechanism. NF is an intermediate membrane process between UF and RO. It is typically used for the separation of

multivalent ions and uncharged organic solutes with molecular weight in the range of 100 and 1000 Da. NF membranes are characterized by pore diameters in the range of 1–3 nm. They operate at lower pressures (generally in the range of 3–30 bar) than RO membranes. Polymeric NF membranes contain ionisable groups as carboxylic or sulphonic acid groups, which result in a surface charge in the presence of a feed solution. The separation mechanism is based on steric, Donnan and dielectric exclusion effects [96].

Energy requirements are potentially much lower for NF than for conventional separation processes, leading to significant cost savings. Separation by NF membranes occurs primarily due to size exclusion and electrostatic interactions [97]. For uncharged molecules, sieving or size exclusion is the major factor responsible for separation [98].

The membrane performance in pressure-driven membrane processes can change very much with time due to membrane fouling and concentration polarization. The general effect of these phenomena is a reduction of the permeate flux through the formation of an additional barrier due to the retention of feed components with a consequent increases of the mass transfer resistance. Membrane fouling may be defined as a deposition of retained particles (colloids, emulsions, macromolecules, and dissolved organics) onto the membrane surface or inside the pores, resulting in a long-term flux decline. Concentration polarization refers to an accumulation on the membrane surface of compounds that are partially or completely rejected by membranes with the formation of a viscous or gelatinous cake layer and to a reduction of the permeating components in a region close to the membrane surface, called the boundary layer. The control of both phenomena is of crucial importance for the industrial application of membrane processes [96].

5. Biorefinery concept for the valorization of olive leaves

Within the last two decades, plant material components such as lignocellulosic residues and their potential biofuel production have increasingly received more attention in the science community, as well as their new value-added compounds and biomaterials utilizing “biorefinery” approach. This concept is comprised of a wide range of

technologies able to separate biomass components in to their building blocks (carbohydrates, proteins, fats, etc.), which can be converted to value-added products, biofuels, and chemicals. Separation takes place in a facility, or network of facilities, that integrates biomass conversion processes and equipment to produce transportation biofuels, power, and chemicals from biomass [8].

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 (Figure. I5). 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 [99].

5.1. Production of bioenergy and biofuels

Olive mill wastes (OMW) 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 [100] and biohydrogen [101,102] production, studies have been focused on biomethane production by anaerobic digestion (AD). Among bioprocesses to transform OMW 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, 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 OMW 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. [103].

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 [99,104].

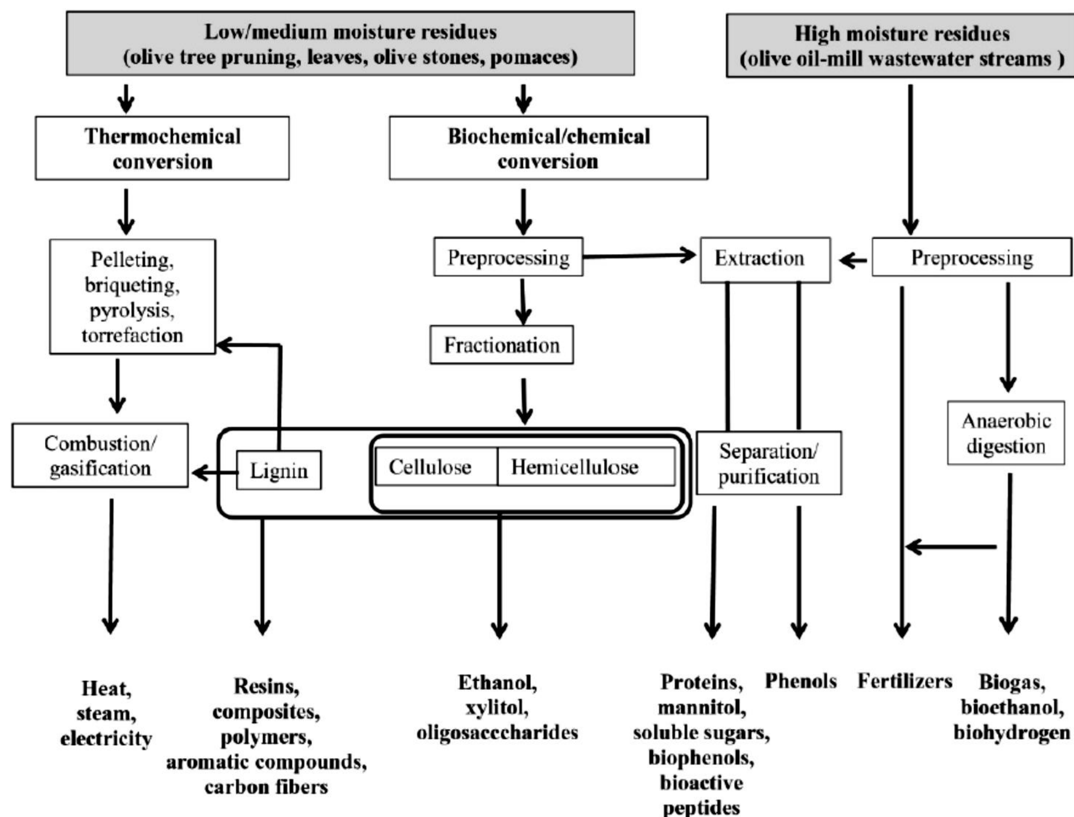


Figure I5. Valorization scheme for different residues in generated in the olive oil industry [99].

6. Clinical and therapeutic applications of olive leaves

Throughout the history, olive (Olea europea L.) leaves have been heavily exploited for the prevention or the treatment of hypertension, carcinogenesis, diabetes, atherosclerosis and so many other traditional therapeutic uses. These activities are thought to be the output of olive micronutrients especially polyphenols [105].

6.1. Antioxidant properties

The determination of polyphenols in plants has been of great interest because of the natural antioxidant activity of these compounds [106]. Antioxidants can delay or prevent oxidation of cellular oxidative substrates and hence considered very important additives in food processing. Synthetic antioxidant compounds like BHT and butylated hydroxyanisol (BHA) are cheap, effective and highly stable. However, the use of BHT

and BHA in foods has decreased due to their suspected action as promoters of carcinogenesis as well as the general consumer rejection of synthetic food additives [7], and for the same reason, there has been a great interest in olive leaves due to their high antioxidant potential that has been found to be comparable to the synthetic antioxidants [107]. Oleuropein and hydroxytyrosol of olive leaf extract have been reported to be much more efficient antioxidants than BHT or vitamin E & C and pure oleuropein. The coefficient of inhibition 5 for hydroxytyrosol and 7 for oleuropein is higher than stoichiometric factor of 2 for BHT. These results have been explained by two corresponding phenomena including the oleuropein hydrolysis to hydroxytyrosol with the corresponding increase in the antioxidant capacity of the extract and the synergistic effect of phenols in the olive leaf extract [2]. Olive leaf extracts possess high antioxidant activity (7.52 $mmol_{Trolox}$ equivalent antioxidant capacity per gram), having a linear relationship between the antioxidant activity and the phenolic content, which renders such products useful for the enhancement of the oxidative stability of food products like edible oils [108,109].

6.2. Antimicrobial properties

In addition to the beneficial effects on human health, OLE also has antimicrobial properties. Oleuropein and hydroxytyrosol from olive leaf extracts have also been proved to be a unique class of HIV-1 inhibitors, effective against viral fusion and integration [110]. antibacterial effect of olive leaves extract in raw peeled undeveined shrimp has been studied, That study demonstrates the potential use of OLE formulations to improve the microbial quality of PUD shrimp and OLE might be useful to the seafood industry as a natural preservative [111]. In another report of the determination of phenolic compounds in olive leaves, and the evaluation of their *in vitro* activity against several microorganisms, it is clearly declared that at low concentrations olive leaves extracts had an unusual combined antibacterial and antifungal action, which suggest their great potential as nutraceuticals, particularly as a source of phenolic compounds [112]. Also, an investigation on the antimicrobial effect of OLE against major foodborne pathogens including Listeria monocytogenes, Escherichia coli O_{157}:H_7, and

Salmonella Enteritidis, had the result as follow: at a concentration of 62.5 mg/ml, OLE almost completely inhibited the growth of these three pathogens. OLE, as a natural product, has the potential to be used as an antimicrobial to control foodborne pathogens [113].

6.3. Therapeutic properties

In recent years, olive leaf extract is being used in different pretreatments. One example of that is a study on pretreatment of carbendazim- exposed rats with olive leaves extract that showed marked improvement in both physiological and histopathological alterations. It concluded that olive leaves extract is a promising chemotherapeutic agent for reducing the toxicity of carbendazim and may be for other pesticides and toxicants [114].

Constituents of O. europea have shown very good anticancer activities on various types of cancers [115]. In a work, a hydroxytyrosol rich olive leaves extract was investigated for potential anti-tumoral activities. In vitro cytotoxic effects against MCF-7 breast cancer cells were examined and the anti-tumor activities were further investigated and suggested the probable use of olive leaves extract as a cancer preventive food additive [105].

7. Physico-chemical properties and nutritive importance of Moringa oleifera

Moringa oleifera belonging to the family of Moringaceae is an effective remedy for malnutrition. Moringa is rich in nutrition owing to the presence of a variety of essential phytochemicals present in its leaves, pods and seeds. In fact, moringa is said to provide 7 times more vitamin C than oranges, 10 times more vitamin A than carrots, 17 times more calcium than milk, 9 times more protein than yoghurt, 15 times more potassium than bananas and 25 times more iron than spinach [116]. The fact that moringa is easily cultivable makes it a sustainable remedy for malnutrition. Countries like Senegal and Benin treat children with moringa [117,118].

M.oleifera benefits could also be attributed to their composition of phenolic compounds. The search for new antioxidants and phenolics from herbal sources has garnered great attention in the last decade. In this regard, leaf extracts of *M. oleifera* have been reported to exhibit antioxidant activity both *in vitro* and *in vivo* due to their abundance of phenolic acids and flavonoids [119]. This fact, together with the possibility of the phenolic compounds interacting with other plant components, makes the extraction probably the most important step in sample pretreatment. The extraction of polyphenols depends greatly on the solvent's polarity, method and extraction time, which determine both the quantitative and qualitative composition of those compounds. The polarities of phenolic compounds vary significantly and it is difficult to develop a single method for optimal extraction of all phenolic compounds [120]. The total polyphenol content determined from the same plant and its corresponding antioxidant activity may vary widely depending on the extraction conditions applied. Due to the aforementioned, the necessity of optimizing the extraction method for each sample prior to carrying out the analytical characterization seems indispensable. The optimal extraction method should be simple, rapid and economical.

Another point to keep in mind is that there are considerable variations in the nutritional value of *M. oleifera*, which depend on several factors like genetic background, environment and cultivation methods, as reported by Moya *et al.* [121] and Rodríguez-Pérez *et al.* [122].

7.1. Plantation and soil conditions

M. oleifera can be grown in any tropical and subtropical regions of the world with a temperature around 25–35°C. It requires sandy or loamy soil with a slightly acidic to slightly alkaline pH and a net rainfall of 250–3000 mm. The direct seeding method is followed as it has high germination rates. Since moringa seeds are expected to germinate within 5–12 days after seeding and can be implanted at a depth of 2 cm in the soil. Moringa can also be propagated using containers. The saplings are placed in plastic bags containing sandy or loamy soil. After it grows to about 30 cm, it can be transplanted. However, utmost care has to be taken while transplanting as the tap roots

are tender and tend to get affected. The tree can also be cultivated from cuttings with 1 m length and 4–5 cm in diameter, but these plants may not have a good deep root system. Such plants tend to be sensitive to drought and winds. For commercial purposes large scale intensive and semi-intensive plantation of moringa may be followed. In commercial cultivation, spacing is important as it helps in plant management and harvest. *M. oleifera* differs in nutrient composition at different locations [117,123].

7.2. Nutritive properties

Every part of *M. oleifera* is a storehouse of important nutrients and anti-nutrients. The leaves of *M. oleifera* are rich in minerals like calcium, potassium, zinc, magnesium, iron and copper [118]. Vitamins like beta-carotene of vitamin A, vitamin B such as folic acid, pyridoxine and nicotinic acid, vitamin C, D and E also present in *M. oleifera* [124]. Phytochemicals such as tannins, sterols, terpenoids, flavonoids, saponins, anthraquinones, alkaloids and reducing sugar present along with anti-cancerous agents like glucosinolates, isothiocyanates, glycoside compounds and glycerol-1-9-octadecanoate [125]. Moringa leaves also have a low calorific value and can be used in the diet of the obese. The pods are fibrous and are valuable to treat digestive problems and thwart colon cancer [126]. A research shows that immature pods contain around 46.78% fiber and around 20.66% protein content. Pods have 30% of amino acid content, the leave shave 44% and flowers have 31%. The immature pods and flowers showed similar amounts of palmitic, linolenic, linoleic andoleic acids [127]. Moringa has lot of minerals that are essential for growth and development among which, calcium is considered as one of the important minerals for human growth. While 8 ounces of milk can provide 300–400 mg, moringa leaves can provide 1000 mg and moringa powder can provide more than 4000 mg. Moringa powder can be used as a substitute for iron tablets, hence as a treatment for anemia. Beef has only 2 mg of iron while moringa leaf powder has 28 mg of iron. It has been reported that moringa contains more iron than spinach [128]. A good dietary intake of zinc is essential for proper growth of sperm cells and is also necessary for the synthesis of DNA and RNA. *M. oleifera* leaves show around 25.5–31.03 mg of zinc/kg, which is the daily requirement of zinc in the diet [129]. PUFAs are linoleic acid, linolenic acid and oleic acid; these PUFAs have the

ability to control cholesterol. Research show that moringa seed oil contains around 76% PUFA, making it ideal for use as a substitute for olive oil [130]. A point to note is that the nutrient composition varies depending on the location. Fuglie [128] revealed that seasons influence the nutrient content. It was shown that vitamin A was found abundantly in the hot-wet season, while vitamin C and iron were more in the cool-dry season [131]. The difference in results can be attributed to the fact that the location, climate and the environmental factors significantly influence nutrient content of the tree [117,121].

7.3. Preservation methods

Moringa can also be preserved for a long time without loss of nutrients. Drying or freezing can be done to store the leaves. A report by Yang et al. [131] shows that a low temperature oven used to dehydrate the leaves retained more nutrients except vitamin C than freeze-dried leaves. Hence, drying can be done using economical household appliance like stove to retain a continuous supply of nutrients in the leaves. Preservation by dehydration improves the shelf life of Moringa without change in nutritional value.

An overdose of moringa may cause high accumulation of iron. High iron can cause gastrointestinal distress and hemochromatosis. Hence, a daily dose of 70 g of moringa is suggested to be good and prevents over accumulation of nutrients [132].

7.4. Medicinal properties

M. oleifera is often referred as a panacea and can be used to cure more than 300 diseases. Moringa has long been used in herbal medicine by Indians and Africans. The presence of phytochemicals makes it a good medicinal agent [117].

7.4.1. Anti-diabetic properties

Moringa has been shown to cure both Type 1 and Type 2 diabetes. Type 1 diabetes is one where the patients suffer from non-production of insulin, which is a hormone that maintains the blood glucose level at the required normal value. Type 2 diabetes is one

associated with insulin resistance. Type 2 diabetes might also be due to Beta cell dysfunction, which fails to sense glucose levels, hence reduces the signaling to insulin, resulting in high blood glucose levels [133]. Several studies have shown that, moringa can act as an anti-diabetic agent. A study has shown that the aqueous extracts of *M. oleifera* can cure streptozotocin-induced Type 1 diabetes and also insulin resistant Type 2 diabetes in rats [134]. In another study, the researchers fed the STZ-induced diabetes rats with Moringa seed powder and noticed that the fasting blood glucose dropped [135]. Also, when the rats were treated with about 500 mg of moringa seed powder/kg bodyweight, the antioxidant enzymes increased in the serum. This shows that the antioxidants present in moringa can bring down the ROS caused in the Beta-cells due to the STZ induction [124].

7.4.2. Anticancer properties

Cancer is a common disease and one in seven deaths is attributed due to improper medication. Around 2.4 million cases are prevalent in India, while there are no specific reasons for cancer to develop. Several factors like smoking, lack of exercise and radiation exposure can lead to the disease [136]. Cancer treatments like surgery, chemotherapy and radiation are expensive and have side effects. *M. oleifera* can be used as an anticancer agent as it is natural, reliable and safe, at established concentrations. Studies have shown that moringa can be used as an anti-neoproliferative agent, thereby inhibiting the growth of cancer cells. Soluble and solvent extracts of leaves have been proven effective as anticancer agents. Furthermore, research papers suggest that the anti-proliferative effect of cancer is maybe due to its ability to induce reactive oxygen species in the cancer cells. Researches show that the reactive oxygen species induced in the cells leads to apoptosis. This is further proved by the up regulation of caspase 3 and caspase 9, which are part of the apoptotic pathway [137–139].

7.4.3. Other diseases

Moringa can be used as a potent neuroprotectant. Cerebral ischemia is caused due to obstruction of blood flow to the brain. This leads to reperfusion and lipid peroxidation, which in turn results in reactive oxygen species. Moringa with its antioxidants can reduce the reactive oxygen species, thereby protecting the brain [140,141]. *M. oleifera* is used to treat dementia, as it has been shown to be a promoter of spatial memory. The leaf extracts have shown to decrease the acetylcholine esterase activity, thereby improving cholinergic function and memory [142]. Adeyemiet al. [143] showed that moringa in diet of rats, can increase protein content and decrease levels of urea and creatinine in blood, preventing renal dysfunction. Moringa decreased acidity in gastric ulcers by a percentage of 86.15% and 85.13% at doses of 500 mg and 350 mg, respectively and therefore can be used as an antiulcer agent [144]. Moringa is prescribed by herbal practitioners for patients with AIDS. Moringa is suggested to be included in the diet, with the view of boosting the immune system of HIV positive individuals. However, more research is essential to validate the effect of moringa on anti-retroviral drugs [145]. The hydro-alcoholic extract of moringa flowers reduced the levels of rheumatoid factor, TNF-alpha and IL-1 in arthritic rats. This proves that moringa can be a potent therapy for arthritis [146].

References

[1] S. H. Omar, "Oleuropein in olive and its pharmacological effects," *Sci. Pharm.*, vol. 78, no. 2, pp. 133–154, 2010.

[2] Z. Erbay and F. Icier, "The Importance and Potential Uses of Olive Leaves," *Food Rev. Int.*, vol. 26, no. 4, pp. 319–334, 2010.

[3] A. Dasgupta, "Chapter 4 Antiinflammatory Herbal Supplements," 2021, pp. 69–91.

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

[5] N. Talhaoui, A. Taamalli, A. M. Gómez-caravaca, A. Fernández-gutiérrez, and A. Segura-carretero, "Phenolic compounds in olive leaves : Analytical determination , biotic and abiotic in fl uence , and health bene fi ts," *FRIN*, vol. 77, pp. 92–108, 2015.

[6] S. Şahin and M. Bilgin, "Olive tree (Olea europaea L.) leaf as a waste by-product of

table olive and olive oil industry: a review," *J. Sci. Food Agric.*, vol. 98, no. 4, pp. 1271–1279, 2018.

[7] N. Rahmanian and T. Ahmed, "Bioactive profile , dehydration , extraction and application of the bioactive components of olive leaves," *Trends Food Sci. Technol.*, vol. 42, no. 2, pp. 150–172, 2015.

[8] J. M. Romero-García, L. Niño, C. Martínez-Patiño, C. Álvarez, E. Castro, and M. J. Negro, "Biorefinery based on olive biomass. State of the art and future trends," *Bioresour. Technol.*, vol. 159, pp. 421–432, 2014.

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

[10] A. Lama-muñoz, M. Contreras, F. Espínola, M. Moya, I. Romero, and E. Castro, "Characterization of the lignocellulosic and sugars composition of different olive leaves cultivars," *Food Chem.*, vol. 329, no. November 2019, p. 127153, 2020.

[11] C. Pérez-Rodrigo and J. Aranceta, "Olive Oil: Its Role in the Diet," B. Caballero, P. M. Finglas, and F. B. T.-E. of F. and H. Toldrá, Eds. Oxford: Academic Press, 2016, pp. 158–166.

[12] A. Lama-muñoz *et al.*, "Extraction of oleuropein and luteolin-7-O-glucoside from olive leaves : Optimization of technique and operating conditions," *Food Chem.*, vol. 293, no. January, pp. 161–168, 2019.

[13] A. Lama-Muñoz, M. del M. Contreras, F. Espínola, M. Moya, I. Romero, and E. Castro, "Content of phenolic compounds and mannitol in olive leaves extracts from six Spanish cultivars: Extraction with the Soxhlet method and pressurized liquids," *Food Chem.*, vol. 320, no. September 2019, p. 126626, 2020.

[14] J. M. Romero-garcía *et al.*, "Obtaining sugars and natural antioxidants from olive leaves by steam-explosion," vol. 210, pp. 457–465, 2016.

[15] A. Romani *et al.*, "Health Effects of Phenolic Compounds Found in Extra-Virgin Olive Oil, By-Products, and Leaf of Olea europaea L.," *Nutrients* , vol. 11, no. 8. 2019.

[16] M. Vergara-barberán, M. J. Lerma-garcía, J. M. Herrero-martínez, and E. F. Simó-alfonso, "Classification of olive leaves and pulps according to their cultivar by using protein profiles established by capillary gel electrophoresis," *Anal Bioanal Chem*, pp. 1731–1738, 2014.

[17] L. M. Crawford, D. M. Holstege, and S. C. Wang, "Journal of Food Composition and Analysis High-throughput extraction method for phenolic compounds in olive fruit

(Olea europaea)," *J. Food Compos. Anal.*, vol. 66, no. December 2017, pp. 136–144, 2018.

[18] Ž. Tihana *et al.*, "Trends in Food Science & Technology From extraction of valuable compounds to health promoting bene fi ts of olive leaves through bioaccessibility, bioavailability and impact on gut microbiota," vol. 83, no. July 2018, pp. 63–77, 2019.

[19] M. M. H. Khalil, "Green synthesis of silver nanoparticles using olive leaf extract and its antibacterial activity," *Arab. J. Chem.*, vol. 7, no. 6, pp. 1131–1139, 2014.

[20] S. Souilem, I. Fki, I. Kobayashi, N. Khalid, and M. A. Neves, "Emerging Technologies for Recovery of Value-Added Components from Olive Leaves and Their Applications in Food / Feed Industries," *Food Bioprocess Technol.*, pp. 229–248, 2017.

[21] J. E. Hayes, P. Allen, N. Brunton, M. N. O'Grady, and J. P. Kerry, "Phenolic composition and in vitro antioxidant capacity of four commercial phytochemical products: Olive leaf extract (Olea europaea L.), lutein, sesamol and ellagic acid," *Food Chem.*, vol. 126, no. 3, pp. 948–955, 2011.

[22] S. H. Omar, "Cardioprotective and neuroprotective roles of oleuropein in olive," *Saudi Pharm. J.*, vol. 18, no. 3, pp. 111–121, 2010.

[23] I. Hassen, H. Casabianca, and K. Hosni, "Biological activities of the natural antioxidant oleuropein : Exceeding the expectation – A mini-review," *J. Funct. Foods*, vol. 18, no. 2015, pp. 926–940, 2020.

[24] S. R. Jensen, H. Franzyk, and E. Wallander, "Chemotaxonomy of the Oleaceae: iridoids as taxonomic markers," *Phytochemistry*, vol. 60, no. 3, pp. 213–231, 2002.

[25] M. J. Ram and J. M. Mart, "Protective role of oleuropein and its metabolite hydroxytyrosol on cancer," *Trends Food Sci. Technol*, vol. 31, pp. 92–99, 2013.

[26] E. Fuentes and I. Palomo, "Antiplatelet effects of natural bioactive compounds by multiple targets: Food and drug interactions," *J. Funct. Foods*, vol. 6, no. 1, pp. 73–81, 2014.

[27] M. V Sepporta *et al.*, "Oleuropein inhibits tumour growth and metastases dissemination in ovariectomised nude mice with MCF-7 human breast tumour xenografts," *J. Funct. Foods*, vol. 8, no. 1, pp. 269–273, 2014.

[28] J. Rubio, F. Franco, A. Sánchez, B. Cantos, M. Mendez, and V. Calvo, "Does the presence of hepatitis virus B and C influence the evolution of diffuse large B-cell lymphoma?," *Leuk Lymphoma*, pp. 1–5, 2014.

[29] S. Cicerale, L. J. Lucas, and R. S. J. Keast, "Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil," *Curr. Opin. Biotechnol.*, vol.

23, no. 2, pp. 129–135, 2012.

[30] S. Achat *et al.*, "Direct enrichment of olive oil in oleuropein by ultrasound-assisted maceration at laboratory and pilot plant scale," *Ultrason. Sonochem.*, vol. 19, no. 4, pp. 777–786, 2012.

[31] V. Mancebo-Campos, M. D. Salvador, and G. Fregapane, "Antioxidant capacity of individual and combined virgin olive oil minor compounds evaluated at mild temperature (25 and 40 C) as compared to accelerated and antiradical assays," *Food Chem.*, vol. 150, pp. 374–381, 2014.

[32] H. Zbidi *et al.*, "Olive tree wood phenolic compounds with human platelet antiaggregant properties," *Blood Cells, Mol. Dis.*, vol. 42, no. 3, pp. 279–285, 2009.

[33] M. Bouaziz, I. Fki, H. Jemai, M. Ayadi, and S. Sayadi, "Effect of storage on refined and husk olive oils composition: Stabilization by addition of natural antioxidants from Chemlali olive leaves," *Food Chem.*, vol. 108, no. 1, pp. 253–262, 2008.

[34] O.-H. Lee *et al.*, "Assessment of phenolics-enriched extract and fractions of olive leaves and their antioxidant activities," *Bioresour. Technol.*, vol. 100, no. 23, pp. 6107–6113, 2009.

[35] M. Castellano and T. Delgado-herv, "LWT - Food Science and Technology Determination of major bioactive compounds from olive leaf," vol. 64, 2015.

[36] A. A. Baskar, S. Ignacimuthu, G. P. Michael, and K. S. Al Numair, "Cancer chemopreventive potential of Luteolin-7-O-Glucoside isolated from ophiorrhiza mungos linn," *Nutr. Cancer*, vol. 63, no. 1, pp. 130–138, 2011.

[37] S. Cheol and J. Kim, "Experimental study on gastroprotective efficacy and mechanisms of luteolin-7-O-glucoside isolated from Ophiorrhiza mungos Linn . in," *J. Funct. Foods*, vol. 25, pp. 302–313, 2016.

[38] B. Zhao *et al.*, "Hydroxytyrosol, a natural molecule from olive oil, suppresses the growth of human hepatocellular carcinoma cells via inactivating AKT and nuclear factor-kappa B pathways," *Cancer Lett.*, vol. 347, no. 1, pp. 79–87, 2014.

[39] A. Officioso, "Oxidative stress-mediated metal toxicity and protective role of bioactive compounds," *PhD Thesis, Universita Degli Studi Di Napoli*, December, 2016.

[40] J. M. Romero-García, L. Niño, C. Martínez-Patiño, C. Álvarez, E. Castro, and M. J. Negro, "Biorefinery based on olive biomass. State of the art and future trends," *Bioresour. Technol.*, vol. 159, pp. 421–432, 2014.

[41] A. Garcia-Maraver, D. Salvachúa, M. J. Martínez, L. F. Diaz, and M. Zamorano, "Analysis of the relation between the cellulose, hemicellulose and lignin content and the

thermal behavior of residual biomass from olive trees," *Waste Manag.*, vol. 33, no. 11, pp. 2245–2249, 2013.

[42] Y. Dai, Q. Meng, W. Mu, and T. Zhang, "Recent advances in the applications and biotechnological production of mannitol," *J. Funct. Foods*, vol. 36, pp. 404–409, 2017.

[43] G. Livesey, "Health potential of polyols as sugar replacers, with emphasis on low glycaemic properties," *Nutr. Res. Rev.*, vol. 16, no. 2, pp. 163–191, 2003.

[44] P. Shankar, S. Ahuja, and K. Sriram, "Non-nutritive sweeteners: review and update," *Nutrition*, vol. 29, no. 11–12, pp. 1293–1299, 2013.

[45] K. C. Ellwood, "Methods available to estimate the energy values of sugar alcohols," *Am. J. Clin. Nutr.*, vol. 62, no. 5, pp. 1169S-1174S, 1995.

[46] M. Grembecka, "Sugar alcohols- their role in the modern world of sweeteners: a review," *Eur. Food Res. Technol.*, pp. 1–14, 2015.

[47] S. M. Ghoreishi and R. G. Shahrestani, "Subcritical water extraction of mannitol from olive leaves," *J. Food Eng.*, vol. 93, no. 4, pp. 474–481, 2009.

[48] W. Soetaert, P. T. Vanhooren, and E. J. Vandamme, "The Production of Mannitol by Fermentation BT - Carbohydrate Biotechnology Protocols," C. Bucke, Ed. Totowa, NJ: Humana Press, 1999, pp. 261–275.

[49] P. Rupérez and G. Toledano, "Celery by-products as a source of mannitol," *Eur. Food Res. Technol.*, vol. 216, no. 3, pp. 224–226, 2003.

[50] J. H. Jacobsen and N.-U. Frigaard, "Engineering of photosynthetic mannitol biosynthesis from CO2 in a cyanobacterium," *Metab. Eng.*, vol. 21, pp. 60–70, 2014.

[51] S. H. Song and C. Vieille, "Recent advances in the biological production of mannitol," *Appl. Microbiol. Biotechnol.*, vol. 84, no. 1, pp. 55–62, 2009.

[52] S. M. Ghoreishi and R. G. Shahrestani, "Innovative strategies for engineering mannitol production," *Trends food Sci. Technol.*, vol. 20, no. 6–7, pp. 263–270, 2009.

[53] M. Makkee, A. P. G. Kieboom, and H. Van Bekkum, "Production methods of d-mannitol," *Starch-Stärke*, vol. 37, no. 4, pp. 136–141, 1985.

[54] W. Soetaert, "Production of D-mannitol and D-lactic acid by fermentation with Leuconostoc mesenteroides.," *Agro Food Ind. Hi-Tech.*, vol. 6, pp. 41–44, 1995.

[55] H. W. Wisselink, R. A. Weusthuis, G. Eggink, J. Hugenholtz, and G. J. Grobben, "Mannitol production by lactic acid bacteria: a review," *Int. Dairy J.*, vol. 12, no. 2–3, pp. 151–161, 2002.

[56] N. von Weymarn, M. Hujanen, and M. Leisola, "Production of D-mannitol by heterofermentative lactic acid bacteria," *Process Biochem.*, vol. 37, no. 11, pp. 1207–

1213, 2002.

[57] O. Akinterinwa, R. Khankal, and P. C. Cirino, "Metabolic engineering for bioproduction of sugar alcohols," *Curr. Opin. Biotechnol.*, vol. 19, no. 5, pp. 461–467, 2008.

[58] J. Xiao, X. Li, X. Min, and E. Sakaguchi, "Mannitol improves absorption and retention of calcium and magnesium in growing rats," *Nutrition*, vol. 29, no. 1, pp. 325–331, 2013.

[59] M. E. Ortiz, J. Bleckwedel, R. R. Raya, and F. Mozzi, "Biotechnological and in situ food production of polyols by lactic acid bacteria," *Appl. Microbiol. Biotechnol.*, vol. 97, no. 11, pp. 4713–4726, 2013.

[60] J. Wang, Y. M. Kim, H. S. Rhee, M. W. Lee, and J. M. Park, "Bioethanol production from mannitol by a newly isolated bacterium, Enterobacter sp. JMP3," *Bioresour. Technol.*, vol. 135, pp. 199–206, 2013.

[61] Á. Gombás, P. Szabó-Révész, G. Regdon, and I. Erős, "Study of thermal behaviour of sugar alcohols," *J. Therm. Anal. Calorim.*, vol. 73, no. 2, pp. 615–621, 2003.

[62] P. R. Jamieson, A. S. Le, K. B. Mulderrig, and L. O. Nabors, "Alternative Sweeteners," New York: M. Dekker, 2001.

[63] G. J. M. den Hartog *et al.*, "Erythritol is a sweet antioxidant," *Nutrition*, vol. 26, no. 4, pp. 449–458, 2010.

[64] V. Monedero, G. Pérez-Martínez, and M. J. Yebra, "Perspectives of engineering lactic acid bacteria for biotechnological polyol production," *Appl. Microbiol. Biotechnol.*, vol. 86, no. 4, pp. 1003–1015, 2010.

[65] B. C. Saha and F. M. Racine, "Effects of pH and corn steep liquor variability on mannitol production by Lactobacillus intermedius NRRL B-3693," *Appl. Microbiol. Biotechnol.*, vol. 87, no. 2, pp. 553–560, 2010.

[66] P. Gaspar, A. R. Neves, A. Ramos, M. J. Gasson, C. A. Shearman, and H. Santos, "Engineering Lactococcus lactis for production of mannitol: high yields from food-grade strains deficient in lactate dehydrogenase and the mannitol transport system," *Appl. Environ. Microbiol.*, vol. 70, no. 3, pp. 1466–1474, 2004.

[67] E. Daviskas, S. D. Anderson, and I. H. Young, "Effect of mannitol and repetitive coughing on the sputum properties in bronchiectasis," *Respir. Med.*, vol. 104, no. 3, pp. 371–377, 2010.

[68] E. Daviskas, S. D. Anderson, S. Eberl, and I. H. Young, "Beneficial effect of inhaled mannitol and cough in asthmatics with mucociliary dysfunction," *Respir. Med.*, vol. 104, no. 11, pp. 1645–1653, 2010.

[69] K. Kalimeris *et al.*, “Mannitol and renal dysfunction after endovascular aortic aneurysm repair procedures: a randomized trial,” *J. Cardiothorac. Vasc. Anesth.*, vol. 28, no. 4, pp. 954–959, 2014.

[70] G. Livesey, “Sweeteners and Sugar Alternatives in Food Technology.” Wiley-Blackwell, 2012.

[71] EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). 2011. “Scientific Opinion on the Substantiation of Health Claims Related to the Sugar Replacers Xylitol, Sorbitol, Mannitol, Maltitol, Lactitol, Isomalt, Erythritol, D-Tagatose, Isomaltulose, Sucralose and Polydextrose and Maintenance of Tooth Mineralisation by Decreasing Tooth Demineralisation (ID 463, 464, 563, 618, 647, 1182, 1591, 2907, 2921, 4300), and Reduction of Post-Prandial Glycaemic Responses (ID 617, 619, 669, 1590, 1762, 2903, 2908, 2920) Pursuant to Article 13(1) of Regulation (EC) No 1924/2006.” *EFSA Journal* 9(4): 2076.

[72] J. Azmir *et al.*, “Techniques for extraction of bioactive compounds from plant materials : A review,” *J. Food Eng.*, vol. 117, no. 4, pp. 426–436, 2013.

[73] R. M. Smith, “Before the injection- modern methods of sample preparation for separation techniques,” *J. Chromatogr.A*, vol. 1000, pp. 3–27, 2003.

[74] M. M. Cowan, “Plant Products as Antimicrobial Agents,” *Clin Microbiol Rev*. vol. 12, no. 4, pp. 564–582, 1999.

[75] A. M. D. Luque, “Soxhlet extraction of solid materials: an outdated technique with a promising innovative future,” *Anal. Chim. Acta*, vol. 369, 1998.

[76] I. M. Prado, G. H. C. Prado, J. M. Prado, and M. A. A. Meireles, “Food and Bioproducts Processing Supercritical CO_2 and low-pressure solvent extraction of mango (Mangifera indica) leaves: Global yield, extraction kinetics, chemical composition and cost of manufacturing,” *Food Bioprod. Process.*, vol. 91, no. 4, pp. 656–664, 2013.

[77] S. J. Doicrycz and K. S. Suslick, “Interparticle Collisions Driven by Ultrasound,” School of Chemical Sciences, University of Illinois， vol. 2, no. 67, pp. 1987–1989, 1987.

[78] P. Putnik, F. J. Barba, I. Spani, and V. Dragovi, “Green extraction approach for the recovery of polyphenols from Croatian olive leaves (Olea europea),” *FOOD BIOPROD PROCESS*, vol. 6, pp. 19–28, 2017.

[79] E. Roselló-soto, F. J. Barba, O. Parniakov, and C. M. Galanakis, “High Voltage Electrical Discharges, Pulsed Electric Field, and Ultrasound Assisted Extraction of Protein and Phenolic Compounds from Olive Kernel,” *Food Bioproc Tech*, pp. 885–

894, 2015.

[80] R. Vardanega, P. I. N. Carvalho, D. T. Santos, and M. A. A. Meireles, "Obtaining prebiotic carbohydrates and beta-ecdysone from Brazilian ginseng by subcritical water extraction," *Innov. Food Sci. Emerg. Technol.*, vol. 42, no. October 2016, pp. 73–82, 2017.

[81] S. Essien, B. Young, and S. Baroutian, "The Journal of Supercritical Fluids Subcritical water extraction for selective recovery of phenolic leaves bioactives from k anuka," *J. Supercrit. Fluids*, vol. 158, p. 104721, 2020.

[82] M. Ko, H. Kwon, and M. Chung, "Pilot-scale subcritical water extraction of fl avonoids from satsuma mandarin (Citrus unshiu Markovich) peel," *Innov. Food Sci. Emerg. Technol.*, vol. 38, pp. 175–181, 2016.

[83] J. L. Martinez, "Supercritical fluid extraction of nutraceuticals and bioactive compounds," CRC Press, 2007.

[84] M. Khajeh, Y. Yamini, and N. Bahramifar, "Comparison of essential oils compositions of Ferula assa-foetida obtained by supercritical carbon dioxide extraction and hydrodistillation methods," *Food Chem.*, vol. 91, pp. 639–644, 2005.

[85] P. Bajerová, A. Eisner, V. Ježová, M. Adam, and K. Ventura, "Comparison of supercritical fluid and Soxhlet extractions for the isolation of nitro compounds from soils," *J. Sep. Sci.*, vol. 31, no. 8, pp. 1408–1414, 2008.

[86] F. Temelli and Ö. Güçlü-Üstündağ, "Supercritical Technologies for Further Processing of Edible Oils," *Bailey's Industrial Oil and Fat Products*. Jul. 2005.

[87] A. Rawson *et al.*, "Application of Supercritical Carbon Dioxide to Fruit and Vegetables : Extraction , Processing , and Preservation Application of Supercritical Carbon Dioxide to Fruit and Vegetables: Extraction, Processing," *Food. Rev. Int.*, vol. 9129, 2012.

[88] J. R. Jocelyn Paré, J. M. R. Bélanger, and S. S. Stafford, "Microwave-assisted process (MAP^{TM}): a new tool for the analytical laboratory," *TrAC Trends Anal. Chem.*, vol. 13, no. 4, pp. 176–184, 1994.

[89] M. Letellier and H. Budzinski, "Original articles Microwave assisted extraction of organic compounds," *Analusis*, pp. 259–271, 1999.

[90] G. Cravotto, L. Boffa, S. Mantegna, P. Perego, M. Avogadro, and P. Cintas, "Improved extraction of vegetable oils under high-intensity ultrasound and/or microwaves," *Ultrasonics Sonochemistry*, vol. 15, pp. 898–902, 2008.

[91] C. Li, Y. Zheng, X. Wang, S. Feng, and D. Di, "Simultaneous separation and purification of flavonoids and oleuropein from Olea europaea L. (olive) leaves using

macroporous resin," *J. Sci. Food Agric.*, no. May, pp. 2826–2834, 2011.

[92] X. Geng, P. Ren, G. Pi, R. Shi, Z. Yuan, and C. Wang, "High selective purification of flavonoids from natural plants based on polymeric adsorbent with hydrogen-bonding interaction," *J.Chromatgr. A*, vol. 1216, pp. 8331–8338, 2009.

[93] R. Wang *et al.*, "Preparative purification of peoniflorin and albiflorin from peony rhizome using macroporous resin and medium-pressure liquid chromatography," *J. Sep. Sci*, pp. 1985–1992, 2012.

[94] T. Zhigang, Z. Rongqi, and D. Zhanting, "Adsorption and desorption behaviour of taurine on macroporous adsorption resins," *JCTB*, vol. 756, no. July 2000, pp. 752–756, 2001.

[95] R. van Reis and A. Zydney, "Bioprocess membrane technology," *J. Memb. Sci.*, vol. 297, no. 1–2, pp. 16–50, 2007.

[96] C. Conidi, E. Drioli, and A. Cassano, "Membrane-based agro-food production processes for polyphenol separation, purification and concentration," *Curr. Opin. Food Sci.*, pp. 1–16, 2017.

[97] A. E. Yaroshchuk, "Negative rejection of ions in pressure-driven membrane processes," *Adv. Colloid Interface Sci.*, vol. 139, pp. 150–173, 2008.

[98] B. Qi, J. Luo, X. Chen, X. Hang, and Y. Wan, "Bioresource Technology Separation of furfural from monosaccharides by nanofiltration," *Bioresour. Technol.*, vol. 102, no. 14, pp. 7111–7118, 2011.

[99] 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.

[100] M. I. Massadeh and N. Modallal, "Ethanol Production from Olive Mill Wastewater (OMW) Pretreated with Pleurotus sajor-caju," *Energy & Fuels*, vol. 22, no. 1, pp. 150–154, Jan. 2008.

[101] E. Eroğlu, U. Gündüz, M. Yücel, L. Türker, and I. Eroğlu, "Photobiological hydrogen production by using olive mill wastewater as a sole substrate source," *Int. J. Hydrogen Energy*, vol. 29, no. 2, pp. 163–171, 2004.

[102] E. Eroğlu, I. Eroğlu, U. Gündüz, and M. Yücel, "Treatment of olive mill wastewater by different physicochemical methods and utilization of their liquid effluents for biological hydrogen production," *Biomass and Bioenergy*, vol. 33, no. 4, pp. 701–705, 2009.

[103] A. Gunay and D. Karadag, "Recent developments in the anaerobic digestion of olive mill effluents," *Process Biochem.*, vol. 50, no. 11, pp. 1893–1903, 2015.

[104] F. Paiva-Martins *et al.*, “Effects of the dietary incorporation of olive leaves on growth performance, digestibility, blood parameters and meat quality of growing pigs,” *J. Sci. Food Agric.*, vol. 94, no. 14, pp. 3023–3029, Nov. 2014.

[105] Z. Bouallagui, J. Han, H. Isoda, and S. Sayadi, “Hydroxytyrosol rich extract from olive leaves modulates cell cycle progression in MCF-7 human breast cancer cells,” *Food Chem. Toxicol.*, vol. 49, no. 1, pp. 179–184, 2011.

[106] F. Le Floch, M. T. Tena, A. Ríos, and M. Valcárcel, “Supercritical fluid extraction of phenol compounds from olive leaves,” *Talanta*, vol. 46, no. 5, pp. 1123–1130, 1998.

[107] Z. Erabay and F. Icier, “Energy and exergy analysis on drying of olive leaves (Olea Europaea L.) in tray drier,” *J. Food Process Eng.*, vol. 34, no. 6, pp. 2105–2123, Dec. 2011.

[108] F. Brahmi, G. Flamini, M. Issaoui, and M. Dhibi, “Chemistry Chemical composition and biological activities of volatile fractions from three Tunisian cultivars of olive leaves,” *Med. Chem. Res.*, pp. 2863–2872, 2012.

[109] H. H. Orak, S. S. Isbilir, and H. Yagar, “Determination of Antioxidant Properties of Lyophilized Olive Leaf Water Extracts Obtained from 21 Different Cultivars,” *Food Sci. Biotechnol.*, vol. 21, no. 4, pp. 1065–1074, 2012.

[110] S. Lee-huang *et al.*, “Discovery of small-molecule HIV-1 fusion and integrase inhibitors oleuropein and hydroxytyrosol: Part I. Integrase inhibition,” *Biochem Biophys Res Commun.*, vol. 354, pp. 872–878, 2007.

[111] A. M. Ahmed, N. S. Rabii, A. M. Garbaj, and S. K. Abolghait, “Antibacterial effect of olive (Olea europaea L.) leaves extract in raw peeled undeveined shrimp (Penaeus semisulcatus),” *Int. J. Vet. Sci. Med.*, vol. 2, no. 1, pp. 53–56, 2014.

[112] A. P. Pereira *et al.*, “Phenolic compounds and antimicrobial activity of olive (Olea europaea L. Cv. Cobrançosa) leaves,” *Molecules*, vol. 12, no. 5, pp. 1153–1162, 2007.

[113] Y. Liu, L. C. McKeever, and N. S. A. Malik, “Assessment of the antimicrobial activity of olive leaf extract against foodborne bacterial pathogens,” *Front. Microbiol.*, vol. 8, no. FEB, pp. 1–8, 2017.

[114] T. A. Zari, “Therapeutic effects of olive leaves extract on rats treated with a sublethal concentration of carbendazim,” *Eur Rev Med Pharmacol Sci.*, no. April, 2011.

[115] M. A. Hashmi, A. Khan, M. Hanif, U. Farooq, and S. Perveen, “Traditional Uses, Phytochemistry, and Pharmacology of Olea europaea (Olive),” *Evidence-Based Complementary and Alternative Medicine*, vol. 2015, 2015.

[116] J. L. Rockwood, B. G. Anderson, and D. A. Casamatta, “Potential uses of Moringa

oleifera and an examination of antibiotic efficacy conferred by M. oleifera seed and leaf extracts using crude extraction techniques available to underserved indigenous populations," *Int. J. Phototherapy Res.*, vol. 3, no. 2, pp. 61–71, 2013.

[117] L. Gopalakrishnan, K. Doriya, and D. Santhosh, "Moringa oleifera: A review on nutritive importance and its medicinal application," *Food Sci. Hum. Wellness*, vol. 5, no. 2, pp. 49–56, 2016.

[118] J. N. Kasolo, G. S. Bimenya, L. Ojok, J. Ochieng, and J. W. Ogwal-Okeng, "Phytochemicals and uses of Moringa oleifera leaves in Ugandan rural communities," *J. Med. Plants Res.*, vol. 4, no. 9, pp. 753–757, 2010.

[119] B. Vongsak, P. Sithisarn, S. Mangmool, S. Thongpraditchote, Y. Wongkrajang, and W. Gritsanapan, "Maximizing total phenolics, total flavonoids contents and antioxidant activity of Moringa oleifera leaf extract by the appropriate extraction method," *Ind. Crops Prod.*, vol. 44, pp. 566–571, 2013.

[120] P. Garcia-Salas, A. Morales-Soto, A. Segura-Carretero, and A. Fernández-Gutiérrez, "Phenolic-compound-extraction systems for fruit and vegetable samples," *Molecules*, vol. 15, no. 12, pp. 8813–8826, 2010.

[121] B. Moyo, P. J. Masika, A. Hugo, and V. Muchenje, "Nutritional characterization of Moringa (Moringa oleifera Lam.) leaves," *African J. Biotechnol.*, vol. 10, no. 60, pp. 12925–12933, 2011.

[122] C. Rodríguez-Pérez, R. Quirantes-Piné, A. Fernández-Gutiérrez, and A. Segura-Carretero, "Optimization of extraction method to obtain a phenolic compounds-rich extract from Moringa oleifera Lam leaves," *Ind. Crops Prod.*, vol. 66, pp. 246–254, 2015.

[123] M. Aslam, F. Anwar, R. Nadeem, U. Rashid, T. G. Kazi, and M. Nadeem, "Mineral composition of Moringa oleifera leaves and pods from different regions of Punjab, Pakistan," *Asian J Plant Sci*, vol. 4, no. 4, pp. 417–421, 2005.

[124] M. Mbikay, "Therapeutic potential of Moringa oleifera leaves in chronic hyperglycemia and dyslipidemia: a review," *Front. Pharmacol.*, vol. 3, p. 24, 2012.

[125] L. Berkovich, G. Earon, I. Ron, A. Rimmon, A. Vexler, and S. Lev-Ari, "Moringa Oleifera aqueous leaf extract down-regulates nuclear factor-kappaB and increases cytotoxic effect of chemotherapy in pancreatic cancer cells," *BMC Complement. Altern. Med.*, vol. 13, 2013.

[126] I. Oduro, W. O. Ellis, and D. Owusu, "Nutritional potential of two leafy vegetables: Moringa oleifera and Ipomoea batatas leaves," *Sci. Res. Essays*, vol. 3, no. 2, pp. 57–

60, 2008.

[127] D. I. Sánchez-Machado, J. A. Núñez-Gastélum, C. Reyes-Moreno, B. Ramírez-Wong, and J. López-Cervantes, “Nutritional quality of edible parts of Moringa oleifera,” *Food Anal. Methods*, vol. 3, no. 3, pp. 175–180, 2010.

[128] L. J. Fuglie, “The Moringa Tree: a local solution to malnutrition,” *Church World Serv. Senegal*, vol. 5, pp. 75–83, 2005.

[129] J. T. Barminas, M. Charles, and D. Emmanuel, “Mineral composition of non-conventional leafy vegetables,” *Plant Foods Hum. Nutr.*, vol. 53, no. 1, pp. 29–36, 1998.

[130] S. Lalas and J. Tsaknis, “Characterization of Moringa oleifera Seed Oil Variety “‘ Periyakulam 1,” *J. Food Compos. Anal.*, vol. 1, pp. 65–77, 2002.

[131] R. Y. Yang *et al.*, “Nutritional and functional properties of Moringa leaves - from germplasm, to plant, to food, to health,” *Moringa Other Highly Nutr. Plant Resour. Strateg. Stand. Mark. A Better Impact Nutr. Africa (Accra, Ghana)*, pp. 1–17, 2006.

[132] I. J. Asiedu-Gyekye, S. Frimpong-Manso, C. Awortwe, D. A. Antwi, and A. K. Nyarko, “Micro-and macroelemental composition and safety evaluation of the nutraceutical Moringa oleifera leaves,” *J. Toxicol.*, vol. 2014, 2014.

[133] M. E. Cerf, “Beta cell dysfunction and insulin resistance,” *Front. Endocrinol. (Lausanne).*, vol. 4, p. 37, 2013.

[134] S. M. Divi, R. Bellamkonda, and S. K. Dasireddy, “Evaluation of antidiabetic and antihyperlipedemic potential of aqueous extract of Moringa oleifera in fructose fed insulin resistant and STZ induced diabetic wistar rats: a comparative study,” *Asian J Pharm Clin Res*, vol. 5, no. 1, pp. 67–72, 2012.

[135] A. L. Al-Malki and H. A. El Rabey, “The antidiabetic effect of low doses of Moringa oleifera Lam. Seeds on streptozotocin induced diabetes and diabetic nephropathy in male rats,” *Biomed. Res. Int.*, vol. 1, pp. 1–13, 2015.

[136] M. K. Nair, C. Varghese, and R. Swaminathan, “Cancer: Current scenario, intervention strategies and projections for 2015,” *NCHM Backgr. Pap. Dis. India*, pp. 219–225, 2005.

[137] S. Leelawat and K. Leelawat, “Moringa olefiera extracts induce cholangiocar-cinoma cell apoptosis by induction of reactive oxygen species production,” *Int J Pharmacog Phytochem Res*, vol. 6, pp. 183–189, 2014.

[138] C. Tiloke, A. Phulukdaree, and A. A. Chuturgoon, “The antiproliferative effect of Moringa oleifera crude aqueous leaf extract on cancerous human alveolar epithelial cells,” *BMC Complement. Altern. Med.*, vol. 13, no. 1, pp. 1–8, 2013.

[139] I. L. Jung, “Soluble extract from Moringa oleifera leaves with a new anticancer activity,” *PLoS One*, vol. 9, no. 4, p. e95492, 2014.

[140] K. Baker, C. B. Marcus, K. Huffman, H. Kruk, B. Malfroy, and S. R. Doctrow, “Synthetic combined superoxide dismutase/catalase mimetics are protective as a delayed treatment in a rat stroke model: a key role for reactive oxygen species in ischemic brain injury,” *J. Pharmacol. Exp. Ther.*, vol. 284, no. 1, pp. 215–221, 1998.

[141] W. Kirisattayakul, J. Wattanathorn, T. Tong-Un, S. Muchimapura, P. Wannanon, and J. Jittiwat, “Cerebroprotective effect of Moringa oleifera against focal ischemic stroke induced by middle cerebral artery occlusion,” *Oxid. Med. Cell. Longev.*, vol. 2013, 2013.

[142] C. Sutalangka, J. Wattanathorn, S. Muchimapura, and W. Thukham-mee, “Moringa oleifera mitigates memory impairment and neurodegeneration in animal model of age-related dementia,” *Oxid. Med. Cell. Longev.*, vol. 2013, 2013.

[143] O. S. Adeyemi and T. C. Elebiyo, “Moringa oleifera supplemented diets prevented nickel-induced nephrotoxicity in wistar rats,” *J. Nutr. Metab.*, vol. 2014, 2014.

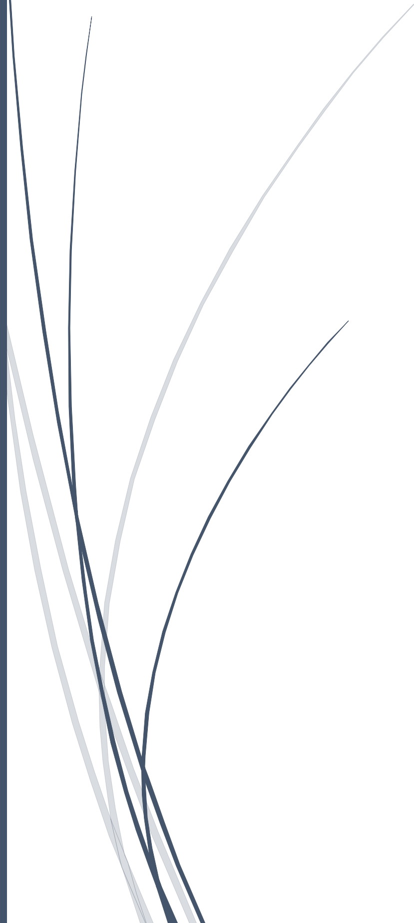
[144] M. K. Choudhary, S. H. Bodakhe, and S. K. Gupta, “Assessment of the antiulcer potential of Moringa oleifera root-bark extract in rats,” *J. Acupunct. Meridian Stud.*, vol. 6, no. 4, pp. 214–220, 2013.

[145] T. G. Monera and C. C. Maponga, “Prevalence and patterns of Moringa oleifera use among HIV positive patients in Zimbabwe: A cross-sectional survey,” *J. Public Health Africa*, vol. 3, no. 1, pp. 22–24, 2012.

[146] S. G. Mahajan and A. A. Mehta, “Anti-arthritic activity of hydroalcoholic extract of flowers of moringa oleifera lam. in wistar rats,” *J. Herbs, Spices Med. Plants*, vol. 15, no. 2, pp. 149–163, 2009.

Objectives

Valorization of plant biomass by emerging technologies: olive and moringa leaves



The general objective of this PhD Thesis is characterization and valorization of olive and moringa leaves in terms of bioactive compounds recovery and purification by using emerging clean technologies 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:

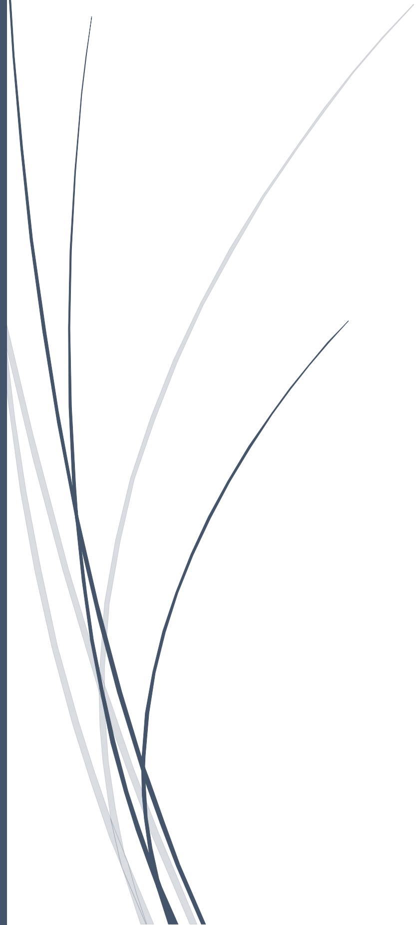
- To obtain a freeze dried extract of olive leaf (OL) rich in bioactive compounds from the Spanish variety "*Serrana de Espadán*". Investigating the effect of the freeze drying technology on individual phenolic profile and antioxidant activity, as well as on other important compounds present in the OL, such as mannitol. Studying the effect of different extraction technologies, specifically, conventional solvent extraction and ultrasound assisted extraction (UAE), type of solvent, OL/solvent ratio and the source of olive leaves on TPC and other bioactive compounds.
- To perform phenolic compounds recovery from moringa leaves by conventional solvent extraction and by using different hydroalcoholic mixture. Obtaining the maximum extraction rate and the time needed to reach equilibrium in an extraction process. Carrying out kinetic study for the different biocompounds in moringa leaf. Comparison of the results obtained by conventional solvent extraction and ultrasound-assisted extraction (UAE). Furthermore, obtaining a freeze-dried powder from an optimized moringa leaf extract and subsequent characterizing of the freeze-dried extract in terms of total and individual phenolic compounds, antioxidant capacity and pigments.
- To study and investigate the effect of process variables (temperature and sample particle size) on the quality and quantity of bioactive compounds extracted from olive leaves by means of subcritical water.
- Purification and selective isolation of phenolic compounds available in olive leaf extract (OLE) by implementing adsorption-desorption process. Utilizing several polymeric resins as adsorbents with two content levels for each and three solvents in desorption stage. Performing analysis of phenolic and sugar content for olive leaf extract, adsorbate and desorbed samples of all test runs.

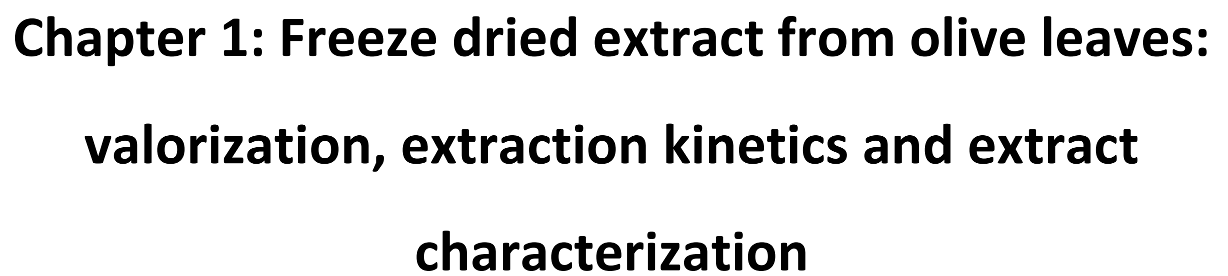
Subsequently, optimization of experimental runs in terms of highest phenol recovery and sugar rejection in order to achieve a phenolic rich composition with minimum impurities.

- Evaluating the effects of membrane filtration on sugar and phenolic content of previously prepared olive leaf extract. Performing a primary microfiltration process for all runs as pretreatment. Then subjecting pretreated extracts as feed to ultrafiltration and nanofiltration processes. Subsequently, characterization of the permeates and retentates obtained from membrane filtration operations to determine their phenolic and sugar profile.

Results

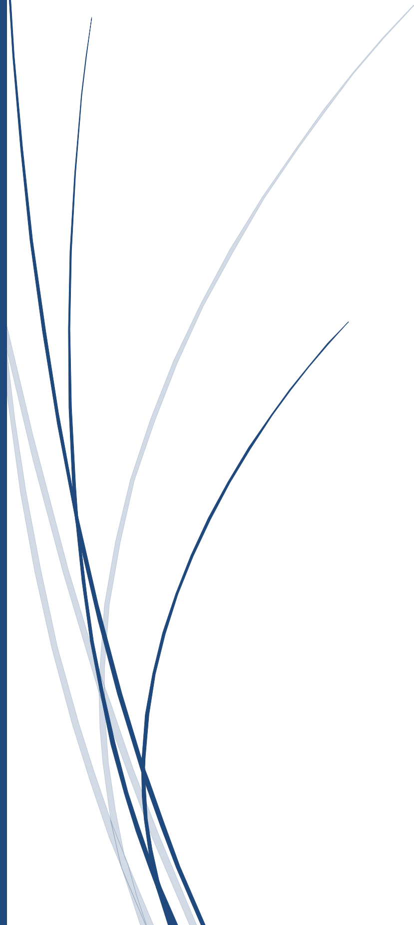
Valorization of plant biomass by emerging technologies: olive and moringa leaves





Chapter 1: Freeze dried extract from olive leaves: valorization, extraction kinetics and extract characterization

Valorization of plant biomass by emerging technologies: olive and moringa leaves



Valorization of olive leaves (OL) in a biorefinery context should include extraction of bioactive compounds, specially taking into account the high content of extractives of this by-product. Extraction of bioactive compounds from Spanish OL (cultivar "*Serrana de Espadán*") was studied by conventional and ultrasound-assisted extraction (UAE). Faster extraction was observed by UAE, although similar final extraction yield was reached by both technologies. The best extraction solvent was an 80 % ethanol hydroalcoholic mixture at a ratio of 20 mL per gram of dried OL (DOL). At these conditions the highest content of oleuropein and luteolin-7-O-glucoside was determined as 31±2 and 4.1±0.2 mg/g_{DOL}. The power law and the Weibull models fitted the total phenolic compounds extraction kinetics quite well. The major soluble carbohydrate was mannitol, with a content of 4.48 ± 0.09 mg/g_{DOL} in the extract. The influence of OL source was also studied and it was concluded that the leaves collected as wastes from the factory presented the highest phenolic yield and antioxidant capacity.

The optimum extract was freeze dried resulting in a solid power with more than 11% of oleuropein and 17% of mannitol. Antioxidant activity of the freeze-dried extract was preserved for two months.

The obtained results of this chapter has been published in journal of Food And Bioproducts Processing, vol. 124, pp. 196-207, 2020.

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La valorización de la hoja de olivo (OL) en un contexto de biorrefinería debe incluir la extracción de compuestos bioactivos, especialmente teniendo en cuenta el alto contenido de extractos de este subproducto. Se estudió la extracción de compuestos bioactivos de OL español (cultivar "*Serrana de Espadán*") mediante extracción convencional y asistida por ultrasonido (EAU). Los EAU observaron una extracción más rápida, aunque ambas tecnologías alcanzaron un rendimiento de extracción final similar. El mejor disolvente de extracción fue una mezcla hidroalcohólica de etanol al 80% en una proporción de 20 ml por gramo de OL seco (DOL). En estas condiciones, el contenido más alto de oleuropeína y luteolin-7-O-glucósido se determinó como 31±2 y 4,1±0,2 mg/g_{DOL}. La ley de potencia y los modelos de Weibull se ajustan bastante bien a la cinética de extracción de compuestos fenólicos totales. El principal carbohidrato soluble fue manitol, con un contenido de 4,48±0,09 mg/g_{DOL} en el extracto. También se estudió la influencia de la fuente de OL y se concluyó que las hojas recolectadas como desechos de la fábrica presentaron el mayor rendimiento fenólico y capacidad antioxidante.

El extracto óptimo se liofilizó dando como resultado un poder sólido con más del 11% de oleuropeína y 17% de manitol. La actividad antioxidante del extracto liofilizado se conservó durante dos meses.

Los resultados obtenidos de este capítulo han sido publicados en revista de Food And Bioproducts Processing, vol. 124, pp. 196-207, 2020.

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PhD Thesis

Chapter 1: Freeze dried extract from olive leaves: valorization, extraction kinetics and extract characterization

1.1. Introduction

The olive tree (*Olea europaea* L.) is one of the oldest known cultivated plants. It is usually native to Mediterranean countries and its cultivation has spread globally during the past two decades due to the healthiness attributed to the consumption of olive oil. More than 8 million of olive trees are cultivated worldwide; almost 98% of them are in the Mediterranean basin [1]. Spain is the country with the largest olive orchard acreage and with the highest number of olive trees [2].

Olive leaves (OL) are one of the byproducts of olive farming and they can be generated during the pruning of olive trees and also in the separation process before olive processing (about 10 % of the weight of the olives) [3]. The production of OL from pruning is about 25 kg per olive tree plus 1 extra kg of leaves that can be collected at the oil mill [4]. Lama Muñoz et al. [5] reported that the amount of OL that accumulate annually from the olive industry may exceed 1 million tons, being this residue an attractive candidate to be considered as raw material in a biorefinery context.

In many countries OL have traditionally been used as animal feed or simply burned them together with the branches from pruning [6]. However, OL present a chemical composition with important amounts of extractives, lignin, protein and carbohydrates [7]. A complete biomass valorization should include first the extractive fraction valorization. Extractives valorization is especially important for OL since they contain an important fraction of extractives reaching values up to 45 % with a high content of polyphenol compounds [8]. Phenolic composition of the OL extracts can vary as a function of the extraction method applied and sample origin [7]. Most of the studies have reported oleuropein as the main phenolic compound in OL. Oleuropein has anti-inflammatory, antioxidant and antimicrobial properties. Other important phenolic compound in OL is luteolin-7-O-glucoside that is a potent drug against colon carcinogenesis [5]. Therefore, due to the increasing demand for the replacement of chemical additives by natural ones, OL extracts could be considered as an important, easily available and inexpensive raw material to be considered as natural antioxidant. Romero-García et al. [9] reviewed different applications of OL, within the context of a biorefinery based on olive biomass. These authors reported health and medical food applications but also in supplemented foods or even nutraceuticals. Other valuable

compounds present in OL are polyols or sugar alcohols, mainly mannitol. Mannitol comprises a significant portion of the soluble carbohydrate pool [10] and presents a sweetness potency equivalent to 70 % sucrose, but with low caloric values (2 kcal/g) [6]. Furthermore, mannitol has also antioxidant properties that make it useful for applications in the food and pharmaceutical industries [11]. Currently mannitol is obtained by catalytic hydrogenation from glucose-fructose mixtures; however, low mannitol yield is reached and high reaction temperature and pressure are used. Therefore, OL can be considered as a natural source of mannitol versus the current industrial production.

To convert the OL into valuable added products, the first step should be the extraction of the bioactive compounds. Different extraction techniques can be applied. The most commonly extraction system used has been the solid–liquid extraction by maceration of the plant biomass in a solvent. Among the different extraction technologies, ultrasound-assisted extraction (UAE) technology has been reported as a clean, green, extraction technique that presents several benefits such as the enhance of the extraction yield or the enhance of the extraction of heat-sensitive components [12]. The industry usually prefers to use powdered extracts instead of phenolic compounds in liquid environments. Furthermore, drying of the extracts with high content of phenolic compounds will help to preserve these bioactive compounds.

The aim of this study was to obtain a freeze dried extract of OL rich in bioactive compounds from the Spanish variety "*Serrana de Espadán*". This variety has been hardly studied for their bioactive compounds and antioxidant capacity. The effect of the freeze drying technology on individual phenolic profile and antioxidant activity, as well as on other important compounds present in the OL, such as mannitol was investigated. First, optimization of the extraction of total phenolic compounds, TPC, from OL was carried out. The effect of different extraction technologies, specifically, conventional solvent extraction and ultrasound assisted extraction (UAE), type of solvent, OL/solvent ratio and the source of olive leaves on TPC and other bioactive compounds was studied. In this work as solvent type different ethanolic aqueous mixtures were selected; however, it must be highlighted that the development of new novel green solvents such as the use of deep eutectic solvents combined with different extraction

technologies is a promising technology in the recovery of bioactive compounds from different plants and crops [13–15]. TPC extraction kinetics were fitted to different empirical extraction kinetic models. Furthermore, other values of TPC and oleuropein content in OL extracts reported previously in the literature have been summarized for a better comparison between the different data taking into account the solvent type, extraction technology, variety and equilibrium time. The optimum extract was freeze dried to protect and concentrate the easily oxidizable phenolic compounds, as well as other valuable compounds such as mannitol and pigments present in the freeze dried extract.

1.2. Materials and methods

1.2.1. Raw material

OL samples were collected from two different sources in three different types: (1) Spanish OL from the pruning of olive trees of the variety "*Serrana de Espadán*", provided by Maicas-Sediles (Teresa, Castellón). (2) Iranian OL from the pruning of olive trees provided by the cooperative “Mamalan” Agriculture & Industry Company Tarom city (Zanjan, Iran) and (3) Iranian OL collected as by product in the oil factory. OL were dried in a convection oven at 45 ºC to reduce the humidity to values lower than 10 %. This initial drying step by hot-air has resulted in higher oleuropein recovery in previous studies reported in the literature [16]. After drying, OL were grounded by using a cutting mill (Retsch SM100, Retsch Technology GmbH, Germany) to obtain a particle size in the range from 0.5 to 0.1 mm to increase the interfacial surface and improve extraction. Samples were stored in the freezer at -18ºC until extraction studies.

1.2.2. Determination of chemical composition of olive leaves

Spanish OL were characterized according to the Standard Biomass Analytical Methods provided by the National Renewable Energy Laboratory (https://www.nrel.gov/bioenergy/biomass-compositional-analysis.html).

The lipid fraction of Spanish OL was analysed by Soxhlet extraction method using a BUCHI 811 extraction system (Buchi Laboratotiums Technik AG, Flawil, Switzerland). The fatty acid profile was determined by the official method of the AOAC. The chromatographic method has been detailed elsewhere [17]. Protein content of OL was determined by elemental analysis by a Shimadzu TOC-V-CSN (Shimadzu, Japan) analyser by applying a conversion factor of 6.25.

The total amount of mannitol in OL was determined by following the same extraction procedure as Oddo et al. [10]. 300 mg of powdered OL were extracted twice with 10 mL of a hydroalcoholic mixture, 80% ethanol (v/v), and once with 10 mL of H_2O, overnight at room temperature. The combined extracts were subjected to mannitol quantification by HPLC.

1.2.3. Extraction procedures

1.2.3.1. Conventional solvent extraction

Extractions were performed in an orbital shaker (Grant instruments-OLS 200, Shepreth Cambridgeshire, England). For Spanish OL, extraction kinetics were carried out for 1 h at 50 ºC and samples were taken periodically. Different ethanol aqueous mixtures were selected as extraction solvents due to their food grade features (100 % ethanol, 80 % ethanol, 50 % ethanol, 20 % ethanol and 100% water). The effect of solvent volume to DOL mass ratio was determined by varying this ratio from 20 mL/g_{DOL} to 3 mL/ g_{DOL} by using an 80 % ethanol aqueous mixture (v/v) as extraction solvent at 50ºC for 1h.

Conventional solvent extractions were also carried out for Iranian OL from two different sources: IOL-T from the pruning of trees and IOL-F from the factory at 50ºC, a ratio of 20 mL:g_{DOL}. Extracts were compared with the results obtained for Spanish OL.

1.2.3.2. Ultrasound assisted extraction (UAE)

Ultrasound assisted extraction (UAE) (VibraCell^{TM} 75042, Bioblock Scientific, U.S.A.) was performed by using water as solvent. UAE was carried out by using a 750 W Sonics Material TM with a 13 mm probe. Samples were processed at a constant ultrasound frequency of 20 kHz and 79 µm of amplitude and a ratio of 20 mL solvent/ g_{DOL} was used. After a short initial period of time, temperature was kept at 50 ºC by using a thermostated vessel where the jacketed water was circulated constant at 30 ºC. OL were introduced in the vessel (Φ = 4.8 cm, V = 199 cm^3) and the probe was submerged in the solution at a constant depth of 2 cm from the bottom of the vessel [18]. The temperature was continuously recorded during extraction and samples were taking periodically to follow the extraction kinetics.

1.2.4. Freeze-drying process

A freeze-dried extract was obtained from the liquid extract obtained at 50 ºC by using 80% ethanol aqueous mixture at 20 mL solvent/g_{DOL} ratio. Before freeze drying, ethanol was removed by a rotary evaporator and afterwards, the remaining extract was submitted to freeze drying. First, samples were frozen with liquid nitrogen (-196 ºC), equilibrated at -80 ºC for 2 h and then submitted to freeze-drying in a Labconco Freeze Dry System (Labconco Corporation, U.S.A.) at $1.5 \cdot 10^{-4}$ mbar during 48 h. The moisture content of the freeze dried particles was determined gravimetrically by weighing small amounts of dried particles (around 0.5 g) before and after drying in an oven at 120 ºC until constant weight.

1.2.5. Modeling of extraction kinetics and statistical analysis

The knowledge of the kinetics of the extraction of the selected compounds is important to reduce the consumption of energy and work-up and to know the time needed to obtained a certain extraction yield [19]. In this chapter, total phenolic content (TPC) data along extraction were fitted to two different kinetic models, the power law and the Weibull models. The power law model can be represented as:

$$Y\ (mg\ GAE/gDOL) = B \cdot t^{n} \quad (1)$$

where B is a constant incorporating the characteristics of the carrier active agent system, and n is the diffusional exponent. This value is lower than 1 for plant material [20]. The Weibull model was expressed as:

$$Y\ (mg\ GAE/gDOL) = A \cdot (1 - exp(kt^{n})) \quad (2)$$

The exponent n indicates the shape of the extraction curve. If $n > 1$, the curve is sigmoid and if $n < 1$, the curve is parabolic. A represents the maximum extraction yield.

The estimation of the kinetics parameters was carried out by non-linear regression by using the Marquardt algorithm (Statgraphics X64). The quality of the fitting was presented by evaluating the root mean square deviation (RMSD):

$$RMS = \sqrt{\frac{\sum_{i=1}^{n}\left(Y_{i,exp} - Y_{i,calc}\right)^{2}}{n}} \quad (3)$$

where $Y_{i,exp}$ and $Y_{i,calc}$ are the experimental and calculated extraction yield and n is the number of experimental data points in each extraction.

Statistical differences were obtained using the software Statgraphics X64. The results were presented as the mean ± standard deviation of at least three replicates. The significance of the differences was determined based on an analysis of the variance with the Fisher's least significant difference (LSD) procedure at p-value ≤ 0.05.

1.3. Results and discussion

1.3.1. Biomass composition

Chemical composition was only performed for the Spanish OL and is presented in Table 1.1. It must be highlighted the high content of extractives, around 25% in a dry basis.

Therefore, within a biorefinery concept, valorization of the extractive fraction of OL is a key initial step in this specific feedstock due also to the high content of TPC in the extractive fraction. Table 1.1 also lists the chemical composition for other olive tree pruning leaves found in the literature, where the presence of extractives was also remarkable reaching values up to 45 % [8,21]. The total content of mannitol in water (0.63 ± 0.02 %, w/w) and ethanol (3.36 ± 0.01 %, w/w) extractives was a bit lower (3.99 ± 0.03 %, w/w) than the content determined by the procedure described by Oddo *et al.* [10], 5.50 ± 0.05 (% w/w). This value was of the same order as the one reported by Oddo *et al.* [10], with a mean value for Olea leaves samples of 277 µmol mannitol/g_{DOL} (5.05 % of mannitol w/w). These authors also found that there were not significant variations in the mannitol content during the year for Olea samples, either due to rainfall or to temperature.

The total structural carbohydrate fraction accounted for nearly 30% (12.5 % hemicellulose and 17.5 % cellulose). Manzanares et al. [8] reported lower values for total structural carbohydrates, but similar values were reported by Gullón et al., (see Table 1.1) [21]. These differences can be attributed to the different olive varieties. Valorization of this carbohydrate fraction is also interesting from a biorefinery point of view, although is not the objective of this work. The content of acid insoluble lignin was 11 %; since lignin in plants burns very effectively it could be used as bio-based alternative to petroleum [8].

The fatty acid profile of the lipid fraction, 2.7 % (w/w), was presented in Table 3. The most abundant fatty acid was the polyunsaturated acid linolenic acid (> 40 %, w/w) while oleic acid, accounted for 13% w/w. Similar profile has been reported by Carvalheiro *et al.* [22] for OL cultivated in Southern Brazil.

Table 1.1. Chemical composition of "*Serrana de Espadán*" OL expressed as g/100 g_{DOL}.

Compound	**This work**	**(Gullón et al., 2018) [21]**	**(Manzanares et al., 2017) [8]**
Cellulose	17.5 ± 0.6	21.6 ± 0.2	9.3 ± 0.4
Hemicellulose	12.5 ± 0.5	14.5 ± 0.2	9.5 ± 0.2
Xylose	7.8 ± 0.3	9.0 ± 0.0	4.5 ± 0.1
Galactose	--	2.0 ± 0.0	2.0 ± 0.1
Arabinose	4.7 ± 0.2	2.8 ± 0.2	4.0 ± 0.4
Mannose	--	0.5 ±0.1	0.3 ±0.0
Extractives	24.6 ± 2	28.6 ± 1.4	45.2 ± 1.5
Water soluble extractives	16.4 ± 0.9	23.5 ± 1.4	
Glucose	2.2 ± 0.2	7.3 ± 0.1	
Sacarose	0.28 ± 0.04		
Phenolics	1.4 ± 0.1	2.9 ± 0.0	
Mannitol*	0.63 ± 0.02		Glucose 7.1 ± 0.1
Ethanol soluble extractives	8 ± 1	5.1 ± 0.2	Phenolics 4.4 ± 0.2
Glucose	0.37 ± 0.01		
Sacarose	0.11 ± 0.01		
Phenolics	1.7 ± 0.1		
Mannitol*	3.36 ± 0.01		
Acid-insoluble lignin	10.76 ± 0.02	15.4 ± 0.4	15.1 ± 0.5
Acid-soluble lignin	6.2 ± 0.3	2.3 ± 0.1	2.6 ± 0.2
Ash	4.69 ± 0.02	3.9 ± 0.6	8.3 ± 0.2
Lipid	2.7 ± 0.2	nr	nr
Proteins	10.3 ± 0.4	3.1 ± 0.2	nr

nr: non reported, phenolics are expressed as mg $_{GAE}$/ g_{DOL}, proteins determined as 6.25×N;

**Total mannitol content as determined by Oddo et al. [10] = 5.50 ± 0.5 % (w/w)*

Table 1.2. Fatty acid profile (%, w/w) for the *Serrana de Espadán* olive leaf.

Fatty acid	**Percentage**	
Myristic acid (C14:0)	2.50 ± 0.01	
Palmitic acid (C16:0)	22.8 ± 0.1	
Palmitoleic acid (C16:1n-7)	1.90 ± 0.05	
Stearic acid (C18:0)	3.50 ± 0.06	
Oleic acid (C18:1n-9)	12.7 ± 0.4	
Linoleic acid (C18:2n-6)	15.9 ± 0.1	
Linolenic acid (C18:3n-3)	40.2 ± 0.4	
SFA: 28.8 ± 0.2	MUFA: 14.6 ± 0.5	PUFA: 56.1 ± 0.5

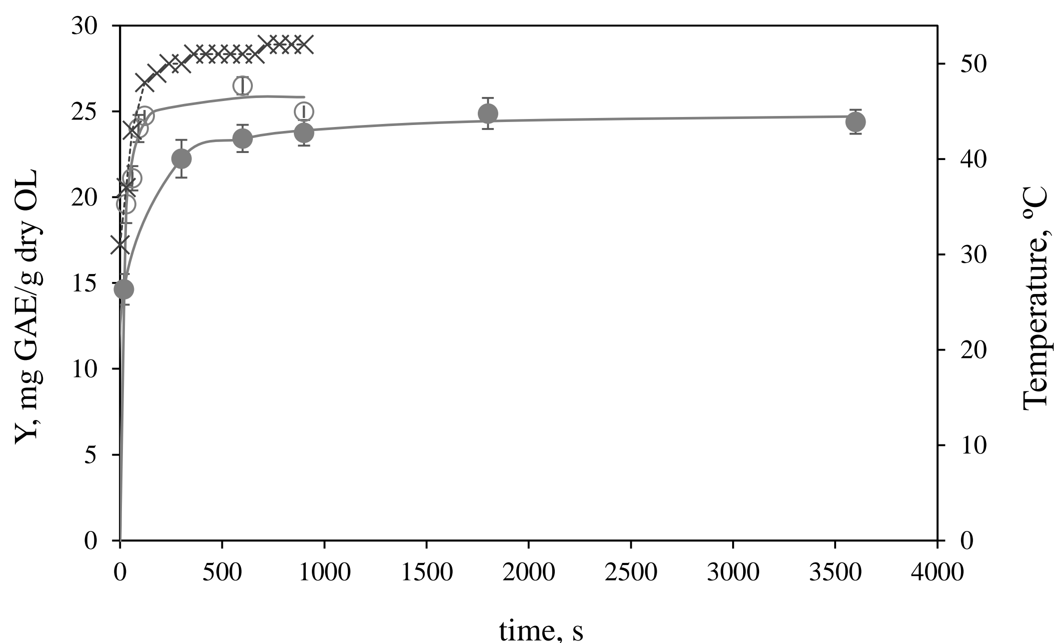


Figure 1.1. TPC extraction kinetics by using water as solvent at 50ºC.

(× Temperature profile in the UAE), 20 mL of solvent/g_{DOL} (●: conventional solvent extraction, ○ ultrasound assisted extraction). Continuous lines represent the Weibull model.

1.3.2. Solid liquid extraction of total polyphenol compounds

1.3.2.1. Comparison of conventional and ultrasound assisted extraction techniques

Figure 1.1 shows TPC extraction kinetics by conventional solvent extraction and by UAE by using water as extraction solvent, at 50 ºC and 20 mL of solvent/g_{DOL}. Other studies on TPC extraction form olive leaves by conventional solvent extraction and UAE have been carried out in the temperature range from 25 to 70 ºC (see Table 1.3). An increase in temperature usually enhances the solubility and diffusivity of the targeted compounds, improving the mass transfer between the matrix and the solvent. However, it could also cause phenolic compounds degradation. Therefore, in this work, 50 ºC was chosen as operating temperature to propose also a more cost-effective process.

Figure 1.1 also shows the temperature profile for the 15 min of UAE A sharp temperature increase was observed during the first 3 min of TS, where most of the extraction took place. The acoustic energy density in the UAE experience has been evaluated according to O'Donnell et al. [23] resulting in 0.84 J/s·g. For both technologies, a fast initial extraction period was observed followed by a second slow period controlled by diffusion. These type of extraction curves have been also described in the literature for bioactive compounds extraction from different plant materials [20]. Faster extraction kinetic was observed by UAE than by conventional extraction. The final extraction yield was reached at 120 s by UAE while ten times longer were needed by conventional extraction, 1200 s. The faster extraction by UAE was reflected in the higher values of the initial extraction rate, 0.21 mg GAE/g_{DOL}·s vs 0.075 mg GAE/g_{DOL}·s by UAE and conventional extraction, respectively. Cavitation created by the ultrasound waves induced a better penetration of the solvent into the OL and improved the diffusion process. Furthermore, the increase in the extraction rates can be attributed to the increased contact surface between the solid and the liquid phase by particle disruption [24]. Despite the faster extraction, similar final extraction yields were reached by both extraction technologies. In any case, fast extraction of TPC was observed by both technologies probably due to the easy extraction of compounds

weakly linked to the cell walls [25]. Kemdhakem *et al.* [26] also reported faster extraction kinetic of TPC from OL from Tunissia by UAE than by conventional extraction, using water as solvent with similar extraction yields by both technologies, 11 mg GAE/g_{DOL} (at 2.5 % w/v and 50 ºC, see also Table 1.3). These authors also observed a fast extraction of TPC since 80 % of TPC were extracted in only 1 min.

Table 1.3 lists other values of equilibrium extraction times reported in the literature regarding TPC from OL for comparison. Lafka *et al.* [27] reported an equilibrium time longer than 180 min for different extraction solvents including pure ethanol and ethanol:water mixtures (1/1) when extraction was performed in an orbital shaker (5:1 v/w). Bilgin and Sahin [28] determined that 60 min were enough to reach the equilibrium time for TPC extraction by using methanol as extraction solvent by UAE.

Based on the results obtained in this work, and taking into account the fast extraction kinetics obtained by both technologies, with similar TPC extraction yields, conventional extraction was considered a suitable technology for TPC extraction from olive leaves and the effect of other extraction variables was only determined for conventional extraction.

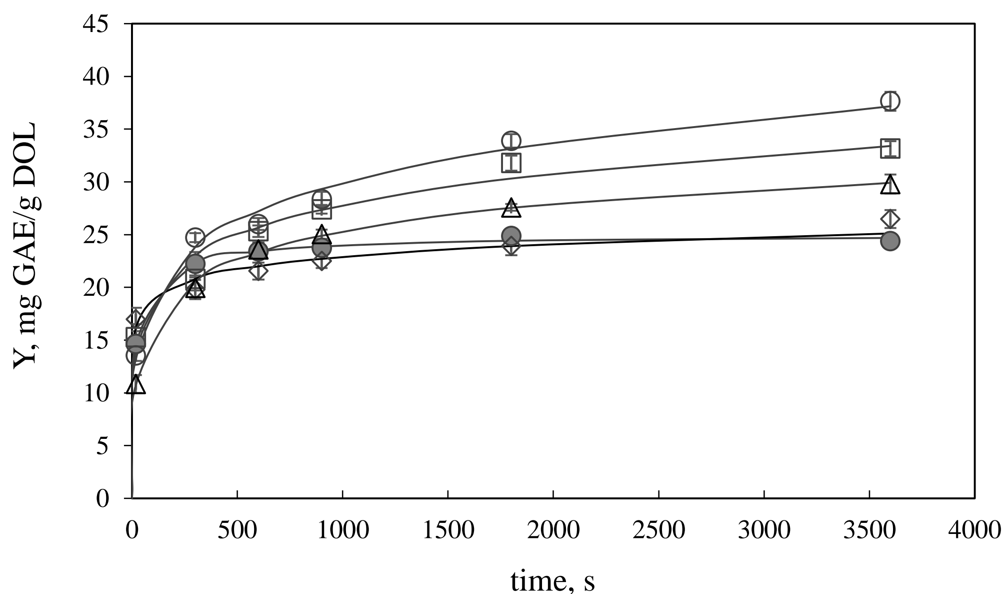


Figure 1.2. Effect of type of solvent on TPC extraction kinetics from Spanish OL (T = 50ºC, 20 mL solvent/g dry OL): ● 100% water, ◇ 20 % ethanol, □ 50 % ethanol, ○ 80 % ethanol, △ 100 % ethanol. Continuous lines represent the Weibull model.

Table 1.3. Comparison of TPC and oleuropein content of OL extracts from different OL cultivars by using different extraction solvents.

Solvent	Extraction method	Variety	solvent:gOL (mL:g)	TPC mg $_{GAE}$/g$_{DOL}$	T, ºC	t, min	Oleuropein mg/g$_{DOL}$	Reference
80 % EtOH	Conventional	"Serrana de Espadán"	20:1	37.6	50	60	31± 2	This work
70 % EtOH	Conventional	Istrsk belica (Slovenia)	5:1	32.7	25	120	27.3	[29]
70 % EtOH	UAE	Istrsk belica (Slovenia)	5:1	138.4^{10min}	25	--	38.1^{120min}	[29]
47 % EtOH	UAE	Picual (Jaen)	17:1	22.3 ± 0.8	room	50	4.2 ± 0.2	[30]
EtOH	UAE	Picual (Jaen)	20:1	14.1 ± 0.9	room	30	13.7 ± 6	[30]
Molasses alcohol	Omni Mixer	Arbequina Hojiblanca Lechin Picual	20:1	--	--	3	45.3 ± 3.4 65.7 ± 6.5 61.3 ± 4.3 87.2 ± 8.2	[31]
60 % EtOH	Dynamic maceration	Picual	12:1	41.1	55		31.8	[5]
60 % EtOH	Soxhlet	Picual	15:1	42.9 ± 0.4	--	240	65.77	[32]
60 % EtOH	UAE	Picual	13:1	35.77 ± 0.6	--	17.9	69.91	[32]
60 % EtOH	Conventional	Picual	--	42.5		240	49.1	[33]
Water	Conventional	Chemchali	40:1	12	70	10	6.5	[26]
Water	UAE	Chemchali	40:1	12	70	10	6.5	[26]
MeOH	Ultrasonic bath	Bursa (Anatolia, Tk) Canakkale (Anatolia, Tk)	20:1	38.66 7.35	25	90	--	[28]
MeOH	HAE	Bursa (Anatolia, Tk) Canakkale (Anatolia, Tk)	20:1	64.66 10.11	25	25000xg (3 times)	--	[28]

HAE: homogenizer assisted extraction

Table 1.4. Phenolic profile of OL extract by using different hydroalcoholic mixtures (mg/g_{DOL}).

Solvent	Hydroxytyrosol	Catechin	Rutin	Verbascoside	Luteolin	Luteolin-7-O-glucoside	Oleuropein
Water	0.13 ± 0.01[a]	0.067 ± 0.001[a]	n.d.	n.d	n.d.	0.09 ± 0.01[a]	0.24 ± 0.03[a]
20 % EtOH	0.27 ± 0.08[a,b]	2.85 ± 0.06[c]	0.28 ± 0.03[b]	n.d	n.d.	2.07 ± 0.05[c]	3.7 ± 0.5[b]
50 % EtOH	0.39 ± 0.09[b]	3.1 ± 0.3[c]	0.5 ± 0.1[c]	1.0 ± 0.2[b]	n.d.	4.2 ± 0.2[d]	15 ± 2[c]
80 % EtOH	0.7 ± 0.2[c]	1.4 ± 0.5[b]	0.22 ± 0.01[b]	1.2 ± 0.1[c]	n.d.	4.1 ± 0.2[d]	31± 2[e]
100 % EtOH	0.17 ± 0.01[a,b]	0.46 ± 0.06[a]	0.026 ± 0.004[a]	0.022 ± 0.002[a]	n.d.	0.9 ± 0.2[b]	26 ± 1[d]

Values with different letters in each column are significantly different when applying the Fisher's least significant difference (LSD) method at p-value ≤0.05.

1.3.2.2. Effect of type of solvent

The effect of type of solvent was studied by conventional solvent extraction by varying the composition of water-ethanol mixtures. Five different mixtures were employed as solvents, water, ethanol and different ethanol/water mixtures: 80/20, 50/50, 20/80 (v/v). The results are presented in Figure 1.2. The fastest kinetic extraction and the highest extraction yield were obtained for an 80 % ethanol aqueous mixture (v/v) with a value of 38 ± 1 mg GAE/g_{DOL}). This result has been also found for other plant matrixes that reported that ethanol alone was less effective than hydroalcoholic mixtures, showing that water plays an important role on phenolic compounds extraction [34]. The better efficiency of water-ethanol mixtures to improve TPC extraction compared to pure ethanol and water solvents has been explained considering the double effect of water and ethanol mixtures, since water swells the plant matrix, while ethanol could disrupt the bonding between the solute and the plant matrices. The addition of water to organic solvents, such as ethanol, help to create a more polar medium that facilitate the extraction of compounds that are soluble in organic solvents and/or water [35]. Therefore, the ratio between ethanol and water is a key factor to be considered to obtain a high extraction yields on phenolic compounds due to their relative polarity.

Other values of TPC extraction yields found in the literature by using ethanol aqueous mixtures as solvent have been collected in Table 1.3. In general, solvent mixtures with ethanol content higher than 50 % (v/v) yielded good results for the different olive cultivars in terms of TPC and the most abundant phenolic compound in OL, oleuropein. Regarding the different extraction methods, those based on the use of pressurized water have not been included in Table 1.3. In general, conventional extraction resulted in good extraction yields compared to other methods more energetically costly, such as UAE. Different TPC was determined for the different olive cultivars, but in most cases values ranged from 30 to 40 mg GAE/g_{DOL} for extraction times between 10 and 120 min, similar to the values obtained in this work.

Figure 1.3. Olive leave extracts by using different solvents. From left to right 100 % water, 20 % ethanol, 50 % ethanol, 80 % ethanol and 100 % ethanol.

1.3.2.2.1. Influence of extraction solvent on phenolic profile

The influence of the type of solvent was also evaluated on the individual phenolic compounds profile in the extracts and on other compounds such as soluble carbohydrates. The extract color by using different ethanol aqueous mixtures can be appreciated in Figure 1.3. The extraction of different pigments can be clearly observed in the variation of the color of the liquid extract from light orange, by using water as solvent, to a more greenish color by using pure ethanol as solvent. The increase in the green color by increasing the amount of ethanol in the extraction solvent indicates the presence of chlorophylls in these extracts.

The individual phenolic compounds that could be identified by HPLC-DAD have been listed in Table 1.4. For all the solvents essayed, oleuropein was the major phenolic component in the OL. It was observed a similar trend for oleuropein content as for TPC, with a maximum content of 31 ± 2 mg oleuropein/g_{DOL} (3.1 % w/w) for an 80 % ethanol aqueous solution (v/v). This result agrees with other literature data that showed that mixtures of organic solvents can lead to higher oleuropein extraction compared to pure solvents [29].

In Table 1.3, the content in oleuropein obtained in this work can be compared with other values reported in the literature. Values ranged between 13.7 up to 87 mg oleuropein/g_{DOL} for different ethanol aqueous mixtures and different OL cultivars.

Oleuropein was also determined as the major phenolic compound by using other extraction solvents. Bouaziz et al. [36] reported a value of 6.8 % (w/w) of oleuropein for OL from Tunisia when using a methanol aqueous mixture (4:1, v/v, 60 g OL/300 mL). Regarding the differences reported in oleuropein content, Erbay et al. [37] reviewed that the most important variables affecting the oleuropein content was the type of cultivar and the different geographical origin, with higher oleuropein content in younger greener leaves.

Other important phenolic compounds determined in this work for OL extracts were luteolin-7-O-glucoside with 4.2 ± 0.2 mg /g_{DOL} and verbascoside, 1.2 ± 0.1 mg / g_{DOL}. Lama et al. [33] reported values between 4.65 and 1.31 mg luteoling7-O-glucoside/g_{DOL} and 0.80 to 2.23 mg verbascoside/g_{DOL} for commercial OL samples from Arbequina and Picual cultivars, respectively. The amount of hydroxytyrosol determined in this work in OL extracts was not very high 0.7 ± 0.2 mg/g_{DOL}, but it was within the range of other varieties (0.08-3.4 mg/g) and was much higher than the amount of hydroxytyrosol found in olive oil of Spanish olive varieties (0.93 mg/kg) [38].

The phenolic profile of OL is known to be affected by several agronomical and technological factors such as leaf age, degree of ripeness, geographical origin, cultivar, phonological stage during sampling, proportion of brunches on the tree, moisture content, degree of contamination with soil and industrial processes employed for extraction [39].

The co-extraction of other valuable compounds from OL such as mannitol has been also determined for the different extraction solvents. The amount of mannitol and other soluble saccharides has been listed in Table 1.5. The mannitol content in the extracts decreased as the amount of ethanol in the extraction solvent increased with no significant difference for water and ethanol aqueous solutions with ethanol content lower than 50 % (v/v). The content of mannitol agrees with the recently reported data of solubility of the different polymorphs of D-mannitol in ethanol-water mixtures that determined that the solubility of the three polymorphs decreased with the rise of the ethanol ratio in the binary solvent mixture [40]. The decrease in the amount of the other soluble saccharides identified in the liquid extracts with the increase of ethanol in the solvent mixture also agreed with the solubility order of glucose and sucrose in ethanol

aqueous mixtures [41,42]. Guinda et al. [31] found that molasses ethanol 96 %, was an adequate solvent for a selective extraction of mannitol, with values between 2.58 to 3.97 % (w/w) for different Spanish Olea europea cultivars with lower values of sucrose and glucose in the ethanolic extracts, from 0.62 to 1.03 % and from 0.22 to 0.46 % (w/w), respectively. In this work, the relative percentage of mannitol considering only the three soluble carbohydrates determined in this work, varied from 67 to 83 % for pure water and ethanol, respectively, with no significant difference for ethanol concentrations higher than 50 % in the extraction medium. According to Oddo et al. [10] mannitol was the main constituent of the soluble carbohydrates in the OL of four species of Oleaceae analysed. This was also confirmed in this work for the different hydroalcoholic mixtures essayed in this work. From Table 6 it can be concluded that mannitol accounted for more than 60 % (w/w) of the soluble carbohydrates in the extracts for any of the solvents studied in this work.

Other values of sugars and mannitol of OL from commercial varieties reported in the literature ranged between the following values in a dry basis: mannitol, 3.7 -8.2 %, glucose, 0.6 -1.8 % and arabinose 0.1 – 0.2 % when using a mixture 60/40 ethanol water (v/v) as solvent [33]. These authors also reported galactose, xylose and mannose as soluble sugars, but in this work these sugars could not be detected when comparing the retention times to those of authentic standards.

Table 1.5. Saccharides and polyols, g/100 g_{DOL}, in the liquid extracts by using different hydroalocholic mixtures as solvent.

Solvent	Mannitol	Glucose	Sucrose
Water	4.8 ± 0.1^{c}	2.21 ± 0.04^{d}	0.96 ± 0.09^{b}
20 % EtOH	4.9 ± 0.1^{c}	1.28 ± 0.05^{c}	0.98 ± 0.08^{b}
50 % EtOH	5.06 ± 0.09^{c}	1.08 ± 0.06^{b}	1.05 ± 0.06^{b}
80 % EtOH	4.48 ± 0.09^{b}	0.99 ± 0.05^{b}	1.01 ± 0.06^{b}
100 % EtOH	2.89 ± 0.05^{a}	0.72 ± 0.06^{a}	0.6 ± 0.1^{a}

Values with different letters in each column are significantly different when applying the Fisher's least significant difference (LSD) method at p-value ≤0.05.

Table 1.6. Kinetic model parameters for TPC extraction at 50ºC for the power law and the Weibull models.

Extraction	**Power law**			**Weibull**			
	B	**n**	**R^2/RMS**	**A**	**k**	**n**	**R^2/RMS**
CE-Water	12.5 ± 1.5	0.091 ± 0.02	0.982 / 1.25	24.8 ± 0.4	0.34 ± 0.03	0.33 ± 0.03	0.995 / 0.21
UAE-Water	16.7 ± 1.8	0.067 ± 0.02	0.980 / 1.30	25.8 ± 0.7	0.23 ± 0.13	0.53 ± 0.15	0.70 / 0.914
20 % EtOH	12.6 ± 0.7	0.082 ± 0.01	0.995 / 0.62	36 ± 30	0.41 ± 0.10	0.13 ± 0.08	0.930 / 0.73
50% EtOH	9.1 ± 0.9	0.161 ± 0.01	0.991 / 1.22	58 ± 41	0.14 ± 0.07	0.22 ± 0.10	0.955 / 1.11
80% EtOh	7.9 ± 0.6	0.191 ± 0.01	0.997 / 0.74	66 ± 37	0.11 ± 0.05	0.25 ± 0.06	0.986 / 0.77
100% EtOH	7.2 ± 0.7	0.178 ± 0.01	0.993 / 0.89	35 ± 2	0.15 ± 0.01	0.31 ± 0.02	0.986 / 0.26

CE: conventional extraction

1.3.2.2.2. Extraction kinetics modelling

Based on the extraction curve shapes, a high initial slope and a slower second period, the power law and the Weibull models were used to fit the experimental extraction kinetic data. Parameters for both models are listed in Table 1.6. Both models can be considered suitable to describe the extraction curves of TPC from OL. The best model was the Weibull with a medium value of the RMS of 0.63 for all the extraction kinetics. The good fitting can be observed in Figures 1.1 and 1.2 where the Weibull model has been represented.

For the power law model, the diffusion coefficient, n, presented values lower than 0.2, which showed that Fickian diffusion controlled TPC extraction from OL. Similar results have been reported by Lafka et al. [27] for the extraction of phenolic compounds from wild olive leaves using different extraction solvents (methanol, ethanol, ethanol:water, n-propanol, isopropanol and ethyl acetate) and for other plant matrixes [34]. The lowest values of n were found for the TPC extraction curves for pure water and ethanol aqueous mixtures with high water content. Regarding the Weibull model, the extraction parameter, A, that can be considered as the maximum extraction yield of TPC, reached a maximum for an 80% ethanol aqueous solution as solvent while the values of k, reached a minimum at this solvent mixture.

1.3.2.3. Effect of solvent/biomass ratio

The effect of solvent:OL ratio was studied for water as solvent and the optimum extraction solvent found in previous section, 80% ethanol (v/v) aqueous solution. For these two solvents extractions were performed for 1 h at 50ºC at different solvent:OL ratios: 3, 5, 8, 10, 15, 20 and 25 mL/g_{DOL}.

The extraction yield, expressed as mg GAE/g_{DOL}, increased by increasing the solvent/OL ratio, reaching a plateau at 15-20 mL of solvent:g_{DOL} for both solvents (Figure 1.4). This behavior can be explained taking into account the higher driving force of the process by increasing the solvent:OL ratio, leading to higher diffusion rate. The

concentration gradient between the solid and the solvent, is higher when a lower amount of solid is used. However, the optimum solvent:OL ratio should also take into account economic factors to provide high recoveries with minimum solvent consumption. Figure 1.4 also shows that the total polyphenol concentration, mg GAE/L, in the extraction medium continuously decreased by increasing this ratio due to the dilution effect. Therefore, 15-20 mL/g_{DOL} can be considered as the optimum solvent:OL ratio for TPC from OL.

Lafka et al. [27] found similar results observing an increase in the extraction efficiency by increasing the ratio solvent:OL due to larger concentration gradients. These authors found an optimum solvent/sample ratio of 5:1(v/w) as the most suitable ratio for TPC extraction. Cifá et al. [29] reported that the ratio 10:1 (v/w) provided the greatest oleuropein extraction yield (29.7 ± 1.7 mg/g) observing no significant differences for the other ratios (3:1, 5:1 and 7:1, 2 h and 25ºC). However, Sifaoui et al. [43] reported a much higher value as the optimum solvent/OL ratio for TPC from OL, 77 mL:g by using water as solvent at 58 °C, for an extraction time of 54 min, at pH of 8 and an agitation speed of 246 rpm.

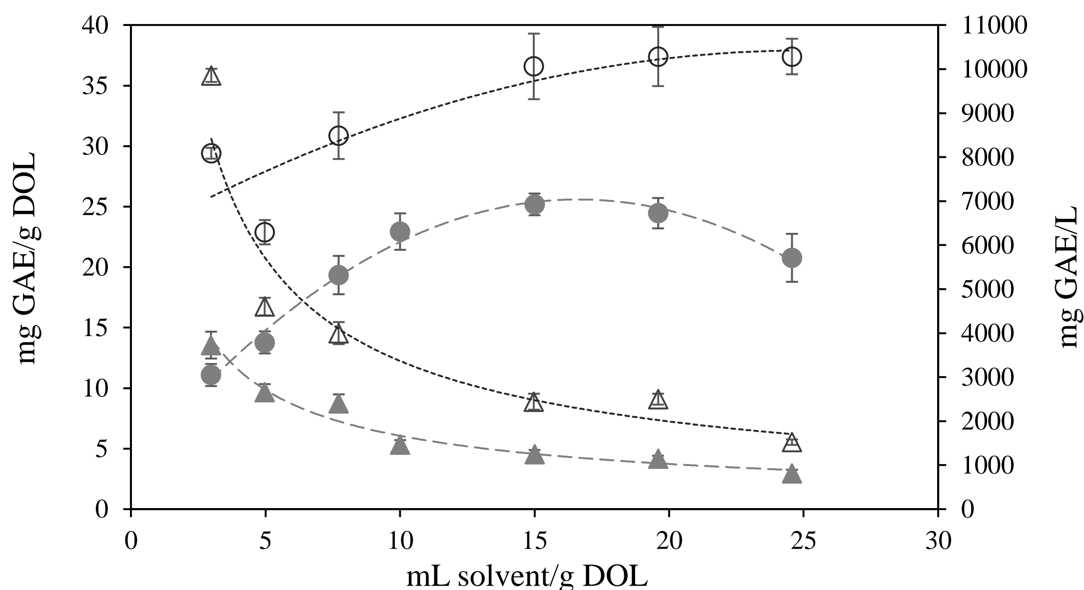


Figure 1.4. Effect of the solvent:DOL ratio on TPC extraction yield (mg GAE/g_{DOL} ○, ●) and total polyphenol concentration in the extraction medium (mg GAE/L, △▲) 80 % ethanol (v/v) (open symbols) 100 % water (filled symbols). Lines are to guide the eye.

Table 1.7. Characterization of OL extracts from different sources, Spanish and Iranian OL from the tree pruning and Iranian OL collected as wastes at the olive oil factory (T = 50 ºC, 80 % ethanol aqueous mixture, 20 mL solvent:gDOL).

Parameter	Spanish OL	Iranian OL	Iranian OL from factory
TPC, mg $_{GAE}$/g $_{DOL}$	37.6 ± 0.8^b	29.1 ± 0.7^a	61 ± 2^c
TFC, mg $_{QE}$/g $_{DOL}$	4.4 ± 0.3^b	2.3 ± 0.3^a	6.9 ± 0.7^c
FRAP, mg $_{Fe2+}$/g $_{DOL}$	70 ± 1^b	60 ± 4^a	134 ± 3^c
DPPH, mg $_{Trolox}$/g $_{DOL}$	62.2 ± 0.2^a	60.4 ± 0.1^a	73 ± 1^b
ABTS, mg $_{Trolox}$/g $_{DOL}$	24 ± 1^a	21.8 ± 0.2^a	37.6 ± 0.7^b

Values with different letters in each raw are significantly different when applying the Fisher's least significant difference (LSD) method at p-value ≤0.05.

1.3.2.4. Effect of olive leaves source

Extractions were carried out by using an 80 % ethanol aqueous solution as solvent at 50 ºC and 20 mL solvent/g_{DOL} ratio. Extracts were characterized by determining TPC, TFC and antioxidant capacity. Results are collected in Table 1.7. The highest content of total bioactive compounds corresponds to Iranian OL collected as waste at the factory, then the Spanish OL and finally the same Iranian OL collected at the tree pruning. The higher content of bioactive compounds led also to higher antioxidant activity for the different tests assayed in this work, DPPH, ABTS and FRAP assays. The high concentration of TPC and TFC of OL collected at the factory could be due to the fact that these OL were in contact for a few days with the olives which could led to higher TPC. Therefore, due to the high antioxidant activity of olive mill leaves, its valorisation could make more profitable the olive sector and reduce the environmental impact, and more attention should be paid to this by-product, specially taking account that the transport cost would be reduced [30].

The variety of OL used in different studies found in the literature was also listed in Table 1.3. For instance in the study of Bilgin & Sahin [28] different cultivars from

Turkey were employed in the extraction of TPC by UAE reporting values in the range from 7.35 to 38.66 mg $_{GAE}$/g$_{DOL}$ by using methanol as solvent. The highest value corresponded to Bursa cultivar, located at high altitude with terrestrial and Mediterranean climate. Ben *et al.* [44] determined TPC for 21 OL cultivars from the different geographical regions of South-Eastern Tunisia. The highest amount was found in Zalmati Zarzis cultivar (20.7 mg $_{GAE}$/g$_{DOL}$) while the lowest was registered in Chemlali ontha cultivar (1.35 mg $_{GAE}$/g$_{DOL}$). The different TPC reported proved the influence of geographical and climate conditions on TPC.

Table 1.8. Characterization of the freeze dried extract (FDE) from "Serrana de Espadán" OL.

Total antioxidants	TPC, mg $_{GAE}$/g$_{FDE}$	113 ± 1
	TFC, mg $_{QE}$/g$_{FDE}$	13.2 ± 0.7
Antioxidant Activity	FRAP, mg $_{Fe2+}$/g$_{FDE}$	435 ±7
	DPPH, mg $_{Trolox}$/g$_{FDE}$	694.2 ± 0.9
	ABTS, mg $_{Trolox}$/g$_{FDE}$	230.4 ± 0.3
Individual phenolic compounds	Hydroxytyrosol, mg/g$_{FDE}$	2.96 ± 0.07
	Catechin, mg/g$_{FDE}$	6.2 ± 0.1
	Verbascoside, mg/g$_{FDE}$	5.74 ± 0.08
	Luteolin-7-O-glucoside, mg/g$_{FDE}$	13.8 ± 0.5
	Oleuropein, mg/g$_{FDE}$	118 ± 7
	Luteolin, mg/g$_{FDE}$	0.51 ± 0.08
	Rutin, mg/g$_{FDE}$	n.d.
Other components	Mannitol, mg/g$_{FDE}$	173.7 ± 0.2
	Sucrose, mg/g$_{FDE}$	78 ± 1
	Glucose, mg/g$_{FDE}$	39 ± 4
	Ch-a, mg/g$_{FDE}$	1.3 ± 0.1
	Ch-b, mg/g$_{FDE}$	0.54 ± 0.05
	Cx+c, mg/g$_{FDE}$	0.49 ± 0.04
Total antioxidants and antioxidant activity after 2 months	TPC, mg GAE/g$_{FDE}$	118 ± 6
	TFC, mg QE/g$_{FDE}$	15 ± 2
	FRAP, mg Fe^{2+}/g$_{FDE}$	505 ± 10

1.3.3. Freeze dried extract (FDE)

A solid extract from Spanish OL was obtained from the liquid extract obtained by using an 80% (v/v) ethanol aqueous mixture at 50 ºC and 20 mL solvent/g_{DOL} ratio This liquid extract was freeze dried previous removal of the ethanol by a rotatory evaporator. The freeze dried extract (FDE) presented a characteristic green color and the humidity content was 7 %. The total yield of the FDE respect to the DOL was 24 %. A complete characterization of the FDE is presented in Table 1.8. FDE presented a high content of bioactive compounds as determined by the TPC and TFC, 113 ± 1 mg $_{GAE}$/g $_{FDE}$ and 13.2 ± 0.7 mg_{QE}/g_{FDE}, respectively, with a high antioxidant capacity as determined by ABTS, DPPH and FRAP tests, respectively.
According to the individual phenolic profile identified in the liquid extract (Table 1.3), the major phenolic compound that could be identified in the FDE was oleuropein with a content of 117 ± 7 mg oleuropein/g_{FDE}, more than 11 % (w/w) of the FDE. Other important phenolic compounds in the FDE were luteolin 7-O-glucoside and verbascoside, 13.8 ± 0.5 mg/g_{FDE} and 5.74 ± 0.08 mg/g_{FDE}, respectively. Rutin was determined in the liquid extract but it could not be quantified in the FDE. Just opposite to luteolin that was determined in the FDE but could not be determined in the liquid extract.
The soluble carbohydrate fraction in the FDE was also determined. Mannitol accounted for 17.4 % (w/w) of the FDE, while sucrose and glucose represented 7.8 and 3.9 % (w/w) of the FDE. The green color of the extract was due to the presence of relative important amounts of chlorophylls as reflected in the content of Chlorophyll-a, followed by Chlorophyll-b and lower amounts of carotenoids.

TPC and TFC were determined after two months of storage at refrigerated conditions, 4 ºC, with values of 118 ± 6 mg_{GAE}/g $_{FDE}$ and 15 ± 2 mg_{QE}/g $_{FDE}$, respectively. Therefore, it can be concluded that TPC and TFC were kept after two months of storage. This could be also observed in the antioxidant activity, as determined by the FRAP assay after two months, with values of 505 ± 10 mg $_{Fe2+}$/ g_{FDE}.

1.4. Conclusions

Chemical composition of OL makes this by-product an attractive raw material to be incorporated into a biorefinery context. Extractives valorisation has been proposed as the first step by using different ethanol aqueous mixtures as solvents. UAE led to faster TPC extraction kinetics but similar extraction yield was reached as for conventional extraction. A 80% ethanol aqueous mixture (v/v) was found to be the best extraction solvent with a TPC yield of 37.6 ± 0.8 mg $_{GAE}$/g$_{DOL}$ and a oleuropein content of 31 ± 1 mg oleuropein/g$_{DOL}$. A ratio of 20:1 (v/w) was selected as an optimum ratio due to an increase in extraction driving force.

A freeze dried extract was obtained with a high content of bioactive compounds, more than 11 % (w/w) of oleuropein and 1.4 % (w/w) of luteolin-7-O-glucoside. This FDE contain also important amounts of mannitol, more than 17 % (w/w).

Scaling-up of the process will be feasible since a cost-effective extraction technology was proposed based on the use of green solvents and the use of an inexpensive and abundant raw material obtaining an extract with good antioxidant properties. As future trends, it can be concluded that phenolic rich food products could be obtained by using freeze-drying process and it is suggested to use the FDE as an additive for food, pharmaceutical or cosmetic industries.

References

[1] Á. Peralbo-Molina and M. D. Luque deCastro, "Potential of residues from the Mediterranean agriculture and agrifood industry," *Trends Food Sci. Technol.*, vol. 32, no. 1, pp. 16–24, 2013.

[2] IOOC, "Introduction," *Gen. Descr. Olive Growning Turkey*, vol. 20, p. 11, 2012.

[3] L. Abaza, A. Taamalli, H. Nsir, and M. Zarrouk, "Olive tree (Olea europeae L.) leaves: Importance and advances in the analysis of phenolic compounds," *Antioxidants*, vol. 4, no. 4, pp. 682–698, 2015.

[4] E. Molina-Alcaide and D. R. Yáñez-Ruiz, "Potential use of olive by-products in ruminant feeding: A review," *Anim. Feed Sci. Technol.*, vol. 147, no. 1–3, pp. 247–264, 2008.

[5] A. Lama-muñoz *et al.*, "Extraction of oleuropein and luteolin-7-O-glucoside from olive leaves : Optimization of technique and operating conditions," *Food Chem.*, vol. 293, no. January, pp. 161–168, 2019.

[6] Á. Guinda, "Use of solid residue from the olive industry," *Grasas y Aceites*, vol. 57, no. 1, pp. 107–115, 2006.

[7] N. Rahmanian, S. M. Jafari, and T. A. Wani, "Bioactive profile, dehydration, extraction and application of the bioactive components of olive leaves," *Trends Food Sci. Technol.*, vol. 42, pp. 150–172, 2015.

[8] P. Manzanares *et al.*, "Residual biomass potential in olive tree cultivation and olive oil industry in Spain: valorization proposal in a biorefinery context," *Spanish J. Agric. Res.*, vol. 15, no. 3, pp. 1–12, 2017.

[9] J. M. Romero-García, L. Niño, C. Martínez-Patiño, C. Álvarez, E. Castro, and M. J. Negro, "Biorefinery based on olive biomass. State of the art and future trends," *Bioresour. Technol.*, vol. 159, pp. 421–432, 2014.

[10] E. Oddo, F. Saiano, G. Alonzo, and E. Bellini, "An investigation of the seasonal pattern of mannitol content in deciduous and evergreen species of the Oleaceae growing in northern Sicily," *Ann. Bot.*, vol. 90, no. 2, pp. 239–243, 2002.

[11] S. M. Ghoreishi and R. G. Shahrestani, "Subcritical water extraction of mannitol from olive leaves," *J. Food Eng.*, vol. 93, no. 4, pp. 474–481, 2009.

[12] B. K. Tiwari, "Ultrasound: A clean, green extraction technology," *TrAC - Trends Anal. Chem.*, vol. 71, pp. 100–109, 2015.

[13] S. Sahin, "Tailor-designed deep eutectic liquids as a sustainable extraction media: An alternative to ionic liquids," *J. Pharm. Biomed. Anal.*, vol. 174, pp. 324–329, 2019.

[14] S. Şahin, A. G. Pekel, and İ. Toprakçı, "Sonication-assisted extraction of Hibiscus sabdariffa for the polyphenols recovery: application of a specially designed deep eutectic solvent," *Biomass Convers. Biorefinery*, 2020.

[15] E. Kurtulbaş, A. G. Pekel, M. Bilgin, D. P. Makris, and S. Şahin, "Citric acid-based deep eutectic solvent for the anthocyanin recovery from Hibiscus sabdariffa through microwave-assisted extraction," *Biomass Convers. Biorefinery*, 2020.

[16] G. Difonzo *et al.*, "Green extracts from Coratina olive cultivar leaves: Antioxidant characterization and biological activity," *J. Funct. Foods*, vol. 31, pp. 63–70, 2017.

[17] Á. G. Solaesa, S. L. Bucio, M. T. Sanz, S. Beltrán, and S. Rebolleda, "Characterization of triacylglycerol composition of fish oils by using chromatographic techniques," *J. Oleo Sci.*, vol. 63, no. 5, 2014.

[18] A. E. Illera *et al.*, “Effect of thermosonication batch treatment on enzyme inactivation kinetics and other quality parameters of cloudy apple juice,” *Innov. Food Sci. Emerg. Technol.*, vol. 47, 2018.

[19] L. Galván D’Alessandro, K. Dimitrov, P. Vauchel, and I. Nikov, “Kinetics of ultrasound assisted extraction of anthocyanins from Aronia melanocarpa (black chokeberry) wastes,” *Chem. Eng. Res. Des.*, vol. 92, no. 10, pp. 1818–1826, 2014.

[20] S. Kitanović, D. Milenović, and V. B. Veljković, “Empirical kinetic models for the resinoid extraction from aerial parts of St. John’s wort (Hypericum perforatum L.),” *Biochem. Eng. J.*, vol. 41, no. 1, pp. 1–11, 2008.

[21] B. Gullón *et al.*, “Valorisation of olive agro-industrial by-products as a source of bioactive compounds,” *Sci. Total Environ.*, vol. 645, pp. 533–542, 2018.

[22] C. V. Cavalheiro *et al.*, “Olive leaves offer more than phenolic compounds - Fatty acids and mineral composition of varieties from Southern Brazil,” *Ind. Crops Prod.*, vol. 71, pp. 122–127, 2015.

[23] C. P. O’Donnell, B. K. Tiwari, P. Bourke, and P. J. Cullen, “Effect of ultrasonic processing on food enzymes of industrial importance,” *Trends Food Sci. Technol.*, vol. 21, no. 7, pp. 358–367, 2010.

[24] S. R. Shirsath, S. H. Sonawane, and P. R. Gogate, “Intensification of extraction of natural products using ultrasonic irradiations-A review of current status,” *Chem. Eng. Process. Process Intensif.*, vol. 53, pp. 10–23, 2012.

[25] D. Amendola, D. M. De Faveri, and G. Spigno, “Grape marc phenolics: Extraction kinetics, quality and stability of extracts,” *J. Food Eng.*, vol. 97, no. 3, pp. 384–392, 2010.

[26] I. Khemakhem *et al.*, “Kinetic improvement of olive leaves’ bioactive compounds extraction by using power ultrasound in a wide temperature range,” *Ultrason. Sonochem.*, vol. 34, pp. 466–473, 2017.

[27] T.-I. Lafka, A. Lazou, V. Sinanoglou, and E. Lazos, “Phenolic Extracts from Wild Olive Leaves and Their Potential as Edible Oils Antioxidants,” *Foods*, vol. 2, no. 1, pp. 18–31, 2013.

[28] M. Bilgin and S. Şahin, “Effects of geographical origin and extraction methods on total phenolic yield of olive tree (Olea europaea) leaves,” *J. Taiwan Inst. Chem. Eng.*, vol. 44, no. 1, pp. 8–12, 2013.

[29] D. Cifá, M. Skrt, P. Pittia, C. Di Mattia, and N. Poklar Ulrih, “Enhanced yield of oleuropein from olive leaves using ultrasound-assisted extraction,” *Food Sci. Nutr.*, vol.

6, no. 4, pp. 1128–1137, 2018.

[30] M. del M. Contreras, A. Lama-Muñoz, F. Espínola, M. Moya, I. Romero, and E. Castro, “Valorization of olive mill leaves through ultrasound-assisted extraction,” *Food Chem.*, vol. 314, no. January, 2020.

[31] A. Guinda, J. M. Castellano, J. M. Santos-Lozano, T. Delgado-Hervas, P. Gutierrez-Adanez, and M. Rada, “LWT - Food Science and Technology Determination of major bioactive compounds from olive leaf,” *LWT - Food Sci. Technol.*, vol. 64, pp. 431–438, 2015.

[32] A. Lama-Muñoz, M. Del Mar Contreras, F. Espínola, M. Moya, I. Romero, and E. Castro, “Optimization of oleuropein and luteolin-7-o-glucoside extraction from olive leaves by ultrasound-assisted technology,” *Energies*, vol. 12, no. 13, 2019.

[33] A. Lama-Muñoz, M. del M. Contreras, F. Espínola, M. Moya, I. Romero, and E. Castro, “Content of phenolic compounds and mannitol in olive leaves extracts from six Spanish cultivars: Extraction with the Soxhlet method and pressurized liquids,” *Food Chem.*, vol. 320, no. September 2019, p. 126626, 2020.

[34] P. Alonso-Riaño, M. T. Sanz Diez, B. Blanco, S. Beltrán, E. Trigueros, and O. Benito-Román, “Water Ultrasound-Assisted Extraction of Polyphenol Compounds from Brewer’s Spent Grain: Kinetic Study, Extract Characterization, and Concentration,” *Antioxidants*, vol. 9, no. 3, p. 265, 2020.

[35] S. A. Socaci, A. C. Fărcaş, Z. M. Diaconeasa, D. C. Vodnar, B. Rusu, and M. Tofană, “Influence of the extraction solvent on phenolic content, antioxidant, antimicrobial and antimutagenic activities of brewers’ spent grain,” *J. Cereal Sci.*, vol. 80, pp. 180–187, 2018.

[36] M. Bouaziz and S. Sayadi, “Isolation and evaluation of antioxidants from leaves of a Tunisian cultivar olive tree,” *Eur. J. Lipid Sci. Technol.*, vol. 107, no. 7–8, pp. 497–504, 2005.

[37] Z. Erbay and F. Icier, “The Importance and Potential Uses of Olive Leaves,” *Food Rev. Int.*, vol. 26, no. 4, pp. 319–334, Sep. 2010.

[38] T. A. Wani *et al.*, “Olive oil and its principal bioactive compound: Hydroxytyrosol – A review of the recent literature,” *Trends Food Sci. Technol.*, vol. 77, no. February, pp. 77–90, 2018.

[39] S. N. El and S. Karakaya, “Olive tree (Olea europaea) leaves: potential beneficial effects on human health,” *Nutr. Rev.*, vol. 67, no. 11, pp. 632–638, 2009.

[40] W. Su, J. Liu, H. Wang, C. Li, and N. Jia, “Thermodynamic study of three anhydrous

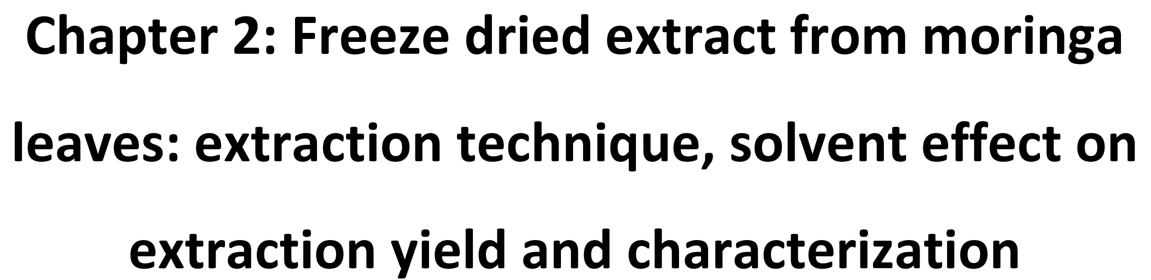
polymorphs of D-mannitol in different binary solvent mixtures from T = (258.15 to 278.15) K," *J. Chem. Thermodyn.*, vol. 141, 2020.

[41] A. C. Galvão, W. S. Robazza, G. N. Sarturi, F. C. Goulart, and D. Conte, "Sucrose Solubility in Binary Liquid Mixtures Formed by Water-Methanol, Water-Ethanol, and Methanol-Ethanol at 303 and 313 K," *J. Chem. Eng. Data*, vol. 61, no. 9, pp. 2997–3002, 2016.

[42] L. A. Alves, J. B. Almeida E Silva, and M. Giulietti, "Solubility of D-glucose in water and ethanol/water mixtures," *J. Chem. Eng. Data*, vol. 52, no. 6, pp. 2166–2170, 2007.

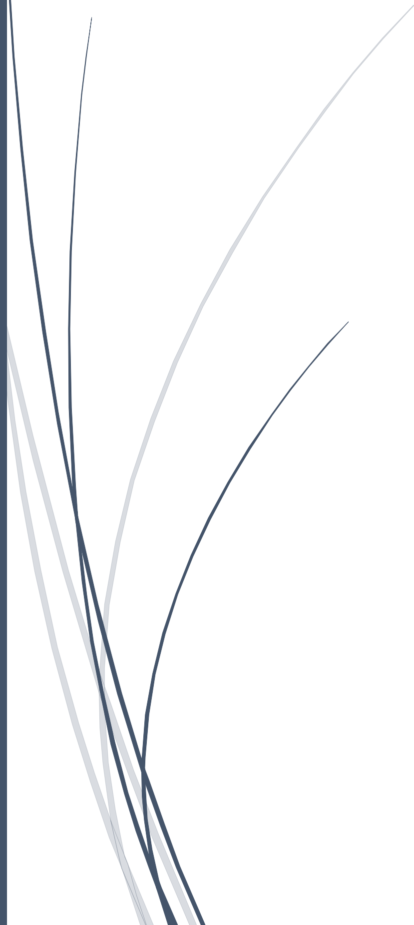
[43] I. Sifaoui, N. Chammem, M. Abderrabba, and M. Mejri, "Optimization of phenolic compounds extraction from olive leaves using experimental design methodology," *J. Mater. Environ. Sci.*, vol. 7, no. 4, pp. 1119–1127, 2016.

[44] M. Ben *et al.*, "The LC-MS / MS characterization of phenolic compounds in leaves allows classifying olive cultivars grown in South Tunisia," *Syst. Ecol.*, vol. 78, no. July 2017, pp. 84–90, 2018.



Chapter 2: Freeze dried extract from moringa leaves: extraction technique, solvent effect on extraction yield and characterization

Valorization of plant biomass by emerging technologies: olive and moringa leaves



A complete chemical characterization of moringa oleifera leaves has been carried out showing a higher content of the extractive fraction as usually observed for other green leaves. Extraction of bioactive compounds present in this fraction has been performed by conventional and ultrasound-assisted extraction (UAE). A 50 % (v/v) hydroalcoholic mixture led to the highest total phenolic compounds yield by conventional solvent extraction, 29.5 ± 0.3 mg per gram of dried moringa leaves. However, UAE did not bring any improvement when using hydroalcoholic mixtures probably due to the physical properties of the ethanol aqueous mixture that affect the UAE performance, such as viscosity and vapor pressure of the mixture. In this study, the retention index of the different solvents has been determined revealing higher retention index for water, 9.5, and a continuous decrease by increasing ethanol concentration, down to 2.9 for ethanol. This a key parameter in a solvent extraction process since it determines the number of stages in an industrial separation process and it is not usually reported in bioactive extraction compounds. Consequently, the retention index will determine the final freeze dried extract yield.

Se ha realizado una caracterización química completa de hojas de moringa oleifera mostrando un mayor contenido de la fracción extractiva que se observa habitualmente para otras hojas verdes. La extracción de compuestos bioactivos presentes en esta fracción se ha realizado mediante extracción convencional y asistida por ultrasonido (EAU). Una mezcla hidroalcohólica al 50% (v/v) condujo al rendimiento de compuestos fenólicos totales más alto por extracción con solvente convencional, 29,5 ± 0,3 mg por gramo de hojas secas de moringa. Sin embargo, los EAU no aportaron ninguna mejora al utilizar mezclas hidroalcohólicas probablemente debido a las propiedades físicas de la mezcla acuosa de etanol que afectan el desempeño de los EAU, como la viscosidad y la presión de vapor de la mezcla. En este estudio se determinó el índice de retención de los diferentes solventes revelando un índice de retención de agua más alto, 9.5, y una disminución continua al aumentar la concentración de etanol, hasta 2.9 para el etanol. Este es un parámetro clave en un proceso de extracción por solventes, ya que determina el número de etapas en un proceso de separación industrial y generalmente no se reporta en los compuestos de extracción bioactivos. En consecuencia, el índice de retención determinará el rendimiento final del extracto liofilizado.

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2.1. Introduction

Moringa oleifera Lam (syn. M. pterygosperma Gaertn.) is a type of medium-sized, evergreen tree belonging to the Moringaceae family. Moringa is an edible and medicinal perennial plant that presents high nutritional and medicinal value. It is widely cultivated in tropical and sub-tropical regions around the world, due to its ability to grow on both humid and hot dry lands, although nutritional composition of Moringa olifeira varies depending on cultivar and climate conditions [1]. All parts of this plant, leaves, fruit, flowers and immature pods can be used for different purposes being defined as a multi-purpose tree [2].

Moringa leaves (ML) have been reported as a natural source of antioxidant compounds, including various phenolic compounds, mainly flavonoids. González-Romero et al. [3] found that ML presented the highest antioxidant capacity and total polyphenol content compared with different vegetable leaves pre-packaged for consumption as salad. Considering the composition and properties of ML, it is certainly important to find the most practical and cost-effective extraction method to obtain extracts from ML rich in antioxidant compounds. Phenolic compounds content and antioxidant capacity of extracts from ML depend on the extraction method, type of solvent, solvent-to-solid ratio, temperature, stirring rate and particle size [4,5]. Extracts of ML have been described as complex mixtures of a large number of phytochemicals, mainly phenolic acids and complex flavonoids, like glucosides, rutinosides, malonyl glucosides and acetyl glucosides of quercetin, isorhamnetin and kaempferol [4]. A subsequently drying step of the liquid extracts will help to obtain a dry powder with a high phenolic concentration that can be used as additive to foods, avoiding its degradation.

In this work, a complete chemical characterization of ML has been carried out. Similar to other green leaves, a high amount of easily extractive compounds can be expected being worth studying its extraction and valorisation. The simplest method of solid liquid extraction is the single contact batch operation and it takes place in two steps: (1) contact of the solvent and the solid material to transfer the solute to the solvent (2) separation of the solution from the remaining solid. In a real extraction process, it is impossible to completely separate the liquid phase from the solid fraction. As a result, two phases are obtained after a solid liquid extraction stage, a liquid phase with the

solute, the extract phase, and a solid phase composed by the exhausted solid and the adhered liquid solution, the raffinate phase. The effect of solvent type on bioactive compounds extraction yield has been usually considered in terms of polarity of the solvent, since it determines the composition of the extracts. However, the liquid solution retained in the solid phase by using different solvents is usually not reported in the extraction studies of biocompounds from green leaves, although this parameter determines economic aspects such as the number of stages of the process and the solvent recovery.

In this work, phenolic compounds recovery by conventional solvent extraction and by using different hydroalcoholic mixture has been performed. The solution retention in the raffinate phase was determined for the different solvents since it determines the real extraction yield of the process. The higher the dissolution retention, the higher loss of solvent and solute in the raffinate phase that determines an increase in the separation stages. Other important parameters to consider in an extraction process is the maximum extraction rate and the time needed to reach equilibrium. Therefore, a detailed kinetic study for the different biocompounds in ML has been also carried out. Antioxidant capacity and individual phenolic compounds profile have been also determined. Results obtained by conventional solvent extraction have been compared with the results obtained by ultrasound-assisted extraction (UAE). UAE technology has been reported as a clean, green, extraction technique that presents several benefits such as the enhancement of the extraction of heat-sensitive components and the global extraction yield [6]. The results obtained by UAE have been discussed considered the physical properties of the hydroalcoholic mixtures.

The final aim of this work was to obtain a freeze-dried extract of ML from an optimized solid-liquid extraction process, considering extract composition and solution retention index. Characterization of the freeze-dried extract was carried out in terms of total and individual phenolic compounds, antioxidant capacity and pigments.

2.2. Materials and methods

2.2.1. Raw material

ML have been purchased from Waka brand in powder form. Moisture content of ML, 5.61 ± 0.02 % (w/w), was determined gravimetrically by weighing it before and after drying in an oven at 105 ºC until constant weight. Results will be expressed as mass of dry moringa leaves, DML. ML were stored in the freezer at -18 ºC until extraction studies.

2.2.2. Determination of chemical composition of moringa leaves

ML were characterized according to the Standard Biomass Analytical Methods provided by the National Renewable Energy Laboratory [7]. After water and ethanol extractives removal, two hydrolysis steps with sulphuric acid were carried out to determine the structural components of the biomass. Carbohydrates were quantified by high-performance liquid chromatography (HPLC) (Agilent 1260, Agilent Technologies, Inc. U.S.A.) equipped with an Aminex-HPX-87H column (Bio-Rad Laboratories Inc. U.S.A.), a variable wavelength detector (VWD) and a refractive index detector (RID) using a mobile phase constituted by 0.005M sulphuric acid. The column and refractive index detector were maintained at 40 ºC. The lipid fraction of ML was measured by Soxhlet extractio using a BUCHI 811 extraction system (Buchi Labortechnik AG, Flawil, Switzerland).

Elemental composition (C, H, N, S) of the ML was determined by an elemental micro-analyzer equipment (Model Flash 2000, Thermo Scientific, Massachusetts, U. S. A.). Protein content of ML was calculated from the nitrogen content by applying a conversion factor of 6.25.

2.2.3. Extraction procedures

2.2.3.1. Conventional solvent extraction

Extractions were performed in an orbital shaker (Grant Instruments-OLS 200, Shepreth Cambridgeshire, England) for 1 h at 50 ºC. First, the effect of solvent to solid ratio, from 10 to 20 mL/g_{ML} was analysed by using a hydroalcoholic solution with 20 % (v/v)

ethanol as solvent. Afterwards, the effect of solvent type was studied by using different hydroalcoholic mixtures (100% ethanol, 80% ethanol, 50% ethanol, 20% ethanol and 100% water, v/v) at 50ºC and a constant liquid/solid ratio of 10 mL/g_{ML}. Extraction kinetics were followed by withdrawing samples at regular time intervals and further analysis.

After extraction, the solid and liquid phases were separated by centrifugation and weighed. The liquid phase was considered as the extract phase (EP) and the remaining solid phase was the raffinate phase (RP).

The global extraction yield was determined by taking an aliquot of the extract phase and weighed (m_{EP}). The extract sample was oven dried at 105 ºC to evaporate the solvent and it was weighed again to determine the mass of soluble solids extracted (m_{SS-EP}) and by difference, the mass of solvent evaporated (m_{EP-D}) can be calculated. The mass fraction of solvent (w_{S-EP}) and total soluble solids (w_{SS-EP}) in the extract phase could be determined:

$$w_{soluble\ solids,EP} = w_{SS,EP} = \frac{m_{SS,EP}}{m_{EP}} \quad (1)$$

$$w_{solvent,EP} = w_{S,EP} = 1 - w_{SS,EP} \quad (2)$$

The global extraction yield for soluble solids was determined by considering as the ratio of the total amount of soluble solids in the extract phase and the initial mass of DML:

$$Yield = \frac{w_{SS,EP} \cdot mass_{EP}}{initial\ mass_{DML}} \cdot 100 \quad (3)$$

The raffinate phase was dried, m_{RP}, until constant weight to remove the solvent. Once solvent was evaporated, the samples were weighed again, m_{RP-D}, allowing the calculation of the solvent mass fraction retained in the raffinate phase.

$$w_{solvent,RP} = \frac{m_{RP} - m_{RP,D}}{m_{RP}} \quad (4)$$

An important parameter in solid liquid extraction is the retention index (RI) evaluated as the solution adhered to the insoluble solids from the raffinate phase [8].

$$\mathrm{RI} = \frac{\mathrm{m_{SS,RP}} + \mathrm{m_{S,RP}}}{\mathrm{m_{IS,RP}}} \tag{5}$$

where $m_{ss,RP}$ is the mass of soluble solids, $m_{s,RP}$ is the mass of solvent and $m_{IS,RP}$ is the mass of the insoluble solids in the raffinate phase. This parameter affects the design of industrial extractors since it determines the solvent recovery and the number of stages. The RI is related to the solution viscosity, particle size, and to the physicochemical affinity between the solvents, cosolvents, and the solid matrix [9]. The higher the RI, the higher is the operational cost of the extraction process [8].
The amount of soluble solids in the raffinate phase can be evaluated taking into account the amount of solvent, assuming equilibrium stage in a single stage solid liquid extraction defined as one in which the extract phase has the same composition as the solution adhered to the solids [10]:

$$m_{SS,RP} = \frac{w_{SS,EP}}{w_{S,EP}} m_{S,RP} \tag{6}$$

$$m_{IS,RP} = 1 - m_{S,RP} - m_{SS,RP} \tag{7}$$

2.2.3.2. Ultrasound-assisted extraction (UAE)

Ultrasound assisted extraction (UAE) was performed by using water, 50 % and 20 % (v/v) ethanol aqueous solutions as solvents at 50ºC. UAE was performed by using a 750 W Sonics Material TM with a 13 mm probe (VibraCell™ 75042, Bioblock Scientific, U.S.A.). ML were processed at a constant ultrasound frequency of 20 kHz and 79 µm of amplitude and a ratio of 10 mL solvent/g_{ML}. ML were introduced in a jacketed vessel (Φ_i = 4.8 cm, V = 199 cm^3) and the probe was submerged in the solution at a constant depth of 2 cm from the bottom of the vessel. Temperature was kept at 50 ºC in the vessel by circulating water through the jacket [11]. Samples were taken periodically to follow the extraction kinetics.

2.2.4. Freeze-drying process

A freeze-dried extract (FDE) was obtained from the liquid extract obtained at 50ºC by using 50% ethanol aqueous mixture (v/v) and a ratio of 10 mL solvent/g_{ML}. Before freeze-drying, ethanol was removed by a rotary evaporator and the remaining extract was submitted to freeze drying. First, samples were frozen with liquid nitrogen (-196 ºC), equilibrated at -80 ºC for 2 h and then submitted to freeze-drying in a Labconco Freeze Dry System (Labconco Corporation, U.S.A.) at $1.5 \cdot 10^{-4}$ mbar during 48 h. The moisture content of the freeze-dried particles was determined gravimetrically by weighing small amounts of dried particles (around 0.5 g) before and after drying in an oven at 105 ºC until constant weight. Solid particles were analyzed in terms of antioxidant activity, individual phenolic compounds and pigments.

2.2.5. Pigments in the freeze-dried extract

The amount of chlorophylls and carotenoids in the FDE was determined according to Sumanta *et al.* [12] and described by Kashaninejad *et al.* [13] for pigments in a freeze dried extract from olive leaves extract. 0.5 g of the FDE were dissolved in 10 mL of diethyl ether. The sample was homogenized and then centrifuged for 10.000 rpm for 15 min at 4 ºC. 0.5 mL of the supernatant were mixed with 4.5 mL of diethyl ether and the absorbance was determined at different wavelengths to determine the concentration of Chlorophyll-a (Ch-a), Chlorophyll-b (Ch-b) and carotenoids (Cx+c), μg/mL, according to the equations collected by Sumanta *et al.* [12]:

$$\text{Ch-a} = 10.05A_{660.6} - 0.97A_{642.2} \quad (8)$$

$$\text{Ch-b} = 16.36A_{642.2} - 2.43A_{660.6} \quad (9)$$

$$\text{Cx+c} = (1000A_{470} - 1.43\text{Ch-a} - 35.87\text{Ch-b})/205 \quad (10)$$

2.2.6. Modeling of extraction kinetics and statistical analysis

TPC data along extraction were fitted to the Weibull model:

$$Y\ (mg\ GAE/L) = A \cdot (1 - exp(-kt^n)) \tag{11}$$

The exponent n indicates the shape of the extraction curve. If $n > 1$, the curve is sigmoidal and if $n < 1$, the curve is parabolic. A represents the maximum extraction yield at a certain specific extraction condition.

The estimation of the kinetic parameters was carried out by non-linear regression by using the Marquardt algorithm. The quality of the fitting was evaluated by the root mean square deviation (RMSD):

$$RMSD = \sqrt{\frac{\sum_{i=1}^{n}\left(Y_{i,exp} - Y_{i,calc}\right)^2}{n}} \tag{12}$$

where $Y_{i,exp}$ and $Y_{i,calc}$ are the experimental and calculated TPC concentrations and n is the number of experimental data points in each extraction.

The results were presented as the mean ± standard deviation of at least three replicates. To confirm significant differences, the Fisher's least significant difference (LSD) procedure at p-value ≤ 0.05 was applied. The fitting procedure and analysis were carried out by the Centurion Statgraphics software.

2.3. *Results and discussion*

2.3.1. *Biomass composition*

Table 2.1 presents the chemical composition of ML. According to the two-step extractives determination, extractives soluble in water were 37.2 ± 0.5 % (w/w) while in ethanol were found to be 11.7 ± 0.4 % (w/w). Water and ethanol extractive fractions accounted for soluble proteins (water or ethanol soluble), different carbohydrates (with higher solubility in water) and TPC. High amount of extractives has been also found in other green leaves such as olive leaves [13] and it supports the importance of the study and optimization of different extraction strategies for ML valorization. The polysaccharide fraction (glucans and hemicellulose) measured was 18.7 ± 0.8 %.

The lipid fraction was 4.04 ± 0.07 % (w/w). It must be highlighted the high protein fraction of the ML, 31.0 ± 0.2 %. This value was of the same order as the values reported by Leone *et al.* [2] for different ML from different sources Chad, Sahhrawi camps and Haiti, with values of 31.5 ± 0.1, 27.98 ± 0.12 and 20.80 ± 0.01 %, respectively. This offers a great alternative to consider ML as a good source of protein.

Table 2.1. Chemical composition of moringa leaves expressed as % ± standard deviation in a dry weight basis.

Compound		Composition, %
Extractives		48.9 ± 0.9
Water soluble extractives		37.2 ± 0.5
Carbohydrates	Suc / Glu / Man	0.40 ± 0.08 / 3.20 ± 0.02 / 1.84 ± 0.02
	Gal / Xyl / Ara	0.66 ± 0.03 / 3.79 ± 0.01 / 0.69 ± 0.01
Proteins		7.6 ± 0.2
TPC		2.4 ± 0.1
Ethanol soluble extractives		11.7 ± 0.4
Carbohydrate:	Glu / Man	1.06 ± 0.06 / 0.57 ± 0.03
	Gal, Xyl, Ara	0.21 ± 0.01 / 1.12 ± 0.07 / 0.64 ± 0.01
Proteins		2.2 ± 0.1
TPC		0.07 ± 0.01
Glucans		9.3 ± 0.4
Hemicellulose		9.4 ± 0.4
Xyl / Gal / Ara / Man		1.72 ± 0.04 / 2.0 ± 0.1 / 3.16 ± 0.04 / 2.9 ± 0.2
Acetate		0.01 ± 0.005
Lignin		10.0 ± 0.1
Soluble		7.99 ± 0.03
Insoluble		2.0 ± 0.1
Proteins*		31.0 ± 0.2
Lipids		4.04 ±0.07
Ashes		8.3 ± 0.5
Elemental composicion		
N		4.95 ± 0.03
C		45.3 ± 0.1
H		6.14 ± 0.04

Suc: sucrose, Glu: glucose, Man: mannose, Gal: galactose, Xyl: xylose, Ara: arabinose, TPC: total phenolic compounds
** proteins determined as 6.25×N*

2.3.2. Conventional solid-liquid extraction of phenolic compounds

2.3.2.1. Effect of solvent to mass ratio

The effect of solvent to ML mass ratio was studied by using a 20 % ethanol aqueous solution as solvent (v/v).

Figure 2.1 shows the TPC extraction kinetics at 50 ºC at two different solvent to ML mass ratios, 10 mL/g_{ML} and 20 mL/g_{ML}. A fast initial extraction period was observed at both solvent to mass ratios followed by a second slow period controlled by diffusion. These type of extraction curves have been also described in the literature for bioactive compounds extraction from different plant materials [14]. For ML, more than 90 % of the TPC extraction was reached after 10 min of extraction time. The final TPC concentrations in the extracts were 2235 ± 104 and 1127 ± 58 mg GAE/L at 10 and 20 mL/g_{ML}, respectively. To compare the results on a dry biomass basis, the maximum extraction yield that could be reached was evaluated assuming that no extract was retained in the raffinate phase after phases separation, yielding values of 23.7 ± 1.1 and 23.9 ± 1.2 mg GAE/g_{DML}, respectively. These two values were not significantly different by applying the Fisher's least significant difference (LSD) procedure at p-value ≤ 0.05, proving that, for this solvent, there was no limitation by the saturation of the solutes that are soluble in the solvent or mass transfer limitations at any of the ratios essayed. In literature, it is usually reported that high liquid/solid ratio results in a greater driving force for diffusion of compounds to the solvent obtaining higher extraction efficiencies and rates [15]. As previously shown, no effect on the extraction yield was found. To evaluate the effect on the extraction rate, the initial extraction rate was determined from the initial linear part of the extraction curve. The values of the initial extraction rate were 807 ± 182 and 381 ± 48 mg GAE/(L·min), or 8.5 ± 1.9 and 8.1 ± 1.0 mg GAE/(g_{DML}·min), at 10 and 20 mL/g_{ML}, respectively. There were not statistically significant differences among the initial slopes expressed as mg GAE/(g_{DML}·min) at the two solvent to mass ratios used in this work. Therefore, the lowest ratio value of 10 mL/g_{ML} was selected in this work, since higher solvent to mass ratios did not result in higher extraction rates or final yields, on a biomass basis, but higher solvent consumption and a more diluted extract phase.

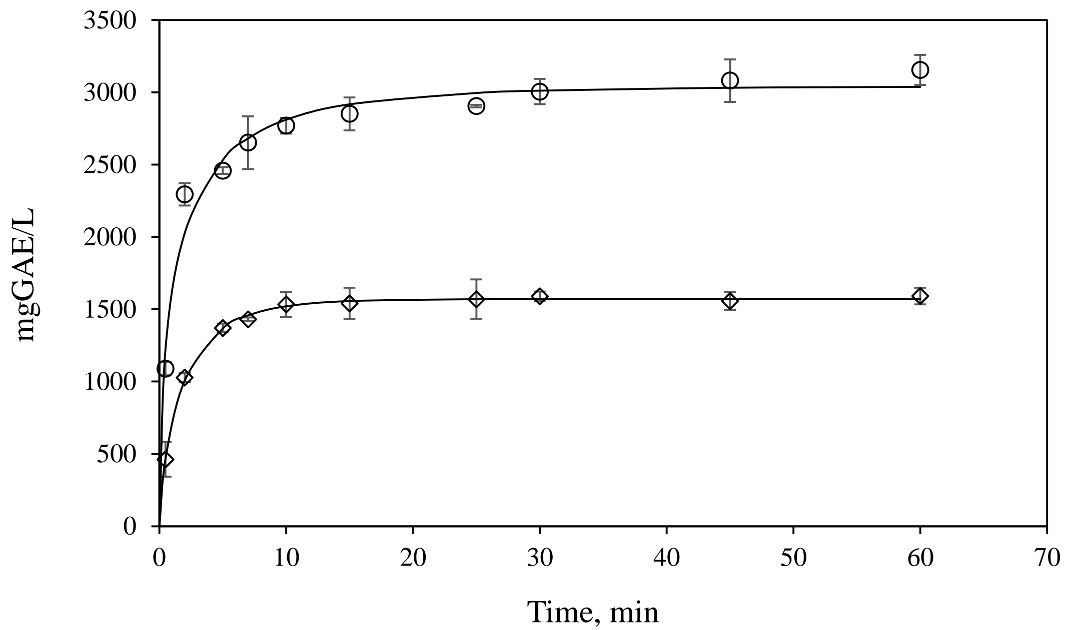


Figure 2.1. Effect of solvent to mass ratio on extraction kinetics by using a 20 % (v/v) ethanol aqueous solution as solvent at 50 ºC: (○) **10 mL:g$_{DML}$** (◇) 20 mL:g$_{DML}$. The continuous lines represent the Weibull model.

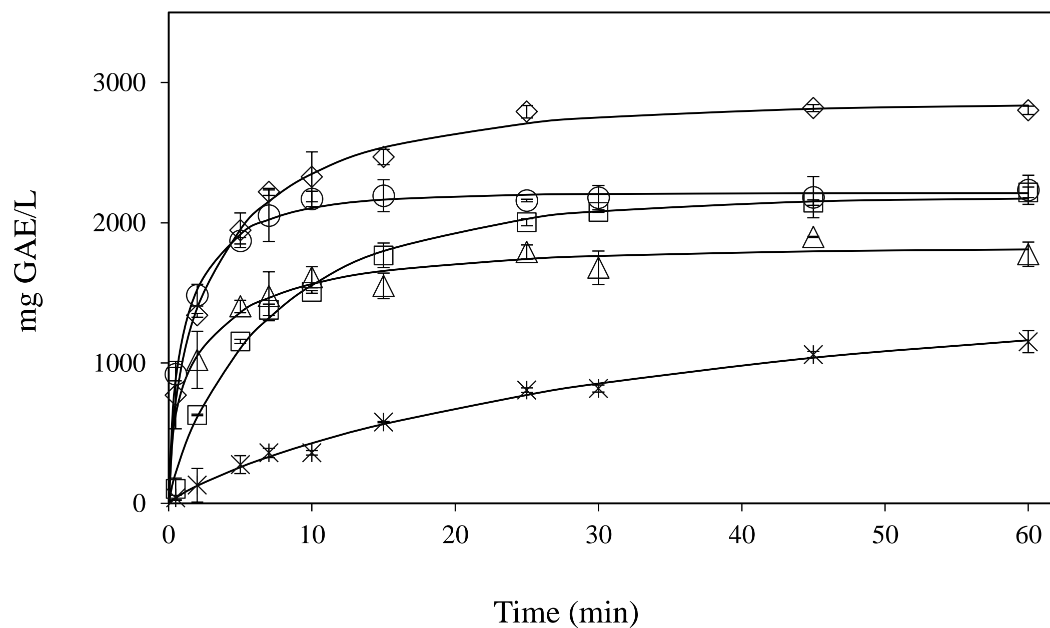


Figure 2.2. Effect of solvent type on extraction kinetics at 50ºC and a ratio of 10 mL/g$_{ML}$: (△) H_2O, (○) 20 % EtOH, (◇) 50 % EtOH, (□) 80 % EtOH, (×) EtOH. The continuous lines represent the Weibull model.

Table 2.2. Raffinate and extract phase composition expressed as weight fraction. Global extraction yield (%) and maximum theoretical TPC yield, assuming no solution retention, and real extraction TPC yield (mg TPC/g_{DML}). Retention index (RI) and mass balance (MB) for the extraction process.

Solvent	Raffinate phase			Extract phase		Yield (%)	$TPC_{max-yield}$, mg TPC/g_{DML}	$TPC_{real-yield}$ mg TPC/g_{DML}	RI	MB, %
	w_{is}	w_{ss}	w_s	w_{ss}	w_s					
Water	0.095	0.034	0.870	0.038	0.962	19.1 ± 0.1^c	18.7 ± 0.2^b	9.0 ± 0.1^a	9.5	95.3
20 % EtOH	0.133	0.030	0.837	0.034	0.966	20.3 ± 0.2^d	23.5 ± 0.4^c	13.7 ± 0.2^b	6.5	94.3
50 % EtOH	0.205	0.022	0.773	0.028	0.972	18.9 ± 0.2^c	29.5 ± 0.5^d	20.8 ± 0.4^d	3.9	96.2
80 % EtOH	0.230	0.021	0.749	0.028	0.972	17.8 ± 0.2^b	23.3 ± 0.4^c	16.9 ± 0.3^c	3.4	96.4
Ethanol	0.254	0.012	0.734	0.015	0.985	9.0 ± 0.4^a	12.1 ± 0.1^a	8.5 ± 0.1^a	2.9	96.4

w_{is}: mass fraction of insoluble solids, w_{ss}: mass fraction of soluble solids, w_s: mass fraction of solvent

2.3.2.2. Effect of type of solvent

The effect of type of solvent was studied by varying the composition of water-ethanol mixtures. Five different mixtures were used as solvents, pure water and ethanol and different ethanol/water mixtures: 80/20, 50/50, 20/80 (v/v).

The results are presented in Figure 2.2. The ethanol:water ratio has been shown to be a key factor to obtain a high concentration of TPC in the extract. By using different ethanol concentration, the solvent extraction capacity changed since it depends mainly on the solubility of the components in the solvent and on the interaction of the components with other constituents of the sample [4]. The fastest extraction kinetic and the highest TPC concentration were achieved for a 50 % ethanol aqueous mixture (v/v) with a value of 2802 ± 29 mg GAE/L. For conventional solvent extraction of TPC from green leaves, better extraction efficiency has been usually reported for water-ethanol mixtures compared to pure ethanol or water as solvents [16]. This fact has been attributed to the double effect of both solvents, since water swells the plant matrix an helps to create a more polar medium that facilitates the extraction of organic compounds, either soluble in ethanol and/or water [17]. Rodríguez Pérez *et al.* [18] also found that TPC from ML was lower when using pure organic solvents, such as methanol, ethanol and acetone than when using these solvents mixed with water. These authors reported a TPC of 3.82 ± 0.09, 27 ± 2 and 24.3 ± 0.3 mg GAE/g_{DML} for pure ethanol, 50 % and 70 % (v/v) ethanol aqueous mixtures, respectively, when using 0.5 g of moringa leaves with 25 mL of the corresponding solvent for 1h. Lin et al. [19] reported a value of the same order for TPC extraction, 33.3 ± 1.8 mg GAE/g_{DML}, when using stirring-assisted extraction at 30 ºC for 30 min and 100 mg ML in 4mL of a 52 % ethanol concentration solvent. In agreement to the TPC concentration in the extract phase plotted in Figure 2.2, the maximum theoretical extraction yield achieved by using the different solvents were 18.7 ± 0.9, 23.5 ± 1.1, 29.5 ± 0.3, 23.3 ± 0.4 and 12.1 ± 0.8 for pure water, 20 % ethanol, 50 ethanol, 80 % ethanol and pure ethanol, respectively.

In most of literature works, the effect of solvent has been considered only in terms of TPC concentration in the extract, and the maximum theoretical extraction yield, expressed as mg TPC/g_{DML}, assuming complete phase separation. However, in this work, the effect of the solvent was analysed also considering the retention index. After

extraction, phase separation was carried out by centrifugation. The solvent retained in the raffinate phase was determined by evaporation and the composition of the phases and retention index were evaluated according to equations 1-7 (Table 2.2). It can be clearly observed that the type of solvent used in the extraction process has a clear impact on the amount of extract phase obtained since it determines the losses of the extract (the solution) in the raffinate phase. The effect of type of solvent in the RI in the raffinate phase is usually reported in other types of extraction process [8,20], but it usually not reported for extraction of bioactive compounds. The RI indicates the amount of solution retained in the raffinate phase, the lower the RI, the more efficient is the extraction process in terms of losses of the solution. The highest RI was obtained for water and it decreased continuously by increasing the amount of ethanol in the mixture solvent. The RI also determines the cost of the downstream separation process in the raffinate phase for solvent recovery. The RI for other extraction processes reported in literature, such as the extraction of soluble solids from murici and pequi seeds by using different types of solvents including ethanol, isopropanol, acetone and hexane [9], reached values between 0.51 to 1.28. Lower RI were also reported for the extraction of oil from semi-defatted babassu bagasse with ethanol (from 0.644 to 0.784) [21]. The values found in this work for the hydroalcoholic mixtures can be attributed to trend high trend of green leaves to adsorb water in its structure not being possible the comparison with the previous mentioned raw material. The higher retention index of water from ML could be observed visually during extraction due to the swelling of leaves in presence of water that resulted in high water retention by green leaves and a lower amount of the extract phase after the centrifugation separation process.

Table 2.2 also lists the extraction yield of the total soluble solids, as determined by Equation 4, considering the separated extract phase and not the initial solvent amount used in the extraction that is usually employed in literature. Table 2.2 also presents the real TPC yield in the extract phase taking into account the losses of the solution in the raffinate phase. These values are lower than the maximum values considering no solution retention ($TPC_{max\text{-}yield}$) and these are the values usually reported in literature, which do not take into account the solution retention in the raffinate phase. It can be concluded that solution retention for the different solvents should be evaluated since it

determines the number of stages in an industrial extraction process. Mass balances considering the initial mass of solvent and the initial ML mass, split up into the extract and the raffinate phases were above 94 % in all cases.

2.3.2.2.1. Influence of extraction solvent on extract composition and antioxidant capacity

The influence of the type of solvent on the profile of individual phenolic compounds and antioxidant capacity of the liquid extracts was also evaluated. The individual phenolic compounds that could be identified and quantifed by HPLC-DAD have been listed in Table 2.3. Identification of the phenolic compounds was done based on the spectrum of the pure standard compounds and the corresponding retention time. In general, the highest concentration of individual phenolic compounds was determined for the 50 % (v/v) ethanol aqueous mixture. Pure quercetin was only found at ethanol concentration higher than 80 % (v/v) while apigenin was found for ethanol aqueous mixtures with ethanol content lower than 50 % (v/v). The main phenolic compounds that could be identified and quantified in this work were neochlorogenic and chlorogenic acids and quercetin 3-O glucoside with maximum concentrations of 424 ± 41, 167 ± 18 and 212.9 ± 0.8 mg/L (4.5 ± 0.4, 1.8 ± 0.2 and 2.24 ± 0.01 mg/g_{DML}) respectively in a 50 % (v/v) ethanol aqueous solvent. Other phenolic compounds determined in this work for ML extracts were kaempherol, kaempherol 3-O-glucoside, vitexin, isovitexin, D (+) catechin, and rutin (with 0.40 ± 0.01, 0.71 ± 0.01, 0.23 ± 0.03, 0.15 ± 0.02, 1.23 ± 0.04, 0.16 ± 0.01 mg/g_{DML}, respectively for a 50 % (v/v) ethanol aqueous mixture).

The comparison of results among authors is hard since the nature of the ML used in the extraction experiments strongly affects the phenolic compounds profile. However, in the literature it has been consistently reported that the most abundant phenolic acids in moringa leaves are chlorogenic acid (and its isomers) whereas the most abundant flavonoids are kaempferol, quercetin, isorhamnetin, and apigenin, generally in the glycoside form, attached to a wide spectrum of sugar substituents [22]. In this sense, Rodríguez-Pérez *et al.* [23] identified more than 60 compound by HPLC-MS, being

kaempferol-3-glucoside and quercetin-3-gluoside the most abundant compounds extracted from ML. Lin *et al.* [19] also identified neochlorogenic and chlorogenic acids as two of the major phenolic compounds in ML together with hyperoside (quercetin 3-D galactoside) and D-(+) catechin, with concentrations of 4.2 ± 0.3, 1.61 ± 0.07, 4.6 ± 0.2, 3.7 ± 0.2 mg/g_{DML}, respectively. These authors also identified kaempferol-3-rutinoside and kaempferol-3-glucoside, with concentrations of 2.2 ± 0.15 mg/g_{DML} and 1.33 ± 0.05 mg/g_{DML}, respectively, as the most abundant flavonoids. Vongsak *et al.* [24] found the best results for cryptochlorogenic and quercetin-3-glucoside extraction when using aqueous ethanol (70%, v/v) with concentrations of 13.2 ± 0.6 and 5.3 ± 0.1 mg/g raw material, respectively. When aqueous ethanol (50%, v/v) was used, the concentration of these two compounds was decreased by 25%, demonstrating the importance of the solvent selection in the extraction of phenolic compounds from ML. Bennet *et al.* [25] tried different moringa varieties, identifying mainly chlorogenic acid (in the range from 0.38 ± 0.02 to 8.9 ± 0.3 mg/g_{DML}), neochlorogenic acid (up to 4.10 ± 0.08 mg/g_{DML}), kaempferol 3-O-glucoside (up to 1.80 ± 0.02 mg/g_{DML}), quercetin 3-O-glucoside (in the range from 0.08 ± 0.01 to 6.3 ± 0.3 mg/g_{DML}), and other more rare flavonoids, such as quercetin 3-O-(6''malonylglucoside) (up to 10.8 ± 0.4 mg/g_{DW}) and kaempferol 3-O-(6''malonylglucoside) (up to 3.1 ± 0.1 mg/g_{DW}). Nouman *et al.* [26] also studied the effect of the Moringa oleifera cultivar on the phenolic compounds profile, finding that chlorogenic acid was in the range from 57.1 ± 1.2 to 103 ± 3 $\mu g/g_{DML}$ and cryptochlogenic acid was found in concentrations up to 9.1 ± 0.5 $\mu g/g_{DML}$ for the cultivars studied. These authors also found kaempferol 3-O-glucoside (from 25.7 ± 2.6 to 39.8 ± 0.8 $\mu g/g_{DW}$), as well as quercetin 3-O-glucoside (from 3.9 ± 0.8 to 8.8 ± 0.4 $\mu g/g_{DML}$) and isovitexin (up to 47.8 ± 1.2 $\mu g/g_{DML}$).

Table 2.3. Concentration of individual phenolic compounds (mg/L) and antioxidant capacity determined in different hydroalcoholic mixture.

Compound / Antioxidant test	**Conventional solvent extraction**					**UAE**	
	100 % water	**20 % EtOH**	**50 % EtOH**	**80 % EtOH**	**100 % EtOH**	**20 % EtOH**	**50 % EtOH**
Neochlorogenic acid	240± 8^{b}	403 ± 2^{c}	374 ± 41^{c}	223 ± 38^{b}	70 ± 2^{a}	392 ± 2^{c}	386 ± 15^{c}
D (+) Catechin	121 ± 4^{b,c}	129 ± 2^{b}	127 ± 5^{c}	115 ± 14^{c}	64 ± 5^{a}	118 ± 1^{b,c}	110 ± 9^{b}
Chlorogenic acid	97 ± 2^{b}	160 ± 2^{c}	167 ± 18^{c}	87 ± 21^{b}	33 ± 3^{a}	175 ± 16^{c,d}	199 ± 6^{d}
Rutine	4.2 ± 0.8^{a}	13± 1^{b,c}	15.9 ± 0.4^{c,d}	15 ± 3^{b,c,d}	11± 2^{b}	15 ± 0.2^{b,c,d,}	17 ± 2^{d}
Apigenin	n.d.	5± 3^{a}	20.5 ± 3.0^{b}	n.d.	n.d.	n.d.	n.d.
Quercetin 3-glucoside	49 ± 2^{a}	115 ± 1^{b}	212.9 ± 0.8^{c}	215 ± 1^{c}	113± 4^{b}	129 ± 20^{b}	219 ± 6^{c}
Quercetin	n.d.	n.d.	n.d.	4 ± 1^{a}	9 ± 5^{a}	n.d.	n.d.
Kaempherol 3-glucoside	13.8 ± 0.7^{a}	38.9 ± 0.1^{b}	71 ± 1^{c}	76 ± 2^{c}	38 ± 10^{b}	37 ± 8^{b}	64.5 ± 0.7^{c}
Kampherol	2.0 ± 0.1^{a}	17.6 ± 0.3^{d}	40.8 ± 0.5^{e}	9.8 ± 0.1^{c}	5 ± 0.4^{b}	16.9 ± 0.5^{d}	10 ± 1^{c}
Vitexin	5.9 ± 0.5^{a}	13 ± 3^{b}	23 ± 3^{c}	17 ± 2^{b}	8.0 ± 0.6^{a}	15.3 ± 0.3^{b}	16 ± 0.4^{b}
Isovitexin	3.2 ± 0.1^{a}	8.7 ± 0.0.8^{b}	15 ± 2^{c}	9.2 ± 0.8^{b}	2.1 ± 0.2^{a}	10.6 ± 0.1^{b}	9.9 ± 0.9^{b}
ABTS, mg Trolox/L	2087 ± 29^{b}	2619 ± 2d	2781 ± 7^{e}	2432 ± 45^{c}	1219 ± 3^{a}	2516 ± 81^{c,d}	2898 ± 124^{e}
FRAP, mg Fe^{2+} /L	1473 ± 7^{b}	2120 ± 53^{c}	2599 ± 89^{e}	2368 ± 57^{d}	998 ± 20^{a}	1994 ± 34^{c}	2731 ± 148^{e}

Values with different letters in each column are significantly different when applying the Fisher's least significant differences (LSD) method at p-value ≤ 0.05.

n.d.: non-detected

The antioxidant capacity of the liquid extracts has been evaluated by the ABTS and the FRAP tests (see Table 2.3). Karthivashan *et al.* [16] essayed three different hydro-ethanolic mixtures (ethanol:water, 50:50, 70:30 and 90:10 %) as solvent and found that 90 % ethanol was the best solvent for bioactive compounds from ML as confirmed by different antioxidant assays, including FRAP and DPPH scavenging activity tests. In this work, the maximum antioxidant activity of the liquid extracts agreed with the maximum concentration of TPC in the extracts for a 50 % ethanol water mixture. According to the Pearson product moment correlation, statistically significant non-zero correlations at the 95 % confidence level were obtained for TPC and the different antioxidants test performed in this work (R^2 = 0.9703 and 0.9770 for FRAP and ABTS tests, respectively)

The variation color of the extract by using different ethanol aqueous mixtures could be clearly observed from light orange, by using water as solvent, to a more greenish color by using pure ethanol as solvent. The increase in the green color by increasing the amount of ethanol in the extraction solvent could indicate the presence of chlorophylls in these extracts. This color trend by using different ethanol aqueous solutions as solvent has been also observed in the extraction of biocompounds from other types of green leaves such olive leaves [13].

2.3.2.3. Comparison of conventional solvent extraction with ultrasound assisted extraction

Figure 2.3 show the extraction kinetics of TPC by using water, and different ethanol aqueous mixtures as solvents (20 % and 50% v/v) by UAE. For a better comparison of the results, extraction kinetics by conventional solvent extraction have been also plotted. The use of UAE when using water as solvent resulted in an increase in the initial extraction rate and a slight increase in TPC yield (from 2105-2209 to 1776-1899 mg GAE/L by UAE and conventional extraction, respectively) due to cavitation created by the ultrasound waves that induced a better penetration of the solvent into the ML and improved the diffusion process. However, when using ethanol aqueous mixtures as solvents the extraction efficiency was lower than by conventional solvent extraction.

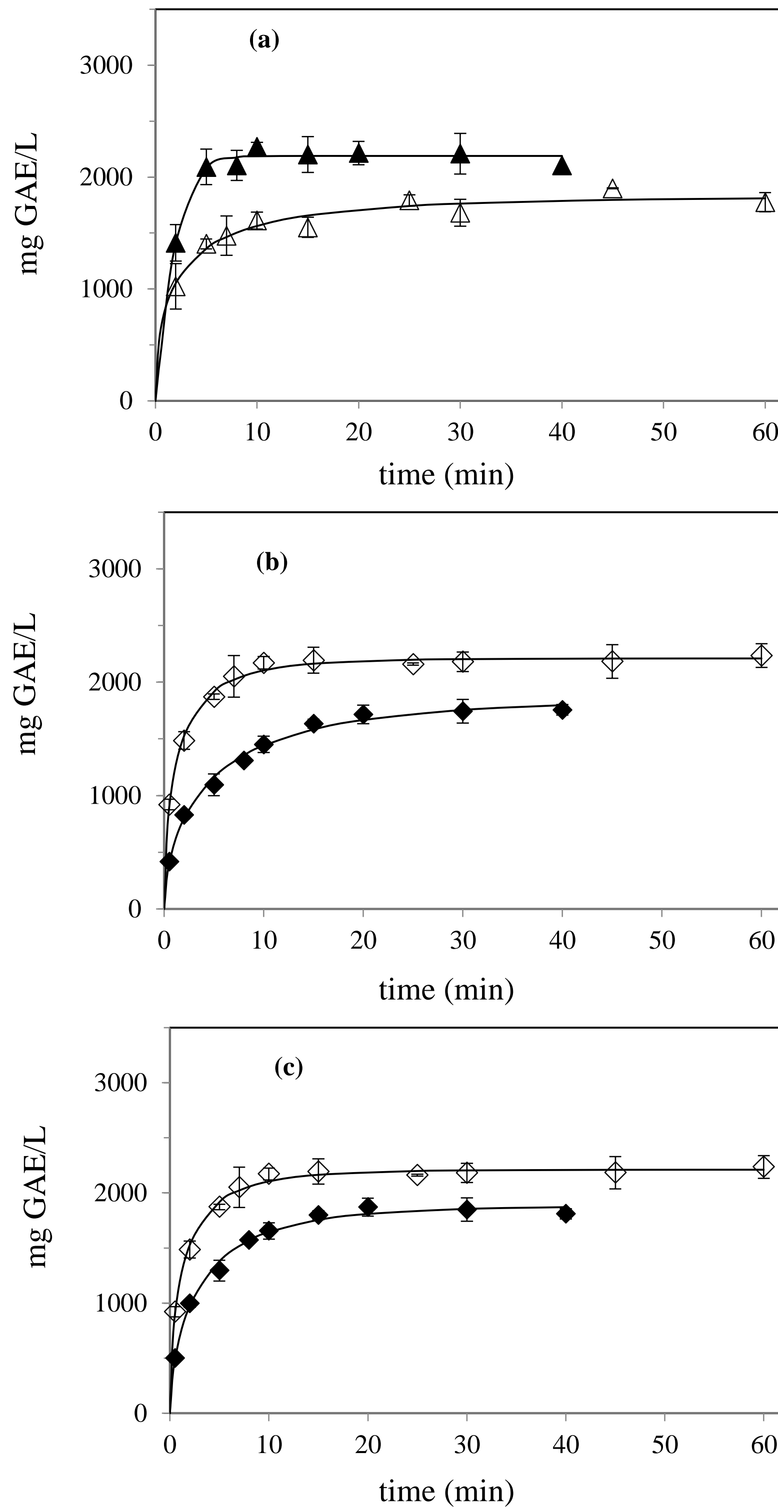


Figure 2.3. Comparison of UAE (solid symbols) and conventional extraction kinetics (empty symbols) at 50 ºC and a ratio of 10 mL/gDML for different solvent types: (a) (△, ▲) water, (b) (○, ●) 20 % EtOH, (c) (◇, ♦) 50 % EtOH. The continuous lines represent the Weibull model.

Rodriguez-Pérez *et al.* have also compared the efficiency of total phenolic compounds extraction from ML by conventional solid-liquid extraction (maceration) and UAE by using different ethanol aqueous mixtures [18]. These authors reported, as a general conclusion, that a higher total phenolic content was obtained with UAE than by using maceration due to the disruption of plant cell walls that allowed an increase in solvent penetration and higher extraction yields. However, a deeper look into their results brings different conclusions. These authors performed conventional solvent extraction in a single stage by using a ratio of 50 mL/g_{ML} for 1 h. On the other hand, UAE was carried out by using the same solvent ratio for 15 min but three more consecutive extractions were carried out after removing the solids by centrifugation and treating the pellets (the solids) with fresh solvent under the same conditions in such way that four consecutive extractions were made to complete 1 h of extraction. Although extraction time was the same, results are not comparable. Conventional solvent extraction was carried out in a single-stage method, while UAE was performed in four successive single-stages adding fresh solvent in the different stages leading to an increasing driving force in each step. These authors reported an extraction yield of 24.3 ± 0.3 mg GAE /g_{DML} in one stage conventional extraction while in the 1st extraction run by UAE they reported a value of 12.6 ± 0.2 mg GAE /g_{DML} when using 70 % (v:v) ethanol aqueous as solvent and 19 ± 1 mg GAE /g_{DML} after the 4th run. Other values reported by these authors when using a 50 % (v/v) ethanol aqueous solution were the following: 27 ± 2 mg GAE/g_{DML} by conventional extraction, while 24 ± 3 mg GAE/g_{DML} after the 1st UAE run (47 ± 4 mg GAE/g_{DML} after the 4th run). Although comparison of results of TPC by maceration and UAE in the 1st run differ in extraction time, it seems more reliable than after the 4th run of UAE, specially taking into account that, these authors determined the equilibrium extraction time needed to extract the different bioactive compounds by performing conventional extractions with a methanol:water (50:50, v:v) mixture at room temperature for 1, 3, 6 and 12 h, for a solvent:biomass ratio of 50 mL/g_{ML} and they found no significant differences among the different times. For that reason, they selected 1 h as extraction for conventional extraction, however, they did not try lower extraction times. In this work, by using different ethanol aqueous

mixtures, 30 min or even shorter times were found to be enough to reach a plateau in the extraction curves (see Figures 2.1 and 2.2).

The effect of fluid properties on cavitation has been studied and reviewed in the literature [27]. It has been reported that low vapour pressure, low viscosity and low surface tension of the solvent can improve the formation of high-intensity cavitation [28]. Based on different data found in the literature for ethanol aqueous mixtures, vapour pressure, surface tension and viscosity have been plotted as a function of ethanol mole fraction at 50 ºC in Figure 2.4 [29–31]. An increase in ethanol concentration led to a decrease in surface tension decreasing the cavitation threshold; while a maximum in viscosity was observed. An increase in viscosity of the medium increased the resistance of the sample to the movement of the ultrasonic probe and higher intensity is necessary to obtain the mechanical vibration [6]. By increasing the ethanol concentration, the vapour pressure of the system increased being also a key factor to explain the decrease in the extraction efficiency by UAE when using ethanol aqueous mixtures as solvents. According to literature [32], at a certain sonic frequency there is an optimum vapour pressure where the impulse pressure and temperature obtained due collapse of a cavity are maximum. Beyond these values, the impulse pressure intensity and the number of cavities decreases and the diameter of the cavity increases leading to disintegration into smaller cavities or dissolution into the medium instead of collapsing violently lowering the effective cavitation damage.

The lower extraction efficiencies obtained by UAE when using ethanol aqueous mixtures could be also due to the low solvent:biomass ratio used in this work. This ratio was lower than the optimum found by other authors when working with UAE that resulted in lower values of specific energies (kJ/g raw material) and higher resistance to the transference of the ultrasonic wave [33].

Individual phenolic compounds for the ethanolic aqueous extracts obtained by UAE have also listed in Table 2.3. Based on the statistical analysis, no difference could be clearly observed in the phenolic profile that could be identified in this work, between conventional solvent and ultrasound assisted extraction.

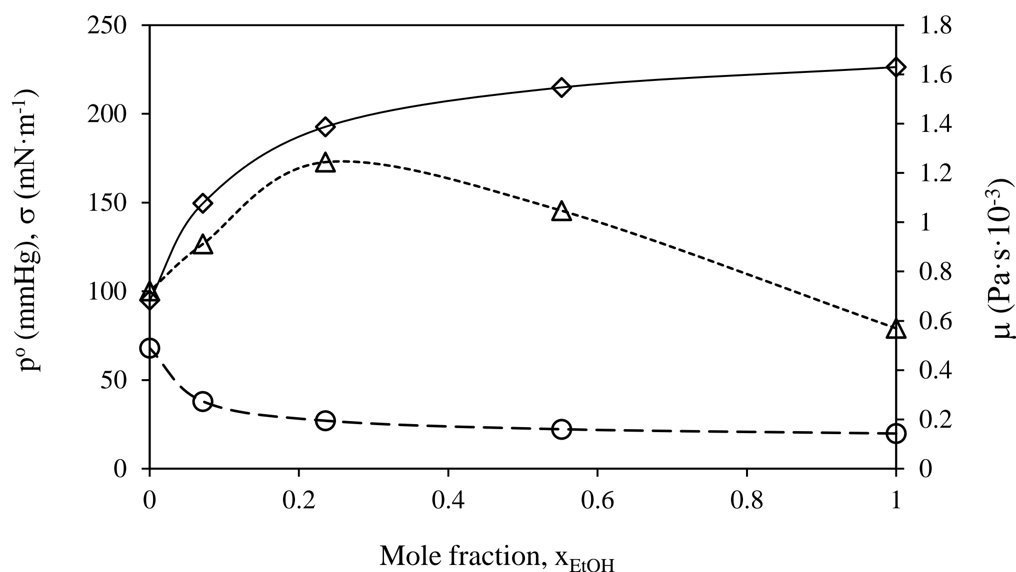


Figure 2.4. Physical properties of ethanol aqueous mixtures at 50ºC. ◊ vapour pressure; Δ viscosity; ○ surface tension.

2.3.2.4. Extraction kinetics modelling

The Weibull model was used to fit all the experimental extraction kinetics data obtained in this work. Parameters for the model are listed in Table 2.4. The Weibull model can be considered suitable to describe the extraction curves of TPC from ML with a medium value of the RMSD of 39 for all the extraction kinetics carried out in this work. The good fitting can be observed in Figures 2.1 – 2.3 where the Weibull model has been represented. The extraction parameter, A, that can be considered as the maximum extraction yield of TPC, reached a maximum for a 50% ethanol aqueous solution as solvent by conventional solvent extraction. The lower yields of TPC by UAE can be also observed in the values of the A parameter, with lower values for ethanol aqueous mixtures by UAE than by conventional extraction. The exponent n, presented values lower than the unity, except for water in UAE that described the parabolic shape of the extraction curves with a high initial slope.

Table 2.4. Kinetic model parameters of Weibull model for TPC extraction from ML.

Extraction kinetic			Kinetic parameters			
Solvent	**Extraction method**	**S/ML**	**A**	**k**	**n**	**RMSD**
20 % EtOH	Conventional	10	2211 ± 22	0.78 ± 0.03	0.59 ±0.03	38
20 % EtOH	Conventional	20	1114 ± 6	0.61 ± 0.02	0.75 ±0.03	12
Water	Conventional	10	1825 ± 69	0.61 ± 0.09	0.51 ±0.11	61
50 % EtOH	Conventional	10	2857 ± 48	0.45 ± 0.02	0.59 ±0.03	47
80 % EtOH	Conventional	10	2183 ± 44	0.19 ± 0.02	0.82 ±0.05	51
EtOH	Conventional	10	1483 ± 221	0.050 ± 0.006	0.84 ±0.08	30
Water	UAE	10	2189 ± 26	0.47 ± 0.09	1.2 ±0.2	47
20 % EtOH	UAE	10	1882 ± 41	0.46 ± 0.03	0.65 ±0.05	44
50 % EtOH	UAE	10	2385 ± 13	0.96 ± 0.03	0.80 ±0.04	26

**S/ML: ratio solvent moringa leaves, mL/g_{DML}*

2.3.3. Freeze dried extract (FDE)

A solid extract was obtained from the liquid ML extract obtained by using a 50% (v/v) ethanol aqueous mixture at 50ºC and 10 mL solvent/g_{DML} ratio This liquid extract was freeze dried previous removal of the ethanol by a rotatory evaporator. The final humidity content was 3.76 ± 0.05 %. The total yield of the FDE respect to the initial ML was 19 %. The low yield obtained compared to the extractive fraction of the ML (nearly 50 % w/w, see Table 2.1) is related to the high dissolution retention in the raffinate phase after extraction and phase separation by centrifugation (see Table 2.2).

Table 2.5. Characterization of the freeze dried extract, FDE; from moringa leaves.

Total antioxidants	TPC, mg GAE/g_{FDE}	82 ± 1
	TFC, mg QE/g_{FDE}	19.6 ± 0.3
Antioxidant activity	FRAP, mg Fe^{2+}/g_{FDE}	127 ± 2
	ABTS, mg FDE/mL	0.474
Individual phenolic compounds, mg/g_{FDE}	Neochlorogenic acid	15.0 ± 0.3
	D (+) Catechin	4.3 ± 0.3
	Chlorogenic acid	5.7 ± 0.6
	Rutin	0.5 ± 0.1
	Quercetin 3-O-glucoside	7.3 ± 0.1
	Kaempherol 3-glucoside	2.07 ± 0.01
	Kampherol	0.48 ± 0.01
	Vitexin	0.95 ± 0.06
	Isovitexin	0.59 ± 0.02
Other components	Ch-a, mg/g_{FDE}	44.8 ± 0.4
	Ch-b, mg /g_{FDE}	20.4 ± 0.1
	Cx+c, mg/g_{FDE}	17.7 ± 0.1

Karthivasan *et al.* [16] reported lower extraction yield of ML by using different ethanol aqueous mixture as solvent after freeze dried of the liquid extracts with an extraction yield of 8.48 % for a 50 % ethanol aqueous mixtures. The lower yield obtained by these authors compared to this work, can be attributed to a poorer separation of the solid residue and the liquid extract since separation was carried out by filtration while in this work was done by centrifugation. Hence, it must be highlighted the importance of the retention index of the selected solvent and an efficient separation process of the liquid and the solid residue to achieve a high final extraction yield in the global process.

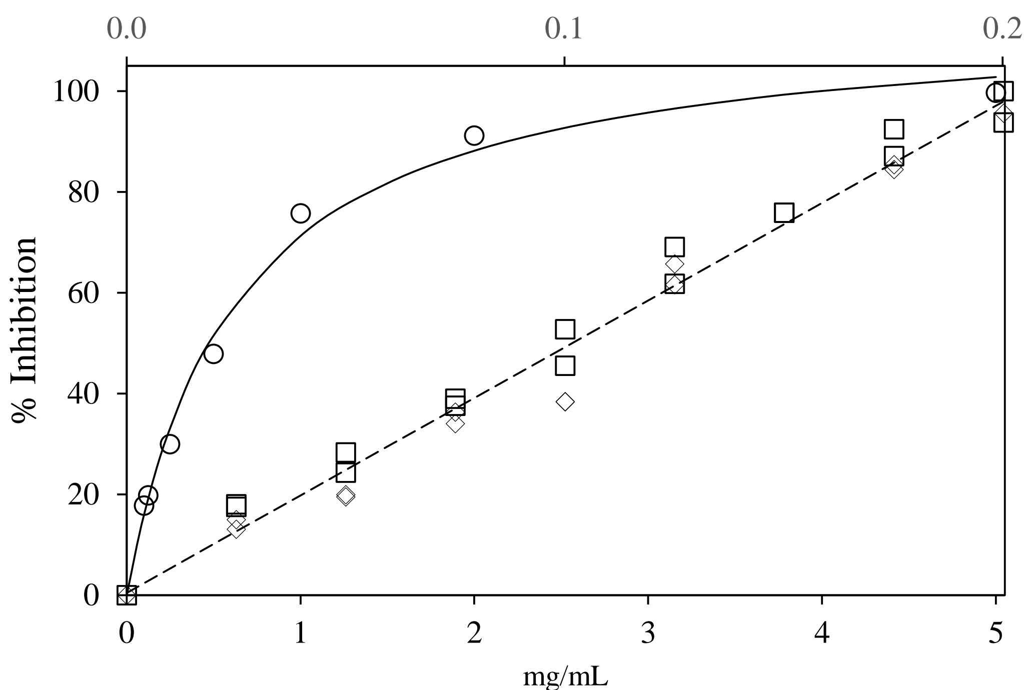


Figure 2.5. Inhibition percentage of FDE from ML (lower x-axis) (○) and comparison with pure TROLOX standard (upper x-axis) dissolved in water (◊) and ethanol (□).

A complete characterization of the FDE is presented in Table 2.5. FDE presented a high content of bioactive compounds as determined by the TPC and total flavonoids compounds, TFC, 82 ± 1 mg GAE/g_{FDE} and 19.6 ± 0.3 mg QE/g_{FDE}, respectively. Antioxidant activity was determined by the FRAP method, with a reducing power of 127 ± 2 mg Fe^{2+}/g_{FDE}. The ABTS test was carried out to obtain the extract concentration to achieve a 50 % inhibition of the radical according to the reaction conditions detailed in appendix chapter. Results were compared with the inhibition response of pure TROLOX in water and ethanol as solvent and are presented in Figure 2.5. The TROLOX standard response was linear and a concentration of 0.102 mg TROLOX/mL was needed to achieve a 50 % ABTS radical inhibition. On the contrary, the FDE of ML exhibited an asymptotic response with a concentration of 0.474 mg FDE/mL to achieve a 50 % ABTS radical inhibition, more than 4.5 times that of TROLOX.

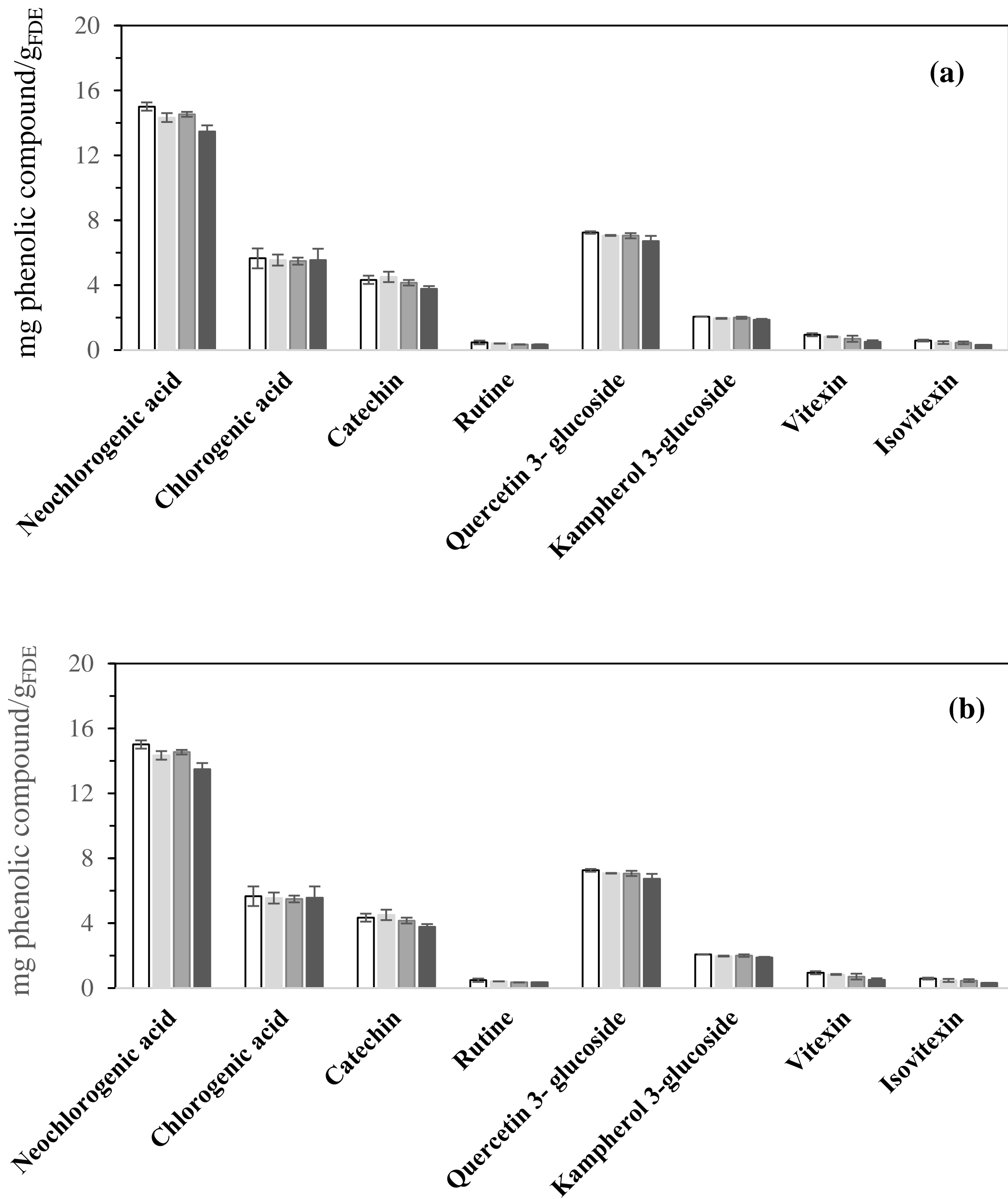


Figure 2.6. Composition of the individual phenolic compounds along storage (a) room temperature and (b) 4ºC: □ fresh prepared FDE, ■ 18 days, ■ 39 days, ■ 101 days.

The individual phenolic compounds that could be identified in the FDE have been also listed in Table 2.5. Similar to the liquid extracts, the main phenolic compounds that could be identified in the FDE were neochlorogenic and chlorogenic acids, quercetin 3-O-β-glucoside and isorhamnetin 3-glucoside with concentrations of 15.0 ± 0.3, 5.7 ± 0.6, 7.3 ± 0.1 and 4.9 ± 0.1 mg /g_{FDE}, respectively. The presence of pigments such as chlorophylls and carotenoids were responsible of the color of the extract. The amount of Chlorophyll-a, Chlorophyll-b and carotenoids was 44.8 ± 0.4, 20.4 ± 0.1 and 17.7 ± 0.1 mg/g_{FDE}, respectively. Vats *et al.* [34] reported a value for β-carotene of 14.1 ± 0.1 mg/g ML, by solvent extraction with a 95 % ethanol aqueous mixture as solvent for 24 h in an orbital shaker at 37ºC.

The individual phenolic compounds concentration was followed in the FDE along storage at room temperature and darkness and at 4 ºC during 101 days (Figure 2.6). Not a significant decrease for the phenolic compounds determined in this work was observed along storage either at room temperature or at cooling conditions. The content of the phenolic compounds varied in the range from 87% to 98 % of the initial content, except for vitexin and isovitexin whose content drop to 55 – 60 of its initial content in the FDE. Vongsak *et al.* [24] also found that after 3 months of storage at 25 ºC, antioxidant capacity and bioactive compounds content were not significantly different in the extracts compared with the freshly prepared extracts.

2.4. Conclusions

Chemical composition of ML showed and important amounts of extractives rich in bioactive compounds that makes this plant an attractive raw material as nutraceutical products source. Extraction of this fraction by using different hydroalcoholic mixture showed the importance of the type of solvent since it determined the amount and type of extracted bioactive compounds but also the retention index of the extract in the solid residue. It was found that by increasing the amount of water in the ethanol aqueous mixture the retention index also increased. A 50 % ethanol aqueous mixture (v/v) was found to be the best extraction solvent with a TPC yield of 29.5± 0.3 mg GAE/g_{DML}, 311 ± 111 and 212.9 ± 0.8 of neochlorogenic aicd and quercetin-3-O-glucoside, respectively. UAE yielded higher extraction yields when using water as solvent, but the presence of ethanol led to lower extraction yields.

A freeze-dried extract was obtained with a high content of bioactive compounds such as neochlorogenic and chlorogenic acids, quercetin 3-O-glucoside and isorhamnetin 3-glucoside, among others. Due to the antioxidant capacity and phenolic composition, the ML freeze-drying extract could be used an additive for food, pharmaceutical or cosmetic industries.

References

[1] M. Valdivié-Navarro, Y. Martínez-Aguilar, O. Mesa-Fleitas, A. Botello-León, C. Betancur Hurtado, and B. Velázquez-Martí, “Review of Moringa oleifera as forage meal (leaves plus stems) intended for the feeding of non-ruminant animals,” *Anim. Feed Sci. Technol.*, vol. 260, no. October 2019, p. 114338, 2020.

[2] A. Leone *et al.*, “Nutritional characterization and phenolic profiling of moringa oleifera leaves grown in chad, sahrawi refugee camps, and Haiti,” *Int. J. Mol. Sci.*, vol. 16, no. 8, pp. 18923–18937, 2015.

[3] J. González-Romero, S. Arranz-Arranz, V. Verardo, B. García-Villanova, and E. J. Guerra-Hernández, “Bioactive compounds and antioxidant capacity of moringa leaves grown in Spain versus 28 leaves commonly consumed in pre-packaged salads,”

Processes, vol. 8, no. 10, pp. 1–20, 2020.

[4] T. L. C. Oldoni *et al.*, “Bioguided extraction of phenolic compounds and UHPLC-ESI-Q-TOF-MS/MS characterization of extracts of Moringa oleifera leaves collected in Brazil,” *Food Res. Int.*, vol. 125, no. March, p. 108647, 2019.

[5] C. Castro-lópez *et al.*, “Impact of extraction techniques on antioxidant capacities and phytochemical composition of polyphenol-rich extracts,” vol. 237, pp. 1139–1148, 2017.

[6] F. Chemat, Zill-E-Huma, and M. K. Khan, “Applications of ultrasound in food technology: Processing, preservation and extraction,” *Ultrason. Sonochem.*, vol. 18, no. 4, pp. 813–835, 2011.

[7] J. B. Sluiter, R. O. Ruiz, C. J. Scarlata, A. D. Sluiter, and D. W. Templeton, “Compositional analysis of lignocellulosic feedstocks. 1. Review and description of methods,” *J. Agric. Food Chem.*, vol. 58, no. 16, pp. 9043–9053, 2010.

[8] R. Oliveira, G. R. Carvalho, M. Cirillo, and F. Queiroz, “Effect of ecofriendly bio-based solvents on oil extraction from green coffee bean and its industrial press cake,” *Brazilian J. Chem. Eng.*, vol. 36, no. 4, pp. 1739–1753, 2019.

[9] A. C. M. A. Araújo, É. Resende de Oliveira, E. G. T. Menezes, B. O. Dias, A. W. C. Terra, and F. Queiroz, “Solvent effect on the extraction of soluble solids from murici and pequi seeds,” *J. Food Process Eng.*, vol. 41, no. 6, 2018.

[10] J. Wlsniak, J. Hillel, and O. Katz, “Holdup and extraction characteristics of jojoba meal,” *J. Am. Oil Chem. Soc.*, vol. 64, no. 9, pp. 1352–1354, 1987.

[11] A. E. Illera *et al.*, “Effect of thermosonication batch treatment on enzyme inactivation kinetics and other quality parameters of cloudy apple juice,” *Innov. Food Sci. Emerg. Technol.*, vol. 47, 2018.

[12] N. Sumanta, C. Imranul Haque, J. Nishika, and R. Suprakash, “Spectrophotometric Analysis of Chlorophylss and Carotenoids from Commonly Grown Fern Species by Using Various Extracting Solvents,” *Res. J. Chem. Sci.*, vol. 4, no. 9, pp. 63–69, 2014.

[13] M. Kashaninejad, M. T. Sanz, B. Blanco, S. Beltrán, and S. M. Niknam, “Freeze dried extract from olive leaves: Valorisation, extraction kinetics and extract characterization,” *Food Bioprod. Process.*, vol. 124, pp. 196–207, 2020.

[14] S. Kitanović, D. Milenović, and V. B. Veljković, "Empirical kinetic models for the resinoid extraction from aerial parts of St. John's wort (Hypericum perforatum L.)," *Biochem. Eng. J.*, vol. 41, no. 1, pp. 1–11, 2008.

[15] R. A. Carciochi, C. A. Sologubik, M. B. Fernández, G. D. Manrique, and L. G. D'Alessandro, "Extraction of antioxidant phenolic compounds from brewer's spent grain: Optimization and kinetics modeling," *Antioxidants*, vol. 7, no. 4, 2018.

[16] G. Karthivashan, M. Tangestani Fard, P. Arulselvan, F. Abas, and S. Fakurazi, "Identification of bioactive candidate compounds responsible for oxidative challenge from hydro-ethanolic extract of moringa oleifera leaves," *J. Food Sci.*, vol. 78, no. 9, 2013.

[17] S. Ancut, A. C. F, D. Cristian, B. Rusu, and M. Tofan, "In fl uence of the extraction solvent on phenolic content , antioxidant , antimicrobial and antimutagenic activities of brewers ' spent grain rcas," vol. 80, pp. 180–187, 2018.

[18] C. Rodríguez-Pérez, R. Quirantes-Piné, A. Fernández-Gutiérrez, and A. Segura-Carretero, "Optimization of extraction method to obtain a phenolic compounds-rich extract from Moringa oleifera Lam leaves," *Ind. Crops Prod.*, vol. 66, pp. 246–254, 2015.

[19] X. Lin, L. Wu, X. Wang, L. Yao, and L. Wang, "Ultrasonic-assisted extraction for flavonoid compounds content and antioxidant activities of India Moringa oleifera L. leaves: Simultaneous optimization, HPLC characterization and comparison with other methods," *J. Appl. Res. Med. Aromat. Plants*, vol. 20, no. September 2020, 2021.

[20] R. E. Treybal, "Treybal, Rober E.; 'Operaciones de transferencia de masa' 2º edition, México: McGraw Hill, cop. 1990," 1990.

[21] O. Z. Sampaio Neto, D. Gonçalves, S. de F. Bergara, E. A. C. Batista, and A. J. de A. Meirelles, "Oil extraction from semi-defatted babassu bagasse with ethanol: Liquid-liquid equilibrium and solid-liquid extraction in a single stage," *J. Food Eng.*, vol. 276, no. July 2019, 2020.

[22] M. Lin, J. Zhang, and X. Chen, "Bioactive flavonoids in Moringa oleifera and their health-promoting properties," *J. Funct. Foods*, vol. 47, no. April, pp. 469–479, 2018.

[23] C. Rodríguez-Pérez, B. Gilbert-López, J. A. Mendiola, R. Quirantes-Piné, A. Segura-

Carretero, and E. Ibáñez, “Optimization of microwave-assisted extraction and pressurized liquid extraction of phenolic compounds from moringa oleifera leaves by multiresponse surface methodology,” *Electrophoresis*, vol. 37, no. 13, pp. 1938–1946, 2016.

[24] B. Vongsak, P. Sithisarn, S. Mangmool, S. Thongpraditchote, Y. Wongkrajang, and W. Gritsanapan, “Maximizing total phenolics, total flavonoids contents and antioxidant activity of Moringa oleifera leaf extract by the appropriate extraction method,” *Ind. Crops Prod.*, vol. 44, pp. 566–571, 2013.

[25] R. N. Bennett *et al.*, “Profiling glucosinolates and phenolics in vegetative and reproductive tissues of the multi-purpose trees Moringa oleifera L. (Horseradish tree) and Moringa stenopetala L.,” *J. Agric. Food Chem.*, vol. 51, no. 12, pp. 3546–3553, 2003.

[26] W. Nouman, F. Anwar, T. Gull, A. Newton, E. Rosa, and R. Domínguez-Perles, “Profiling of polyphenolics, nutrients and antioxidant potential of germplasm’s leaves from seven cultivars of Moringa oleifera Lam.,” *Ind. Crops Prod.*, vol. 83, pp. 166–176, 2016.

[27] J. J. John, S. Kuhn, L. Braeken, and T. Van Gerven, “Effect of fluid properties on ultrasound assisted liquid-liquid extraction in a microchannel,” *Ultrason. Sonochem.*, vol. 42, no. February 2017, pp. 68–75, 2018.

[28] Y. Tao and D. W. Sun, “Enhancement of Food Processes by Ultrasound: A Review,” *Crit. Rev. Food Sci. Nutr.*, vol. 55, no. 4, pp. 570–594, 2015.

[29] J. Gmehling and U. Onken, “Vapor Liquid Equilibrium Data Collection.,” *Vap. Liq. Equilib. Data Collect. Aqueous Syst. Suppl. 3 Chem. Data Ser. Vol. I Part 1c*, vol. 53, no. 9, pp. 1689–1699, 1981.

[30] R. Belda, J. V. Herraez, and O. Diez, “Rheological study and thermodynamic analysis of the binary system (water/ethanol): Influence of concentration,” *Phys. Chem. Liq.*, vol. 42, no. 5, pp. 467–479, 2004.

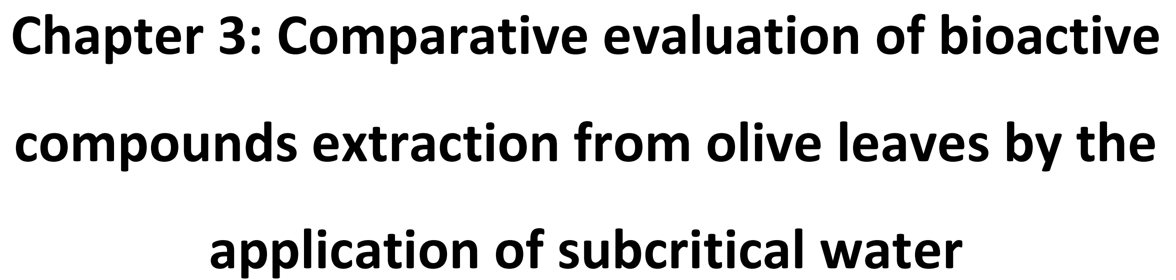
[31] G. Vazquez, E. Alvarez, and J. M. Navaza, “Surface Tension of Alcohol + Water from 20 to 50 °C,” *J. Chem. Eng. Data*, vol. 40, no. 3, pp. 611–614, 1995.

[32] M. M. Chivate and A. B. Pandit, “Quantification of cavitation intensity in fluid bulk,”

Ultrason. Sonochem., vol. 2, no. 1, pp. S19–S25, 1995.

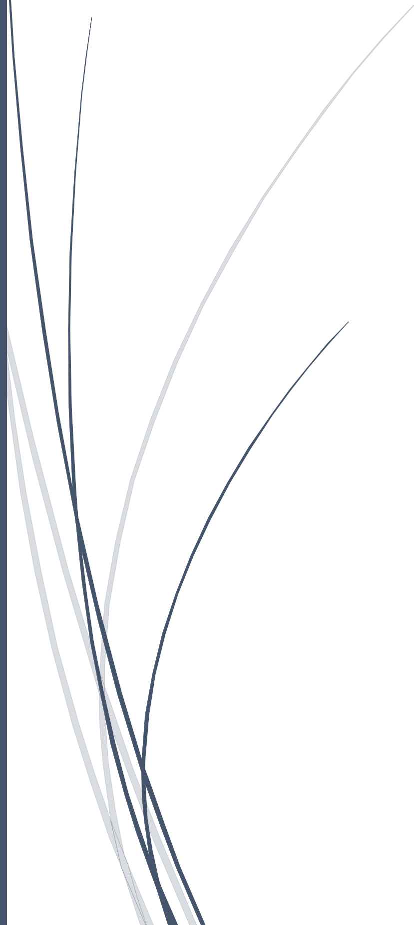
[33] P. Alonso-Riaño, M. T. Sanz Diez, B. Blanco, S. Beltrán, E. Trigueros, and O. Benito-Román, “Water Ultrasound-Assisted Extraction of Polyphenol Compounds from Brewer’s Spent Grain: Kinetic Study, Extract Characterization, and Concentration,” *Antioxidants*, vol. 9, no. 3, p. 265, 2020.

[34] S. Vats and T. Gupta, “Evaluation of bioactive compounds and antioxidant potential of hydroethanolic extract of Moringa oleifera Lam. from Rajasthan, India,” *Physiol. Mol. Biol. Plants*, vol. 23, no. 1, pp. 239–248, 2017.



Chapter 3: Comparative evaluation of bioactive compounds extraction from olive leaves by the application of subcritical water

Valorization of plant biomass by emerging technologies: olive and moringa leaves



In this study, subcritical water extraction was performed on olive leaves by using two different types of configuration a semi-continuous and a discontinuous reactor. Regarding operating conditions, two particle sizes of powdered and comminuted and two different temperature levels of 140 and 180 °C were used in order to obtain liquid extracts. The properties of the obtained extracts of olive leaves were analyzed in terms of total phenolic content, total carbon, and protein recovery yield. Furthermore, identification and quantification of individual phenolic compounds and polysaccharide fractions were performed by HPLC. In addition, The Elemental Microanalysis (CHNS) analysis was performed on the solid residues remained after extraction runs.

Considering overall aspects studied in this study, it can be concluded the considerable advantage of using semi-continuous over the discontinuous system. In addition, extraction temperature of 180 °C and powdered particle size of the sample resulted as optimum operating conditions. Lysine was found to be the major essential amino acid among 17 identified free amino acids in the optimal extraction run. Finally, the highest oleuropein content was obtained at 180 °C with the powdered forms of olive leaves.

En este estudio se ha llevado a cabola extracción de agua subcrítica de hojas de olivo empleando dos sistemas de extracción, un reactor semicontinuo y un reactor discontinuo. En cuanto a las condiciones de operación, se utilizaron dos tamaños de partícula diferente. Por una parte, hojas de olivo en polvo y por otra parte hojas de olivo trituradas con un tamaño de partícula entre 0.5 – 1 cm . En ambos casos, la extracción se realizó a dos niveles de temperatura diferentes 140 y 180 ° C Se analizaron las propiedades de los extractos de hojas de olivo obtenidos en términos del contenido fenólico total, carbono total y rendimientoden la recuperación de proteínas. Además, se llevó a cabo la identificación y cuantificación de compuestos fenólicos individuales, así como las diferentes fracciones de polisacáridos mediante HPLC. Los residuos finales tras el tratamiento con agua subcrítica se analizaron mediante microanálisis elemental (CHNS).

Considerando los resultados obtenidos en este trabajo, se puede concluir que el sistema semicontinuo fue más ventajoso que el sistema en discontinuo. Además, la temperatura de extracción de 180 ° C y el tamaño de partícula en polvo de la muestra resultaron ser las condiciones óptimas de funcionamiento entre las condiciones ensayadas en este trabajo. Se determinó que la lisina fue el principal aminoácido esencial entre los 17 aminoácidos libres identificados en el proceso de extracción. Finalmente, el mayor contenido de oleuropeína se obtuvo a 180 ° C empleando las hojas de olivo en forma de polvo.

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3.1. Introduction

Olive leaves are known as a source of high bioactive compounds. Bioactive compounds are phytochemicals found in foods that are capable of modulating metabolic processes and resulting in the promotion of better health. They exhibit beneficial effects such as antioxidant activity, inhibition or induction of enzymes, inhibition of receptor activities, and induction and inhibition of gene expression [1]. Bioactive compounds are molecules that can present therapeutic potential with influence on energy intake, while reducing pro-inflammatory state, oxidative stress, and metabolic disorders [2]. Today, the public and food producers are paying more attention to natural food additives and if possible, both will choose a food containing natural additives over synthetic ones. Hence, much effort is being made to identify the antioxidants from natural sources such as phenolic compounds [3]. This indicates the growing need for better access to the bioactive compounds in the olive leaves. Olive leaves are considered byproducts of olive farming, one of the most important activities in the Mediterranean region, representing almost 10% of the total weight of materials arriving to the olive mill [4]. There are several extraction methods in order to obtain bioactive compounds such as different phenolic compounds like oleuropein and luteolin-7-O-glucoside or hydroxytyrosol, even other compounds like mannitol which olive leaves are rich in. Pressurized fluid technology (SFT) has received growing interest as a green technology replacing the use of organic solvents, being more and more restricted due to their health and environ-mental related issues. Amon pressurized fluids, the use of subcritical water is attractive since water is the greenest solvent and present unique properties in its subcritical state. The basic principle in pressurized liquid extraction (PLE) is the use of elevated pressure to maintain the liquid state of solvents applied at temperatures above their boiling points. Therefore, this technique is an alternative to a classical version of a solvent extraction of solids that is accomplished in the Soxhlet apparatus under atmospheric pressure. Initially, it was known as accelerated solvent extraction (ASE). The term was derived from the trade name of the first commercially available system made by Dionex, i.e. Accelerated Solvent Extractor (ASE®100). Currently this technique has received different names, such as pressurized solvent extraction (PSE) and pressurized fluid extraction (PFE). When water is employed as the extraction

solvent, to highlight the use of this environmentally friendly solvent, the terms such as pressurized hot water extraction (PHWE), superheated water extraction or subcritical water extraction (SWE) can be found in the literature [5]. Subcritical water extraction (SCWE) is an environmentally friendly process that is increasingly used as an alternative to traditional extraction methods such as solvent extraction [7,8]. In industry the most common extraction solvents are organic. These can be toxic and environmentally damaging, meaning stringent solvent removal procedures are required whenever the products or extracts are to be ingested or pose a potential environmental hazard. There is consequently the need for an extraction technique or solvent that is non-toxic, especially when extracting compounds for people to ingest, such as bioactive polyphenols [8]. SWE is a very promising technique for obtaining bioactive (mainly antioxidants) from natural sources; even if sometimes the high operation temperatures have been suggested as responsible for thermal degradation of bioactive, the fact is that this type of extraction processes may generate new bioactive compounds [9]. SWE takes place at temperatures between the boiling point and critical point of water (100 °C and 374.1 °C), at pressures high enough to keep water in the liquid state (Figure 3.1) [10]. Water is not considered as a suitable extraction fluid for nonpolar organic compounds at room temperature. When the temperature of water is raised, there is a steady decrease in its permittivity, viscosity and surface tension but an increase in its diffusivity characteristics. So, water behaves like certain organic solvents which can dissolve a wide range of medium and low polarity analyte [11]. The most relevant properties of water are those modulated by the extraction process parameters. The main process parameters are pressure, temperature, solvent ratio, particle size, extraction time, mixing and flow rate [12].

The aim of this study was to compare the effectiveness of semi-continuous and discontinuous extraction systems by using subcritical water. Furthermore, temperature and sample particle size as process variables were investigated on the quality and quantity of bioactive compounds extracted from olive leaves.

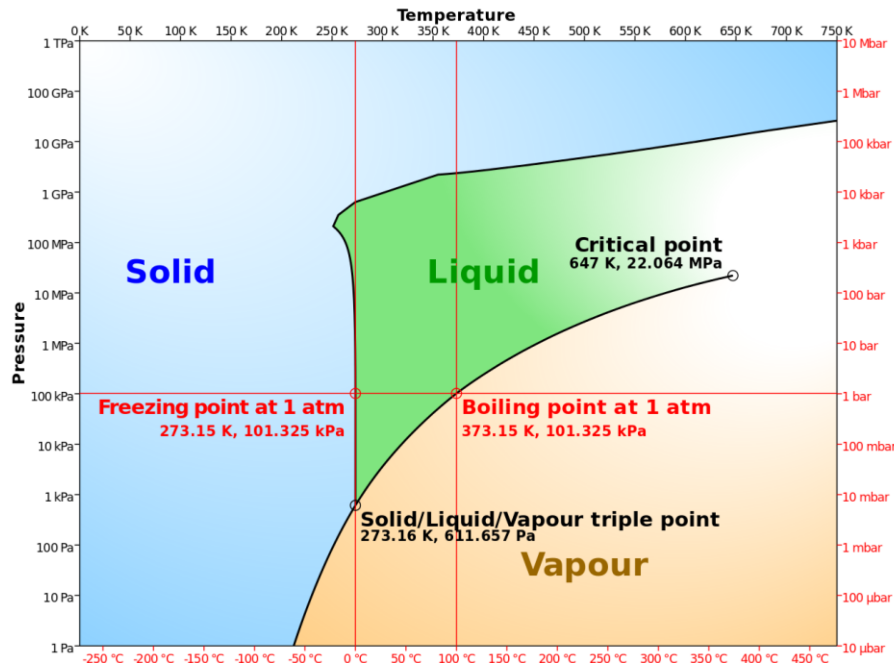


Figure 3.1. Physical state of water at different temperature and pressures (Adapted from Plaza & Turner [13]).

3.2. Material and methods

3.2.1. Plant material

Olive leaf (OL) samples were collected from the pruning of olive trees and were kindly provided by the cooperative "Pagos de Benaval" of the variety "Serrana de Espadán", endemic of the Sierra de Espadán natural park (Castellón, Spain). OL were laid on flat surfaces to dry naturally with exposure to air to reduce the humidity. In this study, two sizes of OL has been used 1) comminuted (pieces between 0.5-1 cm) and 2) Powdered (grounded using an electric grinder to obtain particle size in the range from 0.5 to 0.1 mm) (Figure 3.2). Samples were stored in the refrigerator for extraction tests.

Figure 3.2. Used olive leaf particle sizes: a) Comminuted; b) Powdered.

3.2.2. Subcritical Water Extraction (SWE)

3.2.2.1. Semi-continuous extraction

The semi-continuous reactor consists of a 27 mL extractor (Autoclave Engineers, PA, USA), an HPLC pump (Gilson 305, SC-10 head) to pump the water through the extractor and a back pressure regulator, to keep the working pressure at 5 MPa. The temperature was registered at the inlet and outlet of the extractor and connected to the software acquisition data PicoLog. In this configuration, the solvent is heated by means of a band heater to get the desired temperature at the inlet of the extractor, and another band heater around the extractor keeps such temperature.

In a typical experiment, around 2 g of OL were placed in the extractor mixed with around 4 g of 5 mm glass beads in order to avoid packaging and channeling through the bed. The whole system was filled up with water and pressurized. After that, water was heated by means of the first band heater at the working flow rate, but bypassing the extractor until reaching the desired temperature. Once water reached the working temperature, bypass valve was closed, so water was continuously pumped through the extractor. The extract was cooled down in a chilled water bath right after exiting the extractor and kept at 4 °C until analysis. Extraction experiments lasted 180 min, and the subcritical water experiments were done at two temperatures, 140 and 180 °C. This working procedure, in which only water at the extraction temperature is passed through

the extractor, minimizes the time the raw material is exposed to the high temperature, preserving the integrity of the bioactive compounds.

Table 3.1. Operating conditions of SWE test runs.

Run	Temp (°C)	Flow (mL/min)	Olive leaf size
PLE 1	180	6	Powdered
PLE 2	140	6	Comminuted
PLE 3	180	6	Comminuted

3.2.2.2. Discontinuous extraction

Subcritical water experiments at pilot-scale level were carried out at Hiperbaric's facilities (Burgos, Spain) by using a discontinuous system. The maximum specifications of the prototype were 185 ºC and 20 bar.

The main structural elements of the prototype were a reactor of 25 L capacity, a steam boiler as the heating system, a pump to recirculate and homogenize the biomass inside the reactor, a heat exchanger to avoid coiling during the recirculation process and a solid/liquid separation system. Operation and control of the process was performed by self-built Hiperbaric software.

In a typical run, water was initially pre-heated up to 80 ºC in the steam boiler and circulate it through the heat exchanger, placed in the recirculation pipe, and the extractor. This way, all the system was initially pre-heated at 80 ºC. After this pre-heating period, the system was completely drained and the biomass was charged into the reactor. Then, the reactor was filled with the pressurized water at the working temperature, 180 ºC, achieved by the steam boiler system. The system was pressurized by using nitrogen gas. Recirculation pump was turned on to enhance external mass transfer in the extraction/hydrolysis process. The pump was able to handle up to a biomass concentration of 40 wt% with a maximum particle size of 0.5 mm that determined the particle size of the biomass to be used. The heat exchanger placed in the recirculation pipe allowed contact with the steam boiler outlet pipe avoiding the cooling

in the recirculation process. A sampling system at the bottom of the reactor allowed sample withdrawal to follow the extraction/hydrolysis kinetics.

After extraction was finished a filtration tank, allowed phases separation to obtain a liquid hydrolysate and the solid residue. Experiments were carried out at a biomass loadings of 15 % w/w at 180 ºC and a working pressure of 20 bar. The Scheme of discontinuous SWE procedure can be observed in Figure 3.4.

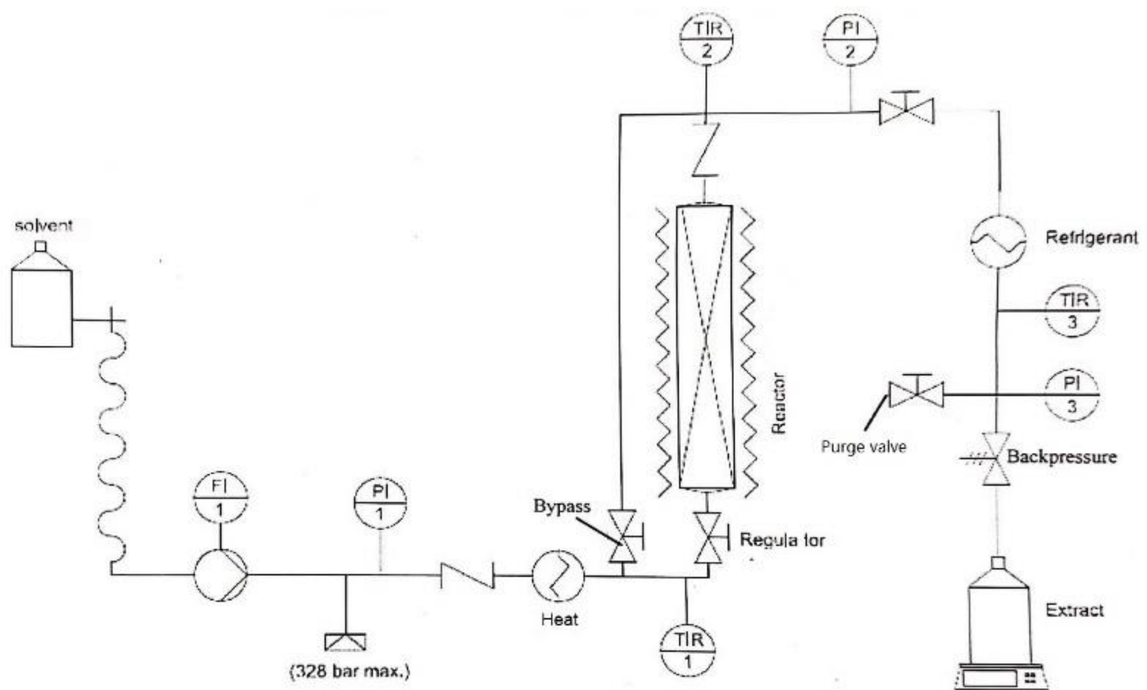


Figure 3.3. Scheme of semi-continuous SWE procedure.

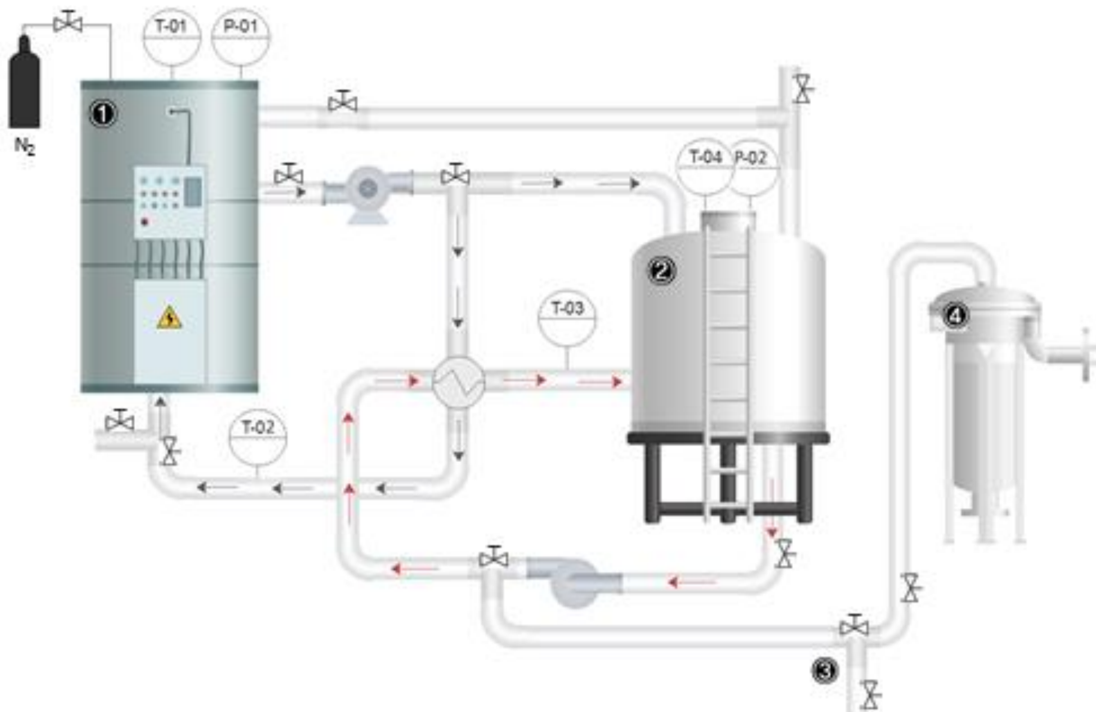


Figure 3.4. Scheme of discontinuous SWE procedure.

3.3. Results and discussion

3.3.1. Controlling the stability of temperature

The temperature profile obtained for both discontinuous and semi-continuous systems for reaching up to 180 °C is shown in Figure 3.5.

In the semi-continuous system, the experiment was continued for more than 60 min, but in the discontinuous configuration the experiment lasted only 45 min. Therefore, comparison between the two temperature profiles was presented form 0 to 45 min. Based on the obtained results, shown in Figure 3.5, average working temperature on discontinuous system was 181.1±0.6 ºC, while in semi-continuous system it was 183.6±2.2ºC. It can be seen that semi-continuous system showed a slightly higher temperature average than discontinuous system. Somehow, temperature was easily controllable until the last operating minute. Therefore, it can be concluded the

advantage of heating jacket system in providing better temperature control in the semi-continuous system. With the semi-continuous system, two more runs were also performed, temperature profile for all runs in the semicontinuous reactor is shown in Figure 3.6. It can be seen that for PLE2 run which was operated at lower temperature (140.4±0.4℃), lower instability at temperature controlling was observed in comparison with PLE1 and PL3 runs which were performed at 182±0.6 ℃ and 183.6±2.2 ℃ respectively.

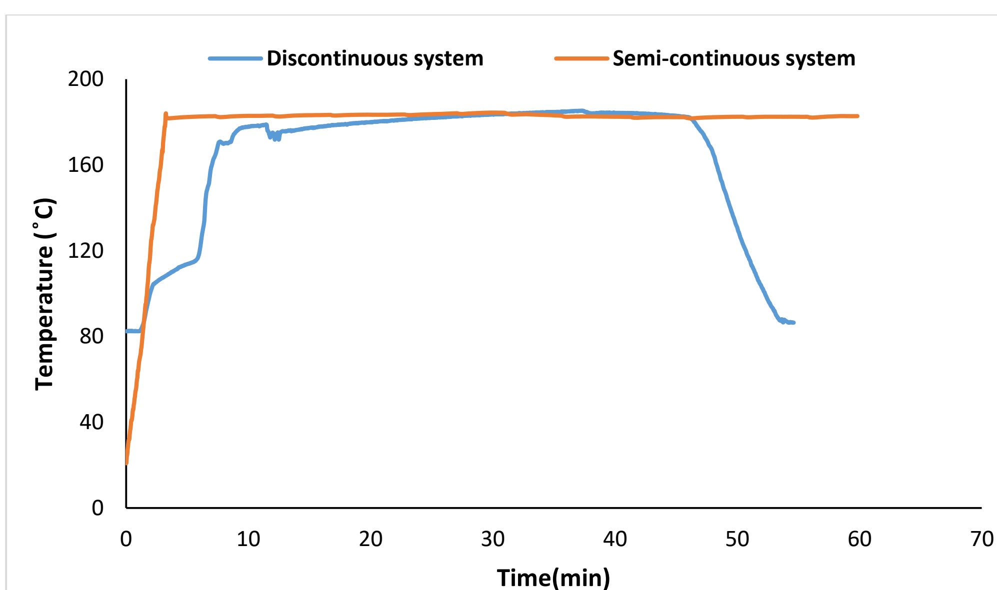


Figure 3.5. Temperature profile in the subcritical reactor for discontinuous (blue line) and semi-continuous (orange line) systems experiments carried out at 180 °C.

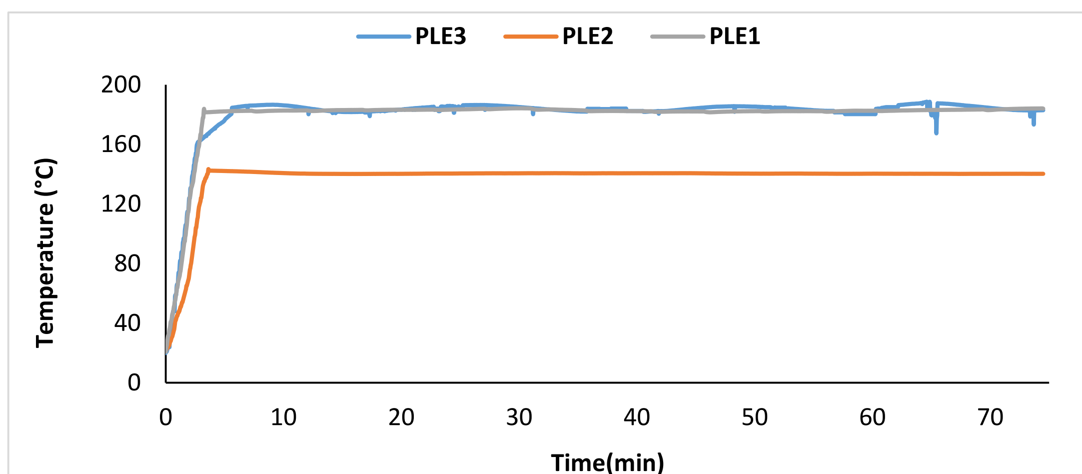


Figure 3.6. Temperature profile in the semi-continuous extraction system tests (PLE1, PLE2, PLE3).

3.3.2. *Evaluation of total and individual phenolic content in SWE tests*

Phenolic compounds are the most important bioactive components in olive leaves from the point of view of biological significance and their total contents vary depending on a number of different factors, such as cultivars, extraction methods, etc. [14].

3.3.2.1. *Effect of reactor configuration*

In order to evaluate the obtained results of two SWE systems, test run PLE1 performed in semi-continuous system was compared with the results obtained in discontinuous system due to similar operating conditions. For this purpose, total phenolic contents (TPC) were measured . Figure 3.7 shows the comparison of two different extraction systems in TPC. The results obtained by means of semi-continuous system showed higher TPC values than discontinuous system. This can be the reason for reaching the desired temperature sooner in semi-continuous system. Results are expressed as mg galic acid per gr of dry OL based on previously prepared calibration curve (R^2=0.99).

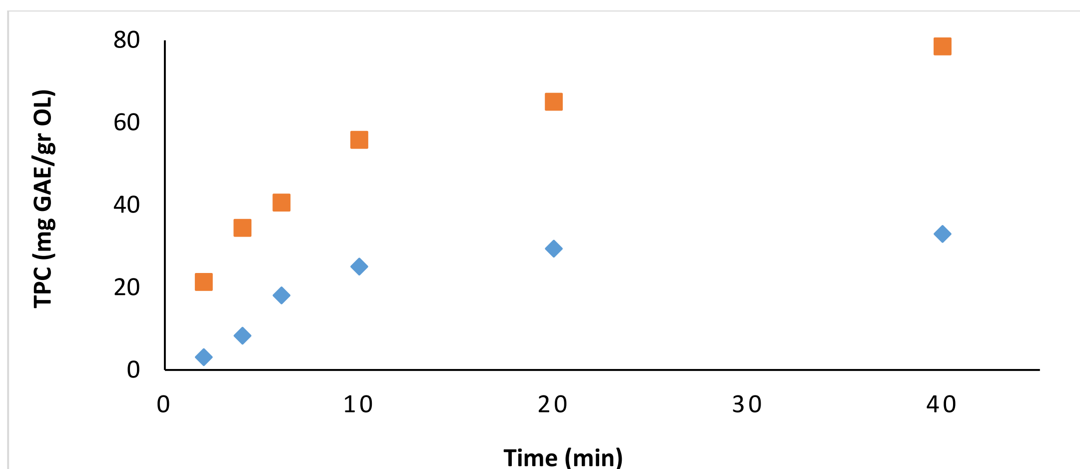


Figure 3.7. Total phenolic content in SWE extraction systems.

Semi-continuous system (■); Discontinuous system (♦)

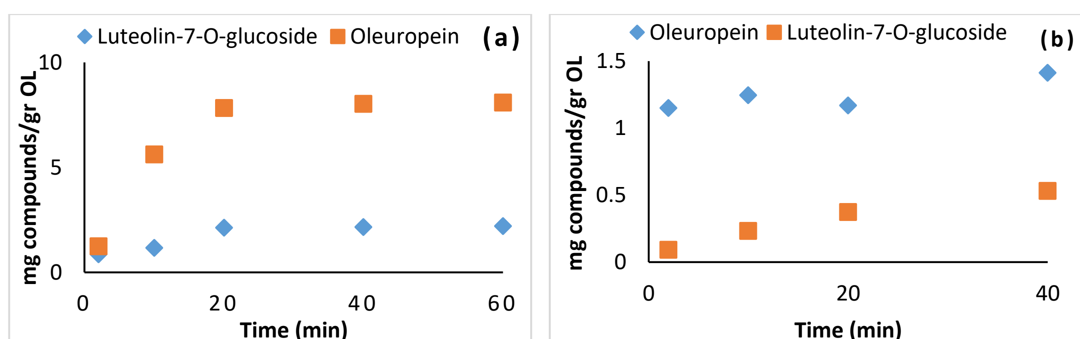


Figure 3.8. Effect of extraction time on content of individual phenolic compounds in SWE systems. **(a)**: Semi-continuous system, **(b)**: Discontinuous system.

Liquid extracts were also injected to HPLC/DAD to identify and quantify the individual phenolic compounds. The compounds determined in the SW extracts were luteolin-7-O-glucoside and oleuropein. These two phenolic compounds are the major and most abundant phenolic compounds in OL. The accumulative profile of these compounds are presented in Figure 3.8 for the semicontinuous and discontinuous configuration. By comparing Figures 3.8a and 3.8b, it can be concluded that that the final value of oleuropein and luteolin-7-O-glucoside in the semi-continuous system, 6000 and 2207 $\mu g/g_{DOL}$, respectively, is considerably higher comparing to the discontinuous system, 1413 and 533. $\mu g/g_{DOL}$, respectively. Figure 3.8a also show that the extraction reaches an equilibrium after about 20 minutes of extraction in the semi-continuous system.

Finer particle sizes reduce the diffusion distance of the compounds in the matrix and increase the contact area between the sample matrix and the extractant, thereby reducing extraction time and increasing extraction efficiency [15]. Particle size is also a key factor in the SWE as it also has an important impact on the extraction process [15,16].

3.3.2.2. Effect of temperature in the semicontinuous reactor

Figure 3.9 shows the effect of temperature on TPC in the semi-continuous extraction runs (PLE1, PLE2, PLE3). For this system, the extractions were carried out for 180 min. PLE2 showed the lowest TPC values which can be the result of the lower operating temperature while PLE1 and PL3 had same operating temperature of 180 °C but with different sample particle size . The slight advantage of PLE1 over PL3 can be attributed to smaller particle size and more surface area of the solid-solvent interaction. PLE 1 presented higher initial extraction rates but also higher final TPC. Based on the literature, it is well known that smaller particle size increases the contact surface area between the solvent and the sample [12]. In general, the extraction yield for small particle sizes is relatively high, while the extraction efficiency for large particle sizes is low and the extraction time needs to be extended [15]. As can be seen in Figure 3.9, PLE3 had a sharp increase in extraction rate until minute 60. Then extraction increasing rate was lowered and reached to an equilibrium after 100 minutes which finally resulted to 97.22 ± 0.02 mg GAE/g_{DOL} of TPC extraction after 180 minutes being the highest value among all test runs.

In addition, M. Herrero *et al.* [18] found that phenolic compounds also differed among the extracts, both quantitatively and qualitatively. Their results stated that by comparing the obtained extracts at 50 and 200 °C, the extracts obtained at 200 °C were more complex due to consisting high variety of individual phenolic compounds. In fact, some compounds present in the extract obtained at 200 °C could not be detected in the extract obtained at 50 °C [18]. This is in accordance with the study performed by M. Plaza *et al.* [9] where increasing extraction temperature, resulted to an enhancement of the total phenols for at least two-fold, and reaching for some samples more than ten-fold

increase. At temperatures above 160 °C, pressurized hot water can degrade cellular walls, built of hemicellulose or lignans, and cleave their covalently bind insoluble phenolic compounds. Meaning, that during the extraction at higher temperatures, hemicellulose can be partially hydrolyzed. Such reactions may contribute to the release of phenolics and other compounds (e.g. reducing sugars) and inflate measurement of total phenols, thus elevating total phenols content in extracts [19]. As previously mentioned, TPC results presented in Figure 3.9 prove the effectiveness of extraction temperature in comparison with sample prticle size.

The comparison of HPLC/DAD quantifications of oleuropein and luteolin-7-O-glucoside for all three PLE runs in semi-continuous system are presented in Figure 3.10. As can be seen in Figure 3.10a, PLE1 showed the optimal oleuropein content with value 8±0.1 and reached the extraction equilibrium after 30 minutes. Regarding luteolin-7-O-glucoside with value 3±0.2, the corresponding data is provided in Figure 3.10b. This Figure shows that PLE1, is again the optimal PLE run in this field where the equilibrium was reached after 40 minutes for all three PLE runs. Based on the obtained results, it can be concluded that higher temperature and lower sample particle size is a priority in order to obtain higher contents of individual phenolic compounds in SWE processes.

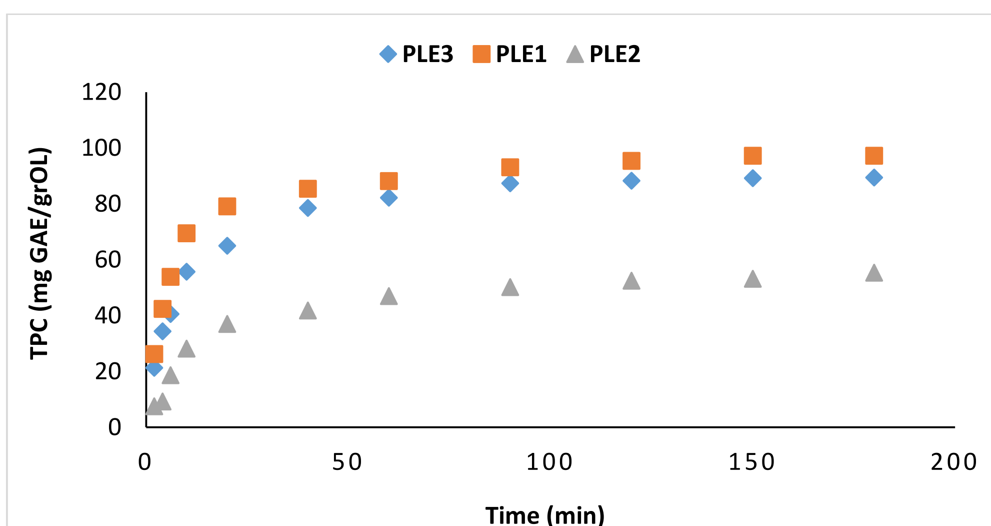


Figure 3.9. Efficiency valuation of PLE test runs in semi-continuous system.

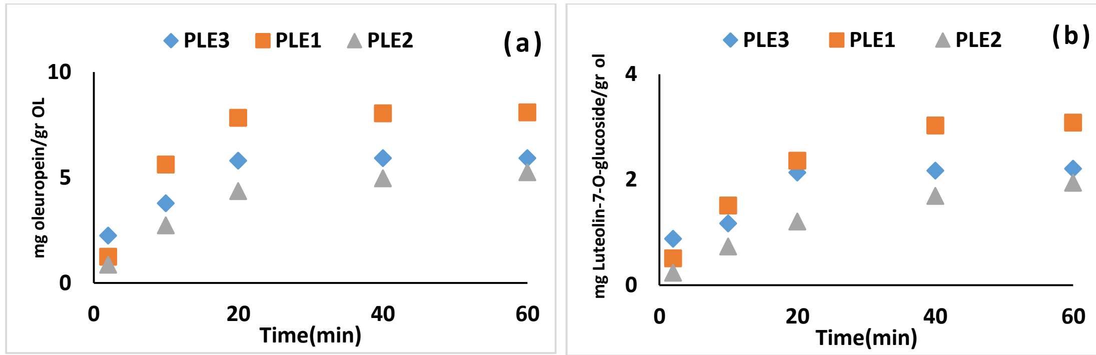
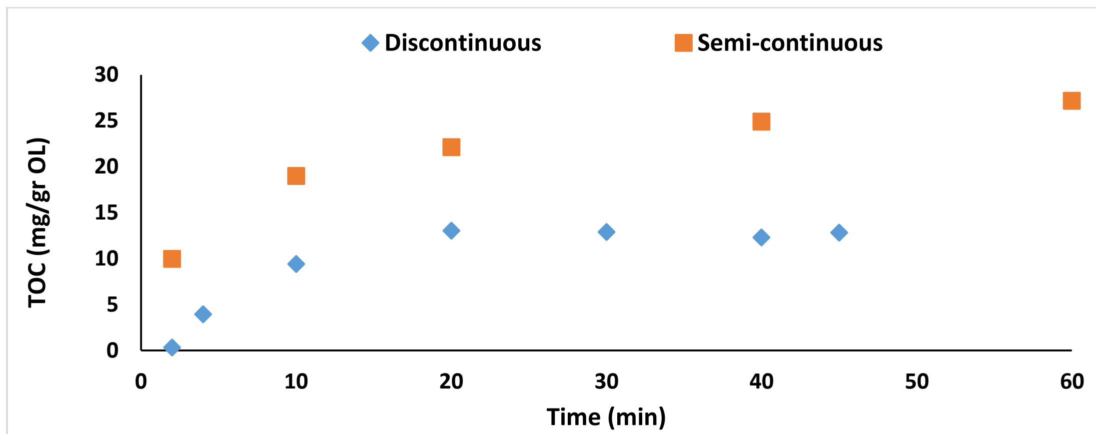
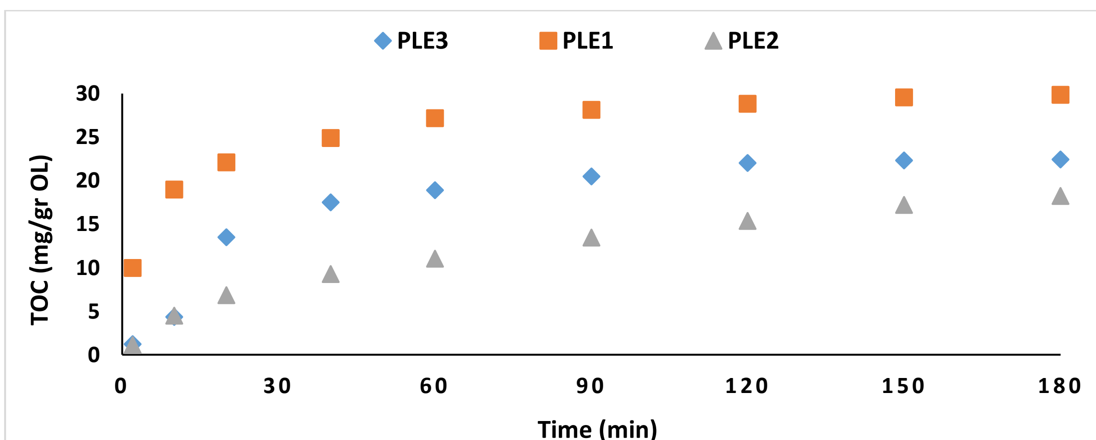


Figure 3.10. Effect of extraction time on content of individual phenolic compounds in semi-continuous system.
(**a**): Oleuropein content; (**b**): Luteolin-7-O-glucoside content



(a)



(b)

Figure 3.11. TOC content of liquid extracts obtained from test runs in SWE processes.

(a): SWE comparison) ; **(b):** Semi-continuous system

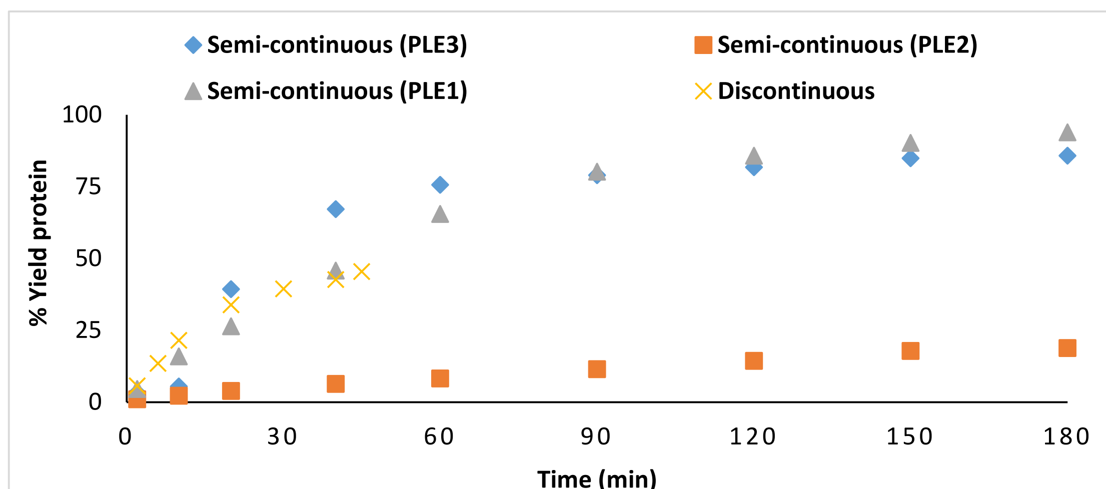


Figure 3.12. Protein yield (%) evaluation in the semi-continuous and discontinuous systems.

3.3.3. Identification and quantification of the amino acid composition

Within the valorization of the protein fraction of the different runs above, in the effluents collected in the treatment with subcritical water, the presence of free amino acids was also determined, which supposes a high added value to the effluents obtained due to the high value in the market for these amino acids, especially when they come from natural sources and have not been obtained through chemical synthesis processes. Amino acids have a central role in all cellular metabolism. Scientists have now discovered more than 500 amino acids in nature, but only 20 participate in DNA translation or human protein synthesis. Aside from their role in making all proteins and all enzymes, amino acids have many other biologically important functions [20]. Amino acids are the essential components of proteins. In living organisms, 20 amino acids make up proteins. There are several hundred more, which perform specialized functions. An amino acid may be essential, which must be directly obtained from food, or nonessential, which can be produced by the body [21]. As can be observed in Table 3.2, the amounts of essential and nonessential amino acids are expressed.

Table 3.2. List of essential and non-essential amino acids [21].

Essential	Nonessential
Valine (hydrophobic)	Glycine (hydrophobic)
Leucine (hydrophobic)	Alanine (hydrophobic)
Isoleucine (hydrophobic)	Serine (hydrophilic)
Phenylalanine (hydrophobic)	Asparagine (hydrophilic)
Tryptophan (hydrophobic)	Glutamine (hydrophilic)
Threonine (hydrophilic)	Tyrosine (charged)
Lysine (charged)	Aspartic acid (charged)
Arginine (charged)	Glutamic acid (charged)
Methionine (special)	Proline (special)
Histidine (special)	Cysteine (special)

The amino acid composition of the OL is presented in Table 3.2. Total of 17 free amino acids were quantified including 9 essential amino acids (Histidine, Leucine, Isoleucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan, Valine) and 8 non-essential amino acids (Alanine, Asparagine, Cysteine, Glutamic Acid, Proline, Serine and Tyrosine, Glycine). It must be highlighted the high content of valine, lysine and glutamic acid with more than 100 mg per g of protein. The obtained results regarding lysine are consistent and in accordance with the results stated in the study of E. H. Ibrahim *et al.* [22] which stated that lysine was the major essential amino acid in olive leaves.

The content of free amino acids has been determined for subcritical water extracts of the discontinuous systems and for PLE1 in the semicontinuous system. The results of the last point of extractions are presented in the Table 3.3. The amounts are expressed as mg of amino acids per grams of protein. Total free amino acids showed optimal value

in semi-continuous system with 91.56 ± 2.55 mg/g, while the value 13.27 ± 0.32 mg/g was obtained by means of discontinuous system.

The highest value of individual amino acids obtained in semi-continuous system was Lysine with 51±1 mg/$g_{protein}$. Lysine is an essential amino acid that is not naturally produced by the body. Many clinical trials have reported that lysine-rich diets and lysine supplements reduce the recurrence, severity, and healing period of herpes simplex virus infections [23].

Table 3.3. Variations of amino acid contents in raw sample and SWE test runs.

	Discontinuous system (mg $_{AA}$/gr $_{protein}$)	**Yield (%)**	**Semi-continuous system- PLE1** (mg $_{AA}$/gr $_{protein}$)	**Yield (%)**	**raw material** (mg $_{AA}$/gr $_{protein}$)
Alanine	1.2±0.1	2.9±0.1	3.3±0.06	7.9±0.2	42±3
Glycine	1.1±0.01	2.9±0.2	0.9±0.07	2.4±0.1	38 ±0.4
Valine	1.3±0.03	1.1±0.1	1.5±0.01	1.3±0.02	120±1
Leucine	0.5±0.01	0.7±0.1	0.7±0.06	0.9±0.01	75±1
Isoleucine	0.8±0.06	0.9±0.1	1±0. 01	1.1±0.1	91±1
Proline	0.4±0.02	1.1±0.1	1.4±0. 05	4±0.3	38±1
Phenylalanine	0.5±0.03	1.2±0.5	0.6±0. 06	1.4±0.1	42±0.5
Threonine	0.8±0.01	1.7±0.2	0	0	48±2
Lysine	0.6±0.07	0.4±0.01	51±1	36±1.2	142±2
Histidine	0.9±0.1	3.2±0.2	7±0.6	25±1.1	28±0.5
Tyrosine	0.3±0.07	2.5±0.1	1.4±0.03	12±0.9	12±0.1
Glutamic acid	1.5±0.01	1.1±0.01	5.1±0.05	4±0.1	132±1
Aspartic acid	1.2±0.01	1.6±0.1	13 ±0. 1	18±1.3	73±1
Methionine	0.5±0.02	7.1±0.2	2.6±0.08	37±1.8	7±1
Serine	1.1±0.01	2.8±0.7	0.9±0.01	2.3±0.1	39. ±2
Tryptophan	0.9±0.01	22.5±0.8	1.1±0.5	28±1.8	4±0.6
Cysteine	0.4±0.01	20±0.9	0.7±0.01	35±1.3	2±0.1
Total free amino acids	14		92.2		

3.3.4. Analysis of total organic carbon and nitrogen in the subcritical water extracts

Total organic carbon (TOC) analysis is specific to organic compounds and theoretically measures all the covalently bonded carbon in water [24]. The analysis of total carbon was performed on the liquid extracts obtained from all test runs, shown respectively in Figure 3.11a (comparison of semicontiuous and discontinuous SWE system) and Figure 3.11b (comparison of semi-continuous PLE runs).

Figure 11.a shows that as the extraction time goes on, the more TOC in subcritical water extracts at the Semi-continuous system while in the discontinuous system, a decrease can be noticed after around 30 minutes of treatment it can be the result of temperature decrease.

According to the Figure 3.11b, the maximum quantified total organic carbon was found in test run PLE1 with the value of 29.9 ±0.9 mg/g_{DOL} after 180 minutes. It can be seen in this figure that the equilibrium was reached after 60-90 min. PLE3 showed 22.43±0.03 mg/g_{DOL} at 180 mi, being the second highest obtained value of TOC. It can be concluded that these two factors have significant effect on total organic carbon extraction. Organic carbon-based compounds provide the materials and energy for subsequent metabolism within the ecosystem [25]. Temperature seems to have the greatest effect on organic carbon extraction.

In addition, the protein content obtained in the SWE was was determined as 6.25×N, and the corresponding results are shown in Figure 3.12. It can be seen observed that a higher equilibrium of solubilized protein fraction was obtained in the semi-continuous system at the highest temperature and the smallest particle size. Although discontinuous system was carried out for only 45 min, it can be observed that already a platform was achieved at this extraction time leading to lower extraction/hydrolysis yield than in the semicontinuous configuration.

The highest yield of protein was achieved at the highest temperature of this study, 180 ˚C. At 180 ˚C a plateau was reached at around 150 min of extraction, while longer times

were needed at 140 ̊C temperature in the semi-continuous system and even in the discontinuous system.3.3.4 Elemental analysis of the subcritical water solid residue

The Elemental Microanalysis (CHNS) analysis was performed on the solid residues Regarding the effect of the sample particle size, the CHN analysis showed that values for the C, H and N are the lowest for the highest temperature and the smallest particle size (PLE1). It should be mentioned that no value for sulfur was identified in the analysis of this study. Regarding PLE1 residue, the hydrogen content reduction should be considered as a result of the optimal bioactive compounds extraction within SWE process. It can be noted that the greater solubilization of the carbon and nitrogen fraction with higher temperature during SWE process will lead to lower N and C content in the solid residue.

Table 3.4. Elemental analysis of dried olive leaves and solid residues after SWE in semi-continuous and discontinuous systems.

SWE process type		N (%)	C (%)	H (%)
Semi-continuous	**PLE1**	0.95±0.13	51.72±0.68	4.88±4.23
	PLE2	2.61±0.03	53.43±0.44	7.56±0.07
	PLE3	1.15±0.6	52.48±1.64	7.65±0.25
Discontinuous		0.95±0.01	60.16±0.03	7.99±0.02
Raw material		1.5±0.09	49±0.66	6.3±0.10

3.3.5. Determination of polysaccharide fraction extraction

Figure 3.13 shows the evaluation of glucose extraction yield for both discontinues and semi-continues systems. A faster glucose extraction was observed for PLE1 in semi-continuous system in comparison with other runs. Within the first 20 minutes of extraction, maximum recovery yield was obtained in both systems and after 30 minutes all extractions reached to an equilibrium. It can be observed that in the discontinuous system a maximum in the glucose recovery was achieved at 20 min, after that glucose content in the discontinuous reaction started to decrease due to glucose degradation as

it is well documented in literature. The high glucose recovery can be attributed to the solubilization of soluble sugars present in the OL and not to cellulose degradation, since cellulose is not hydrolyzed at subcritical water conditions. This can be the reason declared by Mohan et al. [26] that high temperature is needed to hydrolyze cellulose fraction and cellulose does not hydrolyze but dissolves below 250 °C.

Figure 3.14 presents the mannitol extraction curve from olive leaves. In this figure, it can be observed that the equilibrium extraction was achieved at similar working time for type of systems, discontinuous and semicontinuous, aroung 40 minutes. This Figure shows that the modeling extraction yield profile is increasing rapidly in the semi-continuous system until 20 minutes and thereafter It doesn’t change a lot for higher temperature. However, it doesn't show same pattern for the discontinuous system. It goes on until 40 minutes and will be constant then. It shows that hydrolysis in subcritical water is an alternative green process for obtaining this polyol compared to the current process of catalytic hydrogenation of fructose that requires a process of separation of its stereoisomer, sorbitol.

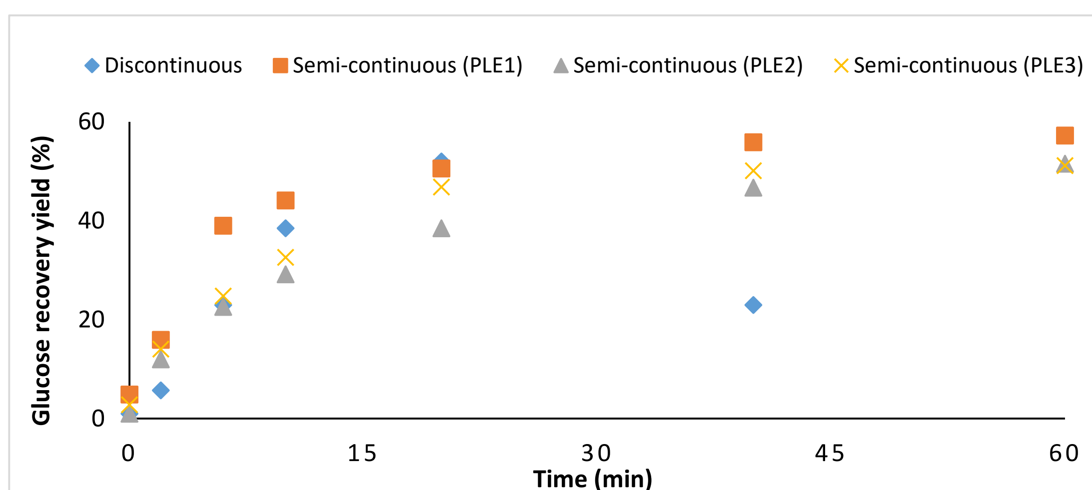


Figure 3.13. Glucose recovery yield evaluation in SWE runs.

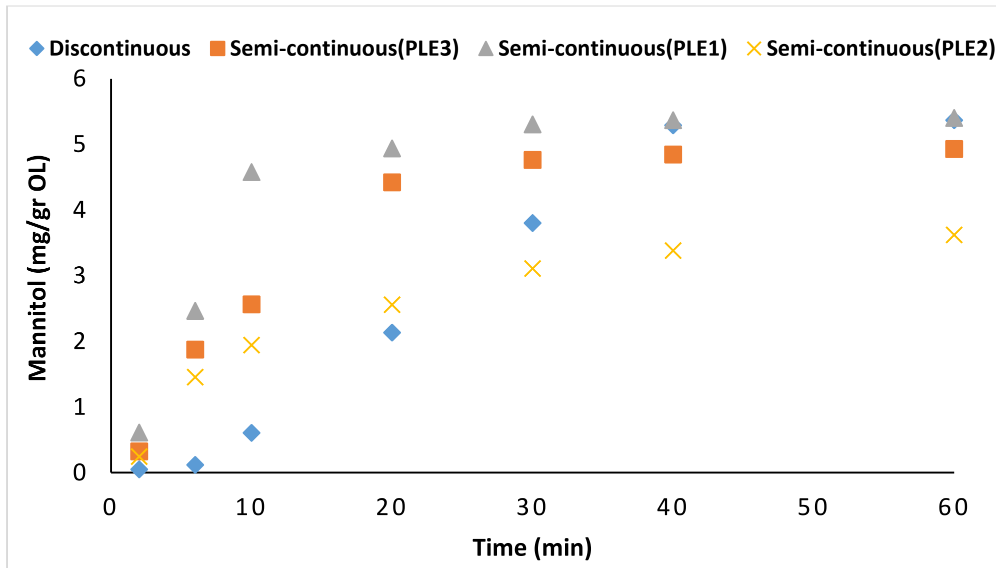
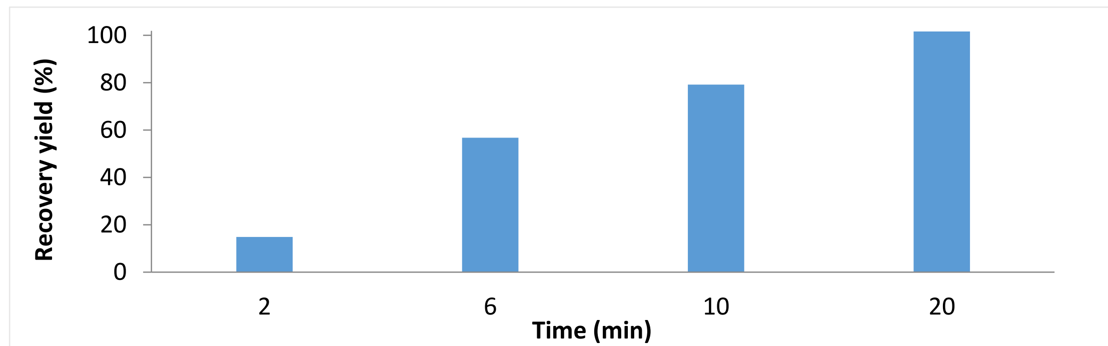
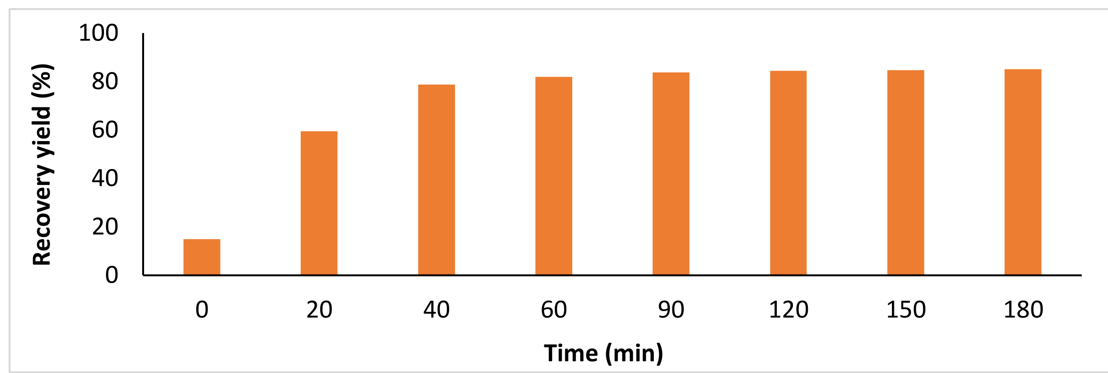


Figure 3.14. Mannitol extraction evaluation in SWE runs.

Figure 3.15(a,b) shows the hydrolysis kinetics of arabinose present in the olive leaves after subcritical water treatment in a discontinuous and a semicontinuous configuration at 180 ºC. Arabinose monomer was not detected in the subcritical water extracts and only after acid hydrolysis, according to NREL protocols, arabinose oligomers were determined in the extracts. At the conditions used in this work, subcritical water produces solubilization of hemicellulose oligomers and not as monomers. After 20 operational minutes in discontinuous system, 100% arabinose recovery yield was achieved while in the semi-continuous system, between 7985% recovery yield was obtained after 40 min of treatment.



(a)



(b)

Figure 3.15. Arabinose yield recovery collected as oligomer fraction in SWE processes. **(a):** Discontinuous system; **(b):** Semi-continuous system

3.4. Conclusions

In this study effectiveness of subcritical water extraction of bioactive compounds from olive leaves by using semi-continuous and discontinuous systems was studied. For this purpose, two temperature levels of 140 and 180 °C and two sample particle sizes (powdered, comminuted) as process variables were investigated. Obtained results indicated that temperature isa key factor in extraction and hydrolysis in subcritical water and higher temperatures and powdered shape of the sample lead to higher process yields specifically enhancement of the total phenols extraction.

Semi-continuous system showed considerably higher results in extraction of TPC and individual phenolic compounds comparing to discontinuous system. In addition, a direct relation was found between extraction temperature and sample particle size increasing with organic carbon extraction rate.. The elemental Microanalysis (CHN) analysis was performed on the solid residues remained after subcritical water treatment and the corresponding results indicated that the greater solubilization of the carbon and nitrogen fraction with higher temperature during SWE processes will lead to lower N and C content in the solid residue. Considering overall aspects studied in this study, it can be concluded the advantage of using semi-continuous over discontinuous system. Furthermore, extraction temperature of 180 °C and powdered particle size of the sample was resulted as optimum operating condition. Whereas, among a total of 17 identified free amino acids in the optimal extraction run, 9 of them were essential amino acids and the rest were non-essential amino acids and lysine was found to be the major essential amino acid in the extracts.

References

[1] R. T. P. Correia and M. I. Genovese, “Bioactive compounds and phenolic-linked functionality of powdered tropical fruit residues,” *Food Sci Technol Int.*, 18 (6), 539–547, 2012.

[2] N. Siriwardhana, N. S. Kalupahana, M. Cekanova, M. Lemieux, B. Greer, and N. Moustaid-moussa, “Modulation of adipose tissue inflammation by bioactive food compounds,” *J. Nutr. Biochem.*, vol. 24, no. 4, pp. 613–623, 2013.

[3] P. Putnik, F. J. Barba, I. Spani, and V. Dragovi, “Food and Bioproducts Processing Green extraction approach for the recovery of polyphenols from Croatian olive leaves (Olea europea) ˇ cevi ´,” vol. 6, pp. 19–28, 2017.

[4] N. Xynos, G. Papaefstathiou, M. Psychis, A. Argyropoulou, N. Aligiannis, and A. Skaltsounis, “The Journal of Supercritical Fluids Development of a green extraction procedure with super / subcritical fluids to produce extracts enriched in oleuropein from olive leaves,” *J. Supercrit. Fluids*, vol. 67, pp. 89–93, 2012.

[5] D. Wianowska and M. Gil, “Trends in Analytical Chemistry Critical approach to PLE technique application in the analysis of secondary metabolites in plants,” *Trends Anal. Chem.*, vol. 114, pp. 314–325, 2019.

[6] M. T. Munir, H. Kheirkhah, S. Baroutian, S. Young, and B. R. Young, “Subcritical water extraction of bioactive compounds from waste onion skin,” *J. Clean. Prod.*, vol. 183, pp. 487–494, 2018.

[7] C. Cheigh, E. Chung, and M. Chung, “Enhanced extraction of flavanones hesperidin and narirutin from Citrus unshiu peel using subcritical water,” *J. Food Eng.*, vol. 110, no. 3, pp. 472–477, 2012.

[8] R. Todd and S. Baroutian, “A techno-economic comparison of subcritical water , supercritical CO 2 and organic solvent extraction of bioactives from grape marc International Panel on Climate Change Internal Rate of Return,” *J. Clean. Prod.*, vol. 158, pp. 349–358, 2017.

[9] M. Plaza, M. Amigo-benavent, D. María, E. Ibáñez, and M. Herrero, “Facts about the formation of new antioxidants in natural samples after subcritical water extraction,” *FRIN*, vol. 43, no. 10, pp. 2341–2348, 2010.

[10] A. M. Baseggio, "The Journal of Supercritical Fluids Subcritical water extraction of fl avanones from defatted orange peel," *J. Supercrit. Fluids*, vol. 138, no. January, pp. 7–16, 2018.

[11] C. Chye, S. Ngin, J. Wan, H. Yong, C. Sin, and E. Shi, "Pressurized hot water extraction (PHWE)," *J. Chromatogr. A*, vol. 1217, no. 16, pp. 2484–2494, 2010.

[12] S. O. Essien, B. Young, and S. Baroutian, "Trends in Food Science & Technology Recent advances in subcritical water and supercritical carbon dioxide extraction of bioactive compounds from plant materials," *Trends Food Sci. Technol.*, vol. 97, no. February 2019, pp. 156–169, 2020.

[13] M. Plaza and C. Turner, "Pressurized hot water extraction of bioactives," *TrAC - Trends Anal. Chem.*, vol. 71, pp. 39–54, 2015.

[14] A. Lama-Muñoz, M. del M. Contreras, F. Espínola, M. Moya, I. Romero, and E. Castro, "Content of phenolic compounds and mannitol in olive leaves extracts from six Spanish cultivars: Extraction with the Soxhlet method and pressurized liquids," *Food Chem.*, vol. 320, no. September 2019, p. 126626, 2020.

[15] W. Jintana and A. Shuji, "Extraction of Functional Substances from Agricultural Products or By-products by Subcritical Water Treatment," vol. 14, no. 4, pp. 319–328, 2008.

[16] M. H. Eikani, "Subcritical water extraction of essential oils from coriander seeds (Coriandrum sativum L .)," *J. Food Eng.*, vol. 80, pp. 735–740, 2007.

[17] M. KHAJENOORI, A. H. ASL, F. HORMOZI, M. H. EIKANI, and H. N. BIDGOLI, "SUBCRITICAL WATER EXTRACTION OF ESSENTIAL OILS FROM ZATARIA MULTIFLORA BOISS," *J. Food Process Eng.*, vol. 32, no. 6, pp. 804–816, Dec. 2009.

[18] M. Herrero, M. Castro-puyana, L. Rocamora-reverte, J. A. Ferragut, A. Cifuentes, and E. Ibáñez, "Formation and relevance of 5-hydroxymethylfurfural in bioactive subcritical water extracts from olive leaves," *FRIN*, vol. 47, no. 1, pp. 31–37, 2012.

[19] J. Pérez-Jiménez and J. L. Torres, "Analysis of Nonextractable Phenolic Compounds in Foods: The Current State of the Art," *J. Agric. Food Chem.*, vol. 59, no. 24, pp. 12713–12724, Dec. 2011.

[20] L. Cole and P. R. Kramer, "Chapter 1.4 - Amino Acid Metabolism," L. Cole and P. R.

B. T.-H. P. Kramer Biochemistry and Basic Medicine, Eds. Boston: Academic Press, 2016, pp. 31–38.

[21] J. Moini, "Chapter 5 - Epidemiology of Diet and Diabetes Mellitus," J. B. T.-E. of D. Moini, Ed. Elsevier, 2019, pp. 57–73.

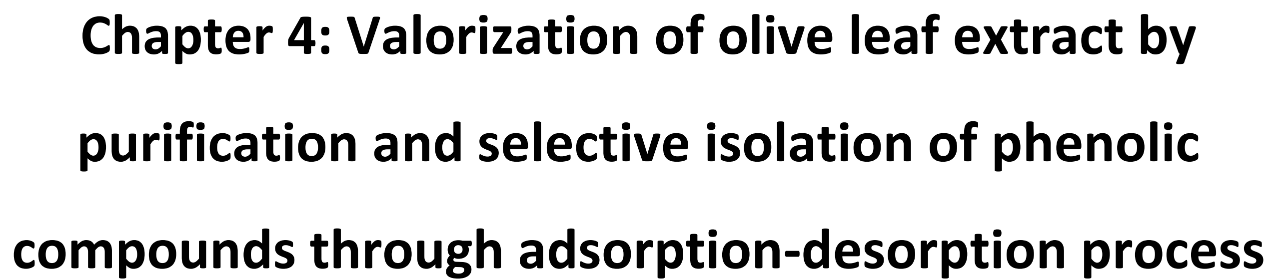
[22] E. H. Ibrahim, M. A. Abdelgaleel, and A. A. Salama, "CHEMICAL AND NUTRITIONAL EVALUATION OF OLIVE LEAVES AND SELECTION THE OPTIMUM CONDITIONS," vol. 42, no. 1, pp. 445–459, 2016.

[23] M. Rossi and B. Jacobs, "Chapter 20 - Herpes Simplex Virus," D. B. T.-I. M. (Fourth E. Rakel, Ed. Elsevier, 2018, pp. 191-197.e2.

[24] D. E. Albert, "Chapter 4 - Methods for Verifying Medical Device Cleanliness," R. Kohli and K. L. B. T.-D. in S. C. and C. Mittal, Eds. Oxford: William Andrew Publishing, 2015, pp. 109–128.

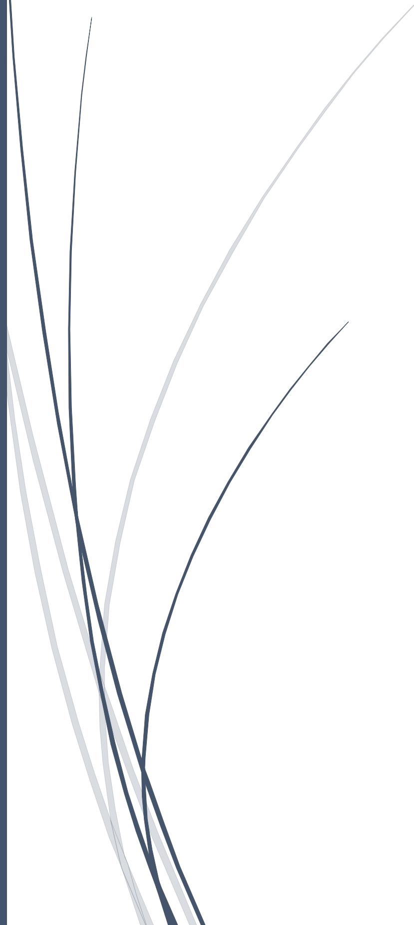
[25] A. C. Dioxide, "The inorganic carbon complex," 1991, doi: 10.1016/B978-0-08-057439-4.50015-0.

[26] M. Mohan, R. Timung, N. N. Deshavath, T. Banerjee, V. V Goud, and V. V Dasu, "RSC Advances," *RSC Adv.*, vol. 5, no. November, pp. 103265–103275, 2015.



Chapter 4: Valorization of olive leaf extract by purification and selective isolation of phenolic compounds through adsorption-desorption process

Valorization of plant biomass by emerging technologies: olive and moringa leaves



Olive leaves are lignocellulosic residues rich in bioactive compounds. They are considered as an olive by-product, and also an excellent source of phenolic compounds. Oleuropein is the main constituent of olive leaf and has been proved to have so many therapeutical properties.

The main objective of the current study was the purification and the selective isolation of phenolic compounds available in olive leaf extract (OLE) from an 80 % ethanolic aqueous solution by implementing adsorption-desorption process. Test runs were performed by using XAD4, XAD16, and XAD7HP polymeric resins each with 5% and 10% (w/v) contents. Three different solvents were used in the desorption stage, 80 % ethanol aqueous solvent at its pH and pH =2 and pure ethanol at pH=2. The analysis of total phenolic content, individual phenolic compounds (oleuropein, luteolin 7-O-glucoside, and catechin) and sugar content (glucose, xylose, mannitol, and arabinose) was performed for all test runs. XAD7HP with 5% (w/v) content showed optimal oleuropein adsorption/desorption ratio of 99.32% by using ethanol (100%) with pH=2 as desorption solvent. Comparison of oleuropein content in the initial OLE with the optimal experimental run indicated 6 fold increase. Furthermore, the content of glucose and mannitol which are the most abundant sugars in the olive leaf extracts was decreased by 25.72% and 36.40%, respectively. Oleuropein/glucose ratio of the optimal test run showed more than 8 fold increase in comparison with initial OLE meaning that selective isolation of oleuropein was achieved.

Las hojas de olivo son residuos lignocelulósicos ricos de la industria del olivo ricas en compouestos antioxidantes tales como compuestos fenólicos. Entre ellos, la oleuropeína es el componente fenólico principal de la hoja de olivo y se ha demostrado que tiene numerosas propiedades terapéuticas.

El objetivo principal del presente estudio fue la purificación y aislamiento selectivo de los compuestos fenólicos presentes en el extracto de hoja de olivo (OLE), obtenido con una mezcla hidroalcohólica al 80% como disolvente,mediante la implementación de un proceso de adsorción-desorción. Las pruebas se realizaron utilizando diferentes resinas poliméricas XAD4, XAD16 y XAD7HP, a dos niveles diferentes de concentración de 5% y 10% (p/v). Se utilizaron tres disolventes diferentes en la etapa de desorción: etanol al 80 %, etanol al 80 % a pH = 2 y etanol puro a pH = 2. El análisis de los compuestos fenólicos totales, compuestos fenólicos individuales (oleuropeína, luteolina 7-O-glucósido y catequina) y el contenido de azúcares (glucosa, xilosa, manitol y arabinosa) se realizó para todas las pruebas. La resina XAD7HP a una concentración del 5% (p/v) mostró una relación óptima de adsorción/desorción de oleuropeína del 99,32% empleando etanol (100%) a pH = 2 como disolvente de desorción. La comparación del contenido de oleuropeína en el extracto inicial con el contenido en el óptimo indicó un aumento de hasta 6 veces en la concentración de oleuropeina. Además, el contenido de glucosa y manitol, que son los azúcares más abundantes en la hoja de olivo, se redujo en un 25,72% y un 36,40%, respectivamente. La relación oleuropeína/glucosa de la prueba óptima mostró un aumento de más de 8 veces en comparación con la encontrada en el extracto original-, lo que significa que una buena separación y concentración selectiva de la oleuropeína en los extractos obtenidos.

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4.1. Introduction

Olive leaves are lignocellulosic residues mainly found in two different points during the process of olive oil production. They are fist found during olive tree pruning (OTP), in which they constitute twenty-five percent of the pruning, but are usually burned or ground together with the remainder of the OTP by-products. The second point is in the olive oil production facility, where leaves are separated from the fruits using a blower machine in the early steps of olive cleaning. Kashaninejad *et al.* [1] carried out a detailed chemical composition determination of olive leaves. These authors also performed a comparison with the chemical composition reported by other authors. In a dry basis, the content of cellulose ranged between 21.6 and 9.3 %, while hemicellulose content was found to vary from 9.5 to 14.5 %. As correspond to a lignocelullosic biomass, acid-insoluble lignin was found to vary between 10.8 and 15.4 %. As it has been also determined for other green leaves, the amount of extractive is quite high ranging from 25 to 45 %. In the studies reported by Kashaninejad *et al.* [1] water and ethanol extractive fractions contained important amounts of total phenolic compounds. Phenolic compounds are responsible for structural and protective functions in plants, contributing to flavor, color, astringency and bitterness of fruits and vegetables. Their role in human health related issues has been acknowledged, fostering an increased demand to produce 'functional' or 'nutraceutical' foods. Some applications proposed for natural polyphenolic compounds are based on their antioxidant activity against reactive species involved in aging and in chronic, autoimmune, inflammatory, coronary and degenerative diseases [4–6]. Their antioxidant properties may explain a part of the potential cancer chemopreventive properties [5], although the antioxidant activity alone is not sufficient to explain their whole set of biological properties [8–11].

Many of the bioactive compounds in olive oils and table olives that provide healthy nutritional benefits, such as phenolic compounds or polyphenols, are also present in olive leaves. Therefore, olive leaves not only are considered as a by-product, but also an excellent source of such compounds. Phenolic composition of olive leaves includes secoiridoids and flavonoids such as oleuropein, verbascoside, luteolin-7-O-glucoside, apigenin-7-O-glucoside and quercetin [10], whose quantities and concentration levels convert them into the major biophenols identified in olive leaves [11]. All of them have

remarkable biological properties [14,15] and their extraction and purification from olive leaves is an excellent option for the exploitation of this waste biomass [14]. Besides phenolic compounds, olive leaves contain other bioactive substances such as sugar alcohols or polyols that contain multiple hydroxyl groups in their structure. Polyols have a large number of commercial applications as building blocks, intermediates in organic synthesis or precursors for production of polyurethanes [17,18].

Oleuropein is the main phenolic compound of olive tree (*Olea europaea*) leaf. It has been proved to have so many therapeutical properties such as antioxidative, antimicrobial, antiviral, antiatherogenic, cardioprotective, antihypertensive, and anti-inflammatory effects [17]. Hence, it is a great value of separating these natural materials rich in biophenols and using them as additives in food, pharmaceutic, and cosmetic industries [18].

Adsorption is an efficient technology for the selective removal, recovery and separation of some compounds that requires a relatively low economic investment [19]. It is one of the most commonly applied processes for the recovery of polyphenols from plant extracts that is gaining increasing importance in food industry. Adsorption is attractive for its relative simplicity of design, operation and scale-up, but its major drawbacks include limited selectivity, the need to regenerate the adsorbent for reuse, and loss of capacity after several cycles [20]. In recent years, several studies dealt with the uptake of polyphenols and other bioactive compounds from oil mill wastes on different adsorbents [23–27].

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 [26]. 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 [29,30]. Ionic strength and pH are key factors influencing the adsorption of phenolic compounds. At acidic pH, the uptake of phenolic compounds by different adsorbents is enhanced because phenols are non-dissociated and the dispersion interactions predominate [11], [31–35].

The main objective of the current study was the purification and selective isolation of phenolic compounds available in olive leaf extract (OLE) by implementing of adsorption-desorption process. Primarily, OLE was prepared by means of conventional solvent extraction methods and then the obtained extract was vacuum concentrated to remove the ethanol. The adsorption-desorption runs were performed by using three polymeric resins each at two different concentrations and three solvents in the desorption stage. The analysis of phenolic content (total phenolic content and individual phenolic compounds such as oleuropein, luteolin 7-O-glucoside, and catechin) and sugar content (glucose, xylose, mannitol, and arabinose) was performed for olive leaf extract, adsorbate and desorbed samples of all test runs. Selection of the optimal process was performed in terms of the highest total phenolic compounds recovery and sugar rejection in order to achieve a final effluent rich in phenolic compounds minimizing the presence of other co-extracted compounds.

4.2. Materials and methods

4.2.1. Sample preparation

Olive leaf (OL) samples were collected from the pruning of olive trees of the variety ¨*Serrana de Espadán*¨, provided by Maicas-Sediles (Teresa, Castellón, Spain). OL samples were dried in a convection oven at 45°C to reduce the humidity to values lower than 10%. This initial drying step by hot-air has resulted in higher oleuropein recovery in previous studies reported in the literature [34]. After drying, olive leaves were grounded by using a cutting mill (Retsch SM100, Retsch Technology GmbH, Germany) to obtain a particle size in the range from 0.5 to 1 mm to increase the interfacial surface and improve extraction. Samples were stored in the freezer at -18 °C for further use.

4.2.2. Chemicals used

All chemicals used in this study were of analytical grade and provided by VWR International Eurolab (Llinars del Vallès, Spain) and Sigma-Aldrich (Darmstadt, Germany).

The chemicals used for each analysis were as follows:

- Folin–Ciocalteu reagent, gallic acid and sodium carbonate were used for total phenolic content (TPC) measurement.
- Acetic acid (CH_3COOH), acetonitrile (C_2H_3N), ammonium acetate (NH_4Ac), oleuropein, luteolin-7-O-glucoside, and catechin were used as standards or solvent in HPLC/DAD for identification and quantification of individual phenolic compounds.
- Sulfuric acid (H_2SO_4), xylose, arabinose, glucose, and mannitol were used as mobile phase or standard solutions in HPLC-RID for identification and quantification of soluble carbohydrates.
- Amberlite® XAD4, XAD16 polymeric resins were provided from Fluka (Germany), and XAD7HP resin was purchased from Sigma-Aldrich (Darmstadt, Germany). These resins have been reported to yield satisfactory adsorption results for phenolic compounds in previous works [22,26,37–39]. Characteristics of all polymeric resins are shown in Table 4.1.

Table 4.1. Physical characteristics of the polymeric resins.

Polymeric resins	Dry density (g/mL)	Mesh	Mean pore size (Å)	Particle diameter (mm)	Specific surface (m^2/g)	Matrix
XAD-4	1.02	20–60	40	0.25–0.84	725	Styrene-divinylbenzene
XAD-16	1.02	20–60	200	0.25–0.84	900	Styrene-divinylbenzen
XAD-7HP	1.05	20–60	90	0.43–0.69	450	Acrylic ester

4.2.3. Conventional solvent extraction

Olive leaf extract (OLE) was prepared by mixing the dried and grinded olive leaves with 80% ethanol hydroalcoholic mixture at a ratio of 20 mL per gram of dried olive leaf (DOL). Extraction was performed in an orbital shaker (Grant instruments-OLS 200, Shepreth Cambridgeshire, England) for 1 h at 50 °C. The mixture was then centrifuged

(Eppendorf centrifuge 5804, Germany) at 4500 rpm for 15 min to separate the liquid extract from the solid residue. The mentioned solvent extraction conditions were proved to be optimal in a previous study of the authors of the current work [1].

4.2.4. Resin pretreatment process

The polymeric resins (XAD4, XAD16 and XAD7HP) were pretreated using the following procedure according to other studies in literature [20,24,37,38]. Resins were first washed with distilled water and after getting vacuum filtered, they were dried at room temperature. Subsequently, resins were rinsed in ethanol with a ratio of 5 $mL_{ethanol}/g_{resin}$ at 150 rpm for 2 h and then left to soak in for 24 h. This step ensured the removal of impurities that could be trapped in the resins. After removal of ethanol by vacuum filtration, resins were dried at room temperature and then milli-Q water was used to wash the resins thrice. Finally, pretreated resins were dried in an oven at 50 °C to constant weight.

4.2.5. Adsorption and desorption processes

Firstly, 1 L of OLE was prepared under optimal conditions previously mentioned in section 4.2.3. The ethanol present in the liquid extract was evaporated by using a Heidolph Laborota 4001 rotary evaporator system (Heidolph, Schwabach, Germany) at 30 °C and 100 mmHg (0.13 bar) for 3 h. The concentrated extract was then diluted to 1 L with distilled water in order to facilitate the adsorption efficiency. Subsequently, the diluted OLE was stored at 4 °C before use in adsorption tests.

In the adsorption experiments, 1.5 and 3 g of pretreated polymeric resins (XAD4, XAD16 and XAD7HP) were mixed with 30 mL of dealcoholized and diluted OLE in 50 mL flasks (20 and 10 mL/g_{resin}) resulting to total of six adsorption runs. All the flasks were shaken in magnetic stirrers at a shaking speed of 150 rpm for 3 h at room temperature, time enough to reach adsorption equilibrium. After adsorption runs reached equilibrium, resins were separated by vacuum filtration and adsorbates were analyzed.

After each run, the adsorbents used were vacuum filtered and chemical analysis was performed in the filtrates. Chemical analysis included phenolic content (total phenolic compounds and individual phenolic compounds oleuropein, luteolin 7-O-glucoside, and catechin) and sugar content (glucose, xylose, mannitol, and arabinose).
Adsorption results were quantified by using the following mass balance according to Eq.(1) [42,43]:

Adsorption ratio: $$A = \frac{(C_0 - C_e)}{C_0} \times 100 \quad (1)$$

where A is the adsorption ratio (%), C_0 and C_e refer to the dealcoholized and diluted OLE and the equilibrium concentrations ($mg_{\text{phenols or surgars}}/mL$), respectively.

In the desorption process, filtered resins from each test run was divided in three equal parts and each part was mixed with a different desorption solvent with the ratio of 8 $mL_{\text{solvent}}/g_{\text{filtered resin}}$ making a total of eighteen desorption runs. Desorption solvents were as follows: ethanol 80% (v/v) with natural pH (solvent A), ethanol 80% (v/v) with pH=2 (solvent B), and ethanol 100% with pH=2 (solvent C), since ethanol has been repeatedly used as desorption solvent in bibliography [26,27,39,42,44]. After mixing filtered resins with desorption solvents, they were mixed in a magnetic stirrer at 150 rpm for 2 h.
After each desorption run, resins were vacuum filtered and the different biocompounds were determined in the liquid effluents. Desorption capacity and total recovery were evaluated using the following Eqs. (2), (3) [39,40]:

Desorption ratio: $$D = \frac{C_D V_D}{(C_0 - C_e) V_0} \times 100 \quad (2)$$

Total recovery: $$R = \frac{C_D V_D}{C_0 V_0} \times 100 \quad (3)$$

where D is the desorption ratio (%), C_D is the concentration of the solute in the filtrate after desorption (mg/mL) and V_D is the volume of this filtrate (mL). V_0 is the volume

of the OLE (mL) and C_o and C_e were defined in equation 1.. R is the total recovery (%), which shows the ratio of final contents of the adsorbates after desorption in the liquid effluents compared to their initial content in dealcoholized and diluted OLE.

4.2.6. Statistical Analysis

All values were expressed as mean ± standard deviation of at least three replicates. The significance of the differences was determined based on an analysis of the variance with the Fisher's Least Significant Difference (LSD) method at p-value ≤ 0.05. The software Statgraphics X64 was used.

4.3. Results and discussion

Characterization of pretreated olive leaf extract before adsorption stage was primarily performed which the corresponding results are shown in Table 4.2. It must be highlighted the high content in total phenolic compounds, especially oleuropein, with important amounts of soluble sugar such a mannitol and glucose. Three polymeric resins, XAD4, XAD16 and XAD7HP were tested in this study which have been used extensively in the bibliography [22,26,37–39]. Two content levels of 5% and 10 % (w/v) was used for each resin, resulting into total of six adsorption series. As mentioned earlier, three different solvents by using ethanol-water solution were tested in desorption stage (solvents A, B and C). The most important advantage of using ethanol as desorption solvent is its high volatility and ease of use in energy efficient vacuum evaporators. Furthermore, the final concentration of valuable phenolic compounds will be significantly increased with economic advantages of paramount importance at industrial scale [42].

By considering six adsorption series and three desorption solvents, a total number of eighteen desorption runs were implemented. Evaluation of adsorption-desorption efficiency was performed by measuring total phenols, and three individual phenolic compounds, catechin, luteolin-7-O-glucoside and oleuropein. In addition, individual sugar content (glucose, xylose, mannitol, arabinose) for all test runs were quantified in order to obtain a better perspective of phenolic compounds purification.

Table 4.2. Characterization of pretreated olive leaf extract before adsorption.

Compounds		Concentration	
		mg/L	mg/g
Phenolic content	Total phenolic content	2136 ± 23	42.7 ± 0.5
	Oleuropein	1839 ± 17	36.8 ± 0.4
	Catechin	30 ± 3	0.59 ± 0.05
	Luteolin-7-O- glucoside	226 ± 3	4.52 ± 0.05
Sugar content	Glucose	577 ± 6	11.6 ± 0.1
	Xylose	185 ± 2	3.69 ± 0.04
	Mannitol	1774 ± 5	35.5 ± 0.1
	Arabinose	44.62 ± 0.05	0.890 ± 0.001

Table 4.3. Phenolic and sugar content of the adsorbates in the liquid effluents after adsorption process.

Compounds		Types and content of polymeric resins (mg/L)					
		XAD4		XAD16		XAD7HP	
		5 % (w/v)	10 % (w/v)	5 % (w/v)	10 % (w/v)	5 % (w/v)	10 % (w/v)
Phenolic content	Total phenols	303 ± 3[d]	222 ± 2[b]	339 ± 1[e]	261 ± 3[d]	236 ± 1[c]	176 ± 2[a]
	Oleuropein	72 ± 2[d]	16.40 ± 0.04[b]	98.2 ± 0.5[e]	37.9 ± 0.2[c]	48.2 ± 0.3[d]	11.8 ± 0.2[a]
	Catechin	14.5 ± 0.2[c]	1.3 ± 0.2[b]	22.2 ± 0.1[d]	0.5 ± 0.1[a]	0.73 ± 0.06[a]	1.45 ± 0.04[b]
	Luteolin-7-O-glucoside	14.5 ± 0.1[e]	3.84 ± 0.01[c]	4.8 ± 0.4[d]	0.7 ± 0.1[b]	0.13 ± 0.09[a]	--
Sugar content	Glucose	480 ± 4[a]	474 ± 2[a]	497 ± 9[b]	478 ± 4[a]	475 ± 8[a]	480 ± 2[a]
	Xylose	140 ± 2[b]	129 ± 4[a]	158 ± 4[c,d]	162.7 ± 0.6[d]	154 ± 1[c]	163.4 ± 0.5[e]
	Mannitol	1565 ± 1[b]	1510 ± 2[a]	1513 ± 16[a]	1522 ± 5[a]	1515 ± 32[a]	1563 ± 3[a]
	Arabinose	18 ± 3[a]	22.3 ± 0.9[b]	29 ± 1[d]	28.2 ± 0.5[c,d]	26.7 ± 0.2[c,d]	25 ± 1[b,c]

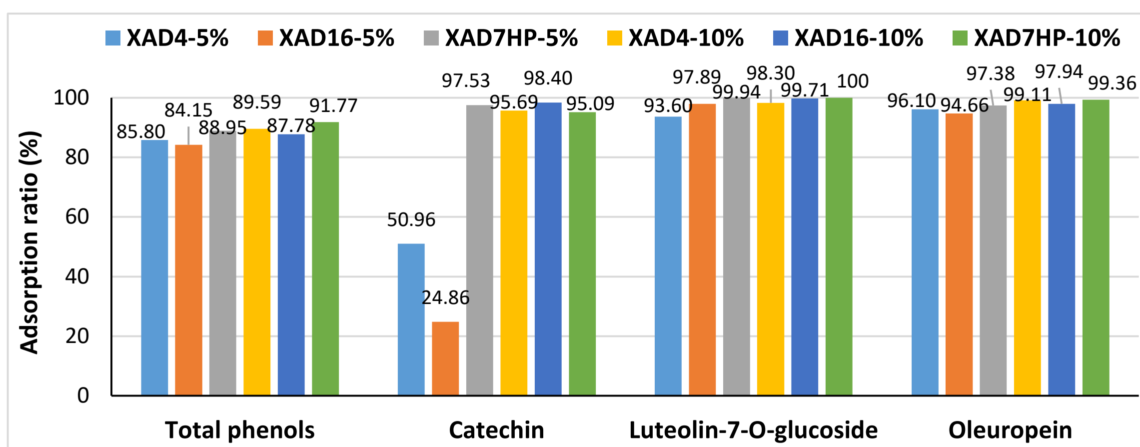
Values with different letters in each raw are significantly different when applying the Fisher's least significant differences (LSD) method at p-value ≤ 0.05

4.3.1. Efficiency of polymeric resins in adsorption process

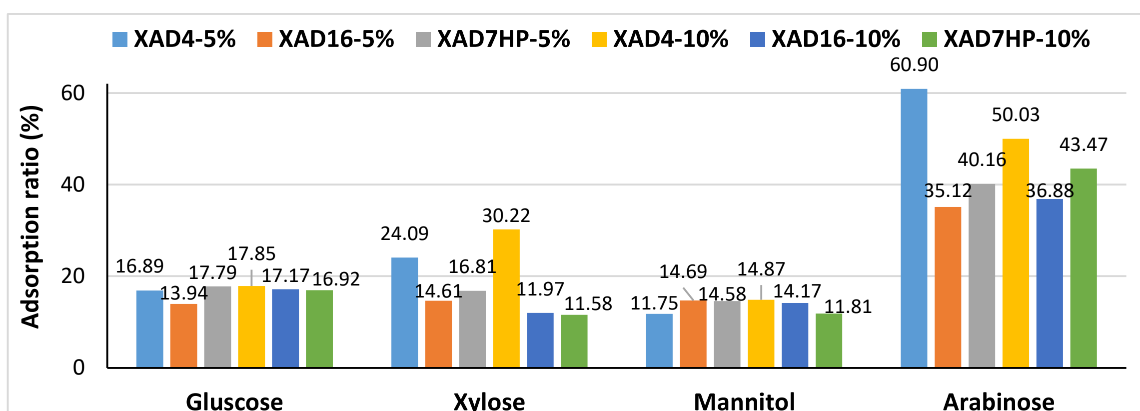
Mean values of quantified phenolic and sugar compounds in the liquid effluents after adsorption, are shown in Table 4.3. In addition, adsorption ratios calculated based on Eq. (1), are depicted in Figure 4.1 (a,b). As it can be seen from Figure 4.1a, considerably high adsorption ratios were obtained for TPC, and for the individual phenolic compounds determined in this work, mainly oleuropein and luteolin-7-O-glucoside. Somehow, all obtained ratios are over 93% which is completely satisfactory considering that the main focus in this study was selective isolation of oleuropein. This is in accordance with the study performed by Fava et al. [41] for the recovery up to 80% of polyphenols from Olive mill wastewater. Regarding adsorption of catechin, XAD4 showed considerably poor results regardless of its content in comparison with other resins. Obtained results of total phenols adsorption showed more than 80% adsorption ratio for all test runs. However, XAD7HP with 10% (w/v) content showed optimal result with 91.77% adsorption ratio. Successful adsorption results for polymeric resins have been published in several studies in the bibliography [37–39].

Results of sugars adsorption ratios are presented in Figure 4.1b. As it can be observed from Table 4.2, mannitol was the most abundant sugar compound measured in the olive leaf extract. Adsorption ratios for all the soluble sugars determined in this work were considerably lower than the adsorption ratios observed for total and individual phenolic compounds. This result showed a high selectivity of the resins for the phenolic compounds that agrees with the overall objective of the adsorption process in this study that was the purification of desired phenols from other coextracted compounds in the OLE such as sugar compounds. The obtained adsorption ratios of mannitol were all below 15%, regardless of resin type or content. This means that the objective of selective isolation of phenolic compounds was performed in a satisfactory level.

Furthermore, adsorption ratios for glucose and xylose were in the range of 13-18% and 11-30%, respectively (Table 4.3). On the other hand, arabinose showed the highest adsorption ratios in sugars with 35-61% range. Nevertheless, since arabinose had the lowest concentration in sugar profile of the olive leaf extract, its high adsorption ratios did not have significant negative effect on the selective isolation of phenolic compounds.



a) Phenolic compounds



b) Sugar compounds

Figure 4.1. Adsorption ratios of phenolic and sugar compounds.

4.3.2. Evaluation of different solvents performance in desorption process

Desorption test runs were performed in equal condition by using previously described solvent types (A = ethanol aqueous mixture 80 % (v/v) at normal pH, B = ethanol aqueous mixture 80 % (v/v) at normal pH =2 and C = pure ethanol at normal pH). The reason for using pH adjusted desorption solvents was due to previously findings which indicated that at acidic pH, the uptake of phenols by different adsorbents is enhanced because phenols are undissociated and dispersion interactions predominate [11,22]. Mean values of analyzed phenolic and sugar compounds in filtrates after desorption stage, are shown in Table 4.4. In addition, the obtained results of desorption ratios are

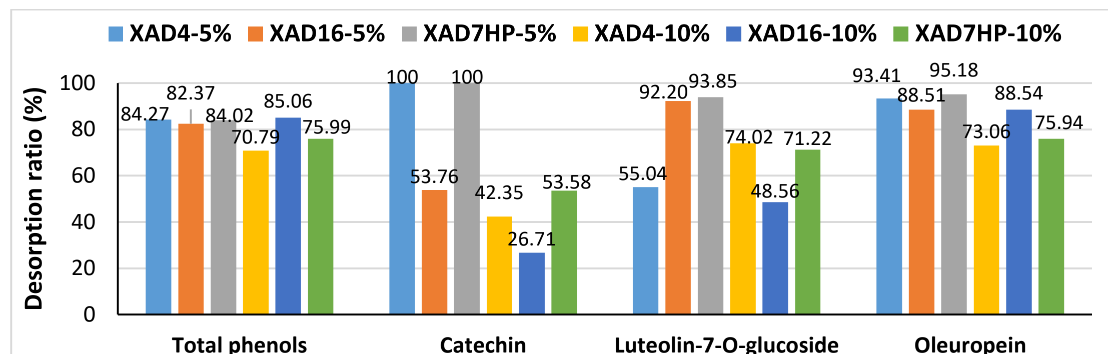
presented in Figures 4.2 (a,b,c) and 4.3 (a,b,c) for phenolic compounds and sugars, respectively. By considering the desorption ratio data with solvent A (ethanol 80% with natural pH), the optimum value for oleuropein desorption was determined l by using XAD7HP resin with 5% (w/v) as presented in Figure 4.2a. Regarding luteolin-7-O-glucoside, XAD7HP and XAD16 with 5% content showed considerably higher desorption ratios than the experiments carried out, with 93.85% and 92.20%, respectively. In addition, XAD4 and XAD7HP with 5% content both showed 100% of desorption ratio for catechin. Total phenolic content (TPC) was also measured for all test runs and the obtained results were in the range of 70.79- 85.06%, with XAD16 (10% w/v) being the optimum.

Supplementary desorption ratios of sugar compounds by using solvent A are presented in Figure 4.3a. It should be mentioned that in the case of sugar compounds, low desorption ratios are desired in order to have better isolation of phenolics from sugars. Among the analyzed sugar compounds, glucose showed considerably higher desorption rates with all resins. Regarding mannitol which was the most abundant sugar compound in olive leaf, the desorption rates were in in the range of 25.70-45.31% except for XAD7HP (10% w/v) which showed unsatisfactory desorption rate of 97.09% (Figure 4.3a). By considering overall results of desorption runs by using solvent A shown in Figures 4.2a and 4.3a, it can be concluded the advantage of using XAD7HP (5% w/v) as the optimal resin and content in comparison with other runs.

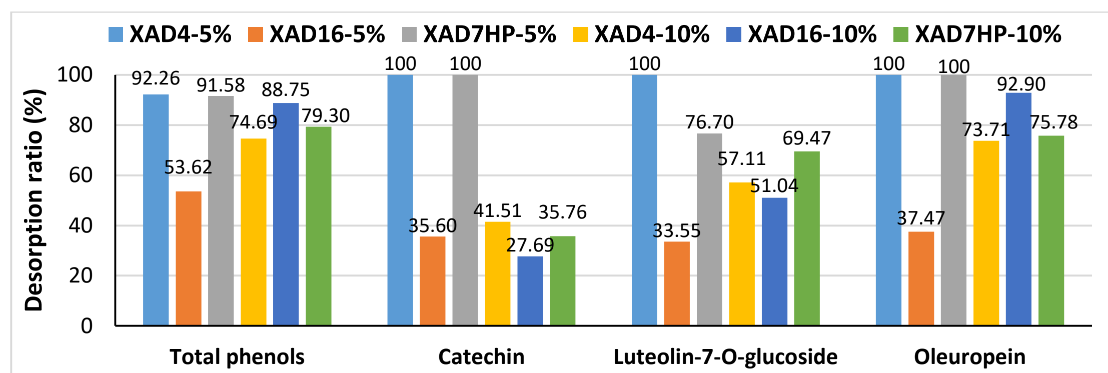
Table 4.4. Phenolic and sugar content of samples after desorption using multiple solvents.

Desorption solvent	Resin		Phenolic content (mg/L)				Sugar content (mg/L)			
	Type	Content (% w/v)	Total phenols	Catechin	Luteolin-7-O-glucoside	Oleuropein	Glucose	Xylose	Mannitol	Arabinose
Ethanol (80%), pH= natural	XAD4	5	3706 ± 50 ^{j}	43 ± 6 ^{b}	280 ± 6 ^{a,b}	3961± 65 ^{c}	166 ± 3 ^{e,f}	13± 0.7^{e}	131.4 ± 0.2 ^{c,d}	21.9± 0.5 ^{f}
		10	1625 ± 161^{a}	14 ± 5 ^{a}	152 ± 64 ^{a}	1598 ± 1 ^{b}	106 ± 13 ^{d}	7 ± 2 ^{c,d}	130 ± 4 ^{c}	0.69± 0.07 ^{a}
	XAD16	5	3553 ± 33 ^{i}	19 ± 6 ^{a}	468 ± 5 ^{c}	3754 ± 21 ^{c}	159 ± 4 ^{e}	33 ± 3^{g}	247 ± 12 ^{h}	14.1± 0.9 ^{d}
		10	1914 ± 8 ^{e,f}	9 ± 5 ^{a}	130 ± 11 ^{a}	1936 ± 30 ^{b}	88 ± 14 ^{a}	2.6 ± 0.2 ^{a}	78± 1 ^{b}	0.24± 0.08 ^{a}
	XAD7HP	5	3831 ± 21 ^{k}	56 ± 5 ^{c}	477 ± 5 ^{c}	4037 ± 54 ^{c}	189 ± 84 ^{h}	53 ± 5^{h}	281 ± 6 ^{i}	37.9± 0.4 ^{g}
		10	1787 ± 4 ^{c}	18 ± 7 ^{a}	190 ± 8 ^{a}	1661 ± 25 ^{b}	102 ± 3 ^{b,c,d}	8 ± 2 ^{c,d}	244.3± 0.4 ^{g,f}	0.78± 0.09 ^{a}
Ethanol (80%), pH= 2	XAD4	5	4057 ± 5 ^{m}	40 ± 6 ^{b}	440 ± 18 ^{c}	4220 ± 61 ^{c}	204 ± 3 ^{i}	28 ± 3^{f}	177 ± 3 ^{f}	45 ± 4 ^{h}
		10	1715 ± 40 ^{b}	15 ± 5 ^{a}	155 ± 54 ^{a}	1593 ± 17 ^{b}	92 ± 1 ^{a}	6.6 ±0.6^{b,c,d}	127 ± 2 ^{c}	4.1± 0.6 ^{b}
	XAD16	5	2312 ± 47 ^{h}	6.1 ± 0.1 ^{a}	178 ± 4 ^{a}	1566 ± 24 ^{a}	95 ± 2 ^{a,b}	12.9 ± 0.4^{e}	127 ± 24 ^{c}	9.8 ± 0.8 ^{c}
		10	1997 ± 29^{e}	10 ± 4 ^{a}	138 ± 6 ^{a}	2008 ± 11^{b}	95 ± 2 ^{a,b}	4 ± 1 ^{a,b,c}	85 ± 2 ^{c}	0.4 ± 0.2 ^{a}
	XAD7HP	5	4175 ± 6 ^{n}	56 ± 5 ^{c}	407 ± 166 ^{b,c}	4326± 45 ^{c}	221 ± 1 ^{j}	53 ± 1^{h}	309 ± 4 ^{j}	45 ± 1 ^{h}
		10	1865 ± 17 ^{d,e}	13 ± 5 ^{a}	188 ± 10 ^{a}	1638 ± 1 ^{a}	104 ± 1 ^{b,c,d}	5 ± 1 ^{a,b,c,d}	237 ± 1 ^{g}	11.7±0.4 ^{c,d}
Ethanol (100%), pH= 2	XAD4	5	3750 ± 10 ^{j}	39 ± 5 ^{b}	507 ± 11 ^{c}	4097 ± 31 ^{c}	170 ± 5 ^{f,g}	2.5 ± 0.5 ^{a}	55 ± 2 ^{a}	1.1 ± 0.2 ^{a}
		10	1964 ± 30 ^{f,g}	14 ±3 ^{a}	209 ± 2 ^{a}	1772 ± 1 ^{b}	107 ± 5 ^{d}	2.9±0.80^{a,b}	141.7 ± 0.8 ^{e}	6.1 ± 0.1 ^{b}
	XAD16	5	3925 ± 11 ^{l}	19 ± 2 ^{a}	510 ± 1 ^{c}	4230 ± 75 ^{c}	176 ± 3 ^{g}	26 ± 3^{f}	249 ± 2 ^{h}	19 ± 1 ^{e}
		10	2131 ± 15 ^{f}	10 ± 3 ^{a}	140 ± 6 ^{a}	2052 ± 24 ^{b}	104 ± 1 ^{b,c,d}	9 ± 1^{d}	134.5 ± 0.8 ^{a}	0.32±0.09^{a}
	XAD7HP	5	4061 ± 12 ^{m}	59 ± 6 ^{c}	397 ± 168 ^{b,c}	4269 ± 79 ^{c}	165 ± 2 ^{e,f}	5.2 ± 0.4 ^{a}	294 ± 1 ^{a}	1.2 ± 0.2 ^{a}
		10	1857 ± 10 ^{d}	17 ± 8 ^{a}	209 ± 6 ^{a}	1755 ± 16 ^{b}	103 ± 6 ^{b,c,d}	8.2 ± 0.4^{d}	252 ± 3 ^{h}	11.3± 0.9 ^{c}

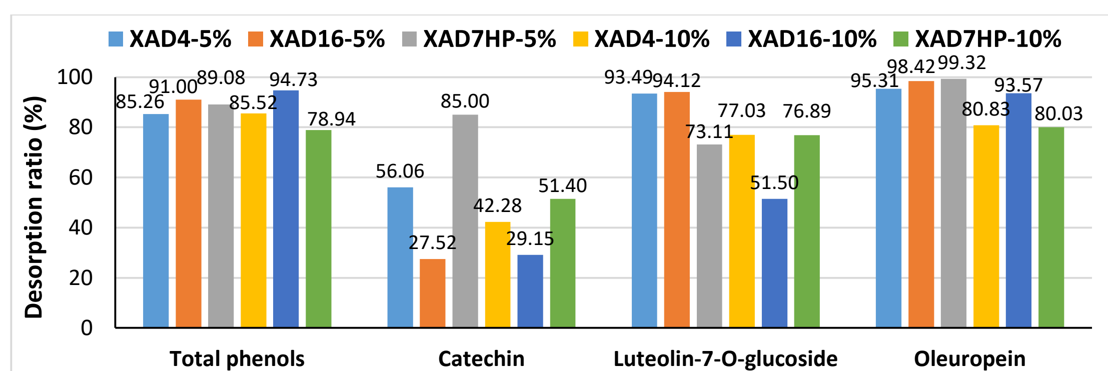
Values with different letters in each raw are significantly different when applying the Fisher's least significant differences (LSD) method at p-value ≤ 0.05



a) Solvent A: Ethanol 80% (v/v) with normal pH

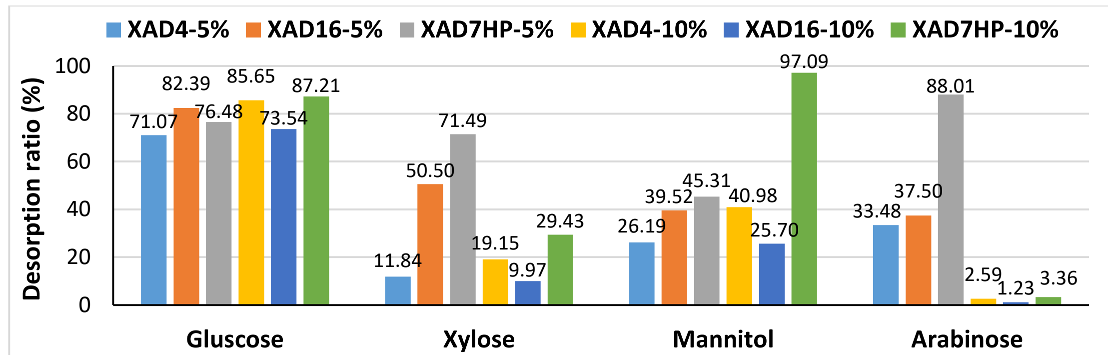


b) Solvent B: Ethanol 80% (v/v) with pH=2

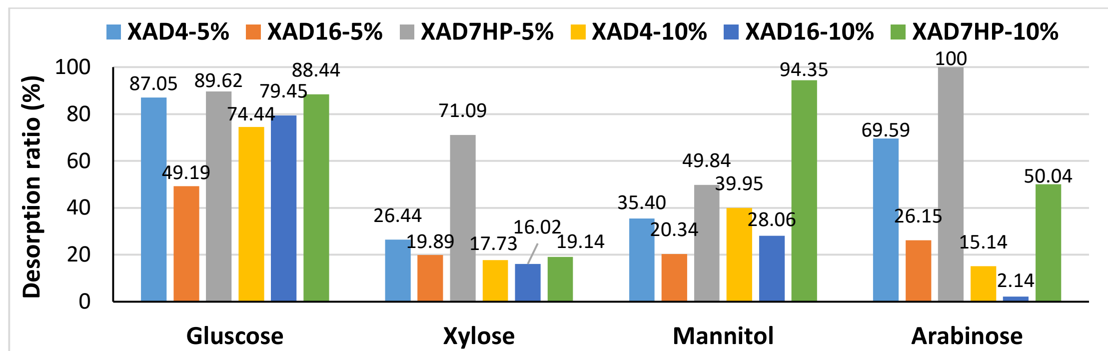


c) Solvent C: Ethanol (100%) with pH=2

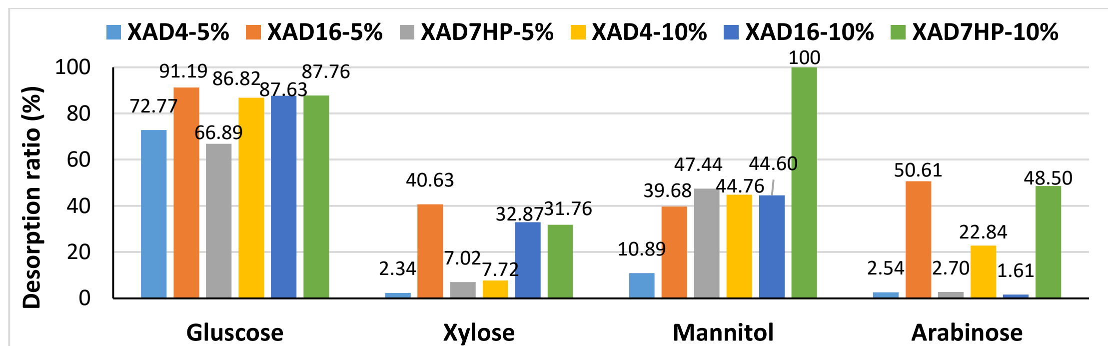
Figure 4.2. Desorption ratios of phenolic compounds.



a) Solvent A: Ethanol 80% (v/v) with normal pH



b) Solvent B: Ethanol 80% (v/v) with pH=2



c) Solvent C: Ethanol (100%) with pH=2

Figure 4.3. Desorption ratios of sugar compounds.

Figure 4.2b shows desorption ratios of phenolics by using solvent B (ethanol 80% v/v with pH=2). XAD4 and XAD7HP with 5% w/v content, showed optimal desorption results comparing to others experiments for all analyzed phenols. Complete desorption ratio of 100% was obtained for oleuropein and catechin with both resins. TPC desorption ratios were 92.26% and 91.58% with XAD4 and XAD7HP with 5% (w/v), respectively. While regarding luteolin-7-O-glucoside, they showed ratios of 100% and 76.70%, respectively (Figure 4.2b). As can be observed in Figure 4.3b, unlike satisfactory results of XAD7HP (5% w/v) in phenol desorption, it did not show satisfactory results in sugar desorption ratios, unless for glucose as for solvent A. However, results obtained with XAD4 (5%) were better compared to XAD7HP (5% w/v). Therefore, it can be concluded that XAD4 (5% w/v) is the optimal test run in the desorption experiments performed with solvent B (Figures 4.2b and 4.3b).

Desorption tests were also performed by using solvent C (ethanol 100% with pH=2) which the corresponding data is shown in Figures 4.2c and 4.3c, for phenolic compounds and sugars, respectively. By evaluating the measurements shown in Figure 4.2c, it can be observed that none of the resins showed the overall highest desorption ratios. However, obtained results proved that all three resins with 5% (w/v) content perform a better desorption ratio comparing to 10% (w/v) content. Besides, XAD7HP (5% w/v) showed the highest oleuropein desorption ratio of 99.32%. Regarding sugar desorption with solvent C, XAD4 (5% w/v) showed optimal results due to considerably lower desorption ratios for xylose, mannitol and arabinose comparing to other resins and contents. Considering quantified phenols and sugars, the advantage and significance of XAD4 with 5% w/v content can be concluded among the test runs performed with solvent C (Figures 4.2c and 4.3c).

After overall evaluation of all performed desorption test runs performed with solvents A, B and C, optimal runs were XAD4 and XAD7HP both with 5% w/v content which showed excellent efficiency. As mentioned earlier, oleuropein was the most desired compound to be purified based on the objective of this study. The concentration ratio of recovered oleuropein over recovered glucose (oleuropein/glucose) in the final effluent were evaluated and are presented in Figure 4.4. The concentration ratio of oleuropein over glucose was the initial feed was also evaluated and presented also in

Figure 4.4. Figure 4.4 reveals the effectiveness of adsorption-desorption processes performed in this study comparing to the initial oleuropein/glucose ratio. Somehow, the initial ratio was 3.18 and the range of the obtained oleuropein/glucose ratio after the desorption test runs were in the range from 15.08-25.89. As can be seen in Figure 4.4, XAD7HP (5% w/v) and solvent C (ethanol 100%, pH=2) showed optimal test runs result with 25.89% oleuropein/glucose ratio. In other words, it showed more than 8 fold increase in comparison with initial OLE. This means that the purification of phenolic compounds and specifically selective isolation of oleuropein has been executed in excellent form.

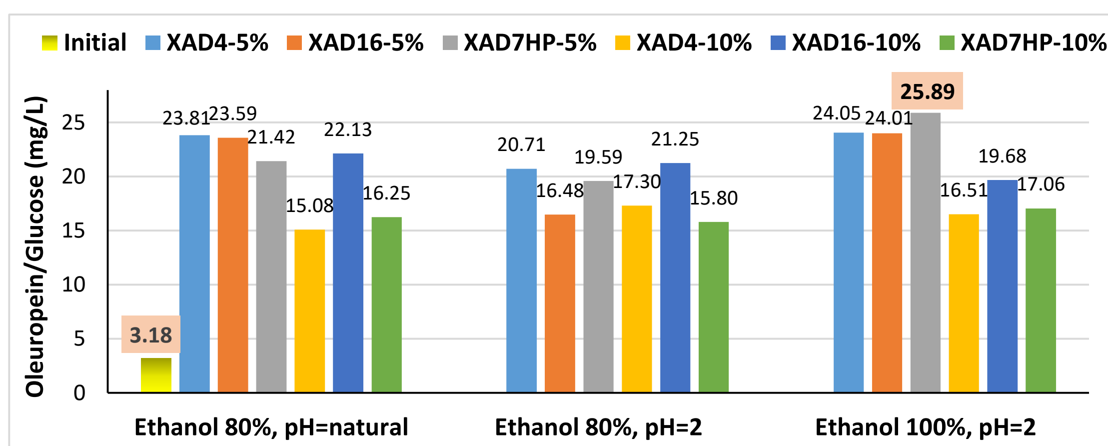


Figure 4.4. Oleuropein to glucose ratio in initial and desorption test runs.

4.3.3. Optimization of phenolic recovery and obtaining dried powders

Corresponding data to total recovery of phenolic compounds (R) are presented in Table 4.5. Total recovery shows the ratio of final content in the effluent after desorption over the initial content in the OLE before adsorption process. Data shown in Table 4.5 indicate that optimal total recovery of oleuropein was obtained by means of XAD7HP (5% w/v) and solvent C resulting to 98.01% recovery. Furthermore, the obtained results with the mentioned test run for total recovery of total phenols, catechin and luteolin-7-O-glucoside were also completely satisfactory with 81.46%, 78.49% and 75.08%, respectively. Therefore, this test run was selected as optimum run and subjected to subsequent drying process.

Table 4.5. Total recovery measurements of phenolic compounds based on Eq. (3).

Desorption solvent	Resin		Total recovery (%)			
	Type	Content (% w/v)	Total phenols	Catechin	Luteolin-7-O-glucoside	Oleuropein
Ethanol (80%), pH= natural	XAD4	5	72 ± 1 [e]	55 ± 8 [c,d]	52 ± 1 [a,b,c]	90 ± 1 [c]
		10	63.4 ± 0.6 [b]	41 ± 9 [b,c]	73 ± 12 [c,d,e,f]	72 ± 5 [b]
	XAD16	5	69.3 ± 0.1 [d]	27 ± 8 [b]	86 ± 1 [d,e,f]	86 ± 1 [c]
		10	74.7 ± 0.3 [f]	26 ± 5 [b]	48 ± 4 [a,b]	88 ± 1 [c]
	XAD7HP	5	74.7 ± 0.4 [f]	79 ± 7 [d,e]	94 ± 4 [g]	91 ± 1 [c]
		10	69.7 ± 0.2[d]	51 ± 10 [c.d]	70 ± 3 [c,d,e,f]	75 ± 1 [b]
Ethanol (80%), pH= 2	XAD4	5	79.2 ± 0.1 [h]	49 ± 9 [a]	99 ± 10[g]	96 ± 1 [c]
		10	67 ± 2 [c]	41 ± 8 [b,c]	57 ± 10 [c,d,e]	72 ± 1 [a]
	XAD16	5	45.1 ± 0.9 [a]	9 ± 1 [a]	33 ± 1[a]	36 ± 1 [a]
		10	78 ± 1 [g,h]	27 ± 8 [b]	51 ± 2 [a,b,c]	91 ± 1 [c]
	XAD7HP	5	81.5 ± 0.1 [i]	79 ± 7[d,e]	75 ± 10 [d,e,f]	98 ± 1 [c]
		10	72.8 ± 0.7 [e]	35 ± 7 [b,c]	69 ± 9[d,e,f]	74 ±10 [a]
Ethanol (100%), pH= 2	XAD4	5	73.2 ± 0.2 [e]	55 ± 8[c,d]	93 ± 2[f]	93 ± 1 [c]
		10	77 ± 1 [g]	40 ± 9 [b,c]	77 ± 1 [d,e,f]	80 ± 1 [b]
	XAD16	5	76.6 ± 0.2 [g]	27 ± 3[b]	94 ± 1 [f]	96 ± 2 [c]
		10	83.1 ± 0.1 [j]	28 ± 9 [b]	51 ± 9 [b,c]	93 ± 1 [c]
	XAD7HP	5	79.2 ± 0.2 [h]	83 ± 8 [e]	35 ± 7 [a]	97 ± 2 [c]
		10	72.4 ± 0.4 [e]	49± 10 [c]	49 ± 11 [a,b]	80 ± 2 [b]

Values with different letters in each column are significantly different when applying the Fisher's least significant differences (LSD) method at p-value ≤ 0.05

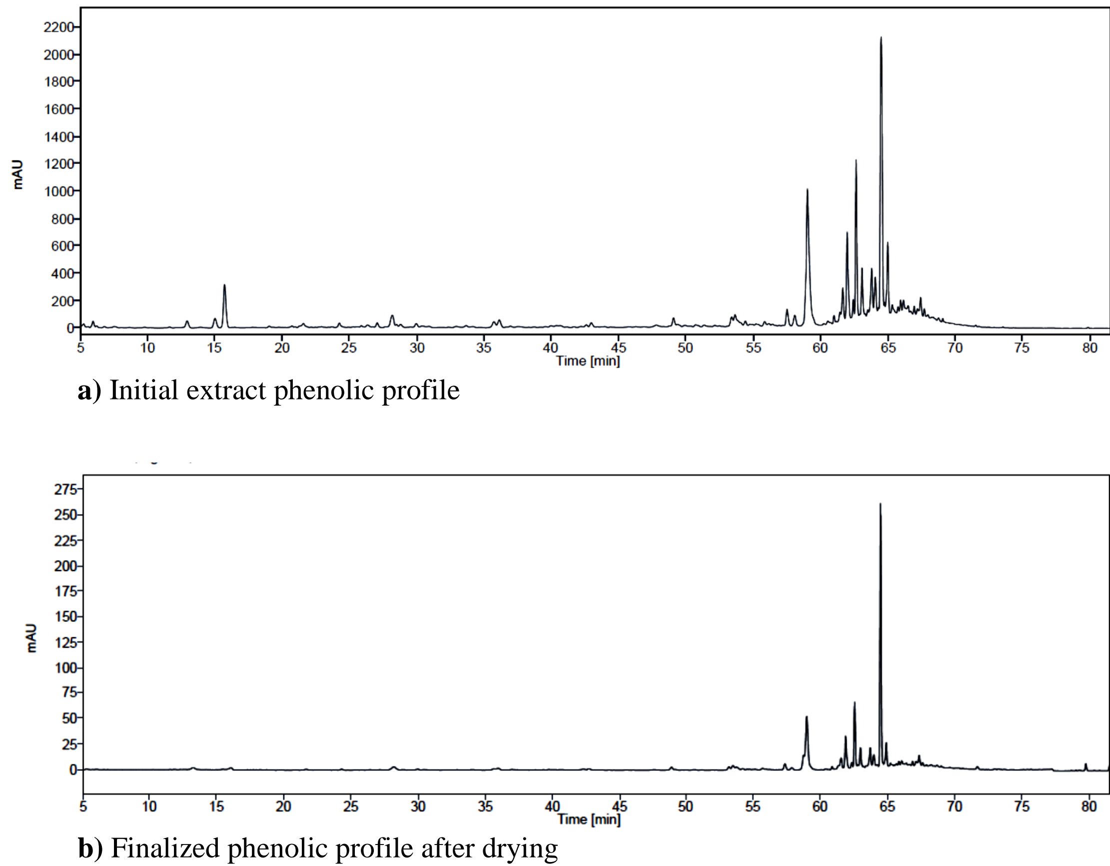
After optimization of the desorption stage, adsorption-desorption process at the determined optimum conditions was performed in larger volume. The ethanolic solvent was removed in a convection oven at 40 °C for 72 h to obtain dried powders.

Dried powders were characterized for their bioactive compounds and are presented in Table 4.6. The initial content of oleuropein in OL, as determined by the ethanolic extract, was 36.8 ± 0.4 while this concentration increase up to 232± 9 mg/g in the final dried extract obtained after purification in this work. The comparison of these values indicated 6 fold increase of oleuropein from initial to final stage. Regarding TPC, initial and final contents shown in Tables 4.2 and 4.6 indicate that TPC increased for almost 4 fold. On the other hand, final content of glucose and mannitol which are the most abundant sugars in the sample have decreased for 25.7 % and 36.4 %, respectively in

comparison with their initial content (Tables 4.2 and 4.6). In a similar study of phenolic purification, Zagklis *et al.* [42] recovered 74% of the initial amount of hydroxytyrosol by vacuum evaporation of ethanol until reach a volume concentration factor of 5.4.
In Figure 4.5 (a,b), HPLC chromatogram obtained from initial OLE and final dried phenolic powders are shown. As can be observed in these figures, phenolic profiles of the initial and final sample are nearly similar and as mentioned earlier, excellent level of purification was executed meaning that phenolic content was increased extensively while the sugar content was reduced. Pictures of the initial olive leaves samples and final product of this study can be seen in Figure 4.6.

Table 4.6. Characterization of the final dried powders after desorption stage.

Characterizations		**content (mg/g)**
Phenolic content	Total phenols	168.0 ± 0.6
	Oleuropein	232 ± 9
	Catechin	0.78 ± 0.14
	Luteolin-7-O-glucoside	25.1 ± 3
	Hydroxytyrosol	0.83 ± 0.13
	Rutin	0.26 ± 0.05
	Verbascoside	2.06 ± 0.16
Sugar content	Glucose	15.6 ± 0.4
	Xylose	4.4 ± 0.2
	Mannitol	22.6 ± 0.1
	Arabinose	0.35 ± 0.01
Chlorophyll	Ch-a	1.69 ± 0.05
	Ch-b	1.54 ± 0.05
	Cx+c	0.025 ± 0.006
Antioxidant activity	Frap assay (mg $_{Fe2SO4}$/ g)	321 ± 9
	ABTS assay (mg $_{Trolox}$/ g)	362 ± 1
Total flavonoid content	(mg $_{quertecine}$/ g)	27.19 ± 0.09
Humidity (%)	**-**	7.2 ± 0.3



a) Initial extract phenolic profile

b) Finalized phenolic profile after drying

Figure 4.5. Chromatogram of initial and finalized phenolic profile of the samples.

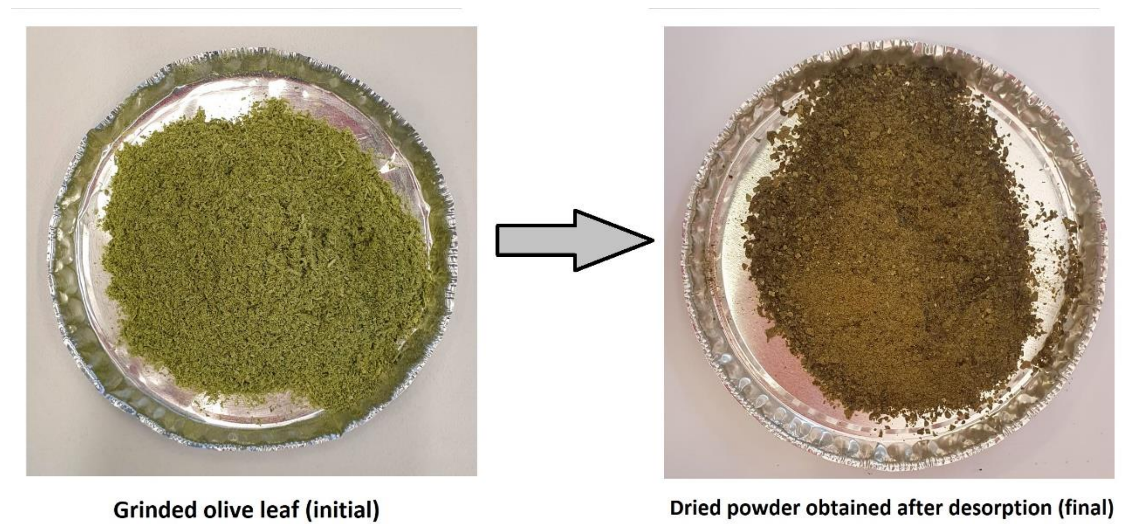


Figure 4.6. Pictures of initial grinded olive leaf and finalized dried phenolic powders.

4.4. Conclusions

This study aimed for purification and selective isolation of phenolic compounds available in ethanolic aqueous olive leaf extract (OLE) by adsorption-desorption process. Optimization of adsorption-desorption test runs was performed in terms of the highest phenolic compounds recovery and sugar rejection in order to achieve a phenolic compounds rich composition with minimum impurities.. XAD7HP with 5% (w/v) content showed optimal oleuropein desorption ratio of 99.32% by using ethanol (100%) at pH=2 as desorption solvent. Initial and the optimal final content of oleuropein were 36.8 ± 0.4 and 232 ± 9 mg/g, respectively, meaning that its content was increased for 6 fold in the proposed adsorption-desorption processes.

In addition, final content of total phenolic content was increased for almost 4 fold in comparison with its initial content. On the other hand, final content of glucose and mannitol which are the most abundant sugars in the olive leaf were decreased for 25.7% and 36.4%, respectively, in comparison with their initial content. Oleuropein/glucose ratio of the optimal test run showed more than 8 fold increase in comparison with the ratio in the initial OLE meaning that the purification of phenolic compounds and specifically selective isolation of oleuropein has been executed in excellent form.

References

[1] M. Kashaninejad, M. T. Sanz, B. Blanco, S. Beltrán, and S. M. Niknam, "Freeze dried extract from olive leaves: Valorisation, extraction kinetics and extract characterization," *Food Bioprod. Process.*, vol. 124, pp. 196–207, 2020.

[2] R. M. Odeh and L. A. Cornish, "Natural Antioxidants for the Prevention of Atherosclerosis," *Pharmacother. J. Hum. Pharmacol. Drug Ther.*, vol. 15, no. 5, pp. 648–659, 1995.

[3] I. Erlund, "Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology," *Nutr. Res.*, vol. 24, no. 10, pp. 851–874, 2004.

[4] H. E. Seifried, D. E. Anderson, E. I. Fisher, and J. A. Milner, "A review of the interaction among dietary antioxidants and reactive oxygen species," *J. Nutr. Biochem.*, vol. 18, no. 9, pp. 567–579, 2007.

[5] L. Bonfili *et al.*, "Natural polyphenols as proteasome modulators and their role as anti-cancer compounds," *FEBS J.*, vol. 275, no. 22, pp. 5512–5526, 2008.

[6] B. Halliwell, "The wanderings of a free radical," *Free Radic. Biol. Med.*, vol. 46, no. 5, pp. 531–542, 2009.

[7] B. H. Havsteen, "The biochemistry and medical significance of the flavonoids," *Pharmacol. Ther.*, vol. 96, no. 2–3, pp. 67–202, 2002.

[8] E. Middleton Jr., C. Kandaswami, and T. C. Theoharides, "The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer," *Pharmacol. Rev.*, vol. 52, no. 4, pp. 673–751, 2000.

[9] M. L. Soto, A. Moure, H. Domínguez, and J. C. Parajó, "Recovery, concentration and purification of phenolic compounds by adsorption: A review," *J. Food Eng.*, vol. 105, no. 1, pp. 1–27, 2011.

[10] S. Souilem *et al.*, "Emerging Technologies for Recovery of Value-Added Components from Olive Leaves and Their Applications in Food/Feed Industries," *Food Bioprocess Technol.*, vol. 10, no. 2, pp. 229–248, 2017.

[11] N. Talhaoui, A. Taamalli, A. M. Gómez-Caravaca, A. Fernández-Gutiérrez, and A.

Segura-Carretero, “Phenolic compounds in olive leaves: Analytical determination, biotic and abiotic influence, and health benefits,” *Food Res. Int.*, vol. 77, pp. 92–108, 2015.

[12] I. Hassen, H. Casabianca, and K. Hosni, “Biological activities of the natural antioxidant oleuropein: Exceeding the expectation - A mini-review,” *J. Funct. Foods*, vol. 18, pp. 926–940, 2015.

[13] I. Samet, M. O. Villareal, H. Motojima, J. Han, S. Sayadi, and H. Isoda, “Olive leaf components apigenin 7-glucoside and luteolin 7-glucoside direct human hematopoietic stem cell differentiation towards erythroid lineage,” *Differentiation*, vol. 89, no. 5, pp. 146–155, 2015.

[14] D. P. Zagklis and C. A. Paraskeva, “Isolation of organic compounds with high added values from agro-industrial solid wastes,” *J. Environ. Manage.*, vol. 216, pp. 183–191, 2018.

[15] O. Gómez-Jiménez-Aberasturi and J. R. Ochoa-Gómez, “New approaches to producing polyols from biomass,” *J. Chem. Technol. Biotechnol.*, vol. 92, no. 4, pp. 705–711, 2017.

[16] A. Lama-Muñoz, M. del M. Contreras, F. Espínola, M. Moya, I. Romero, and E. Castro, “Content of phenolic compounds and mannitol in olive leaves extracts from six Spanish cultivars: Extraction with the Soxhlet method and pressurized liquids,” *Food Chem.*, vol. 320, no. September 2019, p. 126626, 2020.

[17] S. Şahin, M. Bilgin, and M. U. Dramur, “Investigation of oleuropein content in olive leaf extract obtained by supercritical fluid extraction and soxhlet methods,” *Sep. Sci. Technol.*, vol. 46, no. 11, pp. 1829–1837, 2011.

[18] S. Şahin and M. Bilgin, “Selective adsorption of oleuropein from olive (Olea europaea) leaf extract using macroporous resin,” *Chem. Eng. Commun.*, vol. 204, no. 12, pp. 1391–1400, 2017.

[19] J. Kammerer, D. R. Kammerer, U. Jensen, and R. Carle, “Interaction of apple polyphenols in a multi-compound system upon adsorption onto a food-grade resin,” *J. Food Eng.*, vol. 96, no. 4, pp. 544–554, 2010.

[20] Z. Kaleh and S. U. Geißen, “Selective isolation of valuable biophenols from olive mill

wastewater," *J. Environ. Chem. Eng.*, vol. 4, no. 1, pp. 373–384, 2016.

[21] A. De Martino, M. Iorio, P. D. Prenzler, D. Ryan, H. K. Obied, and 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.*, vol. 80, pp. 154–161, 2013.

[22] A. Yangui, M. Abderrabba, and 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.*, vol. 70, pp. 111–118, 2017.

[23] A. Ena, C. Pintucci, and P. Carlozzi, "The recovery of polyphenols from olive mill waste using two adsorbing vegetable matrices," *J. Biotechnol.*, vol. 157, no. 4, pp. 573–577, 2012.

[24] D. P. Zagklis, A. I. Vavouraki, M. E. Kornaros, and C. A. Paraskeva, "Purification of olive mill wastewater phenols through membrane filtration and resin adsorption/desorption," *J. Hazard. Mater.*, vol. 285, pp. 69–76, 2015.

[25] A. Yangui, J. R. Njimou, A. Cicci, M. Bravi, M. Abderrabba, and A. Chianese, "Competitive adsorption, selectivity and separation of valuable hydroxytyrosol and toxic phenol from olive mill wastewater," *J. Environ. Chem. Eng.*, vol. 5, no. 4, pp. 3581–3589, 2017.

[26] D. R. Kammerer, R. Carle, R. A. Stanley, and Z. S. Saleh, "Pilot-scale resin adsorption as a means to recover and fractionate apple polyphenols," *J. Agric. Food Chem.*, vol. 58, no. 11, pp. 6787–6796, 2010.

[27] M. Carmona, A. De Lucas, J. L. Valverde, B. Velasco, and J. F. Rodriguez, "Combined adsorption and ion exchange equilibrium of phenol on Amberlite IRA-420," *Chem. Eng. J.*, vol. 117, no. 2, pp. 155–160, 2006.

[28] 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.*, vol. 338, no. 2, pp. 402–409, 2009.

[29] 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.*, vol. 44, no. 11, pp. 4128–

4138, 2005.

[30] J. F. Garcia-Araya, F. J. Beltran, P. Alvarez, and F. J. Masa, "Activated carbon adsorption of some phenolic compounds present in agroindustrial wastewater," *Adsorption*, vol. 9, no. 2, pp. 107–115, 2003.

[31] V. Caqueret, S. Bostyn, B. Cagnon, and H. Fauduet, "Purification of sugar beet vinasse–adsorption of polyphenolic and dark colored compounds on different commercial activated carbons," *Bioresour. Technol.*, vol. 99, no. 13, pp. 5814–5821, 2008.

[32] M. Ugurlu, A. Gurses, M. Yalcin, and C. Dogar, "Removal of phenolic and lignin compounds from bleached kraft mill effluent by fly ash and sepiolite," *Adsorption*, vol. 11, no. 1, pp. 87–97, 2005.

[33] A. Dąbrowski, P. Podkościelny, Z. Hubicki, and M. Barczak, "Adsorption of phenolic compounds by activated carbon - A critical review," *Chemosphere*, vol. 58, no. 8, pp. 1049–1070, 2005.

[34] G. Difonzo *et al.*, "Green extracts from Coratina olive cultivar leaves: Antioxidant characterization and biological activity," *J. Funct. Foods*, vol. 31, pp. 63–70, 2017.

[35] D. Frascari *et al.*, "Valorisation of olive mill wastewater by phenolic compounds adsorption: Development and application of a procedure for adsorbent selection," *Chem. Eng. J.*, vol. 360, no. November 2018, pp. 124–138, 2019.

[36] D. Pinelli *et al.*, "Batch and Continuous Flow Adsorption of Phenolic Compounds from Olive Mill Wastewater: A Comparison between Nonionic and Ion Exchange Resins," *Int. J. Chem. Eng.*, vol. 2016, 2016.

[37] D. P. Zagklis and C. A. Paraskeva, "Isolation of organic compounds with high added values from agro-industrial solid wastes," *J. Environ. Manage.*, vol. 216, pp. 183–191, 2018.

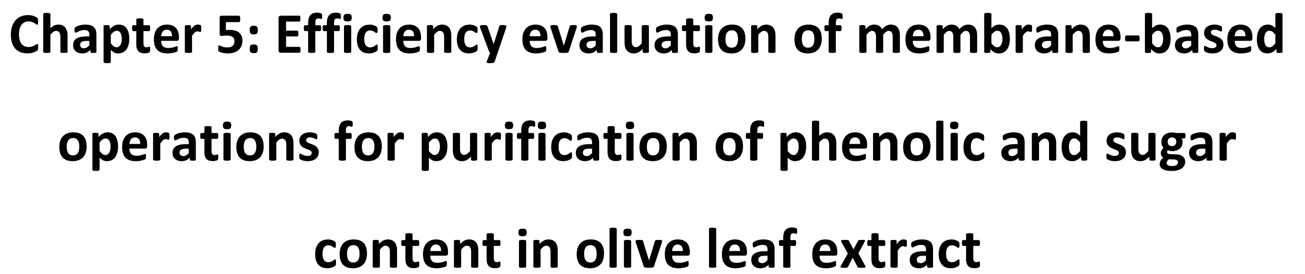
[38] C. Li, Y. Zheng, X. Wang, S. Feng, and D. Di, "Simultaneous separation and purification of flavonoids and oleuropein from Olea europaea L . (olive) leaves using macroporous resin," no. May, pp. 2826–2834, 2011.

[39] Z. Wang, C. Wang, J. Yuan, and 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.*, vol. 1040, pp. 38–46, 2017.

[40] 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.

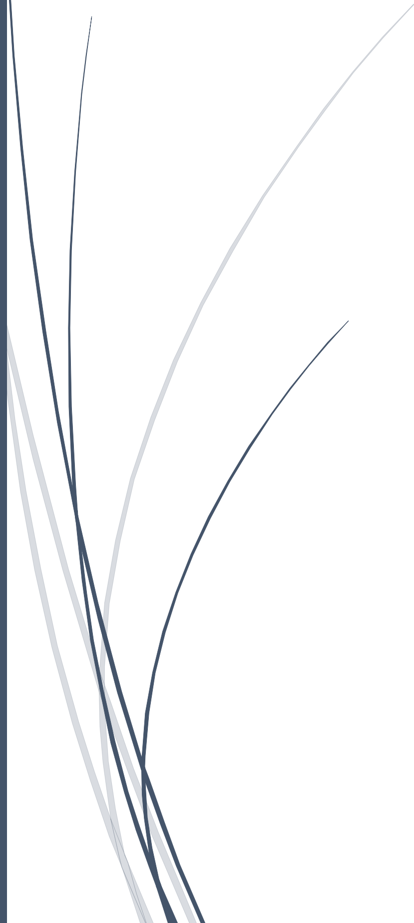
[41] G. Fava *et al.*, "Hydroxytyrosol Recovery From Olive Mill Wastewater: Process Optimization and Development of a Pilot Plant," *Clean - Soil, Air, Water*, vol. 45, no. 4, pp. 1–10, 2017.

[42] D. P. Zagklis, A. I. Vavouraki, M. E. Kornaros, and C. A. Paraskeva, "Purification of olive mill wastewater phenols through membrane filtration and resin adsorption/desorption," *J. Hazard. Mater.*, vol. 285, pp. 69–76, 2015.



Chapter 5: Efficiency evaluation of membrane-based operations for purification of phenolic and sugar content in olive leaf extract

Valorization of plant biomass by emerging technologies: olive and moringa leaves



Olive leaves contain a large variety of bioactive compounds and have been known to have various health enhancing properties. The separation and purification of bioactive compounds from their original sources is a complex procedure since they must be separated from the feedstock in order to increase their purity and antioxidant capacity.

In the current study, the main objective was evaluating the effects of membrane filtration on sugar and phenolic content of the prepared extract from dried olive leaves. Membrane based operations were executed by a primary MF step as pretreatment and then UF and NF processes were performed. Subsequently, characterization of permeates and retentates in terms of their phenolic and sugar content was implemented and the optimal membrane run was subjected to freeze drying process. In a total of five UF test runs, PES membrane with MWCO of 100 kDa showed optimal results. While, NF90 membrane was considered as the optimal choice within four NF runs. Freeze drying process was then performed on the NF90 membrane retentate. Hydroxytyrosol and oleuropein contents increased more than 26 and 17 fold, respectively in freeze dried form in comparison with their initial content. Regarding sugar profile, mannitol content was increased more than 3 fold comparing to initial olive leaf extract.

Las hojas de olivo contienen una gran variedad de compuestos bioactivos y se sabe que tienen varias propiedades que mejoran la salud. La separación y purificación de compuestos bioactivos de sus fuentes originales es un procedimiento complejo, ya que deben separarse de la materia prima para aumentar su pureza y capacidad antioxidante.

En el presente estudio, el objetivo principal fue evaluar los efectos de la filtración por membrana sobre el contenido de azúcar y fenólico del extracto preparado de hojas de olivo secas. Las operaciones basadas en membranas se ejecutaron mediante un paso primario de MF como pretratamiento y luego se realizaron los procesos de UF y NF. Posteriormente, se implementó la caracterización de los permeados y retenidos en términos de su contenido fenólico y de azúcares y el funcionamiento óptimo de la membrana se sometió a un proceso de liofilización. En total de cinco pruebas de UF, la membrana PES con MWCO de 100 kDa mostró resultados óptimos. Mientras que, la membrana NF90 se consideró como la opción óptima dentro de cuatro ciclos de NF. A continuación, se realizó el proceso de liofilización en el retenido de membrana NF90. Los contenidos de hidroxitirosol y oleuropeína aumentaron más de 26 y 17 veces, respectivamente, en forma liofilizada en comparación con su contenido inicial. Con respecto al perfil de azúcar, el contenido de manitol se incrementó más de 3 veces en comparación con el extracto inicial de hoja de olivo.

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5.1. Introduction

By-products derived from olive trees and olive oil extractions are generally known as "olive by-products" [1]. A high number of by-products and residues derived from both olive tree cultivation and the olive processing industry are obtained yearly; most of them have no practical applications. Olive leaves, one of these by-products, can be found in large amounts in olive oil industries.

Leaves represent 10% of the weight of olives collected for oil extraction [2]. Furthermore, they also accumulate in large volumes on farms during the pruning of the trees [3]. It has been estimated that pruning produces 25 kg of by-products (twigs and leaves) per tree annually.

The interest of olive leaves, as a matrix rich in antioxidants, have increased with the aim to be further use in food and food supplements. In food industry, there is an increasing interest in producing functional foods for their health beneficial. The incorporation of such extracts in food industry may contribute to the health benefit of the consumers significantly and also to prolong the shelf life of food products [4].

Historically, olive leaf extract has been widely used as a folk remedy for combating fever and other diseases [5]; today, it is also known to have various health enhancing properties. Several studies have shown that olive leaf extract exhibits a large spectrum of in vitro and in vivo properties, including antioxidant activity [6,7], radio-protective effects [7], anti- proliferative effect on leukaemic cells by inducing apoptosis [8–10], cytotoxic activity against human breast cancer cells [11,12], anti-HIV [13], anti-fungal [14], gastroprotective [15] activities, attenuation of diabetic neuropathic pain [16] and amelioration of gentamicin nephrotoxicity [17].

Olive leaves contain a large variety of phenolic derivatives, and consist of simple phenols (the most common and important low-molecular weight phenolic compounds), flavonoids (flavones, flavanones, flavonols, 3-flavanols), and secoiridoids. Oleuropein, the major constituent of secoiridoid class in olive leaves, has been shown to reduce free fatty acid-induced lipogenesis via lowered extracellular signal-regulated kinase activation in hepatocytes [18]. Moreover, it exhibited antioxidant effects on intestine mucosal damage induced by absolute ethanol [19,20].

Carbohydrates are photosynthesized in green plants initially as monosaccharides, which are then transformed into disaccharides, trisaccharides, and sugar alcohols. The primary function of sugars is as energy source for metabolic changes or as molecular precursors in the biosynthesis of lipids, proteins, antioxidants, and polysaccharides. Thus, olive ripening is characterized by a series of transformations in which sugars are especially involved. Sugars are the main soluble components in olive tissues of leaves and fruits, playing a key role in the cell-wall structure.

Sugars profile in olive tissues has been scarcely reported, with determination limited to a few analytes. Thus, Cataldi et al. [21] reported the determination of eight sugars in olive leaves (namely, myo-inositol, galactinol, mannitol, galactose, glucose, fructose, sucrose, raffinose, and stachyose). Another method reported by Marsilio et al. [22] was focused on predominant sugars such as myo-inositol, mannitol, galactose, glucose, fructose, and sucrose in olive fruits [23].

The separation and purification of phenolic compounds from their original sources is a complex procedure since these molecules are generally present in complex matrices. Indeed, they must be separated from the feedstock in order to increase their purity and antioxidant capacity. The growing interest for the recovery of phenolic compounds from vegetable sources and their utilization as raw materials in cosmetic, pharmaceutical and nutraceutical preparations has urged researchers to develop environmentally friendly extraction and purification procedures in order to preserve their stability.

Membrane separation processes are useful technologies for recovery, fractionation and concentration of phenolic compounds from aqueous and alcoholic processing streams of products, by-products and wastes from biomass processing [24]. Among these processes, pressure driven membrane operations have been successfully employed over the last three decades in food and beverage industries. According to the required transmembrane pressure (TMP) and the pore sizes, pressure driven membrane operations can be divided in: microfiltration (MF, 0.1–5 μm, 1–10 bar), ultrafiltration (UF, 0.5–100 nm, 1–10 bar), nanofiltration (NF, 0.5–10 nm, 10–30 bar) and reverse osmosis (RO,<0.5 nm, 35–100 bar) [25].

Polyphenols are amphipathic molecules, with hydrophobic aromatic rings and hydrophilic (acidic) phenolic hydroxyl groups. Their adsorption on polymeric membranes involves both hydrophobic effects and the formation of hydrogen bonds. A direct relationship exists between membrane polarity of MF membranes and the amount of adsorbed polyphenols: total phenol index increased by increasing the polarity and the basic component of the membrane surface free energy [26]. It is noteworthy that polyphenols may interact together and with other compounds to form large particles which have a negative impact during filtration process [27].

In current study, the main objective was evaluating the effects of membrane filtration on sugar and phenolic content of aqueous prepared olive leaf extract. Primarily, an aqueous olive leaf extract (OLE) was prepared by using conventional solvent extraction. Membrane based operations included a microfiltration process for all runs as pretreatment. Then the pretreated extracts were subjected as feed to ultrafiltration and nanofiltration processes. Subsequently, comprehensive characterization of OLE was performed before and after membrane filtration runs and phenolic and sugar purification levels were evaluated.

5.2. Materials and methods

5.2.1. Sample origin and preparation

Olive leaf (OL) samples were collected from the pruning of olive trees of the variety ¨*Serrana de Espadán*¨, provided by Maicas-Sediles (Teresa, Castellón, Spain). OL were dried in a convection oven at 45°C to reduce the humidity to values lower than 10%. This initial drying step by hot-air has resulted in higher oleuropein recovery in previous studies reported in the literature [28]. After drying, OL were grounded by using a cutting mill (Retsch SM100, Retsch Technology GmbH, Germany) to obtain a particle size in the range from 0.5 to 0.1 mm to increase the interfacial surface and improve extraction. Samples were stored in the freezer at -18 °C for further use.

5.2.2. Chemicals used

All chemicals used in this study were of analytical grade and provided from VWR International Eurolab (Llinars del Vallès, Spain) and Sigma-Aldrich (Darmstadt, Germany).

The chemicals used for each analysis were as follows:

- Folin–Ciocalteu reagent, gallic acid and sodium carbonate were used for total phenolic content (TPC) measurement.
- Acetic acid (CH_3COOH), acetonitrile (C_2H_3N), ammonium acetate (NH_4Ac), oleuropein, luteolin-7-O-glucoside, verbascoside, hydroxytyrosol, catechin, and rutin were used as standards and solvent in HPLC/DAD for identification and quantification of individual phenolic compounds.
- Sulfuric acid (H_2SO_4), xylose, arabinose, glucose, sucrose and mannitol were used as mobile phase or standard solutions in HPLC-RID for identification and quantification of soluble carbohydrates.
- Several types of polymeric membranes were used which their characteristics are shown in Table 5.1.

5.2.3. Conventional solvent extraction

Olive leaf extract (OLE) was prepared by mixing previously dried and grinded olive leaves with distilled water as solvent with a biomass solvent ratio of 1:20 (w/v) which was previously proved to be the optimal ratio [29]. Extractions were then performed in an orbital shaker (Grant instruments-OLS 200, Shepreth Cambridgeshire, England) for 1 h at 50°C. After each extraction, samples were centrifuged (Eppendorf centrifuge 5804, Germany) at 4500 rpm for 15 min to separate the obtained extract from solid residues.

5.2.4. Chemical characterization of extracts

5.2.4.1. Determination of total phenolic content (TPC)

Total phenolic content (TPC) for each extract was measured using Folin-Ciocalateu standard method [30]. Briefly, a standard calibration curve (R^2=0.99) was prepared using gallic acid solution. 100 µl of extracts containing phenolic compounds was added to a test tube. Then 2.8 ml of distilled water, 2 ml of 7.5% sodium carbonate and 100 µl Folin-Ciocalateu 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 (Spectrophotometer V-750, Jasco, Japan). TPC was expressed as milligrams of gallic acid equivalents to per liter of OLE (mg/L) or per g of dried olive leaf (mg GAE/g_{DOL}).

5.2.4.2. Identification and quantification of extract components by HPLC

Chromatographic separation was performed on a HPLC/DAD Agilent 1110 (Agilent Technologies, Inc., U.S.A.) with Kinetex® 5 µm Biphenyl 100 Å, 250 × 4.6 mm column (Phenomenex, Inc. CA, U.S.A.). Phenolic compounds were analyzed by using a linear gradient of two solvents: solvent A (ammonium acetate 5 mM with 1% acetic acid in water) and solvent B (ammonium acetate 5 mM with 1% acetic acid in acetonitrile). The gradient profile involved a linear increase of solvent B: 2%, 7 min; 2–8%, 13 min; 8–10%, 15 min; 10–18%, 20 min; 18–38%, 10 min; 38–65%, 10 min; 65–80%, 5 min; 80%, 10 min. The flow rate was set to 0.8 mL/min and temperature column was 25°C. UV–vis detection was done at 240, 280, 330, 350, and 370 nm. Before injection, extracts were filtered through 0.45 µm pore size. Identification of individual polyphenol compounds was carried out by comparing retention times and spectral data with those of authentic standards. Standard solutions were prepared by dissolution of the compounds in methanol. Results were expressed as mg phenolic compound per liter of the extract (mg/L).

Identification and quantification of soluble carbohydrates was performed by HPLC-RID Agilent 1260 with an AminexHPX-87H column (300 × 7.8 mm, Bio-Rad

Laboratories, Inc., USA.) using H_2SO_4 10 mM as mobile phase with a flow rate of 0.6 mL min^{-1}. The column and detector were maintained at 40°C. Pure soluble carbohydrates were used for calibration (xylose, arabinose, glucose, sucrose and mannitol). Results were expressed as mg carbohydrate per liter of the extract (mg/L).

5.2.5. Membrane treatment process

OLE membrane treatments were performed using microfiltration (MF), ultrafiltration (UF) and nanofiltration (NF) operations in a stainless steel HP4750 stirred batch cell supplied by Sterlitech Corporation (Kent, WA, USA). OLE was primarily pretreated by MF process by using a Synder PVDF membrane with 0.1 µm pore size and then the obtained permeate was used as feed in the subsequent UF or NF processes. The characteristics of the selected flat sheet polymeric membranes used in this work are shown in Table 5.1. Depending on the filtration type, transmembrane pressure (TMP) of 2-25 bar was provided through a nitrogen cylinder to avoid oxidation of the biocompounds. Membrane surface area was 14.6 cm^2 and experiments were performed at room temperature and 300 rpm stirring speed.

Stirred cell modules provide uniform TMP 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 [31].

The permeate flux was monitored by measuring the permeate volume collected at a specific time described by Darcy's equation (Eq.(1)) [32]:

$$J = \frac{1}{A_m} . \frac{V_p}{t} \tag{1}$$

where J is the permeate flux, $L.m^{-2} h^{-1}$, A_m is the effective membrane area, m^2, V_P is the permeate volume, L, and t is the filtration time, h.

Volume Reduction Factor, VRF, is defined as Eq. (2):

$$VRF = \frac{V_F}{V_R} \quad (2)$$

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.

Rejection coefficient percentage (%) of feed components is calculated as Eq. (3):

$$R(\%) = (1 - \frac{C_p}{C_F}) \times 100 \quad (3)$$

where C_P and C_F are the concentration of total phenols and individual phenolic compounds in permeate and feed solutions, respectively [33]. Membrane fouling percentage was calculated by measuring the difference between initial membrane flux (J_0) and steady state flux (J_{ss}). For this purpose, Eq. (3) was used where, C_F and C_P are the initial membrane flux and steady state flux, respectively. Water permeability of each membrane was measured in terms of permeate flux by passing Milli-Q water through the membrane similar to a test run.

Microfiltration (MF) is based on the use of symmetric membranes able to separate particles with diameters of 0.05–10 mm from a solvent or other low molecular weight compounds. Ultrafiltration (UF) involves the use of asymmetric membranes with pore size in the skin layer of 2–10 nm, providing high retention of macromolecules and colloids from a solution. UF membranes are typically characterized by their molecular weight cut-off (MWCO), defined as the equivalent molecular weight of the smallest species that exhibit 90% rejection. For UF membranes, hydrostatic pressures of 2–10 bar are typically used. Nanofiltration (NF) is an intermediate membrane process between UF and reverse osmosis (RO). It is typically used for the separation of multivalent ions and uncharged organic solutes with molecular weight in the range of 100 and 1000 Da. NF membranes are characterized by pore diameters in the range of

1–3 nm. They operate at lower pressures (generally in the range of 3–30 bar) than RO membranes. Polymeric NF membranes contain ionisable groups as carboxylic or sulphonic acid groups, which result in a surface charge in the presence of a feed solution [34].

5.2.6. Freeze-drying process

A freeze-dried extract was obtained from the optimal retentate of the membrane-based operations which showed the highest content of bioactive compounds. First, the sample was frozen with liquid nitrogen (-196 °C), equilibrated at -80 °C for 2 h and then submitted to freeze-drying in a Labconco Freeze Dry System (Labconco Corporation, U.S.A.) at 1.5×10^{-4} mbar during 48 h. The moisture content of the freeze-dried particles was determined gravimetrically by weighing small amounts of dried particles (around 0.5 gr) before and after drying in an oven at 105 °C until constant weight.

5.3. Results and discussion

5.3.1. Effects of transmembrane pressure, molecular weight cut-off and membrane material on permeate flux

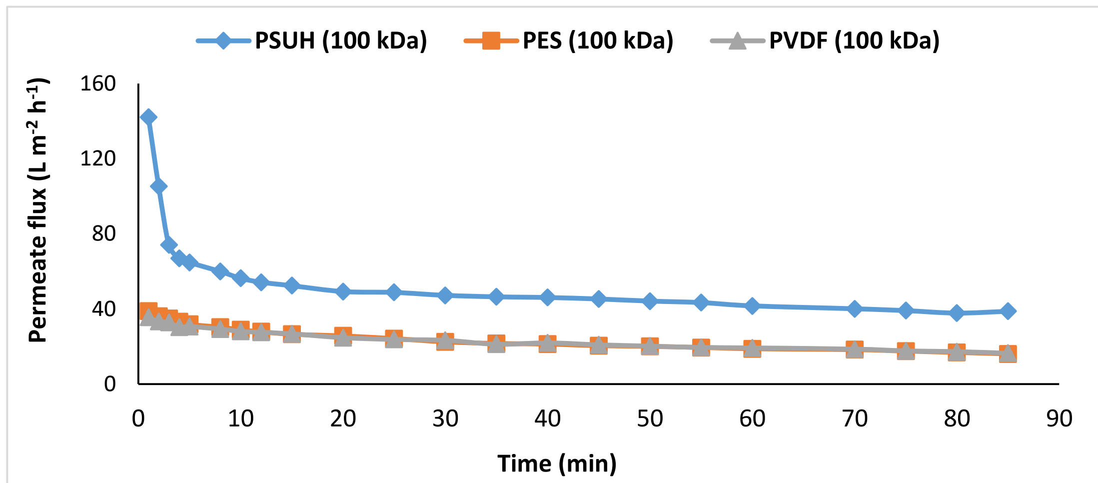
5.3.1.1. Permeate flux evaluation in ultrafiltration runs

Permeate flux evaluation of olive leaf extract (OLE) all ultrafiltration (UF) runs is presented in Figure 5.1. Primarily, the comparison of three different membrane material was performed, PES (Polyether sulfone), PVDF (Polyvinylidene fluoride) and PSU (Polysulfone). Figure 5.1a shows the effect of membrane material on the permeate flux, while molecular weight cut-off (MWCO) and volume reduction factor (VRF) of UF runs were kept constant at 100 kDa and 2, respectively. PES and PVDF membranes showed almost similar permeate flux results with no significant fouling index. A significant difference can be noted between PSU membrane with the others. Although a large fouling index is occurred with PSU in first minutes, but its steady state flux (J_{ss}) is still considerably higher comparing to PES and PVDF membranes (Figure 5.1a). TMP of all UF runs were kept constant at 7 bars.

Table 5.1. Characteristics of the used membranes.

Treatment	NF	NF	NF	NF	UF	UF	UF	UF	UF
Name	NF270	NF90	NP030	N0P10	ST,PES	MK,PES	LY,PES	BY,PVDF	US100
Type	Flat sheet	Flat sheet	Flat sheet	Flat sheet	Flat sheet	Flat sheet	Flat sheet	Flat sheet	Flat sheet
Manufacturer	Dow Filmtec™	Dow Filmtec™	Microdyn-Nadir	Microdyn-Nadir	Synder™	Synder™	Synder™	Synder™	Microdyn-Nadir
Material	Polyamide	Polyamide	PES	PES	PES	PES	PES	PVDF	Polysulfone
Cut-off	200~400 Da	200~400 Da	500 Da	1000-1200 Da	10 kDa	30 kDa	100 kDa	100 kDa	100 kDa
pH range	2-11	2-11	0-14	0-14	2-11	2-11	1-11	1-11	1-14
Water permeability ($L\ m^{-2}\ h^{-1}\ bar^{-1}$)	10.5	6.3	7.1	7.5	25	25	144	140	>100

* *UF: ultrafiltration; NF: nanofiltration*



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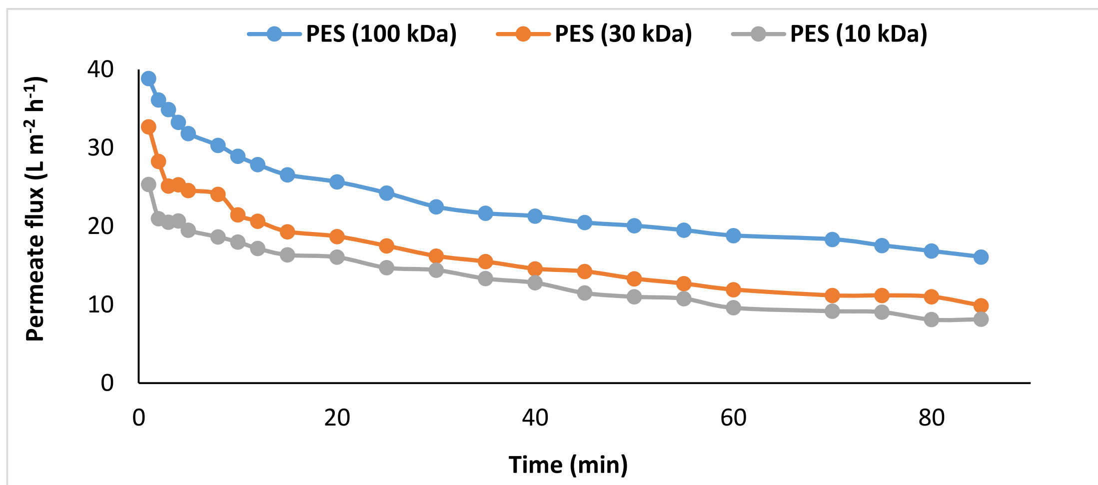


(b)

Figure 5.1. Permeate flux evaluation in Ultrafiltration test runs.

(a): Effect of different membrane materials with same MWCO (100 kDa, VRF=2) ; **(b):** Effect of different MWCO for PES membrane.

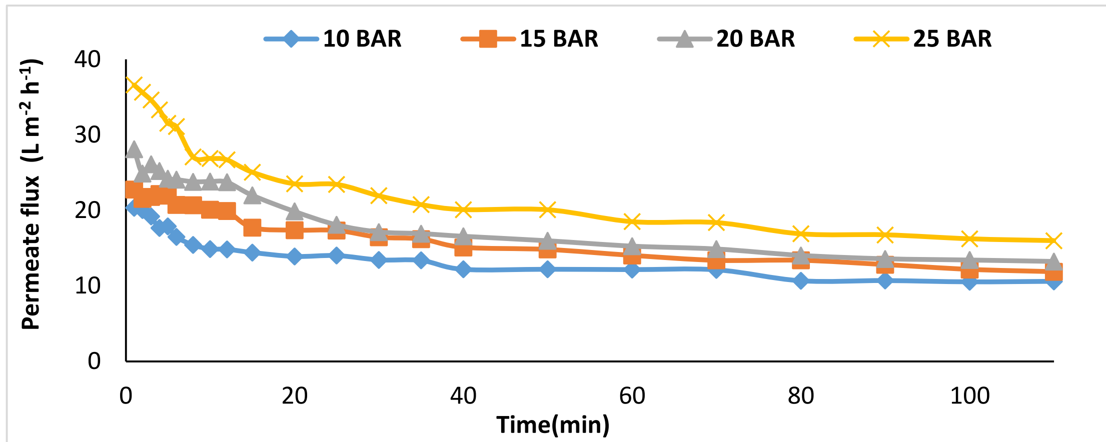
In Figure 5.1b, the effect of membrane MWCO on the permeate flux can be observed. For this purpose, three PES membranes were tested with 100, 30 and 10 kDa MWCO. Results obtained indicate that PES 100 kDa presented a better performance, with higher initial and steady state flux. Regarding PES 30 and 10 kDa membranes, the similar pattern of flux reduction was observed which indicates that membrane's larger pore size shows better results against fouling and flux reduction.

Figure 5.1c shows the overall comparison between all performed ultrafiltration runs. As it is obvious, PSUH membrane with 100 kDa MWCO has the optimal permeate flux. In addition, it can be concluded from Figure 5.1 (a,b,c) that not only the membrane pore size but also the membrane material can have significant effect the permeate flux and the performance of the operation.

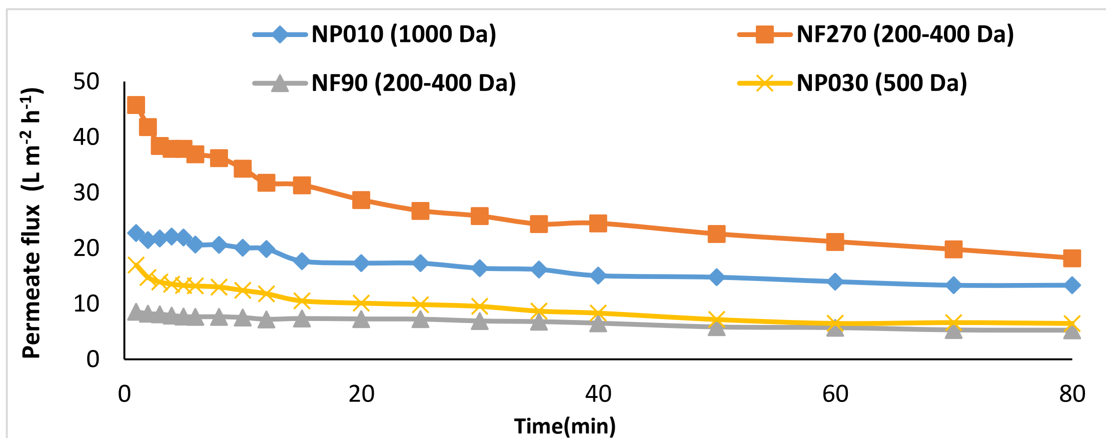
Similar studies has been executed in the literature. Based on the study of Barth *et al.* [35] a comparison between PES and PSU membranes showed that the air fluxes were higher for PSU while liquid fluxes were higher for the latter products [35]. Furthermore, there are other factors that can influence the permeate flux which are transmembrane pressure, and interactions between solutes and membranes [36].

5.3.1.2. Permeate flux evaluation in nanofiltration runs

Effect of pressure on permeate flux in nanofiltration test runs was studied and plotted in Figure 5.2(a,b). As it can be seen in Figure 5.2a, membrane NP010 was tested with different pressures of 10, 15, 20, and 25 bar by using previously microfiltered OLE as feed. This Figure indicates that the test runs with 25 bar pressure showed optimal steady state flux in comparison with other test runs. However, it had the higher fouling percentage with 56.36% measured based on Eq. (3). While, the test runs with 10, 15 and 20 bar pressure showed the fouling percentages of 52.18%, 52.88% and 55.47%, respectively. Test run with 25 bar showed more promising results with about 30% higher steady state flux. However, considering economic and safety considerations, test run with 15 bar pressure was chosed as selected operating pressure for the rest of NF experiments with other membranes.



(a)



(b)

Figure 5.2. Effects of pressure and membrane MWCO on permeate flux in nanofiltration runs. **(a):** transmembrane pressure (TMP); **(b):** membrane MWCO; (TMP=15 bar, VRF=2)

As can be seen in Figure 5.2b, nanofiltration membranes NP010, NF90, NF270 and NP030 were tested at 15 bar pressure and VRF=2. The obtained results showed that NF270 membrane (200-400 Da) had higher initial and steady state flux in comparison with other membranes although it has smaller MWCO than NP010 and NP030 membranes. It also showed the highest fouling index comparing to other membranes. However, this significant difference and advantage in permeate flux of NF270 could be attributed to NF270 membrane material. NF90 (200-400 Da) showed minimum fouling index and also minimum steady state flux among membrane materials. Regarding

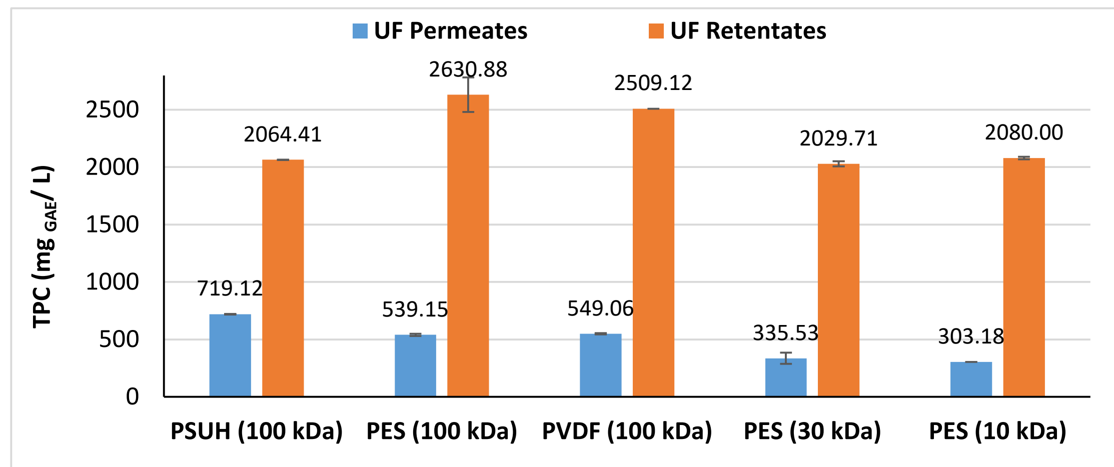
NP010 (1000 Da) and NP030 (500 Da) membranes, NP010 showed more promising results comparing to NP030. They both showed low fouling index but the steady state flux obtained by NP010 was considerably higher than NP030.

5.3.2. Evaluation of bioactive compounds separation in membrane based operations

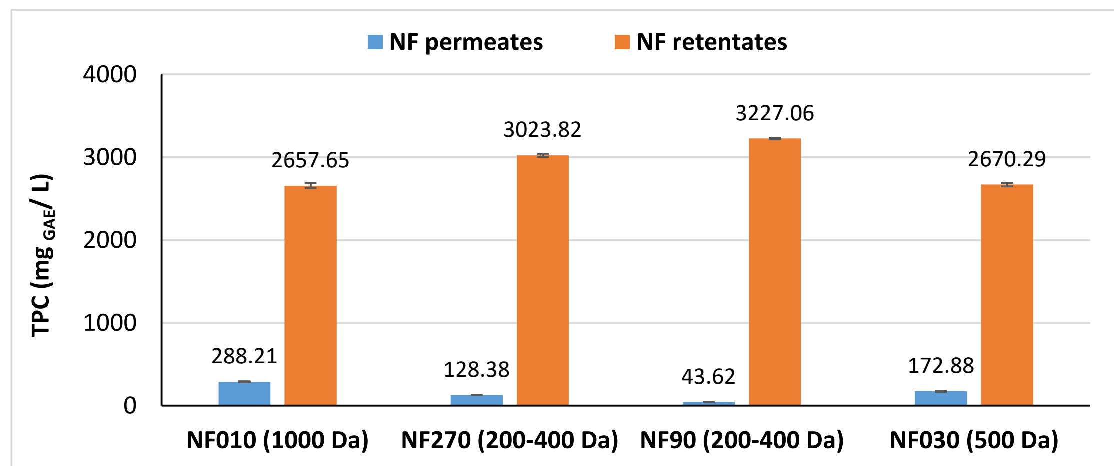
5.3.2.1. Efficiency UF and NF operations in separation of phenolic compounds

Figure 5.3(a,b) shows the effect of different membrane types and MWCO on total phenolic content (TPC) in UF and NF test runs. As can be seen in Figure 5.3a, three UF runs were performed with different materials but same MWCO (100 kDa), while the TMP and VRF were kept constant at 7 bar and 2, respectively. In addition, two UF runs with PES (30 and 10 kDa) were performed. However, optimal TPC retention was obtained by means of PES (100 kDa) with 2631±151 mg_{GAE}/L of TPC in retentate. PES membrane with 30 and 10 kDa MWCO showed nearly similar retention capabilities of TPC and lower TPC content in permeate stream with 303.2±0.7 obtained by PES (10 kDa) being the lowest. However, it can be concluded the advantage of 100 kDa MWCO in comparison with 30 and 10 kDa MWCO in TPC retention since higher results were obtained by them (Figure 5.3a). In other words, shrinkage of the MWCO in the UF operations showed an indirect relation with TPC retention.

The effects of MWCO in NF test runs on total phenol retention is presented in Figure 5.3b. Unlike to UF runs, MWCO reduction showed direct relation with TPC retention in the NF runs. Whereas, NF90 (200-400 Da) showed optimal results with 3227.06±9.98 mg_{GAE}/L in retentate and 43.61±0.62 mg_{GAE}/L in permeate stream. NF90 and NF270 have same MWCO, but the results obtained by NF90 are better. However it should be mentioned the advantage of NF270 in considerably higher permeate flux as already mentioned and can be seen in Figure 5.2b. Regarding NP030 and NP010, since their obtained TPC results are not significantly different (P values ≤ 0.05), but NP010 already showed better permeate flux results (Figure 5.2b). Therefore, NP030 can be considered as a better option in the case of TPC retention.



(a)



(b)

Figure 5.3. Effects of different membrane type and MWCO on total phenolic content (TPC). (a): Ultrafiltration runs; (b): Nanofiltration runs

Table 5.2. Mean values of individual phenolic compounds in UF and NF runs.

	Sample type		**Hydroxytyrosol** (mg/L)	**Catechin** (mg/L)	**Rutin** (mg/L)	**Verbascoside** (mg/L)	**Luteolin-7-O-glucoside** (mg/L)	**Oleuropein** (mg/L)
	Feed		17.4 ± 0.9	9.7 ± 0.9	7.8 ± 0.9	11.9 ± 0.2	71.1 ± 0.2	26.9 ± 0.7
UF	PES (100 kDa)	PE	59.1 ± 0.3	1.6 ± 0.1	0.3 ± 0.05	7.1 ± 0.1	19 ± 0.1	136.9 ± 0.4
		RE	33.7 ± 0.4	2.3 ± 0.2	0.7 ± 0.1	11.9 ± 0.1	48.9 ± 0.2	107.6 ± 0.2
	PVDF (100 kDa)	PE	43.6 ± 0.4	1.2 ± 0.2	0.0	2.1 ± 0.1	2.6 ±0.1	54.8 ± 0.3
		RE	15.9 ± 0.8	3.2 ± 0.1	0.1 ± 0.03	8.4 ± 0.2	44.5 ± 0.9	15.1 ± 0.9
	PSU (100 kDa)	PE	57.7± 0.2	2.8 ± 0.1	0.0	3.1 ± 0.1	8.3 ± 0.2	34.2 ± 0.5
		RE	3.9 ± 0.4	3.6 ± 0.9	0.0	6.2 ± 0.1	39 ± 0.3	4.4 ± 0.1
	PES (30 kDa)	PE	11.9 ± 0.1	3.9 ± 0.1	0.0	2.1 ± 0.1	6.7 ± 0.1	31.2 ± 0.1
		RE	12.3 ± 0.7	23.9 ± 0.9	1.1 ± 0.2	18.2 ± 0.3	117.6 ± 1.2	23.1 ± 0.1
	PES (10 kDa)	PE	27.0± 0.6	0.9 ± 0.1	0.3 ± 0.2	1.7 ± 0.1	4.7 ± 0.1	38.9 ± 0.1
		RE	0.6 ± 0.1	7.5 ± 0.9	11 ± 0.6	20.9 ± 0.2	118.1 ± 0.6	40.4 ± 1.2
NF	NF010 (1000 Da)	PE	18.3 ± 0 .5	0.2 ± 0.3	3.1 ± 0.2	0.9 ± 0.1	1.6 ± 0.2	1.6 ± 0.2
		RE	22.8 ± 0.2	14.4 ± 0.3	19.8 ± 0.9	12.7 ± 0.1	33 ± 0.2	28.6 ± 1.1
	NF030 (500 Da)	PE	8.75 ± 0.01	0.0	0.89±0.18	0.87±0.05	1.48±0.01	6.97±0.15
		RE	22.5 ± 0.9	5.19±0.60	10.34±0.25	18.31±0.06	98.71±0.31	11.90±0.17
	NF270 (200-400 Da)	PE	27.47 ± 0.01	0.03±0.21	0.46±0.16	0.28±0.04	0.57±0.05	2.66±0.32
		RE	152.4 ± 0.5	7.04±0.99	28.80±0.24	16.73±0.03	43.98±0.02	133.51±1.52
	NF90 (200-400 Da)	PE	2.8 ± 0.01	0.0	0.0	0.22±0.01	0.38±0.05	1.78±0.52
		RE	75.5 ± 0.2	4.26±0.21	27.79±0.67	16.90±0.20	43.49±0.01	148.24±2.99

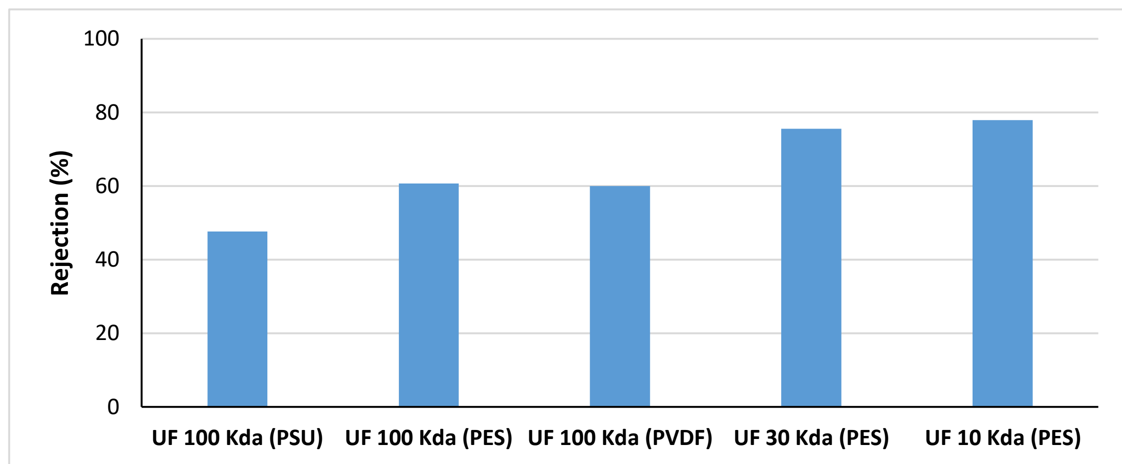
** UF: ultrafiltration; NF: nanofiltration; PE: permeate; RE: retentate*

Table 5.3. Mean values of individual Saccharides and polyols in UF and NF runs.

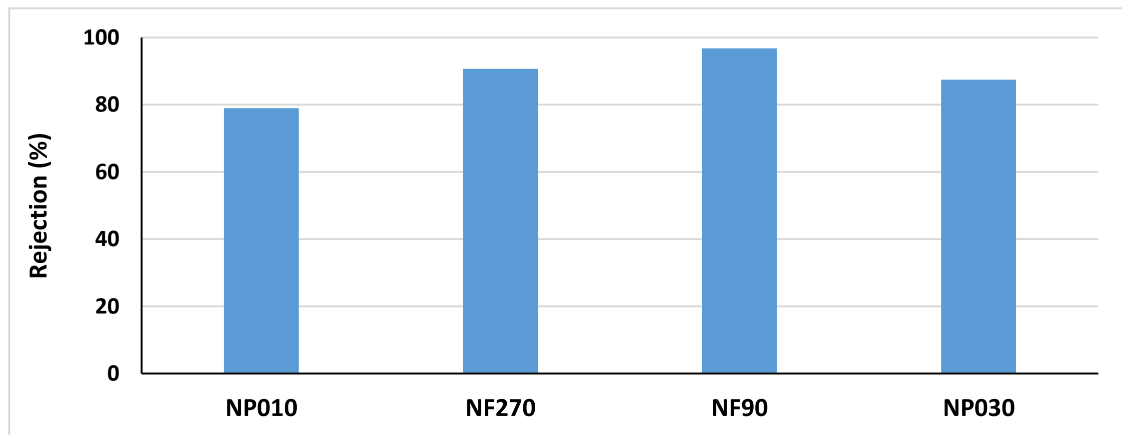
Sample type			Sucrose (mg/L)	Glucose (mg/L)	Xylose (mg/L)	Mannitol (mg/L)	Arabinose (mg/L)
	Feed		318 ± 4	2419 ± 4	480 ± 8	2668 ± 20	270 ± 71
UF	PES (100 kDa)	PE	75 ± 3	1193 ± 15	231 ± 23	1283 ± 26	78 ± 4
		RE	535 ± 81	3604 ± 39	720 ± 83	3352 ± 6	233 ± 5
	PVDF (100 kDa)	PE	129 ± 19	1451 ± 21	314 ±16	1530 ± 10	93 ± 1
		RE	452.4 ± 0.5	3277 ± 88	719 ± 20	3025 ± 20	225 ± 10
	PSU (100 kDa)	PE	188 ± 3	1716 ± 26	326 ± 37	2033 ± 69	108 ± 14
		RE	435 ± 12	3025 ± 39	590 ± 33	3253 ± 24	213 ± 13
	PES (30 kDa)	PE	63 ± 1	724 ± 9	136 ± 13	1012 ± 13	0.00
		RE	655 ± 3	3599 ± 18	804 ± 18	3695 ± 15	275 ± 12
	PES (10 kDa)	PE	38 ± 1	614.6 ± 0.9	139 ± 2	861 ± 3	0.00
		RE	731 ± 2	3855 ± 120	839 ± 34	3866 ± 1	268 ± 4
NF	NF010 (1000 Da)	PE	53 ± 4	831 ± 0.1	171 ± 5	847 ± 2	0.0
		RE	623 ± 51	4209 ± 3	781 ± 13	3937 ± 8	242 ± 10
	NF030 (500 Da)	PE	27 ± 5	367 ± 1	77 ± 3	520 ± 5	0.0
		RE	732 ± 17	3880 ± 57	819 ± 32	4052 ± 26	278 ± 20
	NF270 (200-400 Da)	PE	4.93 ±0.09	72 ± 2	12 ± 0.9	87 ± 0.1	0.0
		RE	613 ± 39	5399 ± 50	961 ± 50	4981 ± 9	278 ± 42
	NF90 (200-400 Da)	PE	4.7 ± 0.3	35 ± 0.1	5.61 ± 0.1	40 ± 0.3	0.0
		RE	525 ± 14	4908 ± 41	994 ± 1	4845 ± 11	339 ± 15

** UF: ultrafiltration; NF: nanofiltration; PE: permeate; RE: retentate*

Figure 5.4 (a,b) shows TPC rejection (%) of each membrane which was calculated based on Eq.(3). As it is presented in Figure 5.4a, PES (10 kDa) showed the highest TPC rejection which proves that MWCO reduction has a direct relation with TPC. Therefore, PES (10 kDa) can be considered as the optimal UF membrane for the recovery of TPC. The similarity of the obtained results of nanofiltration can also be observed in Figure 5.4b, which shows the rejection percentages (Eq.(3)) of TPC compounds. NF90 gained the highest TPC rejection by 96.8% value.



(a)



(b)

Figure 5.4. Rejection percentages of TPC in membrane operations.
(a): Ultrafiltration (TMP=7 bar, VRF=2); **(b):** Nanofiltration (TMP=15 bar, VRF=2)

5.3.2.2. Identification and quantification of individual phenolic compounds by HPLC/DAD

The mean values and standard deviation of individual phenolic compounds in permeate and retentate for of all performed UF and NF runs are presented in Table 5.2. The identified phenolic compounds are hydroxytyrosol, catechin, rutin, verbascoside, luteolin-7-O-glucoside, and oleuropein. It should be mentioned that VRF of all UF and NF test runs were fixed at 2. The operational TMP of all UF runs was fixed at 7 bar and 15 bar for NF runs. In addition, the main focus of this study was the purification of individual phenolic compounds, mainly oleuropein and hydroxytyrosol being the most abundant phenolic compounds in Olive by-products.

As can be seen in Table 5.2, the highest values for oleuropein and hydroxytyrosol in UF test runs were 137+0.4 and 59+0.3 mg/L, respectively, which were obtained from permeate stream of PES (100 kDa). In addition, PVDF (100 kDa) and PSU (100 kDa) membranes showed satisfactory permeability for hydroxytyrosol but considerably lower oleuropein permeability in comparison with PES (100 kDa). Regarding catechin, retentate of PES (30 kDa) showed better results with 24±0.9 mg/L which had considerable difference comparing to other runs. Retentate of PES (10 kDa) showed optimal results for rutin, verbascoside and luteoin-7-O-glucoside, with 10.9±0.6, 20.8±0.2, 118.2±0.7 mg/L, respectively. It is important to note that the membrane operations in this study are single process and the desired fraction in each UF or NF test run is permeate stream, since the separation and purification of bioactive compounds takes place on it and has less impurities comparing to retentate.

Table 5.2 also indicates mean values of individual phenolic compounds in permeate and retentates of NF test runs. Due to lower MWCO of membranes and higher selectivity of bioactive compounds in NF runs, the higher differences between mean values obtained in permeates and retentates are more transparent than UF test runs. NF270 and NF90 which have the lowest MWCO, proved to have optimal results regarding oleuropein and hydroxytyrosol separation. These two membranes showed almost similar behavior against retentions of phenolic compounds. Although, NF90 showed highest oleuropein retention with 149±3 mg/L in its retentate, but NF270

showed optimal hydroxytyrosol value of 152.40±0.52 in its retentate. Retentate of NP030 membrane showed highest retention of luteoin-7-O-glucoside with 98.7±0.3 mg/L. Regarding rest of individual phenolic compounds, no significant difference was observed in membrane retentates or permeate streams.

It can be concluded from the comparison of the UF and NF membrane filtration runs that in overall aspect, NF90 retentate fraction has more satisfactory results in comparison with other fractions. Specifically, it showed optimal hydorxytyrosol and oleuropein content which are the main focus of phenolic recovery in this study.

5.3.2.3. Identification and quantification of individual sugar compounds by HPLC/RID

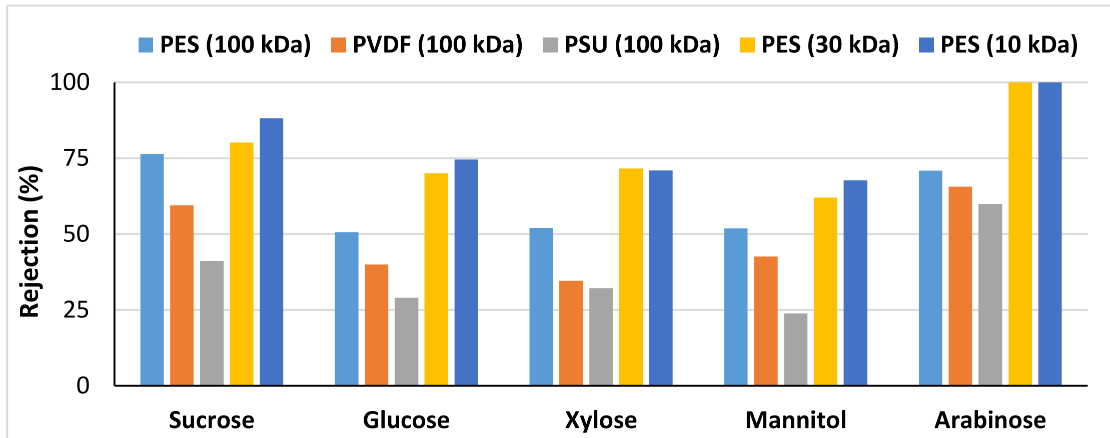
After analyzing the phenolic content of membrane test runs, identification and quantification of individual sugar compounds were performed by means HPLC/RID. For this purpose, the contents of sucrose, glucose, xylose, mannitol, and arabinose were quantified in all UF and NF test fractions. The evaluation of sugar content was performed in two ways. Primarily, by considering the identified sugars as undesired compounds in membrane operations which have to be separated and isolated from major phenolic compounds like oleuropein. On the other hand, olive by-products like OLE are also a source of valuable sugars specially mannitol. Therefore, another focus in this study was dedicated to obtaining a membrane fraction or composition rich in mannitol which can have many further application in multiple industrial sectors.

Mean values of the quantified sugar compounds in all UF and NF tests are presented in Table 5.3. Results obtained in UF test runs indicate that, there is less complication in separation of sugar compounds in comparison with separation of phenolic compounds. As the MWCO of the membranes decreased, the separation efficiency of the sugars was increased considerably regardless of sugar types, increasing the sugar content in the retentate. As it is presented in Table 5.3, PES (10 kDa) showed highest retention of glucose and mannitol with 3855±120 and 3866±1 mg/L, respectively, in the retentate.

Regarding the other sugar compounds (sucrose, xylose, and arabinose), PES (10 kDa) also proved to have high retention capacity comparing to other UF runs.

In addition, sugar rejection (%) of each membrane which was calculated based on Eq.(3) is shown in Figure 5.5(a,b). As it is presented in Figure 5.5a, PES (10 kDa) showed highest glucose and mannitol rejection (Eq.(3)) which were the most abundant sugar compounds in OLE. Therefore, PES (10 kDa) can be considered as the optimal UF membrane for recovery of major sugars. This was in contrary with the optimization of phenolic recovery which PES (100 kDa) showed to have better efficiency over other UF membranes. In the case of UF membranes with similar MWCO of 100 kDa, PES showed considerably better sugar rejection capacity. This means that PES (100 kDa) was optimal UF membrane for selective isolation of phenolic compounds from sugar compounds.

The results of NF test runs shown in Table 5.3 and Figure 5.5b, proved the higher efficiency of NF270 and NF90 membranes in comparison with NP030 and NP010 membranes. Somehow, NF270 showed optimal retention properties for glucose and mannitol with 5399±50 and 4981±9 mg/L, respectively. Although, the obtained results by NF90 were very close to NF270. The similarity of the obtained results of NF270 and NF90 can also be observed in Figure 5.4b, that shows the rejection percentages (Eq.(3)) of sugar compounds. It should be noted the complete rejection of arabinose in all NF runs regardless of membrane type. In addition, comparison of NP030 and NP010 proves the advantage of NP030 for all analyzed sugar compounds in terms of higher rejection.



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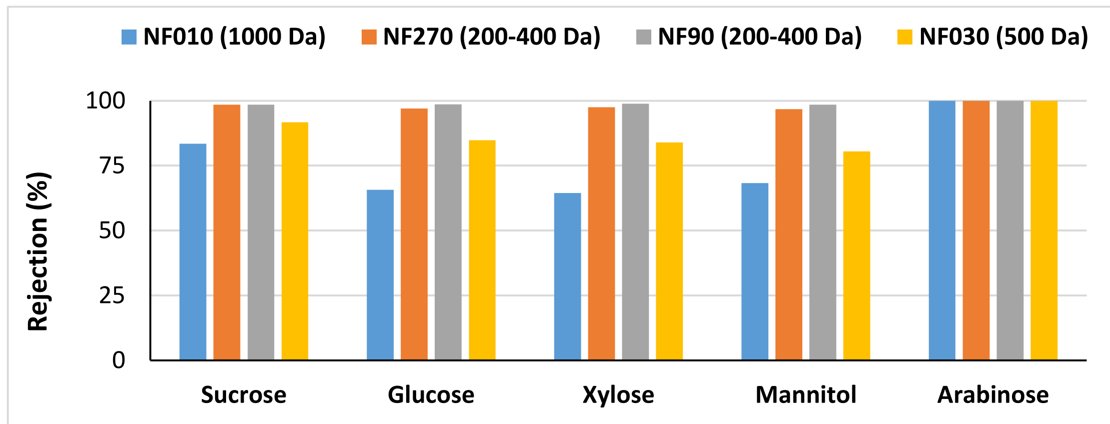


(b)

Figure 5.5. Rejection percentages of individual sugar compounds in membrane operations. **(a):** Ultrafiltration (TMP=7 bar, VRF=2); **(b):** Nanofiltration (TMP=15 bar, VRF=2)

Table 5.4. Characterization of the freeze-dried extract (FDE) from retentate of NF90.

Characterization type	Analyzed compounds	Quantity (mg/g $_{FDE}$)	FDE obtained from conventional liquid extraction (mg/g $_{FDE}$) [29]	Dry powder after adsorption/ desorption process purification (mg/g $_{FDE}$)
Phenolic content	TPC (mg $_{GAE}$/g $_{FDE}$)	120.7 ± 1.3	113 ± 1	168 ± 0.6
	TFC (mg $_{QE}$/g $_{FDE}$)	4.31 ± 0.05	13.2 ± 0.7	27.19 ± 0.09
	Hydroxytyrosol	3.45 ± 0.16	2.96 ± 0.07	0.83 ± 0.13
	Catechin	0.53 ± 0.11	6.2 ± 0.1	0.78 ± 0.14
	Verbascoside	0.78 ± 0.09	5.74 ± 0.08	2.06 ± 0.16
	Luteolin-7-O-glucoside	3.03 ± 0.06	13.8 ± 0.5	25.10 ± 3
	Oleuropein	4.16 ± 0.42	118 ± 7	232 ± 9
	Rutin	0.20 ± 0.21	n.d	0.26 ± 0.05
Sugar content	Glucose	117.0 ± 0.4	39 ± 4	15.6 ± 0.4
	Xylose	19.21 ± 0.15	n.d	4.4 ± 0.2
	Mannitol	150.1 ± 0.2	173.7 ± 0.2	22.6 ± 0.1
	Arabinose	2.91 ± 0.43	n.d	0.035 ± 0.01
Antioxidant activity	FRAP (mg $_{Fe2+}$/g $_{FDE}$)	103.7 ± 3	435±7	321 ± 9
	ABTS (mg $_{Trolox}$/g $_{FDE}$)	373.2 ± 36	230.4±0.3	362 ± 1
Chlorophyll	Ch-a	0.09	1.3 ± 0.1	1.69 ± 0.05
	Ch-b	0.112	0.54 ± 0.05	1.54 ± 0.05
	Cx+c	0.022	0.49 ± 0.04	0.025 ± 0.006

** FDE: freeze dried extract; DOL: dried olive leaf; OLE: olive leaf extract; TPC: total phenolic content; TFC: total flavonoid content*

Values with different letters in each raw are significantly different when applying the Fisher's least significant difference (LSD) method at p-value ≤0.05.

5.3.3. Optimization of membrane filtration and characterization of freeze dried extract

After evaluation of obtained permeate fluxes, identification and quantification of individual phenolic and sugar compounds, the optimal membrane test run which

showed the overall advantage over other test runs was nanofiltration process by means of NF90 membrane. Subsequently, the permeate obtained from this optimal membrane run was subjected to freeze drying process to obtain dried powders. The objective was studying the phenolic and sugar content of the dried powders and compare it with the FDE obtained from conventional liquid extraction (chapter 1) [29] and after adsorption/desorption process purification (chapter 4). In Table 5.4, all the three FDE that have been obtained in this thesis (chapters one, four, and five) are compared. It should be noted that the FDE of the membrane was obtained with water in this chapter while FDE of conventional liquid extraction gained by 80% ethanol hydroalcoholic mixture as a solvent and dry powder after adsorption/desorption process purification had 80% ethanol hydroalcoholic mixture as a solvent and more purification steps. Furthermore, chlorophyll content of the dried powders was measure whereas, its corresponding results are provided in Table 5.4. In addition, a picture of obtained dried powders after freeze drying process can be seen in Figure 5.6. In addition, scheme of the discontinuous membrane module used for test runs in this study is presented in Figure 5.7.

Oleuropein and Luteolin-7-o-glucoside results show that the obtained FDE after adsorption-desorption process was increased around 2-fold than obtained FDE of conventional extraction and in addition, increased almost 57-fold comparing to FDE obtained after membrane filtration. Regarding sugar profile, all the identified sugar compounds are significantly lower in FDE form after adsorption-desorption process compared to other FDEs. It should be mentioned that except mannitol, almost all identified sugars contents are higher in FDE of the membrane filtration than the FDE of conventional extraction which can be justified by the higher solubility of these compounds in water than ethanol. Furthermore, chlorophyll measured from FDE in both conventional extraction and after adsorption-desorption process, indicated a considerable increase comparing FDE of the membrane filtration.

Figure 5.6. Obtained dried powders after freeze drying process of optimal membrane fraction.

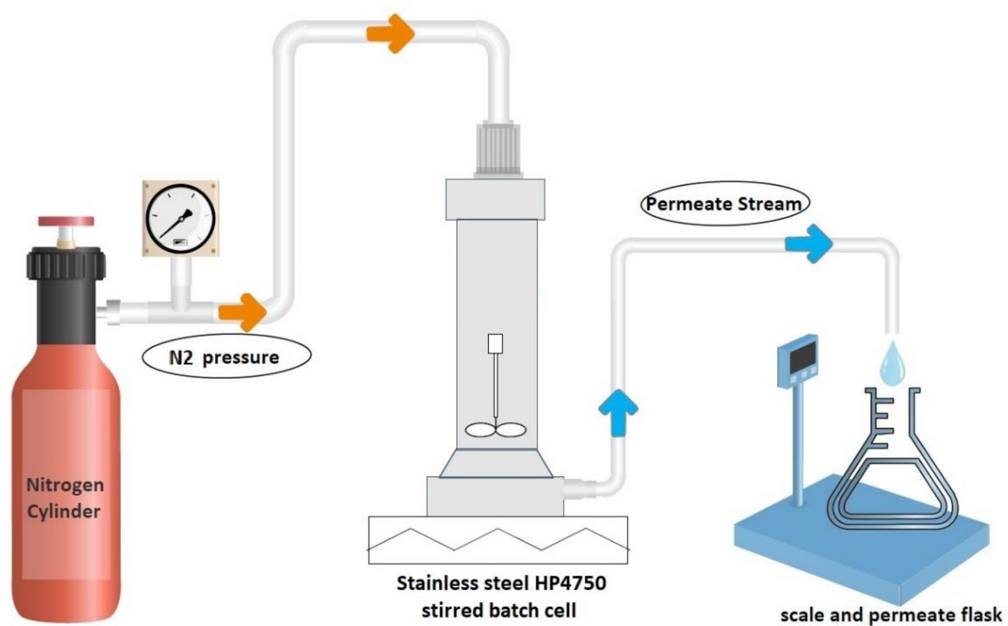


Figure 5.7. Schematic of the discontinuous stirred-cell membrane process.

5.4. Conclusions

The objective of this study was evaluating the effects of membrane filtration on sugar and phenolic content of the prepared extract form dried olive leaves. Optimization of membrane based operations was performed in terms of higher efficiency in separation of valuable bioactive compounds like oleuropein and mannitol in phenolic and sugar profiles of olive leaf extract. From total of nine membrane test runs, five of them was categorized in ultrafiltration (UF) and the other four was in nanofiltration (NF). Analysis of permeate and retentate streams showed that PES membrane with MWCO of 100 kDa was optimal membrane in UF runs since it showed optimum oleuropein content of 137±0.4 mg/L in permeate and quite satisfactory mannitol content (3352±6 mg/L) in retentate. NF90 membrane showed optimal results in NF runs where 148±3 and 4846±11 mg/L of oleuropein and mannitol was measured in retentate, respectively. Freeze drying process was then performed on the NF90 membrane retentate. Analysis of freeze dried extract showed that hydroxytyrosol and oleuropein contents increased more than 26 and 17 fold, respectively in freeze dried powders in comparison with their initial content. Regarding sugar profile, mannitol content was increased more than 3 fold comparing to initial olive leaf extract.

References

[1] E. Molina-Alcaide and D. R. Yáñez-Ruiz, “Potential use of olive by-products in ruminant feeding: A review,” *Anim. Feed Sci. Technol.*, vol. 147, no. 1–3, pp. 247–264, 2008.

[2] M. Herrero, T. N. Temirzoda, A. Segura-Carretero, R. Quirantes, M. Plaza, and E. Ibañez, “New possibilities for the valorization of olive oil by-products,” *J. Chromatogr. A*, vol. 1218, no. 42, pp. 7511–7520, 2011.

[3] A. Govaris, E. Botsoglou, A. Moulas, and N. Botsoglou, “Effect of dietary olive leaves and rosemary on microbial growth and lipid oxidation of turkey breast during refrigerated storage,” *South African J. Anim. Sci.*, vol. 40, no. 2, pp. 145–155, 2010.

[4] M. Bouaziz, I. Fki, H. Jemai, M. Ayadi, and S. Sayadi, “Effect of storage on refined and husk olive oils composition: Stabilization by addition of natural antioxidants from Chemlali olive leaves,” *Food Chem.*, vol. 108, no. 1, pp. 253–262, 2008,.

[5] O. Benavente-García, J. Castillo, J. Lorente, A. Ortuño, and J. A. Del Rio, “Antioxidant activity of phenolics extracted from Olea europaea L. leaves,” *Food Chem.*, vol. 68, no. 4, pp. 457–462, 2000.

[6] A. Čabarkapa *et al.*, “Protective effect of dry olive leaf extract in adrenaline induced DNA damage evaluated using in vitro comet assay with human peripheral leukocytes,” *Toxicol. Vitr.*, vol. 28, no. 3, pp. 451–456, 2014.

[7] J. J. Castillo, M. Alcaraz, and O. Benavente-García, “Antioxidant and radioprotective effects of olive leaf extract,” in *Olives and olive oil in health and disease prevention*, Elsevier, 2010, pp. 951–958.

[8] L. Abaza, T. P. N. Talorete, P. Yamada, Y. Kurita, M. Zarrouk, and H. Isoda, “Induction of growth inhibition and differentiation of Human Leukemia HL-60 cells by a Tunisian Gerboui olive leaf extract,” *Biosci. Biotechnol. Biochem.*, vol. 71, no. 5, pp. 1306–1312, 2007.

[9] R. Fares, S. Bazzi, S. E. Baydoun, and R. M. Abdel-Massih, “The Antioxidant and Anti-proliferative Activity of the Lebanese Olea europaea Extract,” *Plant Foods Hum. Nutr.*, vol. 66, no. 1, pp. 58–63, 2011.

[10] I. Samet, J. Han, L. Jlaiel, S. Sayadi, and H. Isoda, “Olive (Olea europaea) leaf extract

induces apoptosis and monocyte/macrophage differentiation in human chronic myelogenous leukemia K562 cells: insight into the underlying mechanism," *Oxid. Med. Cell. Longev.*, vol. 2014, 2014.

[11] A. Taamalli *et al.*, "Use of advanced techniques for the extraction of phenolic compounds from Tunisian olive leaves: Phenolic composition and cytotoxicity against human breast cancer cells," *Food Chem. Toxicol.*, vol. 50, no. 6, pp. 1817–1825, 2012.

[12] S. Fu *et al.*, "Qualitative screening of phenolic compounds in olive leaf extracts by hyphenated liquid chromatography and preliminary evaluation of cytotoxic activity against human breast cancer cells," *Anal. Bioanal. Chem.*, vol. 397, no. 2, pp. 643–654, 2010.

[13] S. Lee-Huang, L. Zhang, P. L. Huang, Y.-T. Chang, and P. L. Huang, "Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell gene expression by HIV-1 infection and OLE treatment," *Biochem. Biophys. Res. Commun.*, vol. 307, no. 4, pp. 1029–1037, 2003.

[14] M. Korukluoglu, Y. Sahan, A. Yigit, and R. Karakas, "Antifungal activity of olive leaf (Olea Europaea L.) extracts from the Trilye Region of Turkey," *Ann. Microbiol.*, vol. 56, no. 4, pp. 359–362, 2006.

[15] D. Dekanski, S. Janićijević-Hudomal, V. Tadić, G. Marković, I. Arsić, and D. M. Mitrović, "Phytochemical analysis and gastroprotective activity of an olive leaf extract ," *J. Serbian Chem. Soc.*, vol. 74, no. 4, pp. 367–377, 2009.

[16] A. Kaeidi *et al.*, "Olive (Olea europaea L.) leaf extract attenuates early diabetic neuropathic pain through prevention of high glucose-induced apoptosis: In vitro and in vivo studies," *J. Ethnopharmacol.*, vol. 136, no. 1, pp. 188–196, 2011.

[17] M. Tavafi, H. Ahmadvand, and P. Toolabi, "Inhibitory effect of olive leaf extract on gentamicin-induced nephrotoxicity in rats," *Iran. J. Kidney Dis.*, vol. 6, no. 1, pp. 25–32, 2012.

[18] W. Hur *et al.*, "Oleuropein reduces free fatty acid-induced lipogenesis via lowered extracellular signal-regulated kinase activation in hepatocytes," *Nutr. Res.*, vol. 32, no. 10, pp. 778–786, 2012.

[19] M. Alirezaei, O. Dezfoulian, A. Sookhtehzari, P. Asadian, and Z. Khoshdel,

"Antioxidant effects of oleuropein versus oxidative stress induced by ethanol in the rat intestine," *Comp. Clin. Path.*, vol. 23, no. 5, pp. 1359–1365, 2014.

[20] N. Talhaoui, A. Taamalli, A. M. Gómez-Caravaca, A. Fernández-Gutiérrez, and A. Segura-Carretero, "Phenolic compounds in olive leaves: Analytical determination, biotic and abiotic influence, and health benefits," *Food Res. Int.*, vol. 77, pp. 92–108, 2015.

[21] T. R. I. Cataldi, G. Margiotta, L. Iasi, B. Di Chio, C. Xiloyannis, and S. A. Bufo, "Determination of sugar compounds in olive plant extracts by anion-exchange chromatography with pulsed amperometric detection," *Anal. Chem.*, vol. 72, no. 16, pp. 3902–3907, 2000.

[22] V. Marsilio, C. Campestre, B. Lanza, and M. De Angelis, "Sugar and polyol compositions of some European olive fruit varieties (Olea europaea L.) suitable for table olive purposes," *Food Chem.*, vol. 72, no. 4, pp. 485–490, 2001.

[23] S. Gómez-González, J. Ruiz-Jiménez, F. Priego-Capote, and M. D. Luque de Castro, "Qualitative and Quantitative Sugar Profiling in Olive Fruits, Leaves, and Stems by Gas Chromatography−Tandem Mass Spectrometry (GC-MS/MS) after Ultrasound-Assisted Leaching," *J. Agric. Food Chem.*, vol. 58, no. 23, pp. 12292–12299, Dec. 2010.

[24] 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.*, vol. 3, pp. 69–91, 2013.

[25] H. Strathmann, L. Giorno, and E. Drioli, *An Introduction to Membrane Science and Technology*. 2011.

[26] A. Vernhet, M.-N. Bellon-Fontaine, J.-M. Brillouet, E. Roesink, and M. Moutounet, "Wetting properties of microfiltration membrane: Determination by means of the capillary rise technique and incidence on the adsorption of wine polysaccharide and tannins," *J. Memb. Sci.*, vol. 128, no. 2, pp. 163–174, 1997.

[27] A. Cassano, G. De Luca, C. Conidi, and E. Drioli, "Effect of polyphenols-membrane interactions on the performance of membrane-based processes. A review," *Coord. Chem. Rev.*, vol. 351, pp. 45–75, 2017.

[28] G. Difonzo *et al.*, "Green extracts from Coratina olive cultivar leaves: Antioxidant characterization and biological activity," *J. Funct. Foods*, vol. 31, pp. 63–70, 2017.

[29] M. Kashaninejad, M. T. Sanz, B. Blanco, S. Beltrán, and S. M. Niknam, "Freeze dried extract from olive leaves: Valorisation, extraction kinetics and extract characterization," *Food Bioprod. Process.*, vol. 124, pp. 196–207, 2020.

[30] J. E. HOFF and K. I. SINGLETON, *A method for determination of tannins in foods by means of immobilized protein*, vol. 42. 2006.

[31] A. El-Abbassi, H. Kiai, A. Hafidi, M. C. García-Payo, and M. Khayet, "Treatment of olive mill wastewater by membrane distillation using polytetrafluoroethylene membranes," *Sep. Purif. Technol.*, 2012.

[32] J. R. Fried, "Basic Principles of Membrane Technology By Marcel Mulder (University of Twente, The Netherlands). Kluwer Academic: Dordrecht. 1996. 564 pp. $255.00. ISBN 0-7823-4247-X.," *J. Am. Chem. Soc.*, vol. 119, no. 36, p. 8582, Sep. 1997.

[33] A. Zirehpour, M. Jahanshahi, and A. Rahimpour, "Unique membrane process integration for olive oil mill wastewater purification," *Sep. Purif. Technol.*, vol. 96, pp. 124–131, 2012.

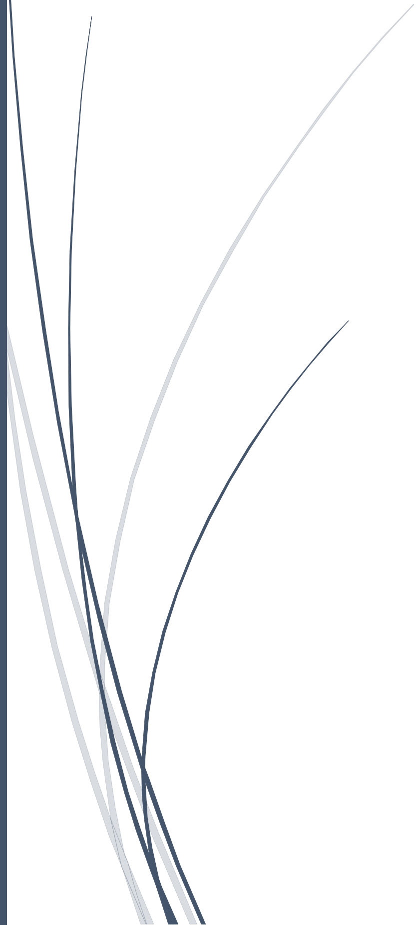
[34] C. Conidi, E. Drioli, and A. Cassano, "Membrane-based agro-food production processes for polyphenol separation, purification and concentration," *Curr. Opin. Food Sci.*, pp. 1–16, 2017.

[35] C. Barth, M. C. Gonçalves, A. T. N. Pires, J. Roeder, and B. A. Wolf, "Asymmetric polysulfone and polyethersulfone membranes : effects of thermodynamic conditions during formation on their performance," vol. 169, pp. 287–299, 2000.

[36] D. Tamires *et al.*, "Concentration of bioactive compounds from grape marc using pressurized liquid extraction followed by integrated membrane processes," *Sep. Purif. Technol.*, vol. 250, no. June, p. 117206, 2020.

Conclusions

Valorization of plant biomass by emerging technologies: olive and moringa leaves



Conclusions

The current PhD Thesis collects several experimental studies aimed on the valorization of by-products generated in olive and moringa leaves processing 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 integrating prism aligned with the proposed objectives, are presented below:

- Chemical composition of olive leaf makes this by-product an attractive raw material to be incorporated into a biorefinery context. Extractive valorisation has been proposed as the first step by using different ethanol aqueous mixtures as solvents.
- Ultrasound-assisted extraction (UAE) method was found to be technically feasible and constitutes a great opportunity for valorization of olive leaf. UAE led to faster phenolic extraction kinetics but similar extraction yield was reached as for conventional extraction.
- Scaling-up of the phenolic extraction process will be feasible since a cost-effective extraction technology was proposed based on the use of green solvents and the use of an inexpensive and abundant raw material resulting to obtaining an extract with good antioxidant properties.
- Extractives rich in bioactive compounds obtained from *Moringa oleifera* leaves make this plant an attractive raw material as nutraceutical products source. Using different hydroalcoholic mixture showed extraction efficiency depends mainly on solvent composition.
- Due to the high antioxidant capacity and rich phenolic composition, *Moringa oleifera* freeze-dried extract could be used an additive for food, pharmaceutical or cosmetic industries.
- Comparison of bioactive compounds extractions in semi-continuous and discontinuous systems by using subcritical water showed results in close range. However, controlling temperature being the most significant factor in extraction efficiency was found to be more accessible in semi-continuous system proposed in this work.

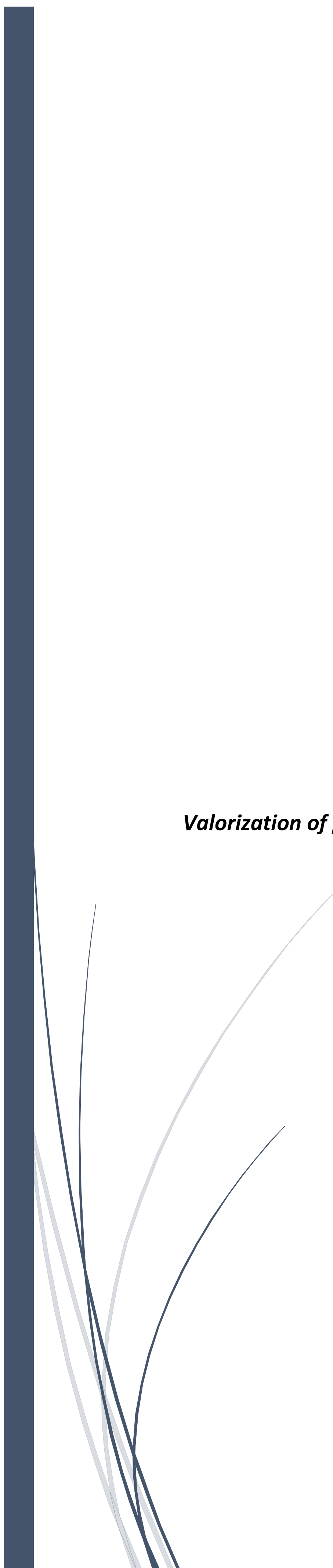
- Adsorption-desorption process was found to be completely efficient in terms of high phenolic recovery and sugar rejection in order to achieve an oleuropein rich composition with minimum impurities. This was led to considerable selective isolation and purification of phenolic compounds from sugar content inside olive leaf extracts.
- Membrane filtration technology performed within ultrafiltration and nanofiltration operations was found to be an efficient method for separation and recovery of bioactive compounds from olive leaf extract. The obtained retentate from optimal nanofiltration run was entered to freeze-drying process. Analysis of freeze dried extract showed that specifically, contents of oleuropein, hydroxytyrosol and mannitol which are valuable bioactive compounds in olive leaf were increased in an extensive level.

Conclusiones

La actual Tesis Doctoral recoge diversos estudios experimentales encaminados a la valorización de los subproductos generados en el procesamiento de hojas de olivo, así como la valorización de hojas de moringa mediante 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, formulados bajo un prisma integrador alineado con los objetivos propuestos:

- La composición química de la hoja de olivo hace de este subproducto una materia prima atractiva para ser incorporada en un contexto de biorrefinería. La valorización de la fracción formada por los extractivos, es decir de aquellos compuestos que se extraen fácilmente, se ha propuesto como primer paso para conseguir la valorización de este subproducto. Para ellos, se propone el uso de diferentes mezclas acuosas de etanol como disolventes.
- Se encontró que la extracción asistida por ultrasonido (EAU) es técnicamente factible y constituye una gran oportunidad para la valorización de la hoja de olivo. La extracción mediante ultrasonidos condujo a una cinética de extracción de compuestos fenólicos totales más rápida. Sin embargo, el rendimiento de extracción fue similar al de la extracción convencional.
- La obtención de extractos de hojas de oliva propuesta en este trabajo es factible ya que se propuso una tecnología de extracción rentable basada en el uso de solventes verdes y el uso de una materia prima abundante y económica obteniendo un extracto con buenas propiedades antioxidantes.
- Los extractos ricos en compuestos bioactivos obtenidos de las hojas de Moringa oleifera hacen de esta planta una materia prima atractiva como fuente de productos nutracéuticos. Al igual que para las hojas de olivo, la variable más importante resultó ser la relación de etanol en las mezclas hidroalcohólicas empleadas como disolvente.

- Debido a la alta capacidad antioxidante y la rica composición fenólica, el extracto liofilizado de Moringa oleifera podría utilizarse como aditivo para las industrias alimentaria, farmacéutica o cosmética.
- La extracción de compuestos bioactivos en hojas de olivo se realizó también empleando agua subcrítica, utilizando tanto un sistema semicontinuos como un sistema en discontinuo.. Se observó un mejor control de la temperatura para el sistema semicontinuo empleado en este estudio. Se observó también que la temperatura fue el factor más importante para la obtención de extractos rico en compuestos bioactivos.
- Se encontró que el proceso de adsorción-desorción fue eficiente en términos de alta recuperación fenólica y rechazo de azúcares para lograr un egluente final con una composición rica en oleuropeína y con un contenido mínimo de impurezas. Esto condujo a un aislamiento selectivo y a la purificación de compuestos fenólicos dentro de los extractos de hojas de olivo.
- Se comprobó que la tecnología de separación por membranas,ultrafiltración y nanofiltración, es una tecnología eficaz para la separación y recuperación de compuestos bioactivos del extracto acuoso de hoja de olivo. El retenido obtenido en la separación óptima de nanofiltración fue liofilizado para obtener un producto sólido. El análisis del extracto liofilizado mostró que e el contenido de oleuropeína, hidroxitirosol y manitol, que son compuestos bioactivos valiosos en la hoja de olivo, aumentaron en el extracto liofilizado final con respecto al extracto acuoso inicial de partida.



Appendix

Valorization of plant biomass by emerging technologies: olive and moringa leaves

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1. Quantification and identification of phenolic content

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 thousands 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 mainly comprise 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 and neurodegenerative diseases) associated with increased cellular oxidation processes, known as oxidative stress.

1.1. Determination of total phenolic content (TPC)

Total phenolic content (TPC) for each sample was measured using Folin-Ciocalteu standard method [1] with some modifications. This 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^- → 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 the aforementioned reagent by the polyphenols to be measured and those non-polyphenolic "interfering" components present in a sample.

Reagents

- Folin-Ciocalteau: commercial reagent, mixture of phosphowolframate and phosphomolybdate (kept at 4 °C)
- Sodium carbonate (Na_2CO_3) 7.5% (w/v) dissolved in distilled water
- Gallic acid
- Distilled water

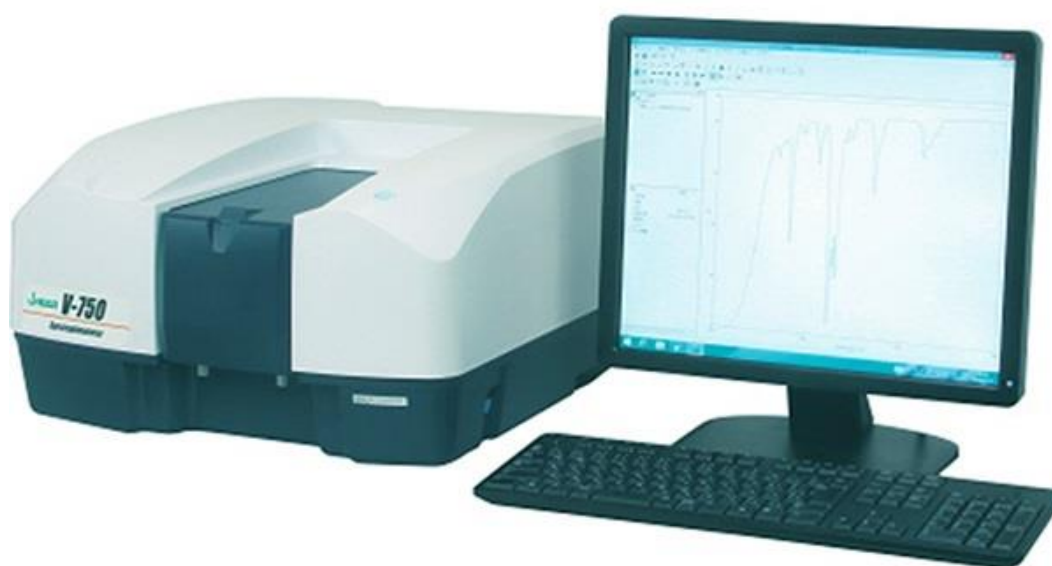

Figure A.1. Spectrophotometer V-750, Jasco, Japan**.**

Development

Total phenolic content (TPC) were determined by using the Folin-Ciocalteau reagent (VWR) [2]. Briefly, 100 µL of the extract were mixed with 2.8 mL of water and subsequently with 100 µl of the Folin-Ciocalteau reagent. After that, 2 mL of sodium carbonate 7.5 % (w/v) were added to the mixture. Absorbance was measured at 750 nm after 60 min of reaction (spectrophotometer V-750, Jasco, Japan- Figure A.1). A calibration curve was prepared with standard solutions of gallic acid and results were expressed as mg gallic acid equivalent (GAE) per gram dried olive leaf (mg/g) or per liter of olive leaf extract (mg/L).

1.2. Determination of total flavonoid content (TFC)

Flavonoids are secondary metabolites synthesized mainly by plants from a phenylalanine molecule and three molecules of malonyl-coA, thanks to the action of an enzyme isomerase, forming a structure containing 2 benzene rings connected by a 3-carbon linking chain (Figure A.2). This structure can undergo many modifications and additions of functional groups, so there are a great variety of groups within this family: flavones, flavonols, flavanones, flavanols, flavanonols, isoflavonoids, anthocyanidins and chalcones. 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.

Principle

Total flavonoid 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*. [3]. The aim is to determine the flavonoid content using a colorimetric method with aluminum chloride: the formed colored compounds have an absorption maximum at 415 nm, which was established as the measurement wavelength. The

absorption measured 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

The following materials, all with analytical grade, were used:

- Quercetin (used as flavonoid standard in this study)
- Ethanol
- Aluminum chloride (10 % aqueous solution): 5 g of $AlCl_3$ were accurately weighed and dissolved with distilled water in a 50 mL volumetric flask under an extractor hood
- Potassium acetate 1 M (4.907 g of potassium acetate (CH_3COOK) diluted in 50 mL of distilled water)
- Distilled water

Preparation of the standard calibration 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 quercetin solution.

Sample preparation

Briefly, 0.5 mL of the OL extract were mixed with 2.8 mL of distilled water and 0.1 mL of $AlCl_3$ (10%, w/v). Finally, 0.1 mL of CH_3COOK (1M) and 1.5 mL of ethanol were added. Absorbance was measured at 415 nm after 30 minutes in darkness (spectrophotometer V-750, Jasco, Japan- Figure A.1). A quercetin standard curve in ethanol was determined and results were expressed as mg Quercetin Equivalent to per gram of dried olive leaf (mg_{QE}/g_{DOL}). To determine the TFC in the freeze-dried extract (FDE), a solution of 1 mg/mL of the FDE in 80 % ethanol was used for the analytical procedures.

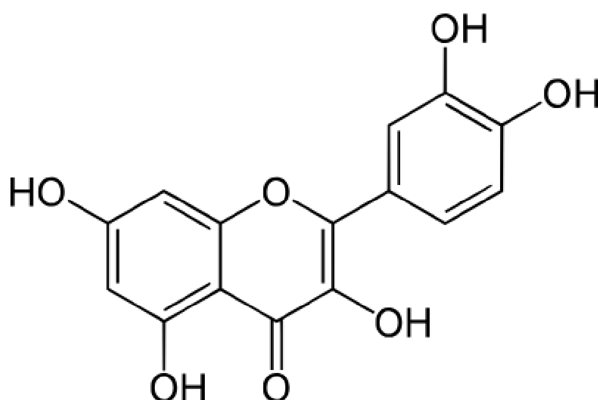

Figure A.2. Quercetin molecule, an example of flavonoid structure.

1.3. Identification and quantification of bioactive compounds by HPLC/DAD Individual phenolic compounds

Chromatographic separation was performed on a HPLC/DAD Agilent 1110 (Agilent Technologies, Inc., USA- Figure A.3) with Kinetex® 5 µm Biphenyl 100 Å, 250 × 4.6 mm column (Phenomenex, Inc. CA, U.S.A.). Phenolic compounds were analyzed by using a linear gradient of two solvents: solvent A (ammonium acetate 5 mM with 1% acetic acid in water) and solvent B (ammonium acetate 5 mM with 1% acetic acid in acetonitrile). The gradient profile involved a linear increase of solvent B: 2%, 7 min; 2–8%, 13 min; 8–10%, 15 min; 10–18%, 20 min; 18–38%, 10 min; 38–65%, 10 min; 65–80%, 5 min; 80%, 10 min. The flow rate was set to 0.8 mL/min and temperature column was 25°C. UV–vis detection was done at 240, 280, 330, 350, and 370 nm. Before injection, extracts were filtered through 0.45 µm pore size. Identification of individual phenolic compounds was carried out by comparing retention times and spectral data with those of authentic standards. Oleuropein, luteolin-7-O-glucoside, verbascoside, hydroxytyrosol, catechin, luteolin and rutin were purchased from Sigma-Aldrich. The injection volume was 10–100 µL depending on the sample content. All tests were performed in duplicate and the results were averaged. Identification of individual polyphenol compounds was carried out by comparing retention times and spectral data with those of authentic standards. Standard solutions were prepared by

dissolution of the compounds in methanol. Results were expressed as mg of phenolic compound per liter of OLE (mg/L) or per g of DOL (mg/g).

Figure A.3. HPLC-DAD Agilent 1100 equipment.

2. *Identification and quantification of individual sugar compounds by HPLC-RID*

Identification and quantification of soluble carbohydrates was performed by HPLC-RID Agilent 1260 with an AminexHPX-87H column (300 × 7.8 mm, Bio-Rad Laboratories, Inc., USA- Figure A.4) using H_2SO_4 10 mM as mobile phase with a flow rate of 0.6 mL min^{-1}. The column and detector were maintained at 40°C. Pure soluble carbohydrates were used for calibration (xylose, galactose, arabinose, glucose, and mannitol). Results were expressed as mg carbohydrate compound per liter of OLE (mg/L) or per g of DOL (mg/g).

Figure A.4. HPLC-RID Agilent 1260 equipment.

3. Antioxidant activity measurement

The purpose of this method is to determine the antioxidant activity (AA) of the products obtained according to different extraction methods. 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.
In this thesis, three following assays were used to determine the antioxidant capacity of the liquid and dried extracts: ABTS assay, DPPH assay and FRAP method. The absorbance in all three assays were measured by using a spectrophotometer (V-750, Jasco, Japan- Figure A.1)

ABTS assay

The $ABTS^+$ radical was prepared according to Re *et al.* [4]. 3 mL of a previously prepared $ABTS^{\cdot +}$ solution were added to 100 µL of the liquid extracts. After 1 h in the dark, the absorbance was measured with the spectrophotometer at 734 nm using ethanol as a blank.

DPPH assay

The method DPPH was performed as described by Brand-Williams *et al.* [5]. 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 *(2,2-diphenyl-1-picrylhydrazyl)* decreases. 60 µL of the liquid extract were mixed with 2940 µL of the DPPH reagent. Subsequently, absorbance was measured at 517 nm after 60 min of reaction in darkness. A Trolox standard calibration curve (R^2=0.99) prepared by using ethanol was used to express the antioxidant capacity of the samples as mg Trolox Equivalent (TE) per g DOL.

FRAP assay

The FRAP method was performed according to Benzie and Strain [6]. 2850 µL of the working FRAP reagent was added to 150 µL of olive leaf extract and was incubated at 37 °C for 30 min. Absorbance was read at 593 nm. As standard, a solution of $FeSO_4 \cdot 7H_2O$ (0.1 M) was used. Results were expressed in mg of $FeSO_4$ per gram of dried OL. To determine the antioxidant capacity of the freeze dried extract (FDE), a solution of 1 mg/ml of the FDE in 80 % ethanol (v/v) was used for the FRAP tests.

4. Pigments in the freeze dried extract

The amount of chlorophylls and carotenoids in the dried sample extracts were determined by dissolving 0.5 g of the sample in 10 mL of diethyl ether according to Sumanta *et al.* [7]. The mixture was homogenized and was centrifuged for 10.000 rpm for 15 min at 4 ºC. The supernatant was separated and 0.5 mL of it were mixed with 4.5 mL of diethyl ether. The equations used for Chlorophyll-a (Ch-a), Chlorophyll-b (Ch-b) and carotenoids (Cx+c) quantification were the ones collected by Sumanta *et al.* [7]:

$$\text{Ch-a} = 10.05A_{660.6} - 0.97A_{642.2} \quad (1)$$

$$\text{Ch-b} = 16.36A_{642.2} - 2.43A_{660.6} \quad (2)$$

$$\text{Cx+c} = (1000A_{470} - 1.43\text{Ch-a} - 35.87\text{Ch-b})/205 \quad (3)$$

5. Elemental analysis

Elemental analysis such as total carbon, total inorganic carbon, and total nitrogen amount was performed on liquid extracts by total organic carbon (TOC) instrument (Shimadzu TOC-V CSN- Figure A.5). The equipment is a total organic carbon and total nitrogen analyzer. It consists of four modules: automatic injector (1) (ASI-V model), total organic carbon analyzer (2) (TOC-V CSN model), total nitrogen analyzer (3) (TNM-1 model) and solids analyzer (4) (model SSM-5000A).

On the other hand, Elemental Microanalysis (CHNS) on the solid residue of the samples was made after the extraction in order to check the changes on the %Carbon, %Nitrogen, %Hydrogen and %Sulfur with the Thermo Scientific Organic FLAN 2000 Elemental Microanalyzer. The analysis technique is fully automated, and is based on the combustion of the samples under optimal conditions (T = 950-1.100 °C, pure oxygen atmosphere) to convert the aforementioned elements into simple gases (CO_2, N_2, H_2O, and SO_2) to obtain a quantitative determination.

Figure A.5. Total Organic Carbon Analyzer (Shimadzu TOC-V CSN).

Figure A.6. Thermo Scientific Organic FLAN 2000 Elemental Microanalyzer.

6. Identification the amino acid composition

The analysis of free amino acids in physiological fluids, grains, fermentation broths and other complex matrices differs from the analysis of protein hydrolysates due to the high concentration of interfering compounds. A relatively large amount of proteins, peptides, urea and other matrix components have to be removed prior to free amino acid analysis by GC or HPLC, otherwise columns deteriorate rapidly, quantitation is poor, and analysis results are not reproducible. Current procedures for de-proteinization and urea removal are labor intensive and recoveries are low for some amino acids. Also, reagents used for de-proteinization can interfere in the amino acid profile. The EZ:faast method, however, does not require traditional de-proteinization or urea removal methods to be followed; proteins are excluded from the sample as it is passes through an SPE sorbent tip. The SPE sorbent binds amino acids while proteins and urea are washed away leaving only the free amino acids.
In this study the extracts have been first prepared by EZ faast™ Amino Acid Analysis Kit for rapid determination of amino acids, this method takes fifteen minutes to prepare the sample for using in gas chromatography. Subsequently, GC Hewlett Packard 6890 was used to obtain the amino acid profiles.

7. 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 B-811 extraction system (BÜCHI Labortechnik AG, Flawil, Switzerland- Figure A.7) was used in this work for the extraction of 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 ºC and 150 ºC can be used. Furthermore, there is the possibility of supplying inert gas for drying thermolabile extracts.

Description

Step 1: 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, BÜCHI B-811 was used for Soxhlet process. This system (Figure A.7) has 4 extraction chambers. The ground olive mill solid waste (cake or pomace), about 19 g (4.7 g in each extraction chamber), 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 by distillation using the same equipment. The obtained oil was weighed and the recovery yield was calculated using Eq. (4):

$$Yield = \frac{Oil\ extract\ obtained\ (g)}{Amount\ of\ olive\ pomace\ used\ (g)} \times 100 \quad (4)$$

Figure A.7. BÜCHI B-811 extraction system.

8. Analysis of fatty acid profile by gas chromatography (GC-FID)

The fatty acid content of the extracted oils was determined by the AOAC 991.39 official method [8], which is based on the breakdown of all glycerides and subsequent derivatization to fatty acid methyl esters (FAMEs). These FAMEs were analyzed by using an Agilent gas chromatograph (6890N Network GC system, Figure A.8) 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. The chromatographic conditions were as follows: initial column temperature of 180 °C for 15 min, heated to 200 ºC at 1 ºC/min, held at 200 ºC for 1 min, heated again to 220 ºC at 5 ºC/min, and finally held at 220 ºC for 15 min. A split injector (50:1) at 250 ºC was used and the FID was also heated to 250 ºC. 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.

Reagents

- Isooctane solution of methyl tricosanoate (C23:0; 1 mg/mL), used as internal standard. It was prepared by accurately weighing 25 ± 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.8. Gas chromatography equipment (GC-FID).

Sample preparation

1 mL of internal standard (IS) solution was accurately added into a glass tube. Then, 25 ± 0.1 mg of extracted oil from olive mill solid wastes was weighed into the glass tube containing the IS and 1.5 mL of NaOH methanolic solution was added. The glass tube was capped and heated for 5 min in a water bath at 100 °C. Then it was cooled down and 2 mL of BF_3 methanolic solution was added. After mixing vigorously, the glass tube was again heated in the water bath at 100 °C for 30 min. Subsequently, the mixture was cooled, diluted with 1 mL of isooctane and then mixed in a vortex for 30 s. Immediately the mixture was diluted in 5 mL of saturated NaCl solution, mixed and left to stand for phase separation. When phases were completely separated, 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 IS and sample weights in mg, respectively; a_x is the slope of the calibration curve of each fatty acid methyl ester (FAME) standard; and $CF_{FAME\text{-}FA}$ is the ratio between molecular weight of each FAME and that of corresponding FA in each case, which is necessary to express result as mg of fatty acid (FA) / g oil rather than as FAME.

$$mg_{\ FA}/g_{\ oil} = \frac{A_x \cdot W_{I.S.}}{A_{I.S.} \cdot W_{sample} \cdot a_x \cdot CF_{FAME-FA}} \times 1000 \qquad (5)$$

References

[1] R. W. Owen, W. Mier, A. Giacosa, W. E. Hull, and B. Spiegelhalder, "Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols , simple phenols, secoiridoids, lignans and squalene," vol. 38, pp. 647–659, 2000.

[2] V. L. Singleton, R. Orthofer, and R. M. Lamuela-Raventós, "Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent," *Methods Enzymol.*, vol. 299, pp. 152–178, 1999.

[3] C. C. Chang, M. H. Yang, H. M. Wen, and J. C. Chern, "Estimation of Total Flavonoid Content in Propolis by Two Complementary Colorimetric Methods," *J. Food Drug Anal.*, vol. 10, pp. 178–182, Sep. 2002.

[4] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, and C. Rice-Evans, "Antioxidant activity applying an improved ABTS radical cation decolorization assay," *Free Radic. Biol. Med.*, vol. 26, no. 9, pp. 1231–1237, 1999.

[5] W. Brand-Williams, M. E. Cuvelier, and C. Berset, "Use of a free radical method to evaluate antioxidant activity," *LWT - Food Sci. Technol.*, vol. 28, no. 1, pp. 25–30, 1995.

[6] I. F. F. Benzie and J. J. Strain, "The Ferric Reducing Ability of Plasma (FRAP) as a Measure of 'Antioxidant Power': The FRAP Assay," *Anal. Biochem.*, vol. 239, no. 1, pp. 70–76, 1996.

[7] N. Sumanta, C. Imranul Haque, J. Nishika, and R. Suprakash, "Spectrophotometric Analysis of Chlorophylss and Carotenoids from Commonly Grown Fern Species by Using Various Extracting Solvents," *Res. J. Chem. Sci.*, vol. 4, no. 9, pp. 63–69, 2014.

[8] AOAC International, "AOAC Official Method 2012.13: Determination of Labeled Fatty Acids Content in Milk Products and Infant Formula," *AOAC Int.*, vol. 2012, pp. 1–9, 2012.