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Recovery of polyphenols by extraction and purification technologies from orange and spinach processing residues

by

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PhD program in Chemical Process Engineering

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¨A Dios sea la gloria, pues por su poder eficaz que actua en nosotros, Él puede hacer muchísimo más de lo que nos podemos imaginar o hacer. ¨

Efesios 3:20

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Abstract

Solid wastes generated during the processing of fruits and vegetables can be a potential source of bioactive compounds such as polyphenols. The use of agri-food wastes within the framework of the circular economy allows minimizing the environmental problems derived from their elimination and increasing the benefit as a source of secondary raw materials.

Orange and spinach are important sources of polyphenols (e.g., hydroxybenzoic acids, hydroxycinnamic acids, and flavonoids), and their beneficial effects on health, due to their antioxidant activity, have been widely studied.

The residues resulting from orange and spinach processing (mainly by peels and seeds, and leaves, respectively) contain significant amounts of polyphenols, so they can be used as raw materials for the recovery of natural antioxidants. In this PhD dissertation, the recovery of polyphenols from orange and spinach residues have been carried out using conventional and innovative technologies. In recent years there has been a special interest in using environmentally friendly solvents, such as water, which allow the extraction of polyphenol from raw materials of plant origin. In addition, ethanol and water, are solvents compatible with food, nutraceutical and pharmaceutical applications, suitable for the extraction of polar compounds such as polyphenols. In this thesis, mechanical stirring extraction (MSE) is used as a conventional method; besides, ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and pressurized liquid extraction (PLE) methods are also considered for the polyphenol extraction from orange and spinach wastes.

In the first instance, orange and spinach wastes were treated with UAE, MAE, and PLE, to evaluate the optimization of the polyphenol extraction using ethanol as the solvent.

UAE was selected for the two matrices, due to its simplicity and low cost and overall extraction performance, which favor its industrial scaling. The best conditions for UAE orange matrix were ethanol/water/HCl in ratio 60/39.9/0.1 (v/v/v) as the solvent at 25 °C for an extraction time of 30 min, providing 0.4 mg gallic acid equivalent (GAE)/g fresh weight (fw). For spinach matrix were ethanol/water/HCl in ratio 80/19.9/0.1 (v/v/v) as the solvent at 25 °C for an extraction time of 30 min, providing 0.82 mg GAE/g fw.

In second instance, the best conditions for orange and spinach polyphenol extraction obtained by UAE were compared with MSE using water as the solvent, obtaining that MSE would be more suitable extraction technique since it is cheaper than UAE. The selected conditions for MSE for orange and spinach wastes extraction were 70 °C, contact time of 15 min, solid/solvent ratio 1:100 and pH 4 without adjustment for orange waste; and 50 °C, 5 min, 1:50 and pH 6 without adjustment, for spinach residue. The total phenolic content (TPC) under these conditions was 1 mg GAE/g fw and 0.8 mg GAE/g fw for orange and spinach, respectively. High performance liquid chromatography (HPLC) was used for characterization of polyphenols, which is the technique of choice for the separation and quantification of polyphenols in agri-food samples. The HPLC was also used for the quantification of the major polyphenols of orange (e.g., 4-hydroxybenzoic acid, hesperidin) and spinach (e.g., ferulic acid, rutin) wastes and for the study of the optimal extraction conditions with the aforementioned extraction methods. The antioxidant activity of the MSE extracts was evaluated using assays based on 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic) acid (ABTS). As a result, extracts rich in polyphenols were obtained, with antioxidant activities of 2.27 mg Trolox equivalent (TE)/g for orange and 0.04 mg TE/g for spinach.

In addition, the orange and spinach extracts from MSE were treated by membrane technology for their separation and concentration. It was concluded that the transmembrane flux depended on the feed flow rate for MF, UF, NF, and RO. The pre-concentration and concentration efficiency were evaluated in terms of TPC and by polyphenols families —hydroxybenzoic acids (HB), hydroxycinnamic acids (HC) and flavonoids (F)—, using HPLC. For the orange and spinach matrices, MF (0.22 μm for both residues) could be used to remove suspended solids and colloids; UF (30 kDa for both residues) could be used for clarification; NF (TFCS for orange and spinach extracts) membranes could be used for the pre-concentration of polyphenols; and RO, (XLE and BW30 for orange and spinach extracts, respectively) membranes could be used for the concentration of polyphenols. Recent research has shown that polyphenol enrichment is possible using membrane technology, as these processes can be carried out at low temperatures, decreasing the thermal destruction of polyphenols. The possibility of introducing and commercializing polyphenols is fully associated with quality concerns in terms of purity. When polyphenols are to be used in food and cosmetic applications [1–3], in order to improve the nutritional profile of food products, to improve food properties, to obtain innovative natural additives for cosmetics, etc., the ideal purity is 95%. When polyphenols have a lower purity and/or are mixtures of different polyphenols, their applications are directed to animal feed [4]. For this reason, developing a membrane train for the recovery and valorization of high purity polyphenols is a major challenge.

Finally, a review was made focused on the antiviral properties of some polyphenols and their mechanism of action against various types of viruses (e.g., coronavirus, dengue, influenza). The relationship between antiviral and antioxidant activity of polyphenols was highlighted, as well as the different mechanisms of action of polyphenols against viral infections, which could be applied as a natural treatment or prevention strategy

Resumen

Los residuos sólidos generados durante el procesamiento de frutas y verduras pueden ser una fuente potencial de compuestos bioactivos como los polifenoles. El uso de los residuos agroalimentarios en el marco de la economía circular permite minimizar los problemas ambientales derivados de su eliminación y aumentar el beneficio como fuente de materias primas secundarias.

La naranja y las espinacas son una importante fuente de polifenoles (por ejemplo, ácidos hidroxibenzoicos, ácidos hidroxicinámicos y flavonoides), y sus efectos beneficiosos para la salud, debido a su actividad antioxidante, han sido ampliamente estudiados.

Los residuos resultantes del procesado de naranjas y espinacas (principalmente las cáscaras y semillas, y las hojas, respectivamente) contienen cantidades significativas de polifenoles, por lo que pueden ser utilizados como materia prima para la recuperación de antioxidantes naturales. En esta tesis doctoral se ha llevado a cabo la recuperación de polifenoles a partir de residuos de naranjas y espinacas utilizando tecnologías convencionales e innovadoras. En los últimos años ha habido un especial interés en el uso de disolventes respetuosos con el medio ambiente, como el agua, que permiten la extracción de polifenoles de materias primas de origen vegetal. Además, el etanol y el agua, son disolventes compatibles con aplicaciones alimentarias, nutracéuticas y farmacéuticas, adecuados para la extracción de compuestos polares como los polifenoles. En esta tesis, se utiliza la extracción mecánica por agitación (MSE) como método convencional; también se consideran los métodos de extracción asistida por ultrasonidos (UAE), extracción asistida por microondas (MAE) y extracción por líquidos presurizados (PLE) para la extracción de polifenoles de los residuos de naranja y espinacas.

En primer lugar, los residuos de naranja y espinacas fueron tratados con UAE, MAE y PLE, para evaluar la optimización de la extracción de polifenoles utilizando etanol como disolvente. Se seleccionó UAE para las dos matrices, debido a su simplicidad, bajo coste y rendimiento global de extracción, que favorecen su escalado industrial. Las mejores condiciones para la matriz de naranja UAE fueron etanol/agua/HCl en proporción 60/39,9/0,1 (v/v/v) como disolvente a 25 °C para un tiempo de extracción de 30 min, proporcionando 0,4 mg de ácido gálico equivalente (GAE)/g de peso fresco (fw). Para la matriz de espinacas se utilizó etanol/agua/HCl en proporción 80/19,9/0,1 (v/v/v) como disolvente a 25 °C durante un tiempo de extracción de 30 min, proporcionando 0,82 mg de GAE/g de peso fresco.

Se compararon las mejores condiciones para la extracción de polifenoles de naranja y espinacas obtenidas por UAE con la MSE utilizando agua como disolvente, obteniendo que la MSE sería la técnica de extracción más adecuada ya que es más económica que la UAE. Las condiciones seleccionadas para la MSE para la extracción de residuos de naranja y espinacas fueron 70 °C, tiempo de contacto de 15 min, relación sólido/disolvente 1:100 y pH 4 sin ajustar para el residuo de naranja; y 50 °C, 5 min, 1:50 y pH 6 sin ajustar, para el residuo de espinacas. El contenido fenólico total (TPC) en estas condiciones fue de 1 mg GAE/g fw y 0,8 mg GAE/g fw para la naranja y las espinacas, respectivamente. Para la caracterización de los polifenoles se utilizó la cromatografía líquida de alta eficacia (HPLC), que es la preferida para la separación y cuantificación de polifenoles en muestras agroalimentarias. La técnica HPLC también se utilizó para la determinación de los principales polifenoles de los residuos de naranja (por ejemplo, ácido 4-hidroxibenzoico, hesperidina) y espinacas (por ejemplo, ácido ferúlico, rutina), y para el estudio de las condiciones óptimas de extracción con los métodos de extracción mencionados. La actividad antioxidante de los extractos de MSE se evaluó mediante ensayos basados en el 2,2-difenil-1-picrilhidrazilo (DPPH), el poder antioxidante férrico reductor (FRAP) y el ácido 2,2'-azino-bis (3-etilbenzotiazolina-6-sulfónico) (ABTS). Como resultado, se obtuvieron extractos ricos en polifenoles, con una actividad

antioxidante de 2,27 mg de equivalentes Trolox (TE)/g para la naranja y de 0,04 mg de TE/g para las espinacas.

Además, los extractos de naranja y espinaca de MSE, fueron tratados por tecnología de membranas para su separación y concentración. Se concluyó que el flujo transmembrana dependía del caudal de alimentación para MF, UF, NF y RO. Se evaluó la eficacia de la preconcentración y la concentración en términos de TPC y por familias de polifenoles —ácidos hidroxibenzoicos (HB), ácidos hidroxicinámicos (HC) y flavonoides (F)—, mediante HPLC. En el caso de las matrices de naranja y espinacas, se podía utilizar MF (0,22 μm para ambos residuos) para eliminar los sólidos en suspensión y los coloides; UF (30 kDa para ambos residuos) para la clarificación; NF (TFCS para los extractos de naranja y espinacas) para los polifenoles preconcentrados; y RO (XLE y BW30 para los extractos de naranja y espinacas, respectivamente) para los polifenoles concentrados. Investigaciones recientes han demostrado que el enriquecimiento de polifenoles es posible utilizando la tecnología de membranas, ya que estos procesos pueden llevarse a cabo a bajas temperaturas, disminuyendo la destrucción térmica de los polifenoles. La posibilidad de introducir y comercializar polifenoles está totalmente asociada al control de calidad en términos de pureza. Cuando los polifenoles se van a utilizar en aplicaciones alimentarias y cosméticas [1-3], por ejemplo para mejorar el perfil nutricional de los productos alimentarios, para mejorar las propiedades de los alimentos, para obtener aditivos naturales innovadores para los cosméticos, etc., la pureza ideal es del 95%. Cuando los polifenoles tienen una pureza menor y/o son mezclas de diferentes polifenoles, sus aplicaciones se dirigen a la alimentación animal [4]. Por esta razón, el desarrollo de un tren de membranas para la recuperación y valorización de polifenoles de alta pureza es un reto importante.

Finalmente, en esta tesis, se hizo una revisión centrada en las propiedades antivirales de algunos polifenoles y su mecanismo de acción contra varios tipos de virus (por ejemplo, coronavirus, dengue, gripe). Se destacó la relación entre la actividad antiviral y

antioxidante de los polifenoles, así como los diferentes mecanismos de acción de los polifenoles contra las infecciones víricas, que podrían aplicarse como tratamiento natural o estrategia de prevención.

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GLOSSARY

UAE: ultrasound-assisted extraction

MAE: microwave-assisted extraction

PLE: pressurized liquid extraction

MSE: mechanical stirring extraction

TPC: total phenolic content

DPPH: 2,2-diphenyl-1-picrylhydrazyl

FRAP: ferric reducing antioxidant power

ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic)

FC: Folin-Ciocalteu

TE: Trolox equivalent

GAE: gallic acid equivalent

RSM: response surface methodology

MF: microfiltration

UF: ultrafiltration

NF: nanofiltration

RO: reverse osmosis

MWCO: molecular weight cut-off

HPLC: High Pressure Liquid Chromatography

HB: hydroxybenzoic acids

HC: hydroxycinnamic acids

F: flavonoids

fw: fresh weight

dw: dry weight

SLE: Solid-liquid extraction

PEF: Pulsed electric field extraction

HHPE: High hydrostatic pressure-assisted extraction

CSE: Conventional solvent extraction

RE: Rotatory extraction

RC: regenerated cellulose

TFC: thin-film composite

PES: polyethersulfone

PVP: polyvinylpyrrolidone

PPA: poly(piperazine-amide)

PA: polyamide

PS: polysulfone

PVDF: polyvinylidene fluoride

MCE: mixed cellulose esters

CA: cellulose acetate

CHAPTER 1

Introduction

1. Introduction

The high demand for natural additives has increased among consumers, encouraging the search for different sources of active compounds [5]. Polyphenols have received greater attention in recent decades, due to their natural origin and antioxidant activity [6]. Natural polyphenols are present in plant biomass providing defense against solar radiation, infections, etc [7].

The interest in natural polyphenols as an alternative to synthetic antioxidants has led to finding abundant and bio-renewable sources of these compounds, especially those of waste origin, such as the residues from the processing of fruits and vegetables [8,9]. Processing of fruit and vegetables generates high amounts of waste [10–12]. Disposal of these residues usually represents a problem and could entail to legal restrictions. Another problem is soil and water contamination because plant waste is prone to microbial spoilage [12]. Moreover, valuable nutrients contained in agri-food residues are lost. Thus, the recovery of high added-value compounds presents in these wastes, such as polyphenols (since they have antioxidant activity) can be used in the production of food, pharmaceutical, and cosmetic products, favoring an attractive circular economy (**Figure 1**).

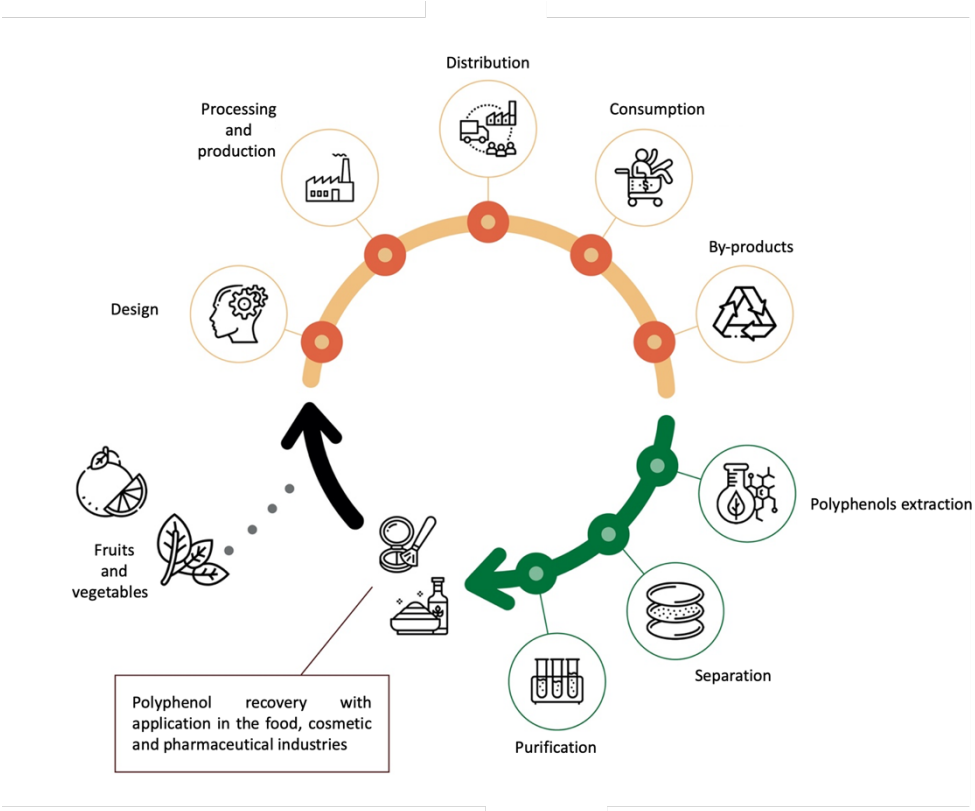


Figure 1. Scheme of the application of fruit and vegetables to the framework of the circular economy.

1.1 By-products of fruit and vegetable processing

Food waste or by-product, is an organic matter that includes a possible raw material that is generated and cannot be introduced into the processing of products [13]. Among the most common wastes generated in fruit and vegetable processing stand out peels, seeds, leaves and stems [14]. These wastes are attractive and natural sources of antioxidant polyphenols. Once they are extracted, they show their high phenolic content. This is the reason why several studies have been carried out to evaluate and apply this antioxidant power in different fields such as food, pharmaceutical and cosmetic industries [15–20]. The percentage of fruit and vegetable wastes generated during different processes are between 5-30% [21].

1.2 Polyphenols as secondary metabolites found in fruit and vegetable wastes

The antioxidants that are ingested in the human diet include different classes of compounds such as polyphenols, that can intervene in oxidative cycles, inhibiting or delaying the oxidative damage of biomolecules [22].

The main classes of antioxidants present in foods of plant origin (including fruits and vegetables) are vitamins, carotenoids and polyphenols; the latter being the most abundant group of bioactive compounds with antioxidant activity in the human diet [23–25]. Polyphenols are secondary metabolites of plants that provide different fundamental physiological functions such as growth and reproduction of plants, protection against pathogenic organisms and ultraviolet radiation; they also contribute to the organoleptic characteristics of foods of plant origin, in their color and taste [21].

Orange peels and seeds have been reported to contain 243 mg/100g fw of total phenolic content [26], besides a wide range of flavonoids like hesperidin, naringenin, hydroxycinnamic acids (e.g., *p*-coumaric acid and ferulic acid) [27]. Also, spinach leaves were found to contain 64 mg GAE/g of dry weight (dw) of total phenolic content and 33 mg/g dw of total flavonoids like rutin, patuletin, spinacetin among others [28,29].

1.2.1 Structure and classification

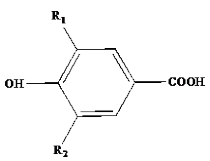
Natural polyphenols constitute one of the most abundant groups of antioxidants in the plant kingdom, with more than 8,000 known structures, of which more than 4,000 are

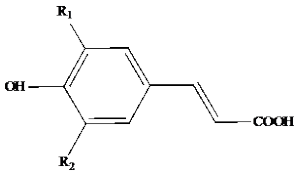
flavonoids [30,31]. Polyphenols are characterized by having one or more phenolic groups in their structure, classified into different families: phenolic acids, flavonoids, stilbenes, lignans and tannins. Of all these, phenolic acids, flavonoids and tannins are considered the main phenolic compounds in the human diet [26,32].

Phenolic acids

The recent interest in phenolic acids can be attributed to their protective role against coronary heart disease, stroke, and cancer [33,34]. These acids can be found in conjugated forms like esters and glycosides. Two subfamilies are distinguished, namely: (i) the benzoic acids if the carboxyl group is directly linked to the aromatic ring; (ii) the cinnamic acids if the carboxylic group is linked to it from a 2-propanyl substituent (**Table 1**). Mango fruit is an important source of hydroxybenzoic acids such as ferulic acid (e.g., 33.75 mg/100g fw, protocatechuic 0.77 mg/100g fw) [35,36]. The fruits that contain the highest content of hydroxycinnamic acids are blueberries, kiwis, plums, cherries, and apples which contain approximately 0.5-2 g/Kg of fresh fruit [6].

Table 1. Main phenolic acids and their structures (adapted from Montenegro et al. [7]).

Class	Structure	Substitutions	Examples
Hydroxybenzoic acids		R1: H, OH, OCH_3 R2: H, OH, OCH_3	Gallic acid Vanillic acid Procyanidin B1 Theogallin

Hydroxycinnamic acids		R1: H, OH, OCH_3 R2: H, OH, OCH_3	Caffeic acid Ferulic acid *p*-coumaric acid Rosmarinic acid

Flavonoids

They consist of the largest group of phenolic compounds in plants (including fruits and vegetables). They represent more than half of the natural polyphenols [37]. They have a wide range of therapeutic, preventive, and antiangiogenic properties, making them a rich class of polyphenols for fighting various diseases [38–40]. The general structure is often schematized as C6-C3-C6 in which the two phenolic rings A and C are linked by a pyran ring B (**Figure 3**). According to the oxidation state of the central C ring, flavonoids are divided into six subgroups: flavonols, flavones, flavanones, anthocyanidins, flavanols and isoflavones (**Table 2**) [18]. The presence of anthocyanidins contributes to the blue, red and violet colors of fruits and vegetables [41].

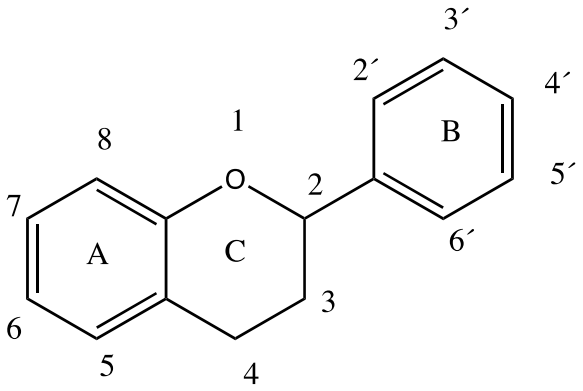
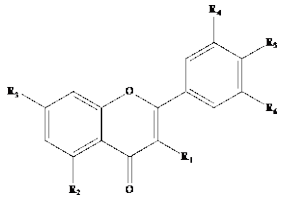
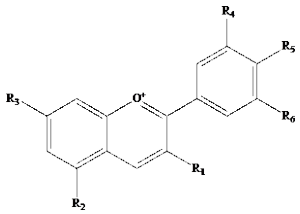
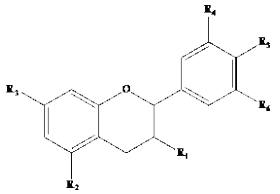
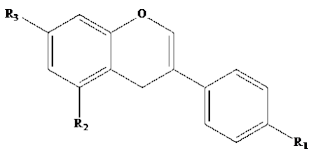
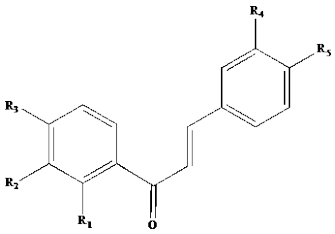


Figure 2. Basic structure of flavonoids

Table 2. Flavonoids classification and the richest food source (adapted from Montenegro et al. [7]).

Class	Structure	Substitutions	Examples	Source
Flavonols *Flavones* *Flavanones*		R1: H, OH R2: H, OH R3: H, OH R4: H, OH R5: OH, OCH_3 R6: H, OH	Hesperidin Naringenin Quercetin Kaempferol Luteolin	Onion, kale, broccoli, tomato, blueberry, apple, parsley, celery, Citrus spp.
Anthocyanidins		R1: H, OH R2: OH, OCH_3 R3: OH R4: H, OH R5: OH R6: H, OH	Cyanidin Pelargonidin	Grape, carrot, tomato

Catechins		R1-R3: OH R4: H, OH R5: OH R6: H, OH	Catechin Epicatechin Epigallocatechin	Peach, grape
Isoflavones		R1: OH R2-R3: H, OH	Genistein Daidzein	Soy bean
Chalcones		R1-R5: H, OH	Xanthohumol Phloretin Isosalipurpurin	Broad bean pod

Stilbenes

In the human diet, stilbenes are found in some plant species. Specifically in peanuts, nuts, grapes skins, leaves, stems and pulp, [7,31].

The most remarkable molecule of this family is resveratrol (**Figure 3**), this polyphenol can prevent cancer, coronary, neurological and digestive diseases [22].

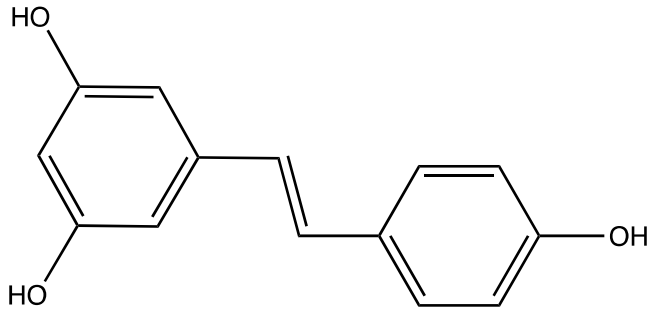


Figure 3. Chemical structure of resveratrol

Tannins and lignans

Tannins are compounds of relatively high molecular weight; they are the third most important group of phenolic compounds. They can be subdivided into hydrolysable tannins (e.g., gallic acid esters) and condensed tannins (e.g., polyhydroxyflavone-3-ol polymers). An important characteristic of tannins is their use in burn therapy, as antimicrobial agents [26].

On the other hand, lignans (**Figure 4**) are formed by oxidative dimerization of two phenylpropane units and are widely distributed in plants. The study of this family has progressed in recent years, due to it interest as a pharmacological alternative in chemotherapy of cancer [41,42].

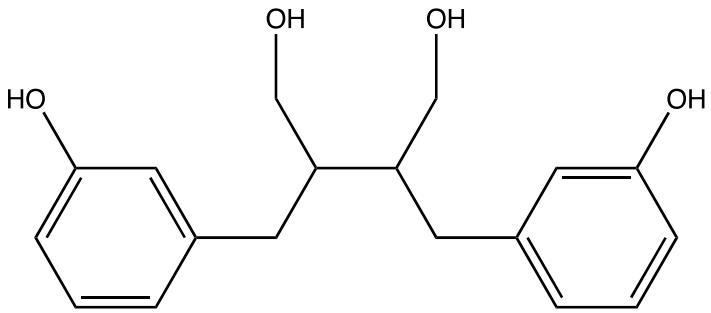


Figure 4. Chemical structure of lignan

1.2.2 Orange and spinach waste as source of polyphenols

In recent years, the world production of fruit juices has been growing. The European Association of Fruit Juices reported a consumption of 9,187 million liters of juice in the European Union in 2017, being Germany the region with the highest consumption (2,342 million liters), followed by France (1,406 million liters), England (1,079 million liters,) Spain and behind Poland with 808 million liters. Among the wide range of juices commercially available, the orange juice is the favorite for Europeans [43].

During the processing of fruits, large amounts of agro-industrial waste such as peels (representing 50%), pulp and seeds (30-35%) are generated [44]. Fruit wastes contain nutrients and biomass, which can be valuable by-products. In particular, the peels have a higher concentration of polyphenols with a higher antioxidant activity than the pulp [45]. To date, this fruit waste has been used as animal feed or disposed as organic waste, and only a small portion is used as a base for beverages or jams. However, now is time to look for new solutions, as some commercially interesting polyphenols –with

antioxidant and antiviral activity, among other properties– can be extracted from citrus peels and reused [46,47].

Vegetables also generate a large amount of agro-industrial wastes. These are still ignored sources of high added-value compounds such as polyphenols, that can contribute to sustainability goals. Especially spinach, a highly consumed vegetable worldwide, generates 25% of wastes. Spinach plays an important role in human health by meeting essential needs from food to medicine [48,49].

Valorization is part of the circular economy framework, which describes an economic system based on business models that replace the end-of-life concept by reducing, reusing, recycling and recovering materials in the production and distribution of consumption processes, achieving sustainable development with environmental quality, economic prosperity and social equality will benefit present and future generations [50,51].

The high costs of managing agri-food waste have led researchers to investigate its potential benefits and to minimize the environmental impact.

1.2.3 Phenolic compounds of orange and spinach waste

The main families of polyphenols present in orange and spinach waste are flavonoids and phenolic acids [11,52].

High amounts of polyphenols are identified in orange peels, mainly up to 72% (w/w) of the total phenolic content (TPC) are hydroxycinnamic acids usually bound to carbohydrate moieties, up to 26% (w/w) flavonoids and their derivates (e.g., hesperidin, narirutin, naringenin, tangeritin, rutin and others) [44,53,54].

According to Derrien et al. [55], spinach waste contains high level of polyphenols, with 200 mg GAE/100g of fw, especially phenolic acids (e.g., *p*-coumaric acid, ferulic acid) and flavonoids derivates (e.g., patuletin, spinacetin, luteolin, kaempferol) [20,56].

1.3 Methods of extraction and characterization of polyphenols

Currently, some research lines are focusing on environmentally friendly technologies that allow the extraction and purification of polyphenols from plant raw materials [22,57]. Extraction and purification are important from an economic, environmental and technical point of view, being non-destructive processes suitable for obtaining large amounts of the extract with high antioxidant activity.

Polyphenols have a structural diversity and complexity; thus, the extraction is the first important step in the recovery of these compounds. Polyphenols are extracted using polar organic solvents (e.g., ethanol, acetonitrile, methanol), water, or their mixtures with water [12,37].

Regarding the environmental impact of an industrial extraction, the concept of green extraction is needed, to enhance an economic (less energy) and ecologic (using environmentally friendly solvents) extraction. In agreement with this green extraction focus, conventional techniques such as mechanical stirring extraction (MSE) [58] or innovative ones such as ultrasound-assisted extraction (UAE) [59], microwave-assisted

extraction (MAE) [60] or pressurized liquid extraction (PLE) [61] can be applied to recover polyphenols (**Table 3**).

Conventional techniques use large amounts of solvent and have lower yields than innovative ones. That is why the demand for innovative techniques with better yields at lower costs has increased. They are also considered "green techniques" because are more sustainable than the conventional ones [62]. Moreover, many innovative techniques have different obstacles such as the degradation of polyphenols due to the high temperatures, the high ultrasonic power applied, very long extraction times and the health danger. Besides, the required equipment and processes have high costs [30].

For the quantification and characterization of polyphenols from fruit and vegetable extracts different spectrophotometric and chromatographic methods have been used. Folin-Ciocalteu (FC) spectrophotometric assay is commonly used to determine TPC [63]. It gives an estimation of TPC, while chromatographic techniques are used for the separation, identification and quantification of individual polyphenols [64].

High performance liquid chromatography (HPLC), is the common technique used to identify and quantify polyphenols from different fruit and vegetables [37,65]. The identification analysis is usually accomplished by diode array detector (DAD) and mass spectrometry (LC-MS) [66].

Table 3. Polyphenol extraction techniques from fruit and vegetables processing residues.

Residue	Technique	Solvent	Sample (g)	Temperature (°C)	time (min)	Reference
Grape (stem)	SLE[1]	10 ml ethanol:water (46.9:53.1 v/v) with 1 g/L citric acid (pH 3.6 adjusted with 1N NaOH)	5	22 ± 2	60-300	[67]
Grape (skin, seeds and stem)	PEF[2]	4.5 ml ethanol:water (50:50 v/v)	1	70	60	[68]
	HHPE[3]	4.5 ml ethanol:water (50:50 v/v)	1	70	60	
	UAE[4]	4.5 ml ethanol:water (50:50 v/v)	1	70	60	
Grape (skin)	SLE	1. 100 ml ethanol:water (3:1 v/v) 2. 100 ml distilled water (75 °C) 100 ml methanol: 0.01% HCl (v/v, methanol HPLC grade)	5	-	120	[69]
Grape (skin and	PLE[5]	ethanol:water (70:30 v/v)	0.5	100	-	[70]

seeds)	SLE	20 ml methanol:water:formic acid (60:37:3 v/v/v)	2	23.5 ± 1.5	0.5	
Blueberry (skin)	SLE	10 ml methanol	1	25	60	[71]
Lemon (peel)	MAE[6]	20 ml ethanol:water (50:50 v/v)	1	-	2	[72]
	UAE	40 ml ethanol:water (50:50 v/v)	1	-	10	
	CSE[7]	50 ml ethanol:water (50:50 v/v)	1	60	120	
Mandarin (peel)	SLE	100 ml distilled water	5	-	30	[73]
Mandarin	MAE	80 ml methanol:water (66:34 v/v)	5	1-120	0.02-16.65	[74]
	UAE	80 ml methanol:water (80:20 v/v)	4	25	60	
	RE[8]	80 ml methanol:water (80:20 v/v)	4	-	-	

Mandarin (peel)	Maceration	40 ml methanol:water (80:20 v/v)	2	40	60	[75]
	UAE	40 ml methanol:water (80:20 v/v)	2	15, 30 and 40	10, 20, 30, 40, 50 and 60	
Orange (peel)	MAE	25 ml acetone:water (50:50 v/v)	2	≤ 80	1.5-4	[76]
	UAE	50 ml acetone:water (75.8:24.2 v/v)	1	27 ± 2	8.33	
	PLE	acetone:water (50:50 v/v, HPLC grade)	1	120	21	
	CSE	50 ml acetone:water (50:50 v/v)	1	60	120	
Tomato (skin)	UAE	5ml methanol:water (80:20 v/v)	0.5	25	80	[77]
Apple (skin)	SLE	100 ml ethanol:water (50:50 v/v)	1	80	160	[78]
Apple (seeds)	SLE	16 ml acetone:water (30:70 v/v)	2.5	25	60	[79]
Avocado (skin and kernel)	SLE	25 ml ethanol:water (80:20 v/v)	1	25	120	[25]

Artichoke (stem, flower and leaves)	SLE	25 ml methanol:water (60:40 v/v)	4	25	60	[80]
Artichoke (stem, flower and leaves)	UAE	10 ml methanol:water (75:25 v/v) with 0.1% formic acid	0.5	25	20	[81]
Broccoli (stem and leaves)	SLE	1.5 ml methanol:water (70:30 v/v)	0.05	70	30	[82]
Broccoli (stem and leaves)	UAE	10 ml methanol:water (80:20 v/v) + 2 ml HCl 1M (pH=2)	0.1	-	20	[83]
Cabbage (leaves)	PLE	water:ethanol:formic acid (95:4.9:0.1 v/v)	2.5	25-100	1	[84]
Cabbage (outer leaves)	UAE	50 ml ethanol (99.9% purity)	5	-	2	[85]

Cabbage (leaves)	PLE	Hexane:dichloromethane (1:1 v/v)	1	70	20	[86]
	SLE	Methanol:water:acetic acid (90:9.5:0.5 v/v)	1	80	10	
Lettuce (leaves)	SLE	10 ml ethanol:water with 0.1% citric acid (70:30 v/v)	1	42	120	[87]
Onion (outer layers)	SLE	30 ml ethanol:water (90:10 v/v) with 0.1% acetic acid	3	60	30	[88]
Spinach (leaves)	PLE	Hexane HPLC grade	1	100 and 150	10	[56]
Spinach (leaves)	SLE	10 ml ethanol:water (70:30 v/v)	100	40	120	[20]
Spinach (leaves)	SLE	25 ml ethanol:water (80:20 v/v)	1	30	240	[89]

[1]SLE: Solid-liquid extraction

[2]PEF: Pulsed electric field extraction

[3]HHPE: High hydrostatic pressure-assisted extraction

[4]UAE: Ultrasound-assisted extraction

[5]PLE: Pressurized liquid extraction

[6]MAE: Microwave-assisted extraction

[7]CSE: Conventional solvent extraction

[8]RE: Rotatory extraction

1.4 The antioxidant activity of polyphenols from fruit and vegetable wastes and their evaluation

Humans, plants and foods are the systems where the antioxidant activity of polyphenols is framed [7,23,24]. In particular, the inhibition of lipid oxidation in foods, while the protection against oxidative stress from human and plant physiology (**Figure 5**) [90–92]. Various methods have been used to determine the antioxidant activity of polyphenols from fruit and vegetable wastes [63,93,94]. Several spectrophotometric methods (e.g., FRAP, DPPH, ABTS) are used to quantify this activity [11,63].

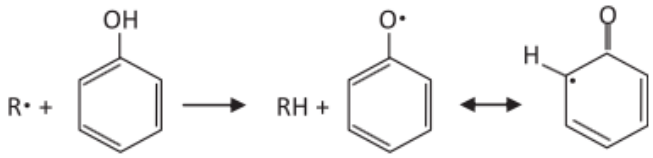


Figure 5. Reaction mechanism of phenolic antioxidants [95].

1.4.1 Methods to evaluate the antioxidant activity of polyphenols

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

This method is the most widely used test to evaluate the free radical-scavenging capacity of fruit and vegetable extracts that the assay relies on the H-transfer from a polyphenol to the DPPH radical. The interaction of the purple DPPH radical with a polyphenol, leads to the formation of pale-yellow hydrazine (see **Figure 6**) since the polyphenol is capable of neutralizing its free radical character [96].

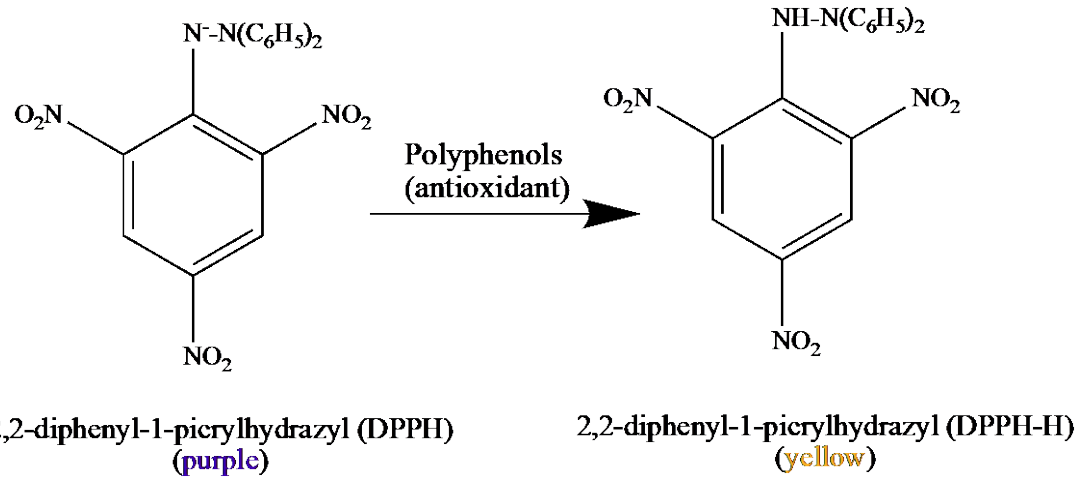


Figure 6. Chemical reaction between the DPPH radical and the antioxidant compound [97].

FRAP (Ferric ion reducing antioxidant power) assay

FRAP is electron transfer (ET)-based test, which changes color when Fe^{3+} (2,4,6-tripyridyl-s-triazine)$_2Cl_3$ probes is reduced [98]. The antioxidants can reduce the Fe^{3+} in the solution to Fe^{2+}, which binds the ferricyanide to yield Prussian blue, or reduce the ferricyanide to ferrocyanide, which binds the free Fe^{3+} in the solution and forms Prussian blue [94].

ABTS (2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation reduction assays

ABTS is also an ET-ased test, when the probe 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid is reduced, the color change [98]. The reduction is visualized as a discoloration corresponding to when the ABTS radical is reduced by the antioxidant (the blue ABTS radical cation is reactive towards most antioxidants colorless neutral form). In this way, the degree of discoloration allows evaluating the percentage of inhibition of the ABTS radical cation, which is determined based on the antioxidant concentration and the reaction time [99].

Folin-Ciocalteu (FC) redox assay

The FC method is based on a single electron transfer and is used to quantify the TPC in fruit and vegetable extracts, using gallic acid as a standard. Due to its oxidation/reduction reaction mechanism can be considered also a method for antioxidant activity quantification. The FC fundament is to reduce the molybdenum component in the phosphotungstic/phosphomolybdic complexing reagent [63,100].

1.5 Separation and purification/concentration techniques by membrane technology

The extract obtained after the extraction step, not only contains polyphenols but also other components such as mono and polysaccharides, as well as the solvent used for the extraction. Therefore, a separation step is important not only to remove the solvent but also to concentrate the bioactive compounds [101].

Traditional polyphenol purification methods include coagulation and precipitation of impurities, adsorption of polyphenols with resins and elution of purified polyphenols, and their concentration by solvent evaporation [102]. These traditional purification methods represent a significant part of the total costs of polyphenols production. Besides, some of them are based on a phase change that involves high temperatures that can produce a decrease in the bioactivity of polyphenols [101].

Currently, pressure-driven membrane processes such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) are well-established systems in different productive sectors, in which the separation process does not use phase changes based on temperature nor chemical agents [103]. Furthermore, they are characterized by high efficiency and simplified operation [104]. Anyway, one of the main problems is the fouling of the membrane which causes a reduction of its permeability.

The visible sign of fouling is the reduction in the permeate flux at constant pressure or the increase in the transmembrane pressure (TMP) at constant filtration flux. Therefore,

pre-treatment of the samples, such as centrifugation, is recommended to limit the fouling of the membrane [105]. In a typical polyphenol recovery process, membrane processes can be combined with different extraction techniques and/or other preprocessing approaches in integrated systems to remove the organic solvent, large molecules such as proteins or polypeptides, and small ones such as salts or simple sugars; besides, others impurities can be cleaned with the aim of producing relatively pure mixtures of polyphenols [101,106].

Membranes are selective barriers that allow the transport of certain components while retaining others [107]. The membrane can separate a feed solution into two fractions (streams) which are the permeate/filtrate and the retentate/concentrate (**Figure 7**). The permeate contains the solvent and solutes pass through the membrane while the retentate contains the particles and compounds that have not been transported through the membrane [95].

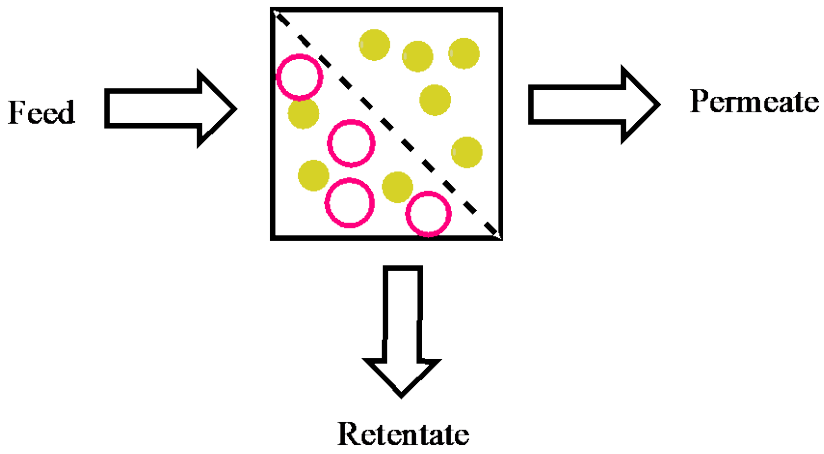


Figure 7. General scheme of a membrane module.

Table 4 lists the main pressure-driven membrane process with the corresponding pore size, molecular weight cut-off (MWCO) and required TMP (the pressure difference between the permeate and retentate).

Table 4. Membrane types and their characteristics using pressure as driving force (adapted from [101]).

Membrane process	**Pore size (nm)**	**MWCO (Da)**	**TMP (bar)**
Microfiltration (MF)	100 – 10,000	> 100,000	0.1 – 2
Ultrafiltration (UF)	2 – 100	1,000 – 100,000	0.1 – 7
Nanofiltration (NF)	0.5 – 2	120 – 1,000	3 – 25
Reverse osmosis (RO)	< 0.5	< 300	35 – 100

MF membranes are symmetrical and capable of separating particles with diameters from 100 to 10,000 nm. They have high permeability, therefore a high flux of water at low pressure can be obtained. Polyphenols have molecular masses between 100 and 30,000 Da [108], thus passing smoothly across the membrane since MF membranes only reject compounds larger than 100 kDa. The adsorption of polyphenols on polymeric membranes involves hydrophobic effects and the formation of hydrogen bonds. There is a direct relationship between the polarity of MF membranes and the amount of adsorbed polyphenols [109].

UF membranes are asymmetric with pores from 2 to 100 nm and are able to retain macromolecules. The mechanism of separation of MF and UF processes is based on particle size [108]. UF membranes with a MWCO of 50 to 100 kDa recover different types of macromolecules such as suspended solids and proteins. The 4 to 30 kDa membranes

concentrate high molecular weight components such as tannins, proteins, and high molecular weight phenolic compounds. The membranes from 1 to 3 kDa are very effective for concentrating low molecular weight polyphenols such as anthocyanidins, low molecular mass polyphenols, and high molecular weight sugars [101].

NF is an intermediate process between UF and RO. This membrane process is used for the separation of ions and uncharged organic solutes with molecular weight from 120 to 1,000 Da. The pore size is between 0.5 to 2 nm. NF polymeric membranes contain ionizable groups such as carboxylic or sulfonic groups; this fact causes the loading of the surface membrane. The separation is based on electrical forces [110].

RO separates compounds with low molecular weights of < 300 Da relying on membranes with a dense structure with no defined pores. The permeability is slow and the rejection is not related to the size of the molecules but with a solution-diffusion mechanism [107].

Hydraulic pressure higher than the osmotic pressure is applied to a solution with a high concentration of solute so the water crosses the membrane from the high- to the low-solute zone [105].

Thus, MF and UF are mainly used for primary treatment purposes, while NF and RO are used for final treatment [109].

Table 5 list some processing examples of polyphenol recovery from fruit, vegetables, plant products and wastes.

Table 5. Polyphenol recovery by membrane techniques from fruit, vegetables and plants products and processing residues (adapted from [30]).

Source	Membrane process	Membrane configuration	Membrane material	Membrane area (m^2)	Pore size (μm)	MWCO (kDa)	Operating conditions	Reference
Apple juice	UF	Flat-sheet	RC[1]	0.1	-	1	4 bar, 0.06-0.108 m^3/h. 30 °C	[111]
Apple juice	NF	Spiral-wound	TFC[2]	1.2	-	1	5, 10, 20 and 30 bar, 30, 40 and 50 °C, VRF 10	[112]
	NF	Spiral-wound	TFC	1.2	-	0.25	10 bar, 40 °C, VRF 10	
Apple juice	UF	Flat-sheet	PES[3]/PVP[4]	0.00287	-	-	1 bar	[113]
	UF	Flat-sheet	RC	0.00287	-	10	1 bar	
	UF	Flat-sheet	RC	0.00287	-	100	1 bar	
Artichoke brines	NF	Spiral-wound	PES	1.6	-	1	6 bar, 20 °C, VRF 3.5	[114]
	NF	Spiral-wound	PES	1.8	-	0.4	6 bar, 20 °C, VRF 3.5	

	NF	Spiral-wound	PPA[5]	2.6	-	0.3	6 bar, 20 °C, VRF 3.5	
	NF	Spiral-wound	PA[6]	2.5	-	0.15-0.3	6 bar, 20 °C, VRF 3.5	
	NF	Spiral-wound	PA	2.6	-	0.15-0.3	6 bar, 20 °C, VRF 3.5	
Artichoke wastewaters	UF	Hollow-fiber	PS[7]	1.2	-	50	0.31 bar, 0.55 m^3/h, 24 °C, VRF 5.67	[115]
	NF	Spiral-wound	PES	1.8	-	0.4	8 bar, 0.3 m^3/h, 25 °C, VRF 5	
	NF	Spiral-wound	PA	2.6	-	0.15-0.3	8 bar, 0.3 m^3/h, 25 °C, VRF 5	
Artichoke extracts	NF	Spiral-wound	PES	1.8	-	0.4	4 bar, 25 °C, VRF 10	[116]
	NF	Spiral-wound	PA	2.6	-	0.15-0.3	10 bar, 25 °C, VRF 5	
Artichoke wastewaters	UF	Tubular	TiO_2	0.1	-	15	4.3 bar, 4 m^3/h, 25 °C	[117]
	NF	Spiral-wound	PA	2.6	-	0.2-0.3	8 bar, 0.3 m^3/h, 12 °C, VRF 5	
Black tea leaves	UF	Tubular	Ceramic	-	-	40	0.7, 12 and 1.7 bar, 50 °C	[118]

Blood orange juice	UF	Tubular	PVDF[8]	0.23	-	15	0.85 bar, 0.14 m/s, 21 °C	[119]
	RO	Spiral-wound	PA	-	-	-	35 bar, 20 °C	
Castanea sativa leaves	UF	Flat-sheet	PES	0.07	-	5	2 bar, 20 °C, VRF 2-6	[120]
	UF	Flat-sheet	PES	0.07	-	10	2 bar, 20 °C, VRF 2-6	
Cocoa seeds	RO	Flat-sheet	TFC	0.000314	-	-	10 bar, 40 °C	[121]
	RO	Flat-sheet	TFC/PA	0.000314	-	-	10 bar, 40 °C	
	NF	Flat-sheet	TFC	0.000314	-	-	10 bar, 40 °C	
	NF	Flat-sheet	TFC	0.000314	-	-	10 bar, 40 °C	
	NF	Flat-sheet	TFC/PA	0.000314	-	-	10 bar, 40 °C	
	NF	Flat-sheet	TFC/PA	0.000314	-	-	10 bar, 40 °C	
Flaxseed hull	UF	Flat-sheet	PES	0.00031	-	30	4 bar, room temperature	[122]
Grape seeds	UF	Flat-sheet	MCE[9]	-	0.22	-	5 bar, 25 °C	[123]

	UF	Flat-sheet	MCE	-	0.45	-	5 bar, 25 °C	
Grape seeds	NF	Tubular	PA	0.044	-	-	6 bar	[124]
	UF	Tubular	PES	0.044	-	4	5 bar	
	UF	Tubular	PS	0.044	-	8	5 bar	
	UF	Tubular	PS	0.044	-	20	2 bar	
	UF	Tubular	PVDF	0.044	-	200	0.2 bar	
Green tea leaves	UF	-	CA[10]-titanium	-	-	-	1.5-4 bar	[125]
Justicia secunda Vahl leaves	MF	Tubular	Ceramic	0.306	-	-	0.6 bar, 5 m/s, 30 °C	[126]
	RO	Spiral-wound	TFC/PA	2.8	-	-	-	

Orange press liquor	NF	Spiral-wound	PA	2.1	-	0.18	20 bar, 20 °C, VRF 3	[127]
	NF	Spiral-wound	PPA	2.6	-	0.3	20 bar, 20 °C, VRF 3	
	NF	Spiral-wound	PES	1.6	-	0.4	6 bar, 20 °C, VRF 3	
	NF	Spiral-wound	PES	1.6	-	1	6 bar, 20 °C, VRF 3	
Orange press liquor	UF	Hollow-fiber	PS	0.16	-	100	0.2 bar, 0.24 m^3/h, 20 °C	[128]
Orange press liquor	UF	Hollow-fiber	PS	1.2	-	100	0.54 bar, 0.5 m^3/h, 25 °C, VRF 13	[129]
	NF	Spiral-wound	PES	1.6	-	1	8 bar, 0.4 m^3/h, 20 °C, VRF 5	
Pequi fruit	NF	Flat-sheet	PA	0.00159	-	0.2-0.3	8 bar, 25 °C	[130]
Root cortices of mulberry	MF	Flat-sheet	-	-	0.45	-	4.8 bar, 25 °C	[131]
	UF	Flat-sheet	PS	-	-	200	4.8 bar, 25 °C	
	UF	Flat-sheet	PS	-	-	50	4.8 bar, 25 °C	
	UF	Flat-sheet	PA	-	-	20	4.8 bar, 25 °C	

	UF	Flat-sheet	PS	-	-	10	4.8 bar, 25 °C	
Soybeans	NF	Spiral-wound	PVDF	0.9	-	0.15-0.3	7 bar, 16 °C, VRF 4	[132]
Soy processing waste stream	UF	Flat-sheet	CA	-	-	30	3.5 bar, 0.2 m^3/h, 50 °C	[133]
	UF	Flat-sheet	CA	-	-	1	5.5 bar, 50 °C, 0.2 m^3/h, VRF 10	
	RO	plate-and-frame	TFC	-	-	-	14 bar, 0.192 m^3/h	
Fermented spinach	UF	-	Fluoropolymer	0.31	-	100,000	2.75 bar, 30 min, room temperature	[134]
Fermented spinach	UF	-	Fluoropolymer	0.31	-	100,000	2.75 bar, 30 min, room temperature	[135]
Fermented broccoli	UF	-	Fluoropolymer	0.31	-	100,000	2.75 bar, 30 min, room temperature	

[1]RC: regenerated cellulose

[2]TFC: thin-film composite

[3]PES: polyethersulfone

[4]PVP: polyvinylpyrrolidone

[5]PPA: poly(piperazine-amide)

[6]PA: polyamide

[7]PS: polysulfone

[8]PVDF: polyvinylidene fluoride

[9]MCE: mixed cellulose esters

[10]CA: cellulose acetate

1.6 Application of polyphenols from fruit and vegetable wastes

Polyphenol recovery from fruit and vegetable processing wastes has applications in the food, pharmaceutical and cosmetic industries. Their biological activities such as antioxidants, antiviral, etc. have been demonstrated in many *in vitro* and *in vivo* studies [7].

In the food industry, polyphenols provide better bioavailability, increasing the half-life of the product and preserving the oxidation of the product, among other benefits [136].

Regarding the pharmaceutical industry, polyphenols (e.g., gallic acid, ferulic acid, catechin, etc.) can be used as drugs with antitumoral, antidiabetic, hepatoprotective, antiviral effects, etc. For example, the anthocyanidins extracted from pomegranate seeds possess antiviral and anticancer properties [26,41].

Finally, in the cosmetic industry, polyphenols such as resveratrol from grape skin and seeds are important ingredients in cosmetic product formulations providing anti-aging properties [56,137].

1.6.1 Food application

Polyphenols contribute to bitterness, color, taste, smell, astringency, and oxidative stability of food products [138]. Recently, their use as food additives in baked goods, noodles and pasta, beverages and functional foods has been demonstrated increasing the world economy [139–142]. As another advantage, fruit juices [143], powders [144] and extracts rich in polyphenols have been recommended to be applied as functional

ingredients to the food industry. For example, lignans (one of the polyphenol families) can be used in fats and oils to increase their stability during heating and storage. Chlorogenic acid is used as an additive in beverages and tea products [145]. Plant extracts (containing polyphenols) are used effectively in functional drinks based on water or tea, as well as in so-called "shakes" [146].

An important aspect that cannot be overlooked during product development is the inspection of the sensory effects of bioactive substances in food before use. Several methods can be used to control these issues, such as microencapsulation of bioactive extracts to mask their unpleasant taste. Due to concerns about the toxic health influences of synthetic antioxidants, consumers today prefer products containing natural additives [6,147].

1.6.2 Pharmaceutical application

Regarding the pharmaceutical industry, several aspects must be considered, such as the conjugation capacity with other phenolic compounds, the molecular size, and the solubility and the degree of glycosylation and acylation. These aspects affect the absorption and metabolism of phenolics which is important in order to determine their

possible health benefits [148]. The elimination of phenolic metabolites from plasma is very rapid; therefore, it is vitally important to consume fruits and vegetables (including oranges and spinach) on a daily basis. The suggested daily intake of polyphenols is 1 g/day to achieve proper concentrations of metabolites in blood [149].

Rodrigo et al. [150] observed that after 2 or 3 h of quercetin ingestion, it was absorbed reaching its maximum concentration in plasma. They also considered that the antioxidant activity was responsible for its defensive benefits by reducing the level of free radicals in the body.

According to Vauzour et al. [151], flavonoids have the potential to overwhelm neuroinflammation and also have the ability to improve memory, learning, and cognitive functions. Flavonoids can enhance neuroprotective actions within the brain, against injuries caused by neurotoxins. Consumption of foods rich in flavonoids can reduce neurodegeneration and prevent or reverse age-dependent losses of cognitive ability [93]. Therefore, flavonoids can be considered therapeutic agents against neurodegenerative diseases such as Parkinson's and Alzheimer's [152].

1.6.3 Cosmetic application

Polyphenols have also been of great interest to the cosmetic industry in recent years, carrying out cosmeceuticals with product formulations enriched with polyphenol extracts [153], which were previously studied as ingredients in topical lotions and creams due to their antioxidant activity [154].

A key aspect that must be taken into account is the adequate concentration of polyphenols in the target areas of the skin, to avoid their precipitation in the

formulations used as a transmission vehicle. For example, some polyphenols (e.g., naringenin, rutin and quercetin) show a low solubility in water that can be enhanced with surfactants such as polyoxyethylene sorbitan monolaurate or block copolymers [155].

The stability of the emulsion against oxidation may be improved if polyphenols are added, due to their antioxidant characteristics [156] and due to their adsorption at the oil-water interface [157].

1.6.4 Future perspectives of the use of polyphenols from orange and spinach wastes in the food, pharmaceutical and cosmetic industries

Currently, several researchers are working hard to develop new nutritional formulations with polyphenols as one of the ingredients, which contribute not only to the prevention or treatment of diseases but also positively affect the health skin. Additionally, these compounds contribute to the preservation of foods and cosmetics. Since industrially synthetic antioxidants are used, unlike natural antioxidants, they may have potential toxic effects on the health of consumers [93].

Future investigation of the application of polyphenols from orange and spinach waste in the pharmaceutical, cosmetic and food industries is needed.

1.7 References

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CHAPTER 2

Objectives

2. Objectives

The main objective of the current PhD Thesis is the evaluation of the residues obtained from the agri-food sector, in particular, it focuses on the recovery of polyphenols from orange and spinach processing wastes, to stablish the optimal conditions for the extraction and separation of polyphenols with antioxidant activity that allow the recovery of these residues. The characterization of the waste, the evaluation of extraction methods and purification technologies, and the implementation of a treatment process based on membrane technology for the recovery of polyphenols from orange and spinach extracts are also part of the study.

Therefore, the specific objectives are:

- Establish optimal conditions for the polyphenol extraction from orange and spinach wastes using different innovative extraction techniques such as UAE, MAE and PLE, testing different experimental factors including different ethanol and HCl percentages, extraction time, temperature and number of cycles.
- Evaluate the MSE as conventional extraction process studying four experimental parameters: extraction time, temperature, solid/solvent ratio (using water as a solvent) and pH. Compare the optimal condition with those obtained with the innovative techniques.
- Identify and quantify the polyphenol content in orange and spinach waste extracts using high-performance liquid chromatography (HPLC) and spectrophotometric methods (FC, FRAP, DPPH, ABTS). In addition, the

antioxidant indices are compared in order to obtain an overview of the antioxidant activity of orange and spinach extracts by MSE.

- Evaluate the recovery of antioxidants from orange and spinach wastes extracts using membrane technology, through the evaluation of MF, UF, NF and RO membranes. The MF and UF membranes are evaluated in the clarification stage of the extracts, while the NF membranes are evaluated in the preconcentration stage and the RO membranes in the final polyphenol concentration stage.
- Review the antiviral activity of polyphenols, being one of the applications after the polyphenol recovery, besides, review the postulated mechanisms of action of polyphenols to defeat viruses as well as the synergy effect of antiviral and antioxidant properties against viral diseases.

CHAPTER 3

Thesis Overview

3. Thesis overview

In this Thesis, chapters 4 and 5 present the conventional and innovative extraction techniques employed for polyphenol recovery from orange and spinach wastes. Mainly, UAE, MAE and PLE were studied to carry out the polyphenol extraction (Publication 1). In addition, MSE was compared with UAE in order to obtain a suitable extraction technique and to maximize polyphenols extraction (Publication 2). Besides, the MSE extracts were characterized by FRAP, DPPH, and ABTS to evaluate the overall antioxidant activity (Publication 2).

In chapter 6, the purification step was studied by evaluating MF, UF, NF and RO membranes to recover polyphenols from the MSE extracts. MF and UF membranes were used to remove impurities of spinach and orange extracts, while NF membranes were used to pre-concentrate polyphenols from low molecular weight compounds and RO membranes were used to properly concentrate polyphenols and facilitate the removal/recovery of the solvent (Publication 3).

Finally, chapter 7 deals with the extraction, purification and recovery of polyphenols with potential antiviral activity (Publication 4). Special attention is also paid in the action mechanisms which are essential for preventing virus replication and infection without side-effects.

Figure 9 shows a scheme of the thesis overview.

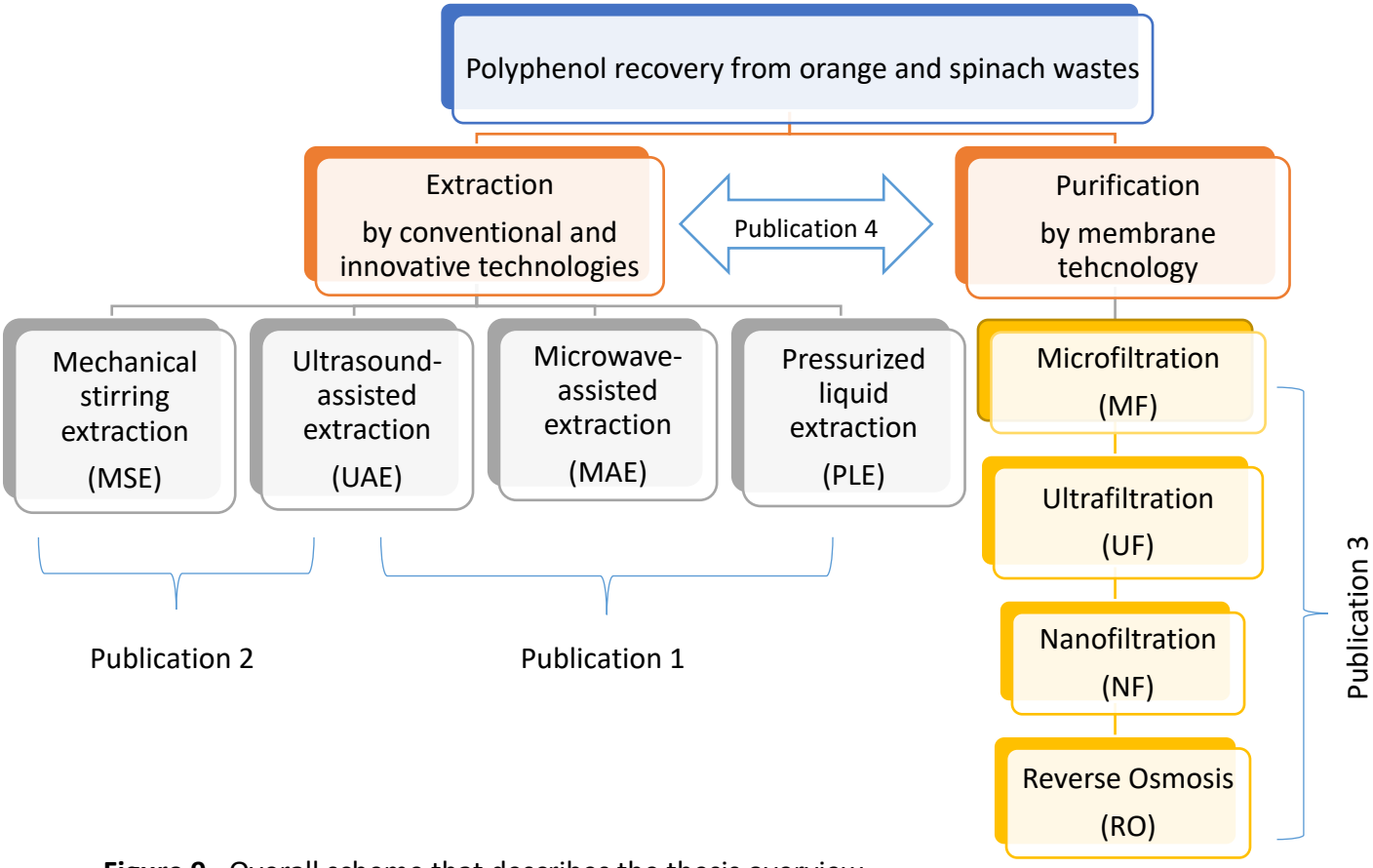

Figure 9. Overall scheme that describes the thesis overview.

Publication 1: María Fernanda Montenegro-Landívar, Paulina Tapia-Quirós, Xanel Vecino, Mònica Reig, César Valderrama, Mercè Granados, José Luis Cortina, Javier Saurina. "*Fruit and vegetable processing wastes as natural sources of antioxidant-rich extracts: Evaluation of advanced extraction technologies by surface response methodology*" Journal of Environmental Chemical Engineering 9 (2021) 105330. CIteScore: 7.5, IF: 5.909, Quartile: Q1, citations: 12 (05/07/2022)

Publication 2: María Fernanda Montenegro-Landívar, Paulina Tapia-Quirós, Xanel Vecino, Mònica Reig, César Valderrama, Mercè Granados, José Luis Cortina, Javier Saurina. "*Recovery of added-value compounds from orange and spinach processing residues: Green extraction of phenolic compounds and evaluation of antioxidant activity"* Antioxidants 10 (2021) 1800. CiteScore: 6.649, IF: 7.38, Quartile: Q1, citations: 2 (05/07/2022)

Publication 3: María Fernanda Montenegro-Landívar, Paulina Tapia-Quirós, Xanel Vecino, Mònica Reig, Mercè Granados, Adriana Farran, José Luis Cortina, Javier Saurina, César Valderrama. *"Recovery of natural polyphenols from spinach and orange by-products by pressure-driven membrane processes"* Membranes 12 (2022) 669. CiteScore: 3.700, IF: 4.106, Quartile: Q1

Publication 4: María Fernanda Montenegro-Landívar, Paulina Tapia-Quirós, Xanel Vecino, Mònica Reig, César Valderrama, Mercè Granados, José Luis Cortina, Javier Saurina. "*Polyphenols and their potential role to fight viral diseases: An overview"* Science of the Total Environment 801 (2021) 149719. CiteScore: 10.5, IF: 7.963, Quartile: Q1, citations: 13 (05/07/2022)

CHAPTER 4

Publication 1

"Fruit and vegetable processing wastes as natural sources of antioxidant-rich extracts: Evaluation of advanced extraction technologies by surface response methodology"

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Fruit and vegetable processing wastes as natural sources of antioxidant-rich extracts: Evaluation of advanced extraction technologies by surface response methodology

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ABSTRACT

This study is focused on the recovery of polyphenols from vegetable and fruit residues and further evaluation of antioxidant features of extracts. Spinach and orange have been selected as representative matrices for a more comprehensive study since they contain significant polyphenols amount that could be used in food, pharmaceutical and/or cosmetic industries. Extraction of polyphenols from spinach and orange waste was performed using three extraction techniques: ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and pressurized liquid extraction (PLE). Tested factors include percentage of organic solvent (ethanol 0–80%), acidity (0–0.5% HCl), extraction time (0–30 min) and temperature (25–120 °C). Optimal extraction conditions for spinach and orange waste have been stablished by design of experiments (DoE). The performance of the extraction process has been preliminarily assessed from the overall polyphenolic content given by high-performance liquid chromatography (HPLC) and Folin–Ciocalteu index. In addition, reducing power and anti-radical capacity of vegetable and fruit extracts have also been determined. For spinach, the best conditions corresponded to UAE with a mixture of ethanol/water/HCl in ratio 80/19.9/0.1 (*v/v/v*) as the solvent at 25 °C for an extraction time of 30 min, providing 0.82 mg gallic acid equivalent (GAE) per g fresh weight (fw). For the orange matrix, PLE has been chosen using 60/39.9/0.1 ethanol/water/HCl (*v/v/v*) solvent at 80 °C for 15 min, providing 3 mg GAE g^{-1} fw. However, UAE is proposed for extraction of polyphenols from spinach and orange waste at industrial scale, due to its simplicity and low cost, among other reasons.

1. Introduction

Agricultural processing inevitably generates large amounts of agri-food residues, which represent an important problem of waste disposal, most of the generated wastes are recycled as animal feed and compost, but the remaining quantities are incinerated and dumped causing greenhouse gas emissions which contributes negatively to climate change [1–3]. Specifically, the vegetable and fruit sector generate around 90 million tons of residues per year in Europe and experts estimate a rise of 40% in the next 4 years [4,5]. In Spain, vegetable and fruit processing are around the 5% of the food industry which generates almost 3 millions tonnes of residues per year [6]. Spinach is one of the most cultivated and consumed vegetables, not only in Spain but also worldwide, which generates between 13% and 25% of waste, basically corresponding to damaged leaves and solid residues from juice production [7]. On the other hand, orange is the main cultivated fruit in Spain [8] that generates around 50% of weight waste composed by peels, pulp and seeds [9].

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According to literature, a plant or vegetable residue is a good natural source of polysaccharides, bioactive molecules (e.g. polyphenols, vitamins) and carbohydrates [10]. Furthermore, according to data of spinach and orange percentage of generated waste, these matrices are attractive for the study of polyphenol recovery [7,9]. Regarding to the powerful antioxidant activity of polyphenols, epidemiological studies have pointed out that diets rich in polyphenols may help to prevent cancer, neurodegenerative and cardiovascular diseases, among others [11].

Polyphenols consist of molecules with one or more phenol groups, classified into different families: phenolic acids, flavonoids, stilbenes and lignans. Among them, phenolic acids (hydroxybenzoic and hydroxycinnamic) and flavonoids (including oligomeric tannins) are the main phenolic components of the human diet [12,13]. Spinach is rich in flavonoids such as luteolin, quercetin, apigenin, among others, whereas orange residues contain hesperidin, vanillic acid, ferulic acid, etc. [14–16].

Thus, polyphenols extracted from spinach and orange residues may be reused in food, cosmetic and pharmaceutical industries [14,17], for example to prepare natural additives, food supplements, or nutritional food ingredients, among other applications [18,19]. Nowadays, the development of a single, efficient and rapid extraction method of polyphenols from such matrices is still a great challenge. This is mainly due to the inherent limitations of various conventional extraction methods [20,21]. The extraction of polyphenols from agri-food wastes can be done using different techniques, including conventional liquid extraction (e.g., mechanical agitation, maceration and Soxhlet) as well as more efficient counterparts (e.g., ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), etc.) [22–24]. UAE offers important benefits such as lower energy consumption in comparison with other techniques (MAE and PLE), being highly recommendable for extraction of polyphenols from food matrices [25,26]. MAE and PLE apply more energy during extraction process, so that higher overall yields may be achieved, but, at the same time, degradation of labile components may occur. Aqueous mixtures containing ethanol have been reported to be suitable for polyphenol extraction and safe for human health [27]. Focusing on spinach, a representative vegetable, ethanol/water mixtures have been assayed and suitable results have been reported [28,29]. Regarding residues from fruit processing, and orange residues in particular, the ethanol/water system has also been explored, showing good performance in the extraction of polyphenols [30–33].

Developing a polyphenol valorization process requires the identification of the most efficient extraction stage, where the issues of characterization of the polyphenols content and their antioxidant capacity, operating conditions and selection of compatible solvents for the subsequent separation and purification stages are critical [34].

The principal objective of the study was the revalorization of agri-food wastes as they may result in a remarkable source of raw products with increasing social and economic impact. In this context, polyphenols are possibly the most relevant group of phytochemicals from these wastes, so the study was focused on obtaining enriched mixtures of polyphenols to be the basis of by-products with great antioxidant features, with potential interest for manufacturing cosmetic and nutraceutical products. The optimization intended to recover the highest amount of phenolic components, and the overall peak area of the chromatogram was found to be a good objective response to compare the recovery yield of those fractions with remarkable antioxidant properties. Polyphenols from vegetable and fruit wastes were extracted by UAE, MAE and PLE techniques, using water-ethanol mixtures, and considering the effect of acidity, extraction time and temperature. For this purpose, factorial design approaches were employed to evaluate the impact of different experimental variables on the recovery. The efficiency of the extraction was accounted from contents of individual and overall phenolics determined by high-performance liquid chromatography with ultraviolet detection (HPLC-UV) and Folin-Ciocalteu (FC) method. Further evaluation of the antioxidant features of extracts relied on ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) indexes.

2. Materials and methods

2.1. Chemicals and reagents

Polyphenols used as standards were as follows: 2-(3,4-dihydroxyphenyl) ethyl alcohol (dihydroxytyrosol), resveratrol, myricetin and catechin from TCI (Japan); 2-(4-hydroxyphenyl) ethanol (hydroxytyrosol), 2,5-dihydroxybenzoic acid, 3,4-dihydroxybenzaldehyde, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, chlorogenic acid, epicatechin, ethyl gallate, ferulic acid, gallic acid, naringenin, *p*-coumaric acid, quercetin, rutin, syringic, caffeic and vanillic acid from Sigma Aldrich (St. Louis, USA); apigenin and caftaric acid from Chengdu Biopurify Phytochemicals (China); hesperidin and kaempferol from Glentham Life Sciences (UK); homogentisic acid from Extrasynthese (France); luteolin from Carbosynth (USA).

Reagents to be used for the extraction process, HPLC method and spectrophotometric indexes were as follows: acetonitrile (ACN, HPLC grade) was purchased from Fisher Scientific (UK). Ethanol (EtOH, HPLC grade), formic acid (98–100%, w/w), hydrochloric acid (32%, w/w), sodium hydroxide, Fe(III) chloride, sodium carbonate and disodium hydrogen phosphate were obtained by Merck (Darmstradt, Germany). Methanol (MeOH, UHPLC Supergradient, ACS) was purchased from Panreac (Spain). Water was purified with a Milli-Q equipment (Merck Millipore). Reagents for antioxidant indexes were potassium peroxodisulfate from Merck, Folin-Ciocalteu (FC) reagent from Panreac, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS), 2-diphenyl-1-picrylhydrazyl (DPPH), and 2,4,6-tripyridyl-S-triazine (TPTZ) from Alfa Aesar (Germany).

2.2. Samples

Spinach, carrot, kale, celery, beet, broccoli, orange, kiwi, strawberry, white grape and red grape samples were purchased from local markets in Barcelona. Industrial solid wastes from fruit and vegetable juices were simulated using the following procedure recommended by the researchers from fruit juice company. Thus, fruits and vegetables were processed with a domestic juicer and the solid residues were used as the samples. No significant differences in terms of physical, chemical and morphological properties were expected among lab wastes and those obtained at industrial scale. Subsequently, solid wastes were milled with a blender to increase the homogeneity of the samples until obtaining a homogeneous paste. Each wet sample was distributed in various containers that were stored in the freezer at −20 °C until use.

2.3. Instruments and apparatus

The determination of the total phenolics content by HPLC-UV was carried out with an Agilent Series 1200 HPLC Chromatograph (Agilent Technologies, Palo Alto, California, USA) with a quaternary pump (G1311A), a degasser (G1322A), an automatic injection system (G1392A) and a diode array detector (G1315B). The Agilent ChemStation software was used for instrument control and data processing.

A double beam Perkin Elmer UV/Vis/NIR Lambda 19 spectrophotometer was used to estimate the antioxidant and antiradical capacities of vegetable and fruit extracts. QS quartz glass high performance cuvettes (10 mm optical path) from Hellma Analytics (Jena, Germany) were used.

The UAE of polyphenols was conducted using an ultrasonic bath (Branson 5510, USA). MAE experiments were performed using a microwave laboratory system (Ethos E, Milestone S.r.l, Italy). Finally, a Dionex ASE 350 apparatus (Dionex Corp., USA) was used for PLE assays. In all the cases, the resulting extracts were centrifuged with a Rotina 420

centrifuge (Hettich, Tuttlingen, Germany).

2.4. Extraction procedures

2.4.1. Ultrasound-assisted extraction (UAE)

Extractions were performed with different ethanol and HCl percentages: ethanol (40%, 60% and 80%) and HCl (0%, 0.1% and 0.5%) in water. Briefly, 1 g of sample was mixed with 20 mL of solvent and was sonicated for 30 min at 25 °C (frequency of 42 kHz and power of 135 W). To minimize the effect of potential heterogeneity of the distribution of ultrasonic waves on each experimental plan, the different replicates of each extraction condition were placed randomly in a proper rack located in the bath. After that, the mixture was centrifuged for 15 min at 3500 rpm and the supernatant was filtered through 0.45 μm nylon membrane (Whatman, Clifton, NJ, USA). To minimize the influence of the adsorption of compounds on the filter, the initial filtered fraction (about 1 mL) was discarded. Subsequently, ca. 1.5 mL of filtrate was collected in an injection vial and was stored at 4 °C until the analyses. In these conditions, extracts were stable for, at least, two weeks. Assays were carried out in triplicate.

Because of the highest simplicity of UAE with respect to the other extraction techniques, the best experimental conditions from this technique were used as the reference for the comparison of the extraction performance of MAE and PLE.

2.4.2. Microwave-assisted extraction (MAE)

The samples were treated under various experimental conditions: ethanol/water mixtures (0%, 40% and 60% ethanol and 0.1% of HCl); temperature (60, 90 and 120 °C) and extraction time (5 and 15 min). In each experimental assay, 1 g of waste sample was mixed with 20 mL of solvent and was placed in an extraction vessel. After MAE treatment, the resulting extracts were centrifuged for 15 min at 3500 rpm. The supernatant was filtered using a nylon filter of 0.45 μm and stored at 4 °C until the analysis as explained in 2.4.1. Extractions were performed in triplicate.

2.4.3. Pressurized liquid extraction (PLE)

Briefly, 1 g of waste sample was mixed with 2 g of diatomaceous earth and then the mixture was placed into a 5 mL extraction cell containing a cellulose filter at the bottom. Different experimental factors were tested, including solvent composition (40%, 60% and 80% ethanol with 0.1% of HCl in water), temperature (80, 100 and 120 °C), extraction time (5, 10 and 15 min) and number of cycles (1 or 2 cycles). Pressure was 145 psi (about 10 bar). Other conditions such as preheating time (5 min), flush volume (60%) and purge time (60 s) were prefixed according to previous studies [35,36]. After PLE, the volume of each extract was adjusted to 20 mL with the extraction solvent. The solutions were centrifuged for 15 min at 3500 rpm, and were processed and stored as explained in 2.4.1. Extractions were made in triplicate.

2.5. Determination of polyphenols by HPLC-UV

Samples were analyzed by HPLC-UV to determine total polyphenols content (TPC) associated to phenolic acids and flavonoids. Working conditions were adapted from the fully validated method by Aznar et al. [37]. A Kinetex C18 column (100 mm × 4.6 mm of internal diameter and 2.6 μm particle size) from Phenomenex (Torrance, CA, USA) was used. The mobile phase was composed of 0.1% formic acid in Milli-Q water (solvent A) and ACN (solvent B). The gradient elution program was as follows: 0 min, 5% B; 25 min, 50% B; 27 min, 90% B; 29 min, 90% B; 29.2 min, 5% B; 35 min, 5% B. The flow rate was 1 mL min^{-1} and the injection volume was 5 μL. UV detection was performed at 280, 310 and 370 nm for a more specific monitoring of target analytes. In more detail, 280 was used for the detection of hydroxybenzoic acids, flavanols and flavanones (e.g. 4-hydroxybenzoic acid, vanillic acid, hesperidin, etc.); 310 for hydroxycinnamic acids (e.g. coumaric and ferulic acids), and 370 nm for other flavonoid families including flavonols, flavones and isoflavones (e.g. quercetin, rutin, etc.). Compounds were quantified using the corresponding pure standards.

The TPC of extracts was estimated according to Tapia-Quirós et al. [38] from the total peak area of the chromatograms at 310 nm with a bandwidth of 33 nm, in the time range of 5–20 min, thus giving simultaneous information on a wide range of phenolic acid and polyphenolic families. The TPC was expressed in terms of gallic acid equivalent (GAE) per g fresh weight (fw), using gallic acid standard solutions to build the calibration curve in the range of concentrations 1–10 mg L^{-1}.

2.6. Identification of targeted polyphenols by HPLC-HRMS

Various phenolic compounds present in the extracts were identified by high performance liquid chromatography-high resolution mass spectrometry (HPLC-HRMS) with an Accela chromatograph coupled to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK). The separation was performed with the Kinetex C18 column (100 mm × 4.6 mm of internal diameter and 2.6 μm particle size) and an elution program based on increasing the methanol percentage. MS detection relied on electrospray ionization (ESI) in negative mode. Mass spectra were acquired in the m/z range 100–1500 at a mass resolution of 30,000 full width at half-maximum (FWHM) at m/z 200. Data was analyzed with XCalibur software v2.0.7 (Thermo Fisher Scientific, USA). Other experimental conditions have been detailed in Ref. [38].

2.7. Determination of antioxidant indexes

Folin-Ciocalteu (FC), ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) methods have been described in detail elsewhere [39]. In any case, spectrophotometric measurements were carried out at the selected wavelengths (765 nm for FC, 595 nm for FRAP, 517 nm for DPPH and 734 nm for ABTS) using a double-beam system in which test and blank solutions were placed in the sample and reference holders, respectively.

2.8. Experimental design and statistical analysis

The influence of experimental variables on the extraction of polyphenols from waste matrices using the proposed techniques (UAE, MAE and PLE) was assessed by factorial design. Depending on the cases, 2- and 3-factor at 2- or 3-level full designs were created to deal with the main effects as well as the possible interactions. In general, variables under study comprised solvent percentage, HCl percentage, temperature and time. Data resulting from these studies were further evaluated statistically to find out the optimal conditions.

All the experiments were performed in triplicate and results were expressed as mean ± standard derivation (SD). The average values of TPC obtained by UAE, MAE and PLE, determined by HPLC-UV, were subjected to analysis of variance (ANOVA) to ascertain the significance of factors and their potential interactions. Mean values of representative cases were also compared using Student's t-test. In any case, $p \leq 0.05$ was considered as the significance criterion.

Extraction values were further processed according to the response surface methodology (RSM) to visualize simultaneously the influence of the experimental factors. The RSM approach was applied to each individual target compound (e.g. hesperidin, ferulic acid, coumaric acid, etc.) as well as to the TPC resulting from the contribution of all the compounds. Extraction values from each studied design were fitted to multilinear expressions including quadratic terms. In the case of the simultaneous influence of ethanol and HCl percentages, for instance, the equation used was $R = a_0 + a_1\ \text{ethanol\%} + a_2\ \text{HCl\%} + a_3\ \text{ethanol\%} + a_4\ \text{ethanol\%}^2 + a_5\ \text{HCl\%}^2$, being a_0, a_1, a_2, a_3 a_4 and a_5 the coefficients to be

fitted, and ethanol% the experimental value of the ethanol percentage, HCl% the HCl percentage, and ethanol%^{2} and HCl%^{2} the corresponding quadratic terms. Subsequently, the significance of the regression and each coefficient were statistically evaluated, and the final model was built including only those relevant terms.

3. Results and discussion

3.1. Extraction of phenolic compounds from spinach waste

Polyphenols were extracted from spinach leave waste using hydro-organic solutions. The optimal working conditions were defined as those leading to the highest total polyphenols recovery. The study of the influence of the experimental variables (extraction time, solvent composition and temperature) on the extraction yield was evaluated for UAE, MAE and PLE techniques.

The principal factors under study were the solvent composition (including solvent type, solvent percentage and HCl percentage), processing temperature and time. Other specific factors, such as number of cycles, were also considered in PLE. With so many factors involved, a full factorial design at several (two or three) levels was considered quite unreasonable because a huge number of experimental runs was required; this was even more dramatic with replicated experiments. Anyway, preliminary evidences suggested that chemical (solvent

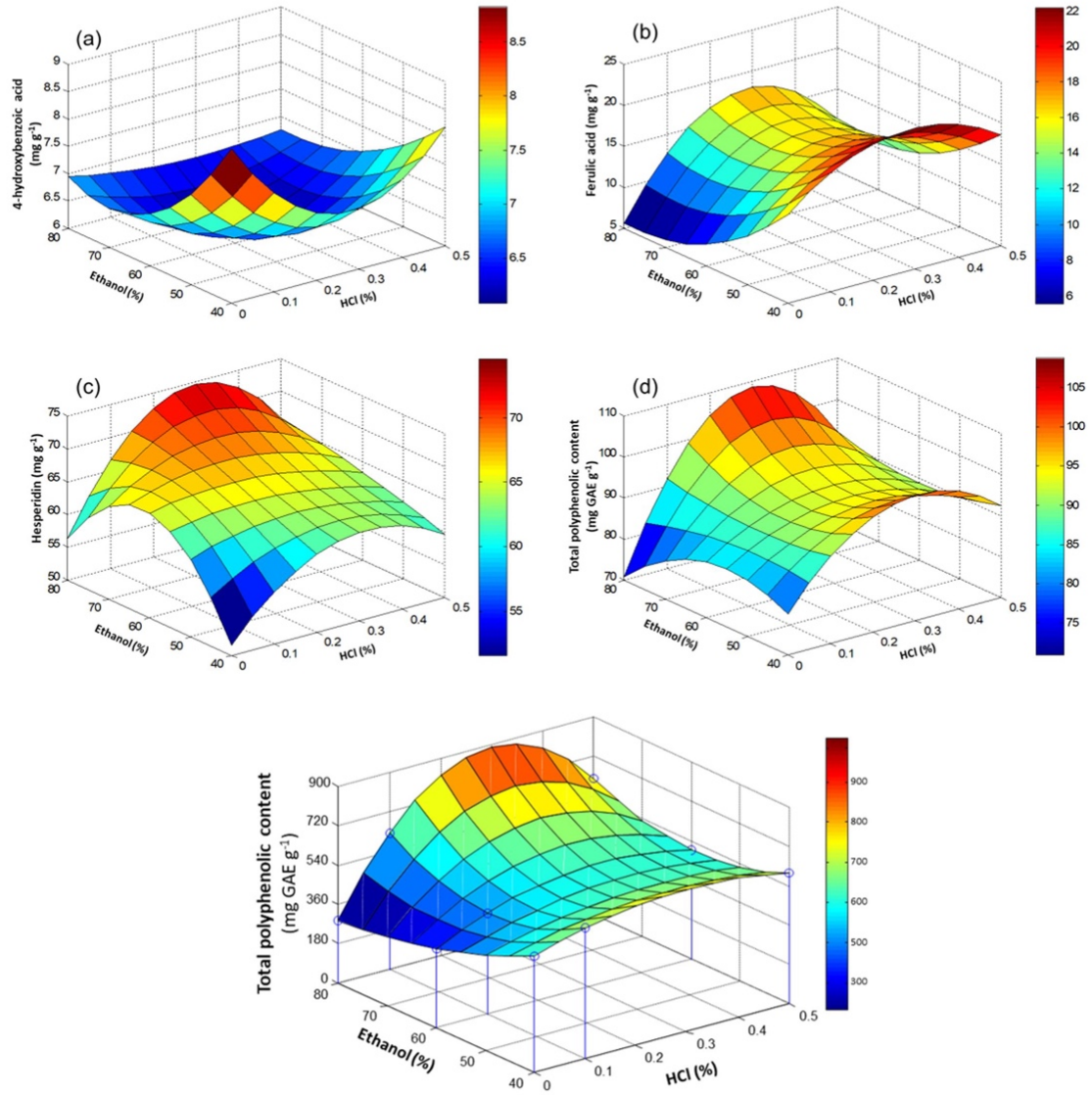

Fig. 1. Surface response depicting the extraction behavior of various individual polyphenols and the total polyphenols content (mg GAE g^{-1} fw) by UAE as a function of ethanol and HCl percentages for spinach waste using UAE. (a) 4-hydroxybenzoic acid; (b) ferulic acid; (c) hesperidin; (d) overall phenolic amount from the addition of each individual compound; (e) total phenolic amount from the overall area at 280 nm. Extraction time: 30 min.

composition) and physical (temperature and time) factors were not interrelated, so independent studies to check the influence of chemical and physical variables on the extraction were designed.

In general, we applied factorial designs (2-factor at 3-level) to explore the influence of the working conditions on the phenolic recovery, so that, a total of 9 experimental conditions were assayed (considering triplicates, 27 runs in each design).

This design was highly versatile and reasonable from the point of view of the experimental effort required as well as the quality of the information provided (it is ideal for the evaluation of the significance of effects and interactions, and data can easily be fitted to multilinear models). Levels and working ranges under study were selected according to our previous experience on the extraction of polyphenols from fruit matrices. These studies were performed at lab scale to propose extraction protocols for further scaling up experiments.

For each extraction technique, the response surface methodology combined with multicriteria decision approach was used to try to find the optimum extraction conditions. For this purpose, each individual compound was considered to fit its surface response. Furthermore, overall responses were established according to two complementary approaches, as a way to estimate the best extraction conditions considering all the polyphenols under study as follows: (i) Combined response considering the amounts of each target compound (here, the addition of the concentrations of 4-hydroxybenzoic acid, coumaric acid, ferulic acid and hesperidin) and (ii) the TPC of extracts was estimated according to the total peak area at 310 nm, in the time range of 5–20 min, which was related to a wide range of phenolic compounds detected under these conditions [38].

Preliminary, spinach extracts were analyzed by LC-HRMS to identify some relevant compounds. 4-hydroxybenzoic acid, coumaric acid, ferulic acid and hesperidin were found thanks to the exact *m/z* values of the corresponding $[M\text{-}H]^-$ ions. The identity of these polyphenols was further confirmed by using standards.

3.1.1. UAE

Initial experiments were performed at room temperature using an extraction time of 30 min [40]. Results of the influence of the ethanol percentage (40%, 60% and 80%, *v:v*) and HCl content (0%, 0.1% and 0.5%, *v:v*) on the extraction of various individual phenolic compounds (4-hydroxybenzoic acid, ferulic acid and hesperidin), on the global phenolic concentration and on the TPC value estimated from the overall area at 310 nm are shown in Fig. 1. As can be seen, the extraction behavior was different depending on the type of compound, being the more polar species better extracted at lower ethanol percentages (e.g. 4-hydroxybenzoic acid, at 40%, see Fig. 1a) while the less polar ones were better recovered at higher percentages (e.g. hesperidin, at 80%, *v:v*, see Fig. 1c). Regarding to the HCl percentage, its influence was noticeable for coumaric and ferulic acids, and the best extraction yields were obtained at intermediate HCl concentrations (see Fig. 1b). These findings were statistically evaluated according to the RSM approach and results given in Table S1 (Supplementary material) confirmed that ethanol percentage was an influencing factor in the extraction of 4-hydroxybenzoic acid and hesperidin, while HCl percentage was relevant for the aforementioned hydroxycinnamic acids.

When the information of compounds belonging to the different families was combined, the best compromise to obtain the maximum extraction recovery has been attained at 80% ethanol and 0.1% HCl (Fig. 1d). This conclusion was in agreement with results from the use of the overall peak area as the data (Fig. 1e). In general, TPC increased with increasing ethanol concentration, whereas the maximum of TPC for HCl was attained at 0.1%. Thus, it was found that higher ethanol concentration and moderate acidity help to increase polyphenols extraction from spinach waste. The optimum UAE conditions for the extraction of polyphenols were 80% ethanol and 0.1% HCl (*v:v*), leading to a TPC of 820 ± 20 mg GAE kg^{-1} fw. Qiu et al. [41] studied the effect of extraction solvent, extraction time, and temperature on Okinawa spinach leaves extraction efficiency, obtaining optimal conditions of 40% ethanol, 30 min extraction time and 40 °C; the TPC under these conditions was 10,380 mg GAE kg^{-1} of dry matter.

A chromatogram showing the polyphenolic profile under the selected extraction conditions is depicted in Fig. 2. The occurrence of various phenolic acids (e.g. 4-hidroxybenzoic, ferulic and *p*-coumaric acids) and hesperidin as relevant components has been confirmed by HPLC-HRMS. Table S2 (Supplementary material) reports the concentration of each one under optimal conditions. These findings were in accordance with data reported in literature indicating that ferulic and *p*-coumaric acids were quite abundant in fresh spinach leaves extracts [42].

3.1.2. MAE

MAE extraction was also investigated with mixtures containing water, ethanol and hydrochloric acid from a factorial design. Levels under study were 0%, 40% and 60% for ethanol *v:v*, 60, 90 and 120 °C for temperature and 5 and 15 min for extraction time (see Table 1). For 5 min, results indicated that TPC improved with the increasing ethanol content, whereas for 15 min the TPC increased in the range of 0–40% ethanol and declined from 40% to 60%. Anyway, no significant ($p > 0.05$) improvement in the extraction yield was observed when increasing extraction time. These results agreed with Fiorito et al. [43], suggesting that a high percentage of ethanol improved the recovery of polyphenols using MAE. This was attributed to the higher solubility of less polar compounds in higher ethanol percentages. For temperature, the extraction yield increased from 60 °C to 90 °C, while decreased at 120 °C. This finding suggested that compound degradation may occurs at high temperatures for this matrix. Therefore, selected MAE conditions for the spinach matrix were ethanol/H_2O/HCl (60/39.9/0.1, *v/v/v*) as the solvent, a temperature of 90 °C and an extraction time of 5 min. The TPC obtained under these conditions was 950 ± 7 mg GAE kg^{-1} fw.

3.1.3. PLE

PLE factors under study comprised solvent composition (40%, 60% and 80% ethanol), temperature (80, 100 and 120 °C), time (5 and 15 min) and number of cycles (1 and 2). Recovery results in terms of overall peak area of polyphenols and PLE/UAE area ratio are summarized in Table 2 for a better comparison of the extraction performance with respect to UAE. ANOVA and t-student tests demonstrated that the effects of temperature, time and solvent composition in the ranges studied were negligible ($p > 0.05$). Therefore, working conditions, selected under the basis of saving solvent, energy and time, were solvent composition of ethanol/H_2O/HCl (40/59.9/0.1, *v/v/v*), temperature of

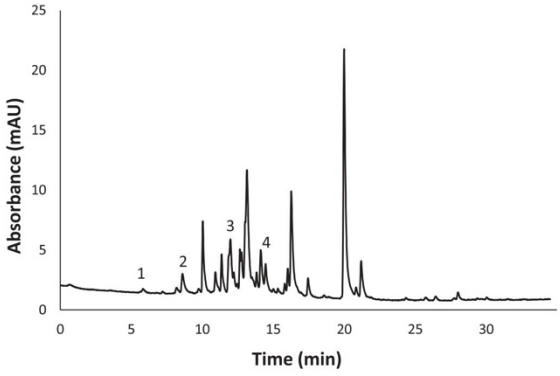

Fig. 2. Chromatogram at 310 nm of the spinach leaf waste extracted by UAE using ethanol/H_2O/HCl (80/19.9/0.1, *v/v/v*) for 30 min of extraction time. Peak assignment: 1 = 4-hidroxybenzoic acid, 2 = *p*-coumaric acid, 3 = ferulic acid, 4 = hesperidin.

Table 1
Results for the experimental design of MAE of spinach matrix. Average of the total area ($n = 3$) with the corresponding standard derivation and MAE/UAE ratio.

Experiment	EtOH (%, *v:v*)	Temperature (°C)	Time (min)	Overall peak area (Average ± SD)	MAE/ UAE
1	0	60	5	330 ± 110	0.49
2			15	350 ± 50	0.52
3		90	5	350 ± 20	0.53
4			15	240 ± 40	0.36
5		120	5	280 ± 80	0.41
6			15	560 ± 440	0.84
7	40	60	5	550 ± 110	0.82
8			15	640 ± 20	0.95
9		90	5	670 ± 70	1.00
10			15	710 ± 170	1.05
11		120	5	610 ± 14	0.90
12			15	580 ± 20	0.87
13	60	60	5	400 ± 70	0.60
14			15	390 ± 140	0.58
15		90	5	780 ± 20	1.16
16			15	630 ± 110	0.94
17		120	5	640 ± 110	0.95
18			15	560 ± 5	0.84

Table 2
Results for the experimental design of PLE of spinach matrix. Average of the total area ($n = 3$) with the corresponding standard derivation and MAE/UAE ratio.

Experiment	EtOH (%, *v:v*)	Temperature (°C)	Overall peak area (Average ± SD)	PLE/ UAE
1	40	80	3600 ± 400	1.23
2		100	3400 ± 200	1.16
3		120	3400 ± 100	1.14
4	60	80	3600 ± 300	1.21
5		100	2980 ± 60	1.00
6		120	2900 ± 200	0.99
7	80	80	3500 ± 200	1.18
8		100	3140 ± 80	1.06
9		120	3300 ± 400	1.12

80 °C and extraction time of 5 min. Besides, the application of further cycles did not improve the recovery yields so that 1 extraction cycle was chosen (see Fig. S1). Hence, a TPC of 1000 ± 130 mg GAE kg^{-1} fw was obtained, being the most effective extraction of polyphenols from spinach leaf waste. Similar conditions (ethanol/H_2O (50/50, *v/v*), temperature of 80 °C and extraction time of 10 min) were reported by Jaime et al. [44] for the extraction by PLE of polyphenols from spinach leaves extract.

3.1.4. Comparison of UAE, MAE and PLE under optimal conditions

In order to evaluate the extraction performance as a function of the extraction technique, recoveries from each option under selected conditions were compared. ANOVA and t-student tests using the TPC values obtained by UAE, MAE and PLE indicated that differences among techniques were statistically not significant ($p > 0.05$). Accordingly, there was not a noticeable improvement in the extraction efficiently of total polyphenols recovery from spinach leaves. Thus, UAE processing could be a simpler and more convenient approach for further scaling up, having an industrial potential, due to its low operating costs [45,46]. In this sense, Talmaciu et al. [47] compared the cost of UAE, MAE and supercritical fluid extraction (SFE) for polyphenol extraction, and concluded that UAE provided the lowest capital cost.

3.2. Extraction of phenolic compounds from orange waste

As for the spinach leave waste, UAE, MAE and PLE were also used to evaluate the extraction of polyphenols from fresh samples of orange peel and seeds. In a similar way, different experimental variables, such as extraction time, solvent composition, and temperature were studied under DoE approaches in order to stablish the optimal conditions and total amount of polyphenols extracted.

Orange extracts were analyzed by HPLC-HRMS to identify the most abundant compounds. In this case, 4-hydroxybenzoic acid, vanillic acid, syringic acid, coumaric acid, ferulic acid, rutin and hesperidin were detected via exact *m/z* values of $[M\text{-}H]^-$ ions. As in the spinach case, the tentative identification was confirmed by using standards. These results agree with some studies of polyphenols in citrus wastes (including orange), in which ferulic acid, *p*-coumaric acid, rutin, vanillic acid and 4-hydroxybenzoic acid were identified as relevant compounds [14, 48–51]. Moreover, Magwaza et al. [52] reported hesperidin as the major flavanone in citrus fruits.

3.2.1. UAE

First studies were focused on the assessment of the solvent composition, in which the ethanol/H_2O/HCl system was studied at 3 levels as follows: 40, 60, 80% (*v:v*) ethanol, and 0, 0.1, 0.5% (*v:v*) HCl. Extractions were carried out at room temperature for an extraction time of 30 min and the extraction performance was evaluated in terms of the TPC.

As in the spinach case, the extraction of individual polyphenols was first studied using RSM. Results summarized in Table S3 (Supplementary material) indicated that ethanol percentage was relevant for the extraction of hydroxybenzoic acids such as vanillic and syringic acids, and the best yields were obtained at low ethanol percentages (40% *v:v* ethanol). Conversely, the influence of HCl percentage was not relevant in this case. For the flavonoids, which are less polar compounds, the ethanol percentage was also noticeable, and the best extractions were attained at 80% ethanol, while the influence of HCl was less important. The extraction behavior of ferulic and coumaric acids was, in this case, dependent on both ethanol and HCl factors. The overall extraction response resulting from the combination of the concentrations of the target compounds also showed the influence of these experimental factors. Results shown in Fig. 3, either from the combined response from each compound (Fig. 3a) or from the TPC values estimated with the total chromatographic area at 310 nm (Fig. 3b), indicated that the maximum recovery corresponded to ethanol/H_2O/HCl (60/39.9/0.1, *v/v/v*), thus giving a TPC value of 400 ± 100 mg GAE kg^{-1} fw by UAE.

Fig. 4 shows the chromatogram obtained from the orange matrix and Table S2 (Supplementary material) reports the concentration of the identified polyphenols in extract obtained under the optimal conditions by UAE (60%, *v:v* ethanol and 0.1%, *v:v* HCl, 30 min extraction time).

3.2.2. MAE

The optimization of the orange waste extraction by MAE was planned in the same way as for the spinach waste. DoE was designed to explore the influence of ethanol (0%, 40% and 60%, *v:v*), temperature (60, 90 and 120 °C) and time (5 and 15 min). In this case, compared with UAE, MAE provided a noticeable increase of extraction, which was attributed to the rupture of the vegetal cells induced by the radiation [31]. The results for orange matrix are summarized in Table 3.

It was found that the extraction improved with the increase of the ethanol content (from 0% to 60%) and temperature (from 60 °C to 120 °C). Time also had a positive effect on this process and, in general, higher TPC values were obtained with 15 min. These conclusions were statistically supported by ANOVA and t-student tests, indicating that the factors studied have a positive influence on the extraction ($p < 0.05$). As a result, selected MAE conditions corresponded to ethanol/H_2O/HCl ratio of 60/39.9/0.1 (*v/v/v*) at 120 °C and with 15 min of extraction time, leading to TPC of 2000 ± 130 mg GAE kg^{-1} fw. At these conditions, the extraction yield improvement was more than 4-fold using MAE with respect to UAE.

Unlike spinach, in this matrix, no degradation of compounds was

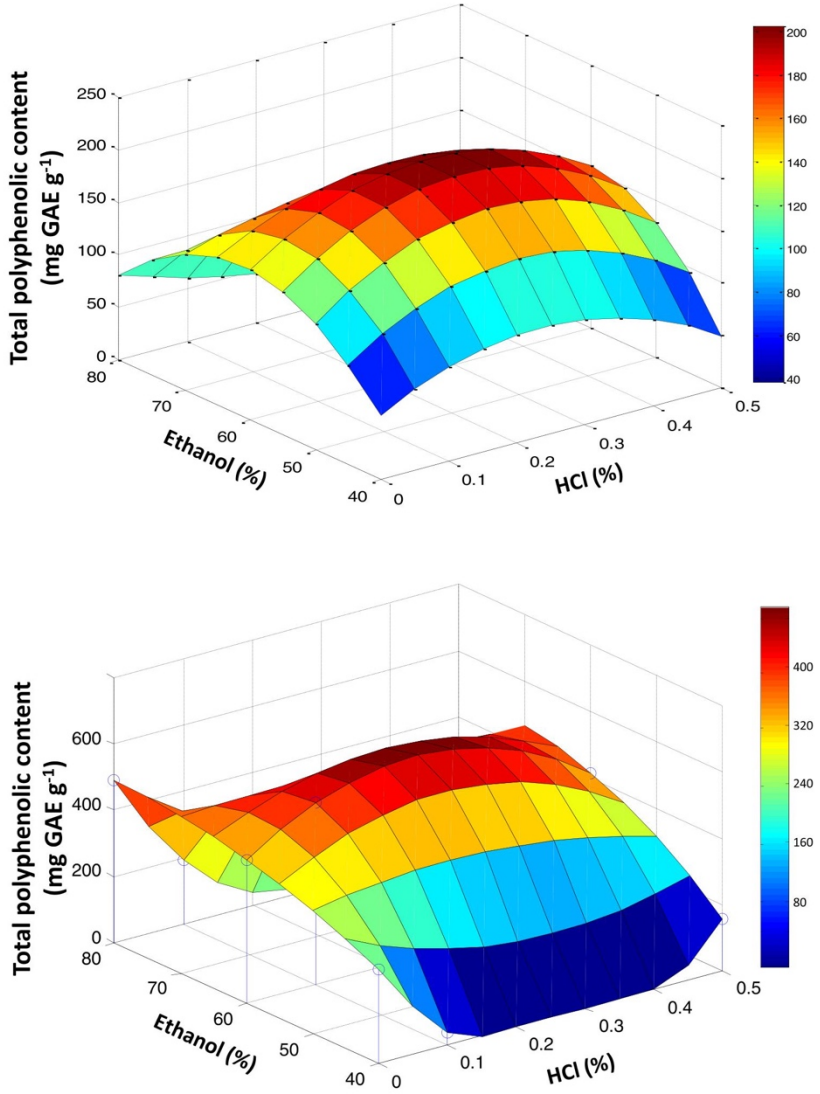

Fig. 3. Surface response of total polyphenols content (mg GAE g^{-1} fw) by UAE as a function of ethanol and HCl percentages for orange waste using UAE. (a) overall phenolic amount from the addition of each individual compound; (b) total phenolic amount from the overall area at 280 nm. Extraction time: 30 min.

observed (it can be mentioned that degradation occurs depending on the nature of the phenolic compounds and of the matrix), even at higher temperatures (120 °C).

3.2.3. PLE

Ethanol percentage, temperature, time and number of cycles were here the variables under study. The DoE design, analogous to that of the spinach case, is detailed in Table 4. In the same table, TPC results indicated that the increase of ethanol percentage slightly increased the extraction yield. However, the influence of temperature was, in general, quite irrelevant. The highest TPC values were obtained at intermediate to high ethanol percentages (60–80% *v:v*) and low to intermediate temperatures (80–100 °C).

Additional studies on process time (see Fig. S2 in Supplementary material) revealed that the extraction increased significantly with time from 5 to 15 min ($p < 0.05$). Besides, the application of further extraction cycles did not improve significantly ($p > 0.05$) the recovery yield (see Fig. S2 in Supplementary material). The same behavior was observed by M'hiri et al. [53], who reported that there is no significant effect of the cycles on the TPC; thus, one cycle is sufficient for polyphenols extraction from orange waste. As a result, conditions selected for the extraction of polyphenols from orange matrix by PLE were as follows: ethanol/H_2O/HCl in ratio of 60/39.9/0.1 (*v/v/v*) as the solvent, 80 °C, 15 min extraction time and 1 extraction cycle. Under these circumstances, TPC of 3000 ± 70 mg GAE kg^{-1} fw was obtained. These results agree with Barrales et al. [33], who determined similar optimal conditions for PLE of polyphenols from orange peels, at 65 °C, ethanol 75% (*v/v*) and extraction time of 20 min.

Finally, the fact that a large extraction time did not decrease the TPC values suggested that polyphenols recovered from the orange matrix

Table 1
Results for the experimental design of MAE of spinach matrix. Average of the total area ($n = 3$) with the corresponding standard derivation and MAE/UAE ratio.

Experiment	EtOH (%, *v:v*)	Temperature (°C)	Time (min)	Overall peak area (Average ± SD)	MAE/ UAE
1	0	60	5	330 ± 110	0.49
2			15	350 ± 50	0.52
3		90	5	350 ± 20	0.53
4			15	240 ± 40	0.36
5		120	5	280 ± 80	0.41
6			15	560 ± 440	0.84
7	40	60	5	550 ± 110	0.82
8			15	640 ± 20	0.95
9		90	5	670 ± 70	1.00
10			15	710 ± 170	1.05
11		120	5	610 ± 14	0.90
12			15	580 ± 20	0.87
13	60	60	5	400 ± 70	0.60
14			15	390 ± 140	0.58
15		90	5	780 ± 20	1.16
16			15	630 ± 110	0.94
17		120	5	640 ± 110	0.95
18			15	560 ± 5	0.84

Table 2
Results for the experimental design of PLE of spinach matrix. Average of the total area ($n = 3$) with the corresponding standard derivation and MAE/UAE ratio.

Experiment	EtOH (%, *v:v*)	Temperature (°C)	Overall peak area (Average ± SD)	PLE/ UAE
1	40	80	3600 ± 400	1.23
2		100	3400 ± 200	1.16
3		120	3400 ± 100	1.14
4	60	80	3600 ± 300	1.21
5		100	2980 ± 60	1.00
6		120	2900 ± 200	0.99
7	80	80	3500 ± 200	1.18
8		100	3140 ± 80	1.06
9		120	3300 ± 400	1.12

80 °C and extraction time of 5 min. Besides, the application of further cycles did not improve the recovery yields so that 1 extraction cycle was chosen (see Fig. S1). Hence, a TPC of 1000 ± 130 mg GAE kg^{-1} fw was obtained, being the most effective extraction of polyphenols from spinach leaf waste. Similar conditions (ethanol/H_2O (50/50, *v/v*), temperature of 80 °C and extraction time of 10 min) were reported by Jaime et al. [44] for the extraction by PLE of polyphenols from spinach leaves extract.

3.1.4. Comparison of UAE, MAE and PLE under optimal conditions

In order to evaluate the extraction performance as a function of the extraction technique, recoveries from each option under selected conditions were compared. ANOVA and t-student tests using the TPC values obtained by UAE, MAE and PLE indicated that differences among techniques were statistically not significant ($p > 0.05$). Accordingly, there was not a noticeable improvement in the extraction efficiently of total polyphenols recovery from spinach leaves. Thus, UAE processing could be a simpler and more convenient approach for further scaling up, having an industrial potential, due to its low operating costs [45,46]. In this sense, Talmaciu et al. [47] compared the cost of UAE, MAE and supercritical fluid extraction (SFE) for polyphenol extraction, and concluded that UAE provided the lowest capital cost.

3.2. Extraction of phenolic compounds from orange waste

As for the spinach leave waste, UAE, MAE and PLE were also used to evaluate the extraction of polyphenols from fresh samples of orange peel and seeds. In a similar way, different experimental variables, such as extraction time, solvent composition, and temperature were studied under DoE approaches in order to stablish the optimal conditions and total amount of polyphenols extracted.

Orange extracts were analyzed by HPLC-HRMS to identify the most abundant compounds. In this case, 4-hydroxybenzoic acid, vanillic acid, syringic acid, coumaric acid, ferulic acid, rutin and hesperidin were detected via exact *m/z* values of $[M\text{-}H]^-$ ions. As in the spinach case, the tentative identification was confirmed by using standards. These results agree with some studies of polyphenols in citrus wastes (including orange), in which ferulic acid, *p*-coumaric acid, rutin, vanillic acid and 4-hydroxybenzoic acid were identified as relevant compounds [14, 48–51]. Moreover, Magwaza et al. [52] reported hesperidin as the major flavanone in citrus fruits.

3.2.1. UAE

First studies were focused on the assessment of the solvent composition, in which the ethanol/H_2O/HCl system was studied at 3 levels as follows: 40, 60, 80% (*v:v*) ethanol, and 0, 0.1, 0.5% (*v:v*) HCl. Extractions were carried out at room temperature for an extraction time of 30 min and the extraction performance was evaluated in terms of the TPC.

As in the spinach case, the extraction of individual polyphenols was first studied using RSM. Results summarized in Table S3 (Supplementary material) indicated that ethanol percentage was relevant for the extraction of hydroxybenzoic acids such as vanillic and syringic acids, and the best yields were obtained at low ethanol percentages (40% *v:v* ethanol). Conversely, the influence of HCl percentage was not relevant in this case. For the flavonoids, which are less polar compounds, the ethanol percentage was also noticeable, and the best extractions were attained at 80% ethanol, while the influence of HCl was less important. The extraction behavior of ferulic and coumaric acids was, in this case, dependent on both ethanol and HCl factors. The overall extraction response resulting from the combination of the concentrations of the target compounds also showed the influence of these experimental factors. Results shown in Fig. 3, either from the combined response from each compound (Fig. 3a) or from the TPC values estimated with the total chromatographic area at 310 nm (Fig. 3b), indicated that the maximum recovery corresponded to ethanol/H_2O/HCl (60/39.9/0.1, *v/v/v*), thus giving a TPC value of 400 ± 100 mg GAE kg^{-1} fw by UAE.

Fig. 4 shows the chromatogram obtained from the orange matrix and Table S2 (Supplementary material) reports the concentration of the identified polyphenols in extract obtained under the optimal conditions by UAE (60%, *v:v* ethanol and 0.1%, *v:v* HCl, 30 min extraction time).

3.2.2. MAE

The optimization of the orange waste extraction by MAE was planned in the same way as for the spinach waste. DoE was designed to explore the influence of ethanol (0%, 40% and 60%, *v:v*), temperature (60, 90 and 120 °C) and time (5 and 15 min). In this case, compared with UAE, MAE provided a noticeable increase of extraction, which was attributed to the rupture of the vegetal cells induced by the radiation [31]. The results for orange matrix are summarized in Table 3.

It was found that the extraction improved with the increase of the ethanol content (from 0% to 60%) and temperature (from 60 °C to 120 °C). Time also had a positive effect on this process and, in general, higher TPC values were obtained with 15 min. These conclusions were statistically supported by ANOVA and t-student tests, indicating that the factors studied have a positive influence on the extraction ($p < 0.05$). As a result, selected MAE conditions corresponded to ethanol/H_2O/HCl ratio of 60/39.9/0.1 (*v/v/v*) at 120 °C and with 15 min of extraction time, leading to TPC of 2000 ± 130 mg GAE kg^{-1} fw. At these conditions, the extraction yield improvement was more than 4-fold using MAE with respect to UAE.

Unlike spinach, in this matrix, no degradation of compounds was

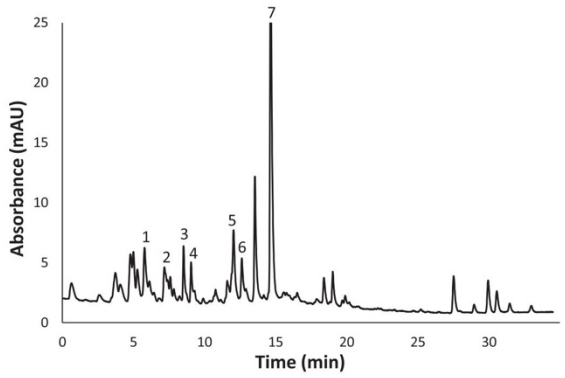

Fig. 4. Chromatogram at 310 nm for the orange matrix extracted by UAE (ethanol:water:HCl 60:39.9:0.1, *v/v/v* and 30 min of extraction time). Peak assignment: 1 = 4-hydroxybenzoic acid, 2 = vanillic acid, 3 = syringic acid, 4 = *p*-coumaric acid, 5 = ferulic acid, 6 = rutin, 7 = hesperidin.

Table 3
Results for the experimental design of MAE of orange matrix. Average of the total area ($n = 3$) with the corresponding standard derivation and MAE/UAE ratio.

Experiment	EtOH (%, *v:v*)	Temperature (°C)	Time (min)	Overall peak area (Average ± SD)	MAE/ UAE
1	0	60	5	780 ± 40	0.51
2			15	800 ± 20	0.52
3		90	5	3000 ± 1000	2.06
4			15	1900 ± 620	1.28
5		120	5	2070 ± 70	1.36
6			15	3000 ± 1000	2.21
7	40	60	5	2600 ± 100	1.70
8			15	2620 ± 60	1.72
9		90	5	2600 ± 200	1.75
10			15	2560 ± 20	1.67
11		120	5	2900 ± 120	1.92
12			15	3100 ± 120	2.04
13	60	60	5	3000 ± 200	1.98
14			15	3300 ± 160	2.21
15		90	5	4100 ± 160	2.73
16			15	4200 ± 180	2.79
17		120	5	5000 ± 400	3.26
18			15	6700 ± 400	4.40

Table 4
Results for the experimental design of PLE of orange matrix. Average of the total area ($n = 3$) with the corresponding standard derivation and MAE/UAE ratio.

Experiment	EtOH (%, *v:v*)	Temperature (°C)	Overall peak area (Average ± SD)	PLE/ UAE
1	40	80	8000 ± 400	4.71
2		100	9500 ± 500	5.63
3		120	10,000 ± 200	5.90
4	60	80	11,500 ± 200	6.76
5		100	10,000 ± 1000	6.01
6		120	10,800 ± 200	6.41
7	80	80	10,500 ± 600	6.21
8		100	11,600 ± 300	6.83
9		120	9000 ± 1000	5.47

were not affected by degradation processes.

3.2.4. Comparison of UAE, MAE and PLE under optimal conditions

A comparison of the three optimized procedures (UAE, MAE and PLE) was performed to propose the most efficient methodology for the extraction of polyphenols from orange wastes of agri-food industry. The TPC values of the extracts obtained by UAE, MAE and PLE were significantly different compared between them ($p < 0.05$). Comparing MAE (2000 ± 130 mg GAE kg^{-1} fw) with UAE (400 ± 100 mg GAE kg^{-1} fw), MAE provided a significant increase ($p < 0.05$) of the extraction (ca. 4-fold higher TPC) without a remarkable alteration of the compositional patterns. In a similar way, PLE yields were ca. 7 times higher than those of UAE ($p < 0.05$), with 3000 ± 70 mg GAE kg^{-1} fw.

Finally, PLE was more efficient than MAE in terms of TPC extraction, providing TPC values in PLE extracts ca. 1.5-fold higher than in MAE extracts. Therefore, in the case of the orange matrix, PLE provided a higher extraction yield of polyphenols, followed by MAE. The use of intensive conditions (ethanol/H_2O/HCl 60/39.9/0.1 (*v/v/v*), 80 °C, 15 min and 1 extraction cycle) was recommendable for the recovery of polyphenols for this matrix. However the scaling of PLE or MAE currently has not been depth studied [54]. On the other side, when considering simplicity, as well as investment and operational costs UAE is the technique of choice [38,47]. For this reason, UAE is proposed as an achievable operational technique for industrial scale-up.

3.3. Antioxidant features of fruit and vegetable waste extracts

The identification of the agri-food wastes with the richest contents of bioactive phytochemicals was the main objective of this paper. Fruit and vegetable extracts could be the basis of by-products with great antioxidant features, with potential interest for manufacturing food supplements and nutraceuticals with increasing social and economic impact. Under this approach, and although UAE conditions chosen above for spinach and orange selected as representatives for vegetable and fruit samples might differ slightly from the optimal ones required for other fruits and vegetables, they could reasonably be extended to other waste matrices to explore the potential of the valorization.

Hence, spinach, carrot, kale, celery, beet, broccoli, orange, kiwi, strawberry, white grape and red grape wastes were processed in the same way as follows. 1 g of each sample residue was extracted with 20 mL solvent solution consisting of 80% (*v:v*) ethanol and 0.1% (*v:v*) HCl. Samples were sonicated for 30 min at room temperature, the supernatant solutions were centrifuged for 15 min at 3500 rpm and were filtered through the nylon membrane. Samples were extracted in duplicate.

The extract solutions from each residue were analyzed chromatographically and spectroscopically according to the different phenolics, antioxidant and antiradical indexes described in Section 2.6. HPLC and FC method were used to determine the TPC in terms of mg GAE kg^{-1} fw, FRAP accounted the reducing power expressed as Trolox equivalent (mg Trolox kg^{-1} fw) and DPPH and ABTS indexes provided an estimation of the antiradical capacity of the samples (also expressed as Trolox equivalent). According to Dzah et al. [55], these assays are used to test the conservation of antioxidant activity of plants extracts after UAE.

Results from these assays are given in Table 5. As can be seen, the values provided by each index differ from the others (even when referred to the same standard). This finding was attributed to the different nature of the redox and antiradical reactions involved. ANOVA was applied to assess the occurrence of significant differences among samples and indexes. Statistical results showed that antioxidant contents differed depending on the samples ($p < 10^{-35}$) since all of them showed different compositional profiles. For the indexes, results were seldom comparable in terms of overall antioxidant capacity ($p < 10^{-17}$). Besides, the interaction between samples and indexes was significant as well ($p < 10^{-19}$), meaning that values from each index depended on the type of sample. These results suggested that understanding the overall antioxidant capacity of fruit and vegetable wastes is still a challenging issue.

Besides, the HPLC method accounts for all absorbing compounds at 310 nm, mainly phenolic compounds although other aromatic molecules may also be detected, thus providing in some cases an

Table 5
TPC, antioxidant and antiradical indexes of the agri-food residues under study. Standard deviations are given in parenthesis.

Sample	HPLC[a]	FC[a]	FRAP[b]	DPPH[b]	ABTS[b]
Spinach	3100 (100)	630 (40)	83 (10)	1005 (80)	1090 (90)
Carrot	360 (20)	60 (1)	14 (10)	*nd*	15 (2)
Kale	3490 (600)	1100 (300)	1192 (1)	108 (2)	1600 (100)
Celery	290 (100)	68 (5)	23 (10)	*nd*	18 (1)
Beet	180 (50)	580 (6)	990 (30)	1450 (30)	1560 (70)
Broccoli	280 (100)	370 (20)	95 (6)	420 (40)	240 (2)
Orange	820 (300)	690 (30)	990 (20)	2700 (200)	570 (30)
Kiwi	390 (10)	200 (20)	144 (10)	890 (40)	305 (9)
Strawberry	1750 (100)	950 (100)	350 (1)	3400 (40)	4900 (100)
Whyte grape	330 (20)	1200 (200)	1360 (50)	6750 (200)	4040 (400)
Black grape	4900 (300)	3450 (20)	5900 (50)	10,600 (600)	8900 (300)

[a] HPLC and FC method data expressed as mg GAE kg^{-1} fw.
[b] FRAP, DPPH and ABTS data expressed as mg Trolox kg^{-1} *fw*; *nd*, non detected.

overestimation of active compounds. Anyway, despite the different scales of assays, results from all these methods were in agreement when comparing the set of samples under study, meaning that those rich in antioxidants displayed high index values for all the methods and vice versa. In the case of vegetables, spinach, kale and beet residues seem to be great sources of antioxidant compounds. In the case of fruit by-products, grape and strawberry matrices provided extracts with high phenolic concentrations.

4. Conclusions

This study is developed in the frame of a more general research project focused on the revalorization of industrial wastes resulting from fruit and vegetable juice processing. Polyphenols have been identified as the most relevant group of phytochemicals from these wastes as a source of antioxidant by-products. The optimization of the extraction conditions has been carried out based on experimental design approaches, multicriteria decision making and response surface methodology. The recovery of remarkable target compounds, such as 4-hydroxybenzoic acid, vanillic acid, coumaric acid and hesperidin, has been successfully evaluated, and specific conditions have been stablished. Anyway, more global criteria have been defined to assess the content of active antioxidant species. In this regard, the overall chromatographic area has been found to be an excellent descriptor of the global phenolic content.

The investigated water-ethanol mixtures, which are permitted by the food industry, showed a good efficiency for the extraction of phenolic compounds from spinach and orange wastes by UAE, MAE and PLE. The comparison of the extraction yields pointed out UAE was a convenient approach for spinach matrix while PLE provided higher performance for the orange matrix, in which ca. 5-fold increase was obtained compared with UAE. Anyway, considering simplicity and operational cost UAE was eventually recommended for industrial waste processing, especially for dealing with labile sample components.

The endorsed procedure was applied to other vegetable and fruit by-products and the resulting ethanolic extracts were analyzed by HPLC and spectrophotometric indexes. Various residues were identified as potential targets of antioxidant compounds, including spinach, kale, grape, strawberry and orange, among others.

CRediT authorship contribution statement

María Fernanda Montenegro-Landívar: Investigation, Formal analysis, Methodology, Writing - original draft. **Paulina Tapia-Quirós**: Investigation, Methodology. **Xanel Vecino**: Conceptualization, Supervision, Writing - review & editing. **Mònica Reig**: Conceptualization, Writing - review & editing. **César Valderrama**: Conceptualization, Supervision, Writing - review & editing. **Mercè Granados**: Conceptualization, Writing - review & editing. **José Luis Cortina**: Resources, Funding acquisition, Writing - review & editing. **Javier Saurina**: Conceptualization, Supervision, Formal analysis, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2021.105330.

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Supplementary material

of

Fruit and vegetable processing wastes as natural sources of antioxidants: evaluation of advanced extraction technologies by surface response methodologies

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Table S1. Concentration (mg of compound per kg of fresh weight) of polyphenols identified in the extracts of spinach and orange wastes under optimal conditions by UAE.

Plant matrix	Optimal conditions	Polyphenols	Concentration	Family
Spinach	$EtOH/H_2O/HCl$ 80/19.9/0.1 *(v/v/v)*, room temperature and 30 minutes	1 = 4-hydroxybenzoic acid	25.35	Hydroxycinnamic acid
		2 = *p*-coumaric acid	23.12	Hydroxycinnamic acid
		3 = ferulic acid	10.51	Hydroxycinnamic acid
		4 = hesperidin	63.94	Flavonoids
Orange	$EtOH/H_2O/HCl$ 60/39.9/0.1 *(v/v/v)*, room temperature and 30 minutes	1 = 4-hydroxybenzoic acid	104.51	Hydroxycinnamic acid
		2 = vanillic acid	394.37	Hydroxycinnamic acid
		3 = syringic acid	37.49	Hydroxycinnamic acid
		4 = *p*-coumaric acid	27.75	Hydroxycinnamic acid
		5 = ferulic acid	25.45	Hydroxycinnamic acid
		6 = rutin	86.12	Flavonoids
		7 = hesperidin	676.68	Flavonoids

2

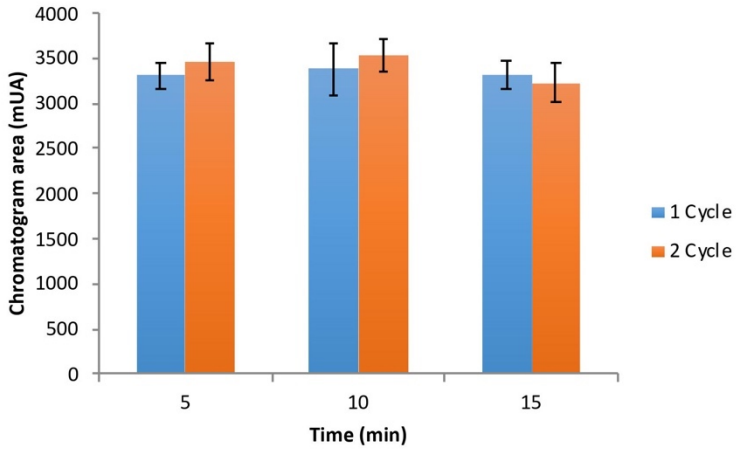

Figure S1. Effect of extraction time and cycles optimization from spinach matrix by PLE.

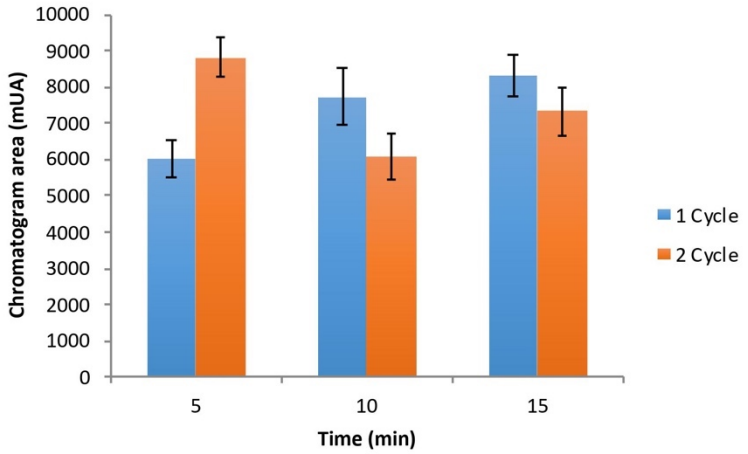

Figure S2. Effect of extraction time and cycles optimization from orange matrix by PLE.

4

CHAPTER 5

Publication 2

"Recovery of add-value compounds from orange and spinach residues: green extraction of phenolic compounds and evaluation of antioxidant activity"

antioxidants

MDPI

Article

Recovery of Added-Value Compounds from Orange and Spinach Processing Residues: Green Extraction of Phenolic Compounds and Evaluation of Antioxidant Activity

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Abstract: Phenolic compounds recovery by mechanical stirring extraction (MSE) was studied from orange and spinach wastes using water as a solvent. The statistical analysis showed that the highest total polyphenol content (TPC) yield was obtained using 15 min, 70 °C, 1:100 (*w*/*v*) solid/solvent ratio and pH 4 for orange; and 5 min, 50 °C, 1:50 (*w*/*v*) solid/solvent ratio and pH 6 for spinach. Under these conditions, the TPC was 1 mg gallic acid equivalent (GAE) g^{-1} fresh weight (fw) and 0.8 mg GAE g^{-1} fw for orange and spinach, respectively. MSE substantially increased the phenolic compounds yields (1-fold for orange and 2-fold for spinach) compared with ultrasound-assisted extraction. Furthermore, the antioxidant activity of orange and spinach extracts was evaluated using DPPH, FRAP and ABTS. The obtained results pointed out that the evaluated orange and spinach residues provided extracts with antioxidant activity (2.27 mg TE g^{-1} and 0.04 mg TE g^{-1}, respectively).

Keywords: agri-food wastes; mechanical stirring extraction; antioxidant activity; waste to resources; resource recovery

1. Introduction

Fruits and vegetables are a rich source of phenolic compounds that provide the plant with protection against harmful ultraviolet radiation and pathogens, among other abiotic and biotic stresses [1–4]. Phenolic compounds are secondary metabolites produced by plants. These act on the plant defense mechanism against, e.g., insects, fungi, drought, and extreme temperatures, among other stress factors [5]. Phenolic compounds, including flavonoids, phenolic acids and others [6], display great antioxidant power (biological and free radical scavenging activity) as one of their principal properties [7], being the reason that the recovery of phenolic compounds has become a key strategy to satisfy the growing demand from the food, cosmetic and pharmaceutical industries [8]. For example, natural

antioxidants have been used to replace the synthetic antioxidants additives (e.g., butylated hydroxytoluene (BTH) or butylated hydroxyanisole (BHA)) used in food products [9–11], since their toxicity has been proven [12,13].

Another source of phenolic compounds is the agri-food processing industries that generate large amounts of by-products and/or wastes (e.g., seeds and peels of Citrus spp., olive mill wastewater, artichoke leaves and stem) [14]. Orange (*Citrus sinensis*) and spinach (*Spinacia oleracea*) crops are among the most abundant in Spain, generating between 50% and 13% of wastes, respectively [15]. It is well established that orange, spinach and their by-products are rich sources of minerals, vitamins and dietary fiber as well as bioactive compounds like polyphenols (specifically flavonoids and phenolic acids) [16], which also provide a high antioxidant activity [8,11].

The antioxidant activity of phenolic compounds is linked to their structure; they generally act by preventing the formation of free radicals involved in the autoxidation process, for which they donate electrons or hydrogen atoms or chelating metal cations [14]. Many studies have found that orange and spinach have antioxidant phenolic compounds (e.g., ferulic acid, luteolin, hesperidin) with promising effects in various diseases such as diabetes, cancer, and hypertension, among others [17,18].

The growing interest for the recovery of phenolic compounds from agri-food wastes and their use as ingredients in cosmetic, pharmaceutical and food preparations has led to develop efficient and cost-effective extraction processes. In this regard, mechanical stirring extraction (MSE) uses low temperatures, requires a simple equipment and the process is not expensive [19]. MSE follows the procedure of shaking the sample in contact with a solvent for a certain time and at a certain temperature to preserve the stability of phenolic compounds [20,21]. The advantage of agitation is that facilitates extraction by increasing diffusion and removing concentrated solution from the sample surface to bring new solvent, and thus achieves an elevated extraction performance [22]. Additionally, the solvent nature plays an important role to obtain a high extraction yield. Methanol, acetone, and ethanol are the most used. Despite their effectiveness, from an industrial point of view, cost, toxicity and safety of other solvents should be considered such as water for high-volume extraction [23–25]. According to Gómez-Mejía et al. [26], research should focus on how to improve the efficiency of aqueous extraction. Additionally, the development of an efficient, energy-saving, and sustainable processes, can also offer advantages to the food industry in terms of energy consumption, time and profitability. In this way, a cleaner production of phenolic compounds can be achieved and thus to achieve a high demand.

Therefore, in view of the above, the aim of the present work is to optimize the phenolic compounds extraction with water, as a solvent, from orange and spinach wastes by mechanical stirring extraction (MSE) and to compare the selected conditions with ultrasound-assisted extraction (UAE), which is one of the most widely used technique for these purposes [17,27]. The total polyphenolic content (TPC) was determined by Folin–Ciocalteu (FC) and by the high-performance liquid chromatography (HPLC-DAD). Furthermore, the antioxidant activity of several fruit and vegetables residues was evaluated by different tests including 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS).

2. Materials and Methods

2.1. Reagents and Solvents

Phenolic compounds used as standards were as follows: 3,4-dihydroxybenzoic acid (>97%), 4-hydroxybenzoic acid (99%), ferulic acid (99%), gallic acid (>97.5), naringenin (>97%), *p*-coumaric acid (>97%), rutin (>94%), syringic acid (>95%), caffeic acid (>98%), and vanillic acid (97%), from Sigma Aldrich (St. Louis, USA); hesperidin (>90%) from Glentham Life Sciences (UK); and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 98% purity) was purchased from Carbosynth (Berkshire, UK).

Acetonitrile (ACN, HPLC grade, >99) was purchased from Fisher Scientific (UK). Ethanol (EtOH, HPLC grade), formic acid (98–100% *w*/*w*) and hydrochloric acid (32% *w*/*w*) were obtained by Merck (Darmstradt, Germany). Water was purified with a Milli-Q equipment (Merck Millipore).

The chemicals used in antioxidant index tests were as follows: formic acid (98-100% *w*/*w*) and potassium peroxodisulfate (>99%) from Sigma Aldrich (St. Louis, MI, USA), hydrochloric acid (32%, *w*/*w*), sodium hydroxide (>99%), Fe (III) chloride (>99%), sodium carbonate (>99%) and disodium hydrogen phosphate (>99%) from Merck (Darmstradt, Germany); Folin-Ciocalteu (FC) reagent was a commercial solution ready to use from Panreac; 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS, 98%), 2,2-diphenyl-1-picrylhydrazyl (DPPH, 95%) and 2,4,6-tripyridyl-S-triazine (TPTZ, 99%) from Alfa Aesar (Kandel, Germany).

2.2. Fruit and Vegetable Samples

Orange (*Citrus sinensis*), kiwi (*Actinidia sinensis*), white and red grape (*Vitis vinifera*), strawberry (*Fragaria vesca*), spinach (*Spinacia oleracea*), carrot (*Daucus carota*), celery (*Apium graveolens*), beet (*Beta vulgaris*), kale (*Brassica oleracea var. sabellica*) and broccoli (*Brassica oleracea var. italica*) were purchased from a local market (Barcelona, Spain). One kg of each one was used in the process of simulating the obtaining of waste from the agri-food industries, specifically in the juice processing. Fruits and vegetables were processed with a domestic juicer. The solid residues obtained (such as orange peel and seeds, and spinach leave waste) were used as representative waste samples and stored in the freezer at −20 °C.

2.3. Instruments and Lab Equipment

The phenolic compounds were determined by HPLC-DAD, with an Agilent Series 1200 HPLC chromatography (Agilent Technologies, Palo Alto, CA, USA) with a quaternary pump (G1311A), a degasser (G1322A), an automatic injection system (G1392A) and a diode array detector (G1315B). The Agilent ChemStation software was used for instrument control and data processing.

The antioxidant and antiradical capacities of vegetable and fruit extracts from the set of samples given in Section 2.2 were estimated with a double beam Perkin Elmer UV/Vis/NIR Lambda 19 spectrophotometer. QS quartz glass high performance cuvettes (10 mm optical path) from Hellma Analytics (Jena, Germany) were used.

The extraction of phenolic compounds was carried out using a magnetic stirred furnished with a heating plate (IKA RCT basic, Staufen, Germany). The pH was measured using a pH-meter from Crison (Alella, Barcelona, Spain). On the other hand, the UAE of phenolic compounds was conducted using an ultrasonic bath (Branson 5510, Danbury CT, USA). The obtained extracts were centrifuged (Rotina 420, Hettich, Tuttlingen, Germany) and the supernatant was filtered through 0.45 μm nylon filters (Whatman, Clifton, NJ, USA).

2.4. Extraction of Phenolic Compounds

2.4.1. Mechanical Stirring Extraction (MSE)

The samples were treated using the conditions listed in Table S1. In brief, 1 g of each by-product sample was mixed with the solvent (Milli-Q water) and placed in the stirring plate. The extraction variables were contact time (5, 15 and 30 min), temperature (25, 50, 70 and 90 °C), solid/solvent ratio (1:10, 1:30, 1:50, 1:100 and 1:200 (*w*/*v*)), and pH (3, unadjusted, and 10). For each assayed condition, experiments were performed in triplicate. After MSE treatment, the resulting extracts were centrifuged for 15 min at 3500 rpm. The supernatant was filtered using a nylon membrane of 0.45 μm. The extracts were stored at 4 °C until the chromatographic and antioxidant analysis.

2.4.2. Ultrasound-Assisted Extraction (UAE)

Extractions were performed the under optimized conditions from previous works [15]. For orange residue, 60:39.9:0.1 ethanol:water:HCl (*v*/*v*/*v*) solvent and contact time of 30 min at 25 °C, and for spinach residue 80:19.9:0.1 ethanol:water:HCl (*v*/*v*/*v*) solvent for an extraction time of 30 min at 25 °C (frequency of 42 kHz and power of 135 W) were used. Briefly, 1 g of orange and spinach samples was mixed with 20 mL of solvent and sonicated. After that, the mixture was centrifuged for 15 min at 3500 rpm. The supernatant was filtered through 0.45 μm nylon filters. The extracts were stored at 4 °C until the chromatographic and antioxidant analysis. Determinations were carried out in triplicate.

2.5. Antioxidant Activity Evaluation

The antioxidant activity of the extracts was determined according to an adaptation of the DPPH, FRAP, ABTS and FC methods described by Alcalde et al. [28].

2.5.1. DPPH

A 0.2 mM DPPH stock solution in 50 mL ethanol was prepared and kept in dark for 2 h. Then, 2 mL of the DPPH solution, 0.8 mL of 0.2 M phosphate buffer (pH 7.4), the necessary volume of standard/sample and Milli-Q water up to 4 mL were mixed and kept in dark for 45 min. The absorbance was recorded at 517 nm using a reagent blank as the reference (the blank absorbance versus water was ca. 1.0 AU). The calibration range was from 0.2 to 10 mg L^{-1} Trolox ($R^2 = 0.984$). The DPPH values were expressed as mg Trolox equivalents/g of fresh weight using the standard curve established previously. All samples were analyzed in duplicate.

2.5.2. FRAP

Required volume of the standard/sample was mixed with 300 μL of FRAP reagent consisting of 20 mM L^{-1} $FeCl_3$, 10 mM L^{-1} TPTZ (containing 50 mM L^{-1} HCl) and 50 mM L^{-1} formic acid solution in a proportion of 1:2:10 (*v*/*v*/*v*) and up to 2.5 mL with Milli-Q water. The absorbance was recorded at 595 nm resulting after 5 min of the reaction, using a blank as the reference. The calibration range was 0.2 to 5 mg L^{-1} Trolox ($R^2 = 0.999$). The FRAP values were expressed as mg Trolox equivalents/g of fresh weight using the standard curve established previously. All samples were analyzed in duplicate.

2.5.3. ABTS

$ABTS^{\bullet+}$ reagent was generated with 20 mL of 7 mM ABTS and 350 μL of 140 mM potassium peroxodisulfate. The mixture was kept in the dark for 16 h before use. A daily working solution was prepared with 600 μL of $ABTS^{\bullet+}$ in 24 mL of EtOH. Then, 1.5 mL of $ABTS^{\bullet+}$ was diluted in the required volume standard/sample and measure up to 2.5 mL with Milli-Q water. The absorbance was measured at 734 nm using the reagent blank as the reference after 25 min of the reaction time. The calibration range was 0.2 to 10 mg L^{-1} Trolox ($R^2 = 0.906$). The ABTS values were expressed as mg Trolox equivalents g^{-1} of fresh weight using the standard curve established previously. All samples were analyzed in duplicate.

2.5.4. FC Assay

Required volume of standard/sample was mixed with 250 μL of commercial FC reagent. After 8 min, 75 μL of 7.5% (*w*/*v*) sodium carbonate aqueous solution and Milli-Q water up to 5 mL were added. The reaction was developed for 2 h and the absorbance was recorded at 765 nm in front of the reagent blank as the reference. TPC was expressed as mg gallic acid equivalents per g of fresh weight (mg GAE g^{-1} fw), and the calibration range was from 1 to 20 mg L^{-1} GAE ($R^2 = 0.966$). All samples were analyzed in duplicate.

2.6. Polyphenolic Content Determination by HPLC-DAD

The extracts of fruits and vegetables indicated in Section 2.2 were analyzed by HPLC with diode array detection (DAD). A Kinetex C18 column (100 mm × 4.6 mm of internal diameter and 2.6 μm particle size) from Phenomenex (Torrance, CA, USA) was used. The mobile phase was composed of 0.1% formic acid in Milli-Q water (solvent A) and Acetonitrile (solvent B). The gradient elution program was as follows: 0 min, 5% B; 30 min, 20% B; 40 min, 45% B; 40.2 min, 5% B; 50 min, 5% B. The flow rate was 1 mL min^{-1} and the injection volume was 5 μL. Chromatograms were recorded at 280, 310 and 370 nm. The total phenolic content (TPC) was estimated from the chromatograms at 280 nm, in the time window between 5 and 36 min, where elution of polyphenols occurs. It is assumed that the peak area is mostly due to polyphenols, and TPC is expressed in terms of gallic acid equivalents (GAE) per g of fresh weight, by calibrating with gallic acid standards in the concentration range 0.5 to 100 mg L^{-1}. In addition, the individual quantification of target analytes, including 4-hydroxybenzoic acid, caffeic acid, ferulic acid, hesperidin, and rutin, was carried out using their corresponding standards in the working range 0.5 to 20 mg L^{-1}. The occurrence of these compounds in the matrices under study was confirmed elsewhere by LC-MS (see ref. [15]). Here, they were checked by HPLC-UV based on retention time and UV spectral features compared with those of the corresponding standards.

2.7. Design of Experiments (DoE)

The optimization of the orange and spinach wastes extraction by MSE was planned. Four independent variables temperature, time, solid/solvent ratio and pH were screened to select the optimal condition used for the extraction and recovery of phenolic compounds. The analysis of variance (ANOVA) was conducted to ascertain the relevance of factors such as temperature, time, solid/solvent ratio and pH of the matrices (orange or spinach). Differences at $p \leq 0.05$ were considered statistically significant.

Principal component analysis (PCA), using the PLS-Toolbox (Eigenvector Research, Inc. Manson, WA, USA), was applied to a global characterization of selected fruits and vegetables according to the antioxidant indexes. The data matrix consisted of 22 rows corresponding to 11 waste by-products extracted in duplicate and 8 columns of the corresponding variable (FRAP, FC, ABTS, DPPH, hydroxybenzoic acids (HB), hydroxycinnamic acids (HC), flavonoids (F) and global TPC). Data was auto scaled to equalize the contribution of the different variables to the model.

3. Results and Discussion

3.1. Optimization of Phenolic Compounds Extraction from Orange and Spinach Wastes

In order to improve the phenolic compounds extraction from orange and spinach wastes, the influence of temperature, time, solid/solvent ratio and pH was assessed. Among these factors, temperature and time were simultaneously studied according to our previous experience in the phenolic compounds extraction from fruit matrices, and taking into account the data reported in literature for similar systems [15,17,26].

3.1.1. Effect of Temperature and Contact Time on TPC

To stablish the optimal MSE conditions for phenolic compounds extraction in orange and spinach, temperature (25, 50, 70 and 90 °C), contact time (5, 15 and 30 min), solid/solvent ratio (1:10, 1:30, 1:50, 1:100 and 1:200 (*w*/*v*)) and pH (3, 10 and without adjust) were varied and their influence on the TPC was studied. The experimental conditions selected were based on a compromise between experimental effort and quality of results. In the case of temperature, preliminary studies suggested that the optimal range was around 50 to 70 °C, although other conditions were checked as well. For the pH, there was clear evidence of compound degradation when increasing pH above 6 or 7, which was more

severe for some kind of extracts. Anyway, in basic medium, phenolic groups are deprotonated, producing anionic species that could better dissolve in the aqueous media. For this reason, pH 10 was investigated as well.

In these studies, the overall area at 280 nm was used as an excellent descriptor of the total phenolic content of extracts. It should be remarked that, for these fruit and vegetable waste matrices, the occurrence of potential interfering (absorbing) species without antioxidant capacity was negligible. In contrast, in other matrices such as tea, coffee, chocolate, rich in other absorbing compounds without antioxidant properties, such as caffeine and theobromine, their contribution to the area at 280 nm should be removed to avoid an overestimation of the antioxidant power. As occurs with other antioxidant indexes such as FC, this overall phenolic concentration was expressed in gallic acid equivalents (GAE) because the content of phenolic acids in these types of samples was relevant. Results are summarized in Tables 1 and 3.

Table 1 shows the Series I of the orange residue, where the TPC values increased with increasing temperature and the maximum yield was achieved at 90 °C (1.40 ± 0.10 mg GAE g-1 fw). Anyways, compared to 70 °C (1.33 ± 0.09 mg GAE g^{-1} fw), the differences were not statistically significant ($p > 0.05$).

Table 1. Assessment of the influence of the experimental factors on the TPC recovery from orange residues.

Series I				
Temperature (°C)	Time (min)	Solid/solvent ratio (*w*/*v*)	pH	TPC (mg GAE g^{-1} fw)
25	5	1:20	4	0.51 ± 0.08 ^{aA}
	15			0.76 ± 0.05 ^{bA}
	30			0.91 ± 0.02 ^{bA}
50	5			0.61 ± 0.04 ^{aAB}
	15			1.00 ± 0.07 ^{bAB}
	30			1.03 ± 0.04 ^{bAB}
70	5			0.65 ± 0.07 ^{aBC}
	15			1.10 ± 0.20 ^{bBC}
	30			1.33 ± 0.09 ^{bBC}
90	5			0.94 ± 0.07 ^{aC}
	15			1.40 ± 0.10 ^{bC}
	30			1.30 ± 0.10 ^{bC}
Series II				
Temperature (°C)	Time (min)	Solid/solvent ratio (*w*/*v*)	pH	TPC (mg GAE g^{-1} fw)
70	15	1:10	4	0.68 ± 0.01 ^{a}
		1:30		0.83 ± 0.02 ^{a}
		1:50		0.81 ± 0.04 ^{a}
		1:100		0.84 ± 0.06 ^{a}
		1:200		NQ
Series III				
Temperature (°C)	Time (min)	Solid/solvent ratio (*w*/*v*)	pH	TPC (mg GAE g^{-1} fw)
70	15	1:100	3	0.94 ± 0.02 ^{a}
			4	1.02 ± 0.09 ^{a}
			10	0.98 ± 0.04 ^{a}

Mean values ($n = 3$) followed by same lowercase letter within the same extraction parameter and capital letter in each column showed no statistically significant difference ($p > 0.05$). NQ below the quantification limit.

Regarding the effect of the contact time, from 5 to 15 min, TPC increased with time, while no significant differences occurred when comparing 15 and 30 min ($p > 0.05$). Thus, 15 min is the selected contact time. In addition, no correlation on TPC was detected in the interaction between temperature and contact time factors. A similar trend from Series I was also observed by Gómez-Mejía et al. [26], that indicated that at higher temperature and contact time may facilitate higher phenolic compounds recovery. Authors studied factors like temperature (62 and 90 °C) and contact time (10 and 15 min) on the extraction of phenolic compounds from orange peels by magnetic agitation with aqueous ethanol (20:80 *v/v*), obtaining as a result that 90 °C and 15 min increased the rutin amount extracted (4.7 mg g^{-1}).

According to data reported in Table 2 (Series I), the study of the effect of the temperature and time on the TPC from spinach residue, shown an increasing trend on the TPC from 25 to 50 °C, while at higher temperatures (70 and 90 °C) a decrease was found, indicating a possible degradation of some phenolic compounds with temperature. Nevertheless, no correlation was detected on the extraction yield in the interaction between temperature and contact time. Thus, the optimum temperature was set at 50 °C; and regarding the contact time at 5 min, was significantly higher (except at 90 °C). At the selected temperature and contact time, the TPC obtained was 0.75 ± 0.04 mg GAE g^{-1} fw.

Table 2. Assessment of the influence of the experimental factors on the TPC recovery from the spinach wastes.

Series I				
Temperature (°C)	Time (min)	Solid/solvent ratio (*w/v*)	pH	TPC (mg GAE g^{-1} fw)
25	5	1:20	6	0.65 ± 0.02 ^{aAB}
	15			0.58 ± 0.01 ^{abAB}
	30			0.47 ± 0.02 ^{bAB}
50	5			0.75 ± 0.04 ^{aA}
	15			0.58 ± 0.02 ^{abA}
	30			0.51 ± 0.01 ^{bA}
70	5			0.51 ± 0.03 ^{aB}
	15			0.48 ± 0.02 ^{abB}
	30			0.46 ± 0.00 ^{bB}
90	5			0.40 ± 0.01 ^{aC}
	15			0.43 ± 0.01 ^{abC}
	30			0.30 ± 0.02 ^{bC}
Series II				
Temperature (°C)	Time (min)	Solid/solvent ratio (*w/v*)	pH	TPC (mg GAE g^{-1} fw)
50	5	1:10	6	0.59 ± 0.09 ^{a}
		1:30		0.67 ± 0.07 ^{a}
		1:50		0.68 ± 0.04 ^{a}
		1:100		0.52 ± 0.04 ^{a}
		1:200		NQ
Series III				
Temperature (°C)	Time (min)	Solid/solvent ratio (*w/v*)	pH	TPC (mg GAE g^{-1} fw)
50	5	1:50	3	0.19 ± 0.01 ^{a}
			6	0.75 ± 0.01 ^{b}
			10	0.48 ± 0.01 ^{c}

Values followed by same lowercase letter within the same extraction parameter and capital letter in each column denote nonsignificant difference ($p < 0.05$). NQ below the quantification limit.

Jaime et al. [29] also determined that temperature and contact time influenced on the phenolic compounds extraction yield from spinach leaves using water as an extractant, being 50 °C and 24 h, respectively, the values selected. This represents a longer contact time than that of our study. Dzah et al. [30] mentioned that long extraction times at high temperatures increase the oxidation rate of phenol and decreases the yield of TPC in the extracts. Hence, efficient extraction temperatures that maintain the stability of the polyphenols are required. It is worth mentioning that the sensitivity of a sample to polyphenol degradation induced by temperature, depends on the polyphenol type in the extract, and their biochemical and physicochemical characteristics, as well as on the interaction between the sample and the solvent. Therefore, results from orange and spinach matrices showed that yield increased with temperature and time due to higher solvation and mass transfer [30,31]. In general, studies on the influence of extraction conditions reveal the importance of the microenvironment effects of variables such as temperature, time, and solid–solvent ratio [15,32,33].

3.1.2. Effect of Solid/Solvent Ratio on TPC

Once, the optima temperature and time were selected from orange and spinach matrices, the solid/solvent ratio was studied between 1:10 to 1:200 (*w*/*v*). As can be seen in the Series II, in Table 1, the TPC increased from 1:10 to 1:30 (*w*/*v*) solid/solvent ratio and then it stabilized. Therefore, statistically the effect of solid/solvent ratio on the extraction of phenolic compounds was not significant ($p < 0.05$), thus, the selected ratio was 1:100 (0.84 ± 0.06 mg GAE g^{-1} fw). Although, lower solid/solvent ratio (e.g., 1:30 (*w*/*v*)) could be selected as optimal due to the TPC concentration, but if we take into account the amount of extracted phenolic compounds, the 1:100 (*w*/*v*) ratio is the most favorable, for example by applying membrane technology, where huge volume of phenolic compounds is needed.

These results are in agreement with the findings of Jovanović et al. [6], who verified an increase in the TPC when increasing the volume of solid/solvent ratio from 1:10 to 1:30 with 50% ethanol using maceration as an extraction technique.

On the other hand, the results of spinach residue from Series II (see Table 2) showed an increase on TPC from 1:10 to 1:50 ratios, and then decreased considerably from 1:100 to 1:200. However, the ANOVA of solid/solvent ratio revealed no significant differences in the TPC values ($p < 0.05$), thus, the 1:50 ratio (0.68 ± 0.04 mg GAE g-1 fw) was chosen, since it achieved a considerable TPC value (0.68 ± 0.04 mg GAE g-1 fw). Some studies have been performed using different ratios of plant material and extraction solvents (solid/solvent ratio). For example, Bokov et al. [34] used a similar solid/solvent ratio to extract flavonoids from spinach leaves, reporting good performance using the 1:40 ratio. For both agri-food matrices, at 1:200 (*w*/*v*) ratio, the sample was very diluted, and thus the TPC was below the limit of quantification of the HPLC method (0.5 mg L^{-1}).

Besides, the characteristics of the solvent in relation to the treated samples, their proportions, their affinities and the extraction conditions are important parameters that should be considered in order to obtain an efficient extraction. Specifically, apart from improving extraction yields, the knowledge of the optimal amount of solvent to use is of economic relevance [30,35].

3.1.3. Effect of pH on TPC

Once temperature, contact time and solid/solvent ratio were established, the effect of pH on TPC was evaluated. For this purpose, acidified or basified solutions were added to adjust the pH to 3 (with HCl), 4 (this is the pH of the orange waste, without adjust) and 10 (with NaOH).

For the orange waste, temperature of 70 °C, contact time of 15 min and solid/solvent ratio of 1:100 were the optima to carried out the Series III. As can be seen in Table 1 (Series III), pH was no significant ($p > 0.05$) from statistical point of view. Therefore, the pH se-

lected was pH 4 (without adjustment) with a TPC of 1.02 ± 0.09 mg GAE g^{-1} fw. Conversely, as can be seen in Table 2 (Series III), the extraction of phenolic compounds from spinach residue reported significant dependence on the pH ($p < 0.05$). Attributing degradations undergoing at very acidic or basic pH. Therefore, pH 6 (without adjustment) was selected, in this case the TPC was 0.75 ± 0.01 mg GAE g^{-1} fw. These results agree with Li et al. [36,37] who found that pH had a significant effect on TPC.

3.2. Total Phenolic Content and Antioxidant Activity of Orange and Spinach Wastes

The Total Phenolic Content: MSE vs. UAE

Orange and spinach waste extracts obtained under optima extraction conditions (70 °C, contact time of 15 min, solid/solvent ratio 1:100 and pH 4 without adjustment for orange waste; and 50 °C, 5 min, 1:50 and pH 6 without adjustment, for spinach residue), by DoE approach in terms of TPC, was compared statistically with UAE as can be seen in Figure 1.

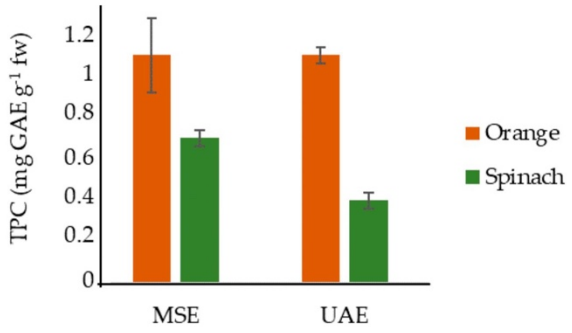


Figure 1. Comparison of TPC yield of MSE and UAE under selected conditions from agri-food residues.

Figure 1 shows that the statistical analysis of the data for orange waste do not present significant differences ($p > 0.05$) between MSE and UAE results (1.1 ± 0 mg GAE g^{-1} fw in both case under optimal conditions). Dahmoune et al. [38] also obtained similar results of TPC without statistical differences among MSE and UAE (15.0 and 15.2 mg GAE g^{-1} dw, respectively).

The TPC obtained from spinach residue by MSE (0.75 ± 0.04 mg GAE g^{-1} fw) compared with the TPC of UAE (0.44 ± 0.04 mg GAE g^{-1} fw), was 41% higher using MSE technique (see Figure 1). However, the opposite trend was reported by Altemimi et al. [17] with higher TPC recoveries by UAE than MSE (0.51 and 0.12 mg GAE g^{-1} dw, respectively) from spinach leaves extraction.

In both orange and spinach matrices MSE would be more suitable extraction technique since it is cheaper than UAE. The UAE could be ruled out since unlike the MSE, it applies ultrasonic energy (135 W) and the contact time is longer (30 min), which may cause inconveniences such as polyphenols degradation. In addition, the required equipment and processes with UAE have high costs [14]. Whatever, Gómez-Mejía et al. [26], reported that MSE is fast, sustainable and economic for the extraction of phenolic compounds compared with UAE.

3.3. Characterization of Antioxidant Activity of Fruit and Vegetable By-Products Extracts

MSE, under the proposed conditions, seems to be a suitable technique to extract phenolic compounds from orange and spinach wastes compared with UAE (see Figure 1). Other fruits and vegetables by-products were selected to evaluate the antioxidant activity

of the extracts by the recommended extraction technique. All of them, including orange, kiwi, strawberry, white and red grape, spinach, carrot, kale, celery, beet and broccoli, were characterized by FRAP, DPPH and ABTS assays expressed as Trolox equivalents (mg TE g^{-1} fw). FC was used to determine the TPC in terms of mg GAE g^{-1} fw.

The natural pH of extract was in the range 3 to 4.5 so that, in some methods, buffer solutions were used to neutralize the excess of acid while providing a proper pH. For a more straightforward procedure focused on routine analysis of large sets of samples, despite the kinetic nature of the reactions absorbances from each index were measured at preselected times leading to steady states. The obtained results of the spectrophotometric assays described in Section 2.5, are reported in Table 3.

Table 3. Comparison of TPC and antioxidant activity of different fruit and vegetable extracts. Data are expressed as mean ± standard derivation (n = 2).

Waste Extracts	DPPH (mg TE g^{-1} fw)	FRAP (mg TE g^{-1} fw)	ABTS (mg TE g^{-1} fw)	FC (mg GAE g^{-1} fw)
Orange	1.31 ± 0.10	2.27 ± 0.25	0.36 ± 0.04	0.51 ± 0.02
Kiwi	0.58 ± 0.02	0.52 ± 0.02	0.28 ± 0.01	0.36 ± 0.04
Strawberry	2.02 ± 0.02	0.78 ± 0.01	0.40 ± 0.02	0.38 ± 0.05
White grape	3.06 ± 0.05	1.97 ± 0.14	1.47 ± 0.07	0.63 ± 0.11
Red grape	3.96 ± 0.16	8.18 ± 0.28	3.37 ± 0.35	2.24 ± 0.02
Spinach	0.70 ± 0.03	0.04 ± 0.00	0.07 ± 0.04	0.47 ± 0.03
Carrot	0.38 ± 0.01	0.09 ± 0.00	0.05 ± 0.00	0.08 ± 0.01
Kale	0.85 ± 0.06	1.57 ± 0.12	1.47 ± 0.03	1.63 ± 0.03
Celery	0.39 ± 0.02	0.04 ± 0.00	0.04 ± 0.00	0.07 ± 0.00
Beet	0.66 ± 0.01	2.28 ± 0.08	1.32 ± 0.06	0.52 ± 0.01
Broccoli	0.48 ± 0.00	0.07 ± 0.01	0.01 ± 0.00	0.20 ± 0.02

GAE (gallic acid equivalents), fw (fresh weight), TE (Trolox equivalents).

A higher value of TE indicates higher antioxidant activity, that is, the samples richest in phenolic compounds present high values for all the indexes and vice versa. In this regarding, it is observed that the most concentrated fruits were orange, white grape and red grape, and for vegetables spinach, kale and beet have the highest antioxidant capacity. In general, Table 3 shows that FRAP generally provides higher values of antioxidant capacity. This may be due to interference issues from non-polyphenolic compounds that may be able to reduce FRAP, but are not as efficient at scavenging radicals. ABTS, as a whole, is the reagent that estimates the lowest antioxidant value, perhaps because it is more stable radical than DPPH.

Moreover, a comparison of data from the four indexes was subjected to a correlation study. For FRAP vs. ABTS reported good correlation coefficient (R^2 = 0.933), followed by FC vs. ABTS (R^2 = 0.905), the correlation coefficient indicates that the antioxidant polyphenols that have been involved in one or the other indexes are similar [28]. Therefore, this may indicate the reduction of Fe^{3+} and $ABTS^{+}$ radical (FRAP vs. ABTS) as well as Mo (VI) and Fe (III) (FC vs. ABTS). About the other antioxidant indexes, lower correlations were obtained (see Table S2).

In order to summarized and to easily visualize all antioxidant activity results, data was subjected to PCA analysis. The principal components (PCs) are mathematical variables that define efficiently the variation of the data. The first principal component (PC1) explained 76.54% and the second principal component (PC2) explained 12.73 % of the data variance. Relationships between samples and indexes were investigated from the scores and loadings plots (see Figure 2). Scores showed the distribution of extracts with respect to PC1 and PC2 (Figure 2a) and loadings explained the behavior of the variables (Figure 2b). As can be seen in Figure 2a, the samples with low activity (e.g., celery and broccoli) are highly grouped due to the fact that they present few differences between them. On the

other hand, samples with higher index values (e.g., red grape, orange and kale) come out to the right and with a lot of dispersion.

Otherwise, Figure 2b provides information on the correlation between the variables. The PC1 and PC2 loadings graphic shows an evident separation between Global TPC and the rest of the variables. Therefore, TPC determines the different behavior between the indexes.

Simultaneous interpretation of the scores and loadings plots suggests that, in Figure 2a, the samples that appear on the right side are the richest in antioxidant compounds. The samples on the left side are poorer in these compounds. Therefore, PC1 explains the antioxidant behavior of the samples. Figure 2b, the samples that are in the upper part show greater radical activity compared to those that are in the lower part.

Regarding to MSE, it seems to be a good technique to extract antioxidant compounds from fruit and vegetables wastes, especially orange and spinach matrices, a very similar index values to that obtained with UAE with ethanol-water mixture by Montenegro-Landívar et al. [15].

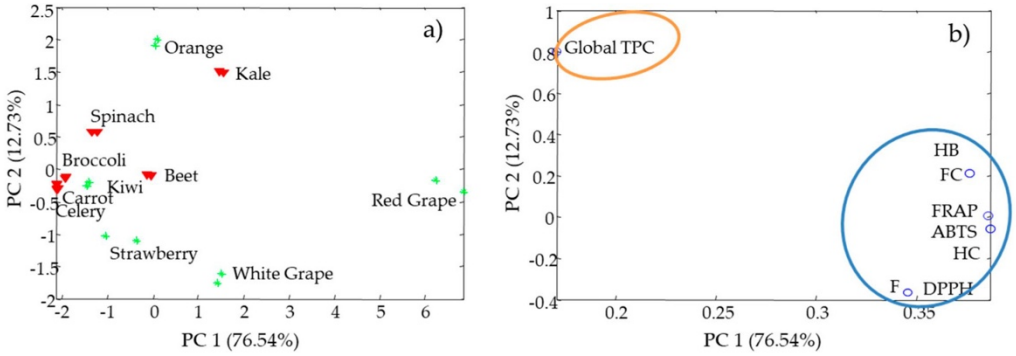


Figure 2. Principal component analysis for the evaluation of the antioxidant features of various fruit and vegetable waste extracts: (**a**) Plot of scores of PC1 vs. PC2 and (**b**) plot of loadings of PC1 vs. PC2. Variable assignation: HB = hydroxybenzoic acids, HC = hydroxycinnamic acids, and F = flavonoids.

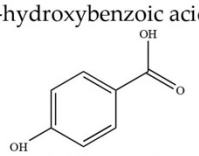
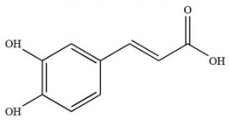
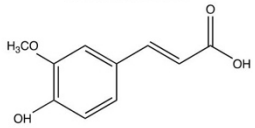
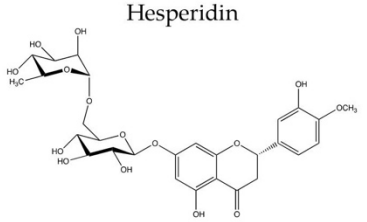
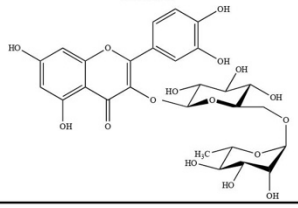
3.4. Characterization of the Phenolic Composition from Orange and Spinach Wastes

Complementary analyses by HPLC-DAD were performed to identify tentatively various phenolic compounds from orange and spinach waste extracts by MSE under the selected conditions (see in Figure S1). In a previous study by Montenegro et al., the principal molecules in these matrices were identified by LC-MS [15]. In this paper, based on those results, compounds were identified tentatively by HPLC-DAD, from the coincidence of retention times and the UV spectra of suspected compounds, with those of the corresponding standards (see Figure S1 in the supplementary material). The identified phenolic compounds can be allocated into three groups: hydroxybenzoic acids, hydroxycinnamic acids and flavonoids as can be seen in Table 4.

Therefore, as derived from HPLC analysis evaluated in orange and spinach waste extracts (see Figure S1), orange waste could be a rich source of 4-hydroxibenzoic acid and hesperidin. Similar results were obtained by Senit et al. [39]. They reported phenolic acids and flavonoids present in orange peel waste with a remarkable antioxidant activity. On the other hand, spinach residue could be considered a suitable source of caffeic acid, ferulic acid and rutin (see Table 4) under the selected extraction conditions evaluated in this study. Bokov et al. [34] and Vázquez et al. [11] also detected that ferulic acid and caffeic acid were present in spinach extract. According to Montenegro-Landívar et al. [15], orange and spinach wastes are good sources of phenolic compounds that could be recovered for the application in the cosmetic, pharmaceutical and food industries. In this regard, the green nature of the extraction method, without using any harmful solvent, is a key aspect

compatible with the production of raw materials for food supplements, nutraceuticals and drugs. Some representative examples of potential applications proposed by other authors were as follows. Papillo et al. [40] suggested that polyphenol extracts from cocoa hulls, can be microencapsulate in order to have heat-stable functional ingredients for bakery products. They used water as solvent and magnetic stirring extraction technique. Additionally, dietary fibers with polyphenols extracted from mango peels were used as functional ingredients in processed foods, due to their potential health benefits (e.g., regulation of blood glucose level, anticarcinogenic effects, antioxidant property) [41].

Table 4. Identified phenolic compounds in the extracts from orange and spinach matrices, their respective family, structure, and concentration under optima conditions.

Polyphenol Family	Structure	Residues	Concentration (mg g^{-1})
Hydroxybenzoic acids	4-hydroxybenzoic acid 	Orange	0.71 ± 0.03
	Caffeic acid 	Spinach	0.04 ± 0.01
Hydroxycinnamic acids	Ferulic acid 	Spinach	0.04 ± 0.01
	Hesperidin 	Orange	4.86 ± 0.09
Flavonoids	Rutin 	Spinach	0.08 ± 0.01

4. Conclusions

Polyphenol extraction from fruit and vegetable wastes was performed using mechanical stirring as a cost-effective technique where water is used as a solvent. Thermo-mechanical treatments of orange and spinach residues, used as model matrices, were applied to evaluate the effect of temperature, time, solid/solvent ratio and pH. Comparing MSE with UAE, the performance was similar for the orange waste; however, for the spinach residue ca. 2-fold improvement was obtained. Therefore, MSE can be postulated to be an efficient technique for the recovery of phenolic compounds from agri-food residues. The

MSE optimal conditions for orange wastes were temperature of 70 °C, solid/solvent ratio 1:100 (*w*/*v*) and pH 4 (without adjustment) in 15 min of contact time, while for spinach residues were temperature of 50 °C, solid/solvent ratio 1:50 (*w*/*v*) and pH 6 (without adjustment) for 5 min. Using the proposed extraction process, under the optimal conditions, each gram of orange and spinach wastes allow obtaining approximately 1 mg of 4-hydroxybenzoic acid and 5 mg of hesperidin per gram of orange waste; and 0.1 mg of rutin per gram of spinach residue. Additionally, the orange and spinach residues presented high antioxidant activity (0.51 ± 0.02 mg GAE/g fw and 0.47 ± 0.03 mg GAE/g fw, respectively) in comparison with carrot, celery, kiwi, strawberry and broccoli, and low antioxidant activity than kale, white and red grape. Some advantages of the proposed method deal with the use of a cheap and green extraction procedure for the recovery of polyphenols, combining water as the solvent with the mechanical stirring. Products obtained in this way will be fully compatible with applications to functional foods, animal feed, dietary supplements or cosmetics thanks to their polyphenolic content and antioxidant activity.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/antiox10111800/s1, Figure S1: Chromatograms of a set of standards at 20 mg L^{-1} each (black lines), an orange extract (brown line), and a spinach extract (green line) recorded at 280 nm to be used for identification purposes. Table S1: Performed variables for the optimization of phenolic compounds extraction, Table S2: Correlation studies among FRAP, DPPH, ABTS and FC.

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Supplementary material of

Recovery of added-value compounds from orange and spinach processing residues: green extraction of phenolic compounds and evaluation of antioxidant activity

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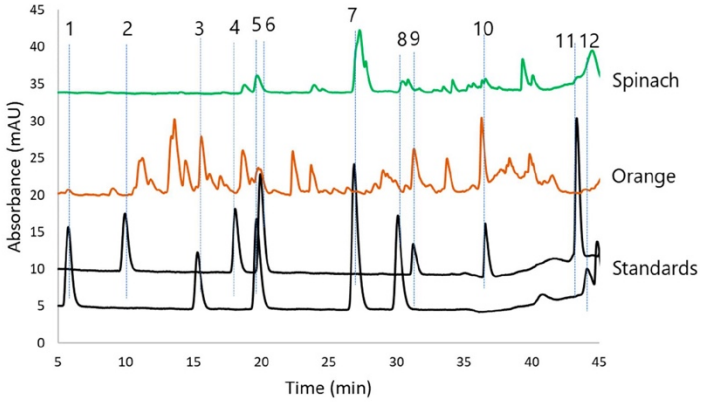

Figure S1: Chromatograms of a set of standards at 20 mg L^{-1} each (black lines), an orange extract (brown line), and a spinach extract (green line) recorded at 280 nm to be used for identification purposes. Selected extraction conditions for orange waste: MSE, temperature 70ºC, solid/solvent ratio 1:100 (w:v) and pH 4, time 15 min. Selected extraction conditions for spinach waste: MSE, temperature 50ºC, solid/solvent ratio 1:50 (w:v) and pH 6, time 5 min). Peak assignment: 1 = Gallic acid, 2 =3,4-Dihydroxybenzoic acid, 3 = 4-Hydroxybenzoic acid, 4 = Syringic acid, 5 = Vanillic acid, 6 = Caffeic acid, 7 = *p*-Coumaric acid, 8 = Ferulic acid, 9 = Rutin, 10 = Hesperidin, 11 = Naringenin, 12 = Kaempferol.

Table S1. Performed variables for the optimization of phenolic compounds extraction.

Assay	R-value
FRAP vs DPPH	0.807
FRAP vs ABTS	0.933
DPPH vs ABTS	0.798
FC vs FRAP	0.845
FC vs DPPH	0.660
FC vs ABTS	0.905

Table S2. Correlation studies among FRAP, DPPH, ABTS and FC.

Experimental variables	Levels	Optima conditions orange waste	Optima conditions spinach residue
Temperature (ºC)	25 / 50 / 70 / 90	70	50
Contact time (min)	5 / 15 / 30	15	5
Solid/solvent ratio *(w:v)*	1:10 / 1:30 / 1:50 / 1:100 / 1:200	1:100	1:50
pH	3 / unadjusted / 10	unadjusted	unadjusted

CHAPTER 6

Publication 3

"Recovery of natural polyphenols from spinach and orange by-products by pressure-driven membrane processes"

membranes

MDPI

Article

Recovery of Natural Polyphenols from Spinach and Orange by-Products by Pressure-Driven Membrane Processes

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Abstract: Spinach and orange by-products are well recognized for their health benefits due to the presence of natural polyphenols with antioxidant activity. Therefore, the demand to produce functional products containing polyphenols recovered from vegetables and fruits has increased in the last decade. This work aims to use the integrated membrane process for the recovery of polyphenols from spinach and orange wastes, implemented on a laboratory scale. The clarification (microfiltration and ultrafiltration, i.e., MF and UF), pre-concentration (nanofiltration, NF), and concentration (reverse osmosis, RO) of the spinach and orange extracts were performed using membrane technology. Membrane experiments were carried out by collecting 1 mL of the permeate stream after increasing the flow rate in 1 mL/min steps. The separation and concentration factors were determined by HPLC-DAD in terms of total polyphenol content and by polyphenol families: hydroxybenzoic acids, hydroxycinnamic acids, and flavonoids. The results show that the transmembrane flux depended on the feed flow rate for MF, UF, NF, and RO techniques. For the spinach and orange matrices, MF (0.22 μm) could be used to remove suspended solids; UF membranes (30 kDa) for clarification; NF membranes (TFCS) to pre-concentrate; and RO membranes (XLE for spinach and BW30 for orange) to concentrate. A treatment sequence is proposed for the two extracts using a selective membrane train (UF, NF, and RO) to obtain polyphenol-rich streams for food, pharmaceutical, and cosmetic applications, and also to recover clean water streams.

Keywords: spinach waste; orange waste; polyphenols recovery; microfiltration (MF); ultrafiltration (UF); nanofiltration (NF); reverse osmosis (RO); integrated membrane processes

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1. Introduction

In recent years, polyphenols have received great interest due to their potential as preventive and therapeutic agents in many diseases such as virus infections, allergies, and diabetes, among others [1,2]. Consequently, the global market for antioxidants is increasing rapidly due to the increasing risk to human health from being in a constantly polluted environment [3]. These agents not only have pharmaceutical applications but

also cosmetic applications, with the objective of developing research at industrial and lab scales to explore the behavior of these molecules and their analogs. Therefore, there is great interest not only in their extraction, but also in the separation, purification/concentration, and recovery of antioxidant compounds from natural sources (including by-products) [4].

Several studies have shown the structure–antioxidant-activity relationship of some families of polyphenols such as hydroxybenzoic acids, hydroxycinnamic acids, and flavonoids extracted from plants [3,5]. Some flavonoids from vegetables such as spinach and from fruits such as oranges have been reported to possess a variety of biological activities and pharmacological properties, including antidiabetic, anti-inflammatory, and anticancer properties, among others [6].

Spinach and orange crops are among the most abundant in the world and are of major interest in Spain, generating between 13% and 50% of waste, respectively [6,7]. There is ample scientific evidence that spinach, oranges, and their by-products are rich sources of minerals, vitamins, and dietary fiber, as well as bioactive compounds such as polyphenols (specifically flavonoids and phenolic acids) [6,8], which also provide a high antioxidant activity [3,6].

The main use of spinach in the food industry is raw or cooked leaves, while oranges are used for fresh juice, juice concentrate, or orange-based drinks. The main residues generated from the processing of spinach and oranges are damaged leaves and the bagasse from the processing of spinach juice, as for orange peels and seeds. These residues can be considered an interesting source of phenolic compounds [3].

In the last decade, different studies have been proposed the recovery of polyphenols from spinach, but greater interest has been shown in oranges since their percentage of waste generation is higher. These natural antioxidants offer interesting perspectives and opportunities in the food industry, for example, in the production of dietary supplements and functional foods, and for their possible use by the pharmaceutical and cosmetic industries. However, the proposed methodologies have some drawbacks [9,10]. For example, extraction with organic solvents is characterized by safety problems such as toxicity, low efficiency, and time consumption. In addition, heat treatment gives rise to pyrolysis, and enzymes can be denatured in enzyme-assisted, ultrasonic, or microwave-assisted extraction. Furthermore, polyphenols are sensitive to heat, light, and oxygen exposure [3]. According to Ameer et al. [11], the antioxidant recovery from agri-food by-products is one of the most important goals.

The recovery of polyphenols from by-products can be carried out using the following main steps: (i) macroscopic pretreatment, (ii) extraction, (iii) isolation (separation of macro- and micro-molecules) and purification, and (iv) product formation [12].

Pressure-driven membrane processes such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO) can be used at different stages: MF in macroscopic pretreatment, UF in the separation of macro- and micro-molecules, and NF and RO in purification/concentration [3,13]. All these processes allow the concentration and separation to be carried out without using heat; another advantage is that the equipment requires little space, running costs and energy consumption are low, and products and co-products are high in quality. All these factors are very important for the recovery of by-products [14].

On the other hand, a disadvantage of membrane processes is related to the sensitivity to concentration polarization and membrane fouling due to chemical interaction with feed components, for which a pretreatment is necessary.

Spinach and orange residues contain soluble sugars (sucrose, glucose, and fructose), insoluble carbohydrates, fibers, organic acids, essential oils, flavonoids, and carotenoids [6]. Therefore, UF can be considered a valid approach to separate and recover valuable compounds from finely divided solid materials present in the extract from the residue of these matrices, with UF being able to remove macromolecules such as pectins and proteins

from the extract, ensuring the production of a clarified solution that contains beneficial compounds for health [15].

Cassano et al. [16,17] used UF membranes to clarify pomegranate and clementine mandarin juices containing phenols (e.g., hydroxycinnamic derivatives) without significantly affecting the antioxidant profile of the permeate, nor the nutritional or physicochemical properties.

Pinto et al. [18] evaluated the performance of different UF and NF membranes in the treatment of ethanolic extract from Eucalyptus globulus bark, obtaining rejection values for total phenolic compounds higher than for total carbohydrates, which indicates the effectiveness of UF and NF process for the removal of sugar residues in the permeate stream.

Another study, by Cassano et al. [19], evaluated UF and NF membranes in flat-sheet configurations (MWCO range 1000 to 4000 Da) to purify polyphenols from sugars in clarified pomegranate juice. The obtained results showed high retentions towards anthocyanins, total polyphenols, and total antioxidant activity (in the range of 80–95). On the other hand, the rejection values towards fructose and glucose were 1–3%, indicating the suitability of the membrane process. In addition, the authors obtained an improved purification of polyphenols by combining the concentration step with discontinuous diafiltration.

There is a great interest in the revalorization of wastes, especially those from the agri-food industry, for use as a direct source of biocompounds, such as polyphenols. First, it should be noted that these biocompounds are usually extracted by a liquid–liquid process with organic solvents. However, in this work, their extraction using water as a green extractant has been proposed. Secondly, it is important to recover these polyphenols from other impurities such as sugars and proteins, and membrane technology is proposed as a sustainable alternative to carry out a separation and concentration in a selective manner, according to the family of polyphenols found in these aqueous extracts.

Therefore, the aim of the present study is to investigate the feasibility of the membrane technology based on MF and UF as a clarification step, NF as a pre-concentration step, and RO for the concentration of polyphenols from spinach and orange aqueous extracts. Furthermore, an integrated treatment train based on membrane technologies for polyphenol separation and concentration from spinach and orange extracts is proposed.

2. Materials and Methods

2.1. Reagents

The main standards used in the experiments were 4-hydroxybenzoic acid, vanillic acid, rutin, syringic acid, gallic acid, ferulic acid, *p*-coumaric acid, and naringenin, purchased from Sigma Aldrich (St. Louis, MO USA); kaempferol and hesperidin were obtained from Glentham Life Sciences (Corsham, UK); and caffeic acid was obtained from Chengdu Biopurify Pytochemicals (Sichuan, China). The solvents were prepared with acetonitrile (ACN, HPLC-UV grade) from Fisher Scientific (UK), formic acid (98–100% *w*/*w*) from Merck (Darmstradt, Germany), and water (purified with a Milli-Q equipment, Merck Millipore, Darmstradt, Germany).

2.2. Samples

Spinach and orange were purchased from a local market in Barcelona (Spain). One kg of each one was used in the process to simulate the waste obtained from agri-food industries, specifically in the juicing process. Spinach and orange were processed with a domestic juicer. The solid residues obtained (spinach leaf waste and orange peel and seeds) were used as representative waste samples and stored in a freezer at −20 °C.

2.3. Extraction Process

The optimization of operational conditions to maximize polyphenol extraction yield from spinach and orange wastes has been studied previously, and it is reported elsewhere [6]. Several parameters were evaluated, e.g., time, temperature, solid-to-solvent ratio, and pH. The optimal conditions obtained by mechanical stirring extraction were 5 min, 50 °C, 1:50 (*w*:*v*), and pH 6 for the spinach matrix; and 15 min, 70 °C, 1:100 (*w*:*v*), and pH 4 for the orange matrix.

2.4. Polyphenol Separation and Concentration by the Membrane Process

2.4.1. Experimental Set-Up and Procedures

Experimental runs were performed using a MemHPLC cell (MMS AG Membrane Systems, Urdorf, Switzerland) with an active membrane area of 28 cm^2. A Waters 515 HPLC pump (Waters, Milford, MA, USA) was used to pump the extract through the membrane system. Additionally, a stirring plate (Heidolph, Schwabach, Germany) placed into the feed tank was used to keep the feed stream constant.

Experiments were performed according to the batch system configuration in which the permeate stream was collected separately, while the retentate was recycled back to the feed reservoir. A variation in the feed flow from 1 mL/min to 10 mL/min was carried out, with 5 min to stabilize the system (between each feed flow); after stabilization, the permeate current was withdrawn. The obtained volume was weighed, and the flow rate of the permeate stream was calculated using the time it took to obtain it. In addition, samples were analyzed by high-performance liquid chromatography (HPLC) to determine the total phenolic content (TPC). All the experiments were performed at room temperature (25 °C). After each test, the membrane module underwent a cleaning procedure with 25 mL of Milli-Q water for 5 min at a flow rate of 5 mL/min.

The membrane performance was evaluated in terms of productivity (permeate flux) and rejection. The permeate flow rate (Q_P), which is the flow that passes through the membrane, was calculated using Equation (1) [13]:

$$Q_p\ (\mathrm{mL/s}) = \frac{M_P\ (\mathrm{g})}{\rho\left(\frac{\mathrm{g}}{\mathrm{mL}}\right) \times t\ (\mathrm{s})} \quad (1)$$

where Q_p is the permeate flow rate (mL/s); M_p is the permeate collected mass (g); ρ is the density (g/mL) of the extract; and t is the time (s) it takes to obtain the sample.

The volumetric flux of permeate (J_v) was calculated using Equation (2) [13]:

$$J_v\left(^{\mathrm{L}}/_{\mathrm{h} \times \mathrm{m}^2}\right) = \frac{Q_P\left(^{\mathrm{L}}/_{\mathrm{h}}\right)}{A\ (\mathrm{m}^2)} \quad (2)$$

where A (m^2) is the membrane permeation area.

The rejection coefficient (R) was calculated using Equation (3) [13]:

$$R\ (\%) = 1 - \frac{C_P}{C_F} \quad (3)$$

where C_P and C_F are the concentrations of solute in the permeate and in feed, respectively.

2.4.2. Membrane Tests

Characteristics of the membranes used in this study are reported in Table 1. Conditioning of the NF and RO membranes consisted of their immersion for 12 hours in Milli-Q water prior to the experiment to remove conservative products. NF and RO dense membranes must be pressurized, where the extract was pumped through the system at 10 mL/min (maximum feed flow rate). The conductivity measurements of the extracts were taken (GLP31 conductivity-meter CRISON (Barcelona, Spain)) every 10 minutes until two measurements were equal and the membrane was considered pressurized.

Table 1. Characteristics of the MF, UF, NF, and RO membranes used in this study.

Membrane		Composition	pH Range (25 °C)	T Max (°C)	P Max (bar)	Iso-Electric Point (IEP)	Contact Angle (°)	Pore Size	MWCO [1] (Da)	Pure Water Permeability (L/m² h bar)	Zeta Potential (mV)	Reference
MF	Filter-Lab 0.22 µm	Mixed cellulose esters (MCE)	2–10	75	<1.4	5.5	31 ± 1 19	0.22 µm	>100,000	7970 ± 290 8090 ± 320 at 1 bar 3770	−21.1 (pH 8) −9.8 (pH 8) +20 (pH 7)	[20,21]
	Filter-Lab 0.45 µm	Mixed cellulose esters (MCE)	4–8	75	<1.8	2–3.3	46.7	0.45 µm		1260 at 0.7 bar	−22.5 (pH 7)	[22,23]
UF	Biomax 30 kDa (Merck, Darmstradt, Germany)	Polyethersulfone (PES)	0–14	95	6	Around 3.5	12 ± 2.94	9.61 nm	30,000	390 ± 20	−16.4 (pH 8)	[24–26]
	Biomax 50 kDa (Merck, Darmstradt, Germany)	Polyethersulfone (PES)	2–13	50	0.5–3	3.05 ± 0.5	68.7 ± 2.2	100 nm	50,000	593.6 ± 84.5 at TMP: 3 bar	Around −15 (pH 7)	[27–29]
NF	NF90 (DuPont, Dellgauw, Netherlands)	Uncoated fully aromatic polyamide TFC [2]	2–11	45	41	4.3 4.0	54 62.7 41.4 63.2 83.4	0.68 0.24 nm	200–400	10.6	+13 (pH 3) −7 (pH 5) −24.9 (pH 7) −28 (pH 9) −29 (pH 10)	[30–34]
	NF270 (DuPont, Dellgauw, Netherlands)	Uncoated semi aromatic polypripierazine amide TFC	2–11	45	41	4.5 4.1	30 27 29 64.1	0.84 nm 0.71 0.42	200–400	17.8	+7 (pH 3) −15 (pH 5) −19, −22 (pH 7) −22 (pH 9) −28 (pH 10)	[31,34–36]
	DURACID (Suez, Trevose, PA, USA)	Sulfonamide-based active layer and polysulfone support	<10 0–9	70	82	4.3	62.2 ± 4.2	0.47 nm	150–300	8 at TMP: 7 bar 17–32 at 15.5 bar	-	[37,38]

	TFCS (KOCH, Cansas, USA)	Proprietary TFC® polyamide	4–11	45	82	3.1	18.7	-	300	49 ± 6 at 5 bar	−6.5 (pH 8)	[39]
	TFC-HR (KOCH, Cansas, USA)	Proprietary TFC® polyamide	4–11	45	41	4.7	35.7	-	300–500	3.5	−9.5 (pH 7) −17 (pH 9)	[40]
RO	SW30HR (DuPont, Delfgauw, Netherlands)	Coated fully aromatic polyamide TFC	2–11	45	69	Always negative	52.8 Around 50	-	100	1.3	−17.8 (pH 10.4)	[41–44]
	BW30LE (DuPont, Delfgauw, Netherlands)	Coated fully aromatic polyamide TFC	2–11	45	41	4.2 Close to 3 4.07	72.2 59.8 55 Around 50 (>SW30HR)	0.32 nm	98 100	2.2	−12.8	[31,32,45,46]
	XLE (DuPont, Delfgauw, Netherlands)	Uncoated fully aromatic polyamide TFC	2–11	45	41	3.5	55 66.3 70.9 65.7	0.89 nm	100	8.8	+13 (pH 3) −17 (pH 5) −33 (pH 7) −38 (pH 9) −38 (pH 10)	[33,34,47,48]

[1] MWCO: molecular weight cut-off. [2] TFC: Thin-film composite.

A 30 mL volume of the extracts from spinach and orange matrices was filtered by MF, UF, NF, and RO membranes. A general scheme of the membrane test employed in this study is shown in Figure 1.

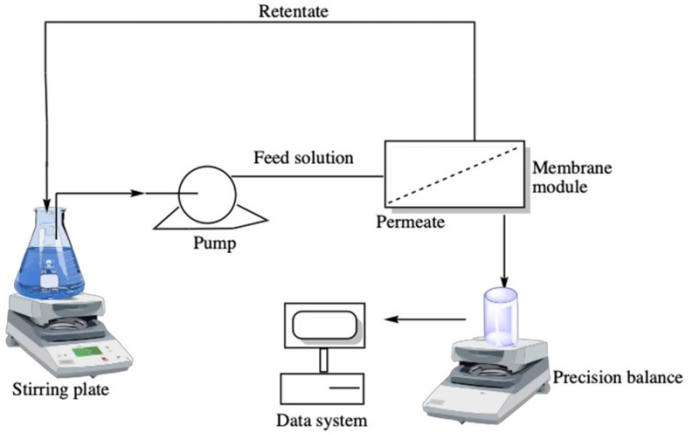


Figure 1. Schematic diagram of the membrane filtration process.

2.5. HPLC Determination of TPC

The TPC was determined by HPLC with a diode array detection (DAD) equipped with a Kinetex C18 column (100 mm × 4.6 mm of internal diameter and 2.6 µm particle size, Phenomenex, Torrance, California, USA). The mobile phase was composed of 0.1% formic acid (Merck, Darmstadt, Germany, 98–100% *w*/*w*) in Milli-Q water (Merck, Darmstadt, Germany) as solvent A and ACN (Fisher Chemical, Leicestershire, UK) as solvent B. The gradient elution program was as follows: 0 min, 5% B; 30 min, 20% B; 40 min, 45% B; 40.2 min, 5% B; 50 min, and 5% B. The flow rate was 1 mL/min, and the injection volume was 5 µL. Chromatograms were recorded at 280, 310, and 370 nm. The TPC and the total hydroxybenzoic acid (HB) contents were estimated at 280 nm and expressed in terms of gallic acid equivalents (GAE) per L, the total hydroxycinnamic acid (HC) contents was determined at 310 nm and was expressed in terms of caffeic acid equivalents (CAE) per L, and the total flavonoid (F) content was estimated at 370 nm and expressed in terms of kaempferol equivalents (KE) per L [6].

2.6. Statistical Analysis

Data were analyzed by two-factor analysis of variance (ANOVA). All the membrane experiments were performed in duplicate, and results are expressed as mean ± standard derivation (SD). The p-values < 0.05 were considered significant.

3. Results and Discussions

3.1. Polyphenol Composition of Extracts of Spinach and Orange Wastes

The TPC in the spinach matrix at optimal extraction conditions (5 min, 50 °C, 1:50 (*w*:*v*), and pH 6) was 5.89 ± 0.88 mg/L. For the orange matrix, the TPC at the optimal extraction conditions (15 min, 70 °C, 1:100 (*w*:*v*) and pH 4) was 2.08 ± 0.07 mg/L [6]. According to Montenegro-Landívar et al. [6], for spinach waste, the main polyphenol families identified were hydroxycinnamic acids (HC, MWCO of 164.15–960.88 Da), specifically caffeic acid (0.93 ± 0.26 mg/L, MWCO of 180.15 Da) and ferulic acid (0.67 ± 0.23 mg/L, MWCO of 194.19 Da), and for the flavonoids (F, MWCO of 254.24–978.85 Da), rutin

(1.47 ± 0.23 mg/L, MWCO of 610.5 Da). For orange waste, the main identified polyphenol family was flavonoids, specifically hesperidin (48.63 ± 0.85 mg/L, MWCO of 610.2 Da).

3.2. Performance of Selected Membranes from Spinach and Orange Extracts

Recovery of polyphenols from spinach and orange extracts were evaluated by different pressure driven membranes such as MF, UF, NF, and RO.

3.2.1. Microfiltration

Spinach and orange extracts were driven towards 0.22 and 0.45 μm MF membranes, to evaluate polyphenols purification. The transmembrane flux increased when the feed flow rate increased (from 1 mL/min to 10 mL/min). This trend was found for both membranes (0.22 and 0.45 μm) and in both matrices. Therefore, the highest transmembrane flux was obtained at the maximal feed flow rate studied. Under these circumstances, the increase in J_v was 93% from 1 to 10 mL/min; therefore, the selected feed flow rate was 10 mL/min for spinach and orange extracts.

The determination of TPC in the spinach extract (5.89 mg/L) and orange extract (2.08 mg/L) revealed that the rejection of the 0.22 μm membrane (23% and 21% averages for spinach and orange matrices, respectively) was higher than the 0.45 μm membrane (17% and 10% averages for spinach and orange matrices, respectively). The ANOVA test results confirmed that there was significant difference between 0.22 and 0.45 μm membranes on the polyphenol recovery from spinach and orange waste extracts ($p = 4.30 \times 10^{-6}$ and $p = 1.63^{-9}$, respectively; see Figure 2a,b). According to Cassano et al. [49], one of the main interactions that occur between polyphenols and MF membranes are steric exclusion and hydrophobic attraction. In fact, the rejection of MF membranes is only based on steric effects, so ions or elements will pass through them depending only on its size and its relation with the membrane's pore size [13,50]. Thus, as shown in Table 1, both MF membranes tested were made of the same material, so one of the differences between them was the pore sizes, which were 0.22 and 0.45 μm. Additionally, the 0.45 μm membrane has a more hydrophobic surface with a contact angle value of 46.7°, while the 0.22 μm membrane is less hydrophobic with a contact angle value of 19–31°. Therefore, the large contact angle of the 0.45 μm membrane is responsible for its lower permeability (see Table 1).

The hydrophobicity of membranes is presented in terms of the contact angle between the water and membrane (contact angle value between liquid and solid). If the contact angle is higher than 90°, the membrane material is considered hydrophobic [51]. In this work, both MF membranes have contact angles lower than 90° (see Table 1). Thus, the membrane surface is less hydrophobic and more hydrophilic, so the membrane wettability rate is faster in the hydrophilic surfaces [52].

Regarding polyphenol families, the rejections of HB, HC, and F for the 0.22 μm membrane were 17%, 20%, and 24%, respectively; analogously, for the 0.45 μm membrane, they were HB 12%, HC 14%, and F 21% (see Table 2) for the spinach matrix; for the orange matrix, they were HB 0%, HC 16%, and F 24% for the 0.22 μm membrane and HB 0%, HC 10%, and F 17% for the 0.45 μm membrane (see Table 3). Therefore, the hydrophobicity of the polyphenol families (HC and F) with molecular weight (see Section 3.1) below the membrane MWCO (see Table 1) could be considered the most important parameter affecting the adsorption of phenolic compounds on the membrane surface.

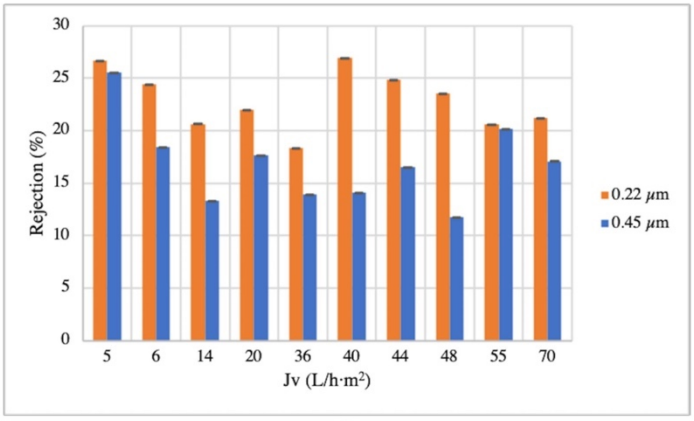


Figure 2. MF (0.22 and 0.45 μm) rejection evolution with permeate flux of spinach and orange matrices (**a**,**b**), respectively.

Table 2. Results of the rejection of each polyphenol family obtained with spinach extracts for the MF, UF, NF, and RO membranes (means of the two repetitions).

Membrane		Retentate Stream			Permeate Stream		
		HB	HC	F	HB	HC	F
MF	0.22 μm	17%	20%	24%	83%	70%	75%
	0.45 μm	12%	14%	21%	88%	86%	79%
UF	30 kDa	36%	32%	40%	64%	68%	60%
	50 kDa	25%	23%	38%	75%	77%	62%
NF	TFCS	100%	100%	81%	0%	0%	19%
	DURACID	100%	90%	73%	0%	10%	17%
	TFC-HR	100%	83%	71%	0%	17%	29%
	NF270	100%	79%	63%	0%	21%	37%
	NF90	100%	78%	66%	0%	22%	34%

	XLE	100%	97%	100%	0%	3%	0%
RO	SW30HR	100%	93%	100%	0%	7%	0%
	BW30LE	100%	93%	100%	0%	7%	0%

Table 3. Results of the rejection of each polyphenol family obtained with orange extracts for the MF, UF, NF, and RO membranes (means of the two repetitions).

Membrane		Retentate Stream			Permeate Stream		
		HB	HC	F	HB	HC	F
MF	0.22 µm	0%	16%	24%	100%	84%	76%
	0.45 µm	0%	10%	17%	100%	90%	83%
UF	30 kDa	0%	67%	29%	100%	33%	71%
	50 kDa	0%	44%	58%	100%	56%	42%
NF	TFCS	100%	89%	72%	0%	11%	28%
	TFC-HR	100%	89%	71%	0%	11%	29%
	DURACID	100%	79%	66%	0%	25%	34%
	NF90	100%	77%	55%	0%	24%	45%
	NF270	100%	76%	68%	0%	36%	32%
RO	BW30LE	100%	94%	80%	0%	6%	20%
	SW30HR	100%	91%	80%	0%	9%	20%
	XLE	100%	90%	80%	0%	10%	20%

With these both membranes (0.22 and 0.45 µm), low rejection percentages were expected, which indicates that the polyphenols passed through the membrane and thus were separated from solid particles and impurities. According to Comite et al. [53], MF is used especially to separate suspended solids in liquid foods. In this work, for example, for the spinach matrix, the suspended solid content of the feed (the extract) was 40 ± 0.0 mg/mL, and its content was completely removed in the permeate for the 0.22 and 0.45 µm membranes. Laorko et al. [54] also managed to remove suspended solids in addition to microorganisms by MF membranes, specifically with the 0.2 µm membrane.

The 0.22 µm membrane was selected to be the optimal membrane pore size for a clean-up stage for both matrices without losing high amounts of polyphenols (lower rejection).

3.2.2. Ultrafiltration

The clarification treatments of spinach and orange extracts were evaluated with the selected UF (30 and 50 kDa) membranes.

In particular, for the spinach extracts, the 50 kDa membrane exhibited the highest permeate flux (10 mL/min), with a value of 63.16 ± 0.46 L/h·m², in comparison with the permeate flux obtained by the 30 kDa membrane (60.49 ± 0.40 L/h·m²). In the case of the orange extracts, the 30 kDa membrane flux (55.90 ± 0.18 L/h·m² (10 mL/min)) was much lower than the flux obtained with the 50 kDa membrane (77.21 ± 0.72 L/h·m² (10 mL/min)). These results are in agreement with Laorko et al. [54], who obtained a lower J_v value with a 30 kDa than a 100 kDa membrane. They also found that the fouling phenomena could significantly affect the flux. Nevertheless, the flux decline is clearly not affected by the membrane material (see Table 1). According to Ahmad et al. [51], the flux decline can be affected by the roughness and porosity of the surface. However, the selected feed flow rate was 10 mL/min, with J_v improvements of 94% and 95% for the spinach and orange matrices, respectively, from 1 mL/min to 10 mL/min.

On the other hand, the rejection levels of total polyphenols from spinach and orange extracts for the selected membranes are also shown in Figure 3a,b, respectively.

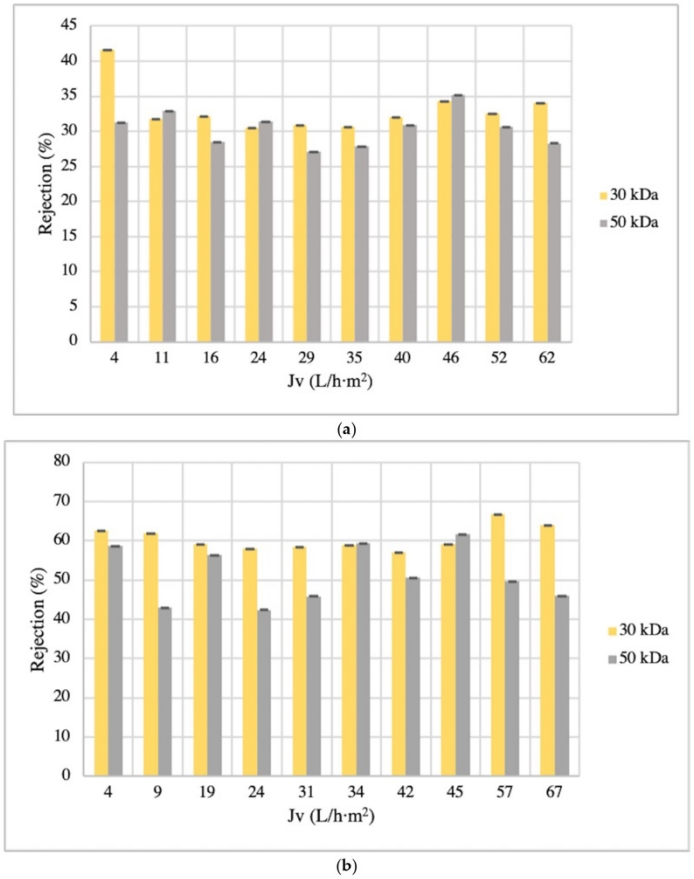


Figure 3. UF (30 and 50 kDa) rejection evolution with permeate flux of spinach and orange matrices ((**a**,**b**), respectively).

In general terms, the greater rejection percentages of polyphenols were observed for the 30 kDa membrane in comparison with the 50 kDa membrane in both matrices' extracts. In the case of spinach extracts, the 30 kDa membrane rejected 33% (on average), and in the 50 kDa membrane, 30% (on average) was rejected (see Figure 3a). For the orange extracts, using the 30 kDa membrane, 61% (on average) polyphenols were rejected, and with the 50 kDa membrane, 51% were rejected (on average). These results were expected, since the behavior of UF membranes can be explained based on steric consideration, and the 30 kDa membrane is the one with the lowest MWCOs (see Figure 3b). Accordingly, ANOVA assessment confirmed that there were significant differences ($p = 0.002$ for spinach and $p = 1.24 \times 10^{-5}$ for orange) between the two tested UF membranes (30 kDa and 50 kDa) on the polyphenol recovery.

Table 1 shows the zeta potential of 30 and 50 kDa membranes. The zeta potential is associated with how the suspension may interact with the surface of the membrane and with the possibility of the formation of films or agglomerates [25]. The zeta potential of the membranes depends on the pH. The feed pHs used in the UF process were 6 and 4 for

spinach and orange extracts, respectively. In fact, at these pH values of feed (which are in the pH range; see Table 1), membranes were negative charged. The negative zeta potential values of 30 and 50 kDa membranes (−16.4 and −15, respectively) indicate higher repulsive electrostatic interactions of spinach and orange components, which could minimize fouling.

Regarding the results of three polyphenol families for the spinach matrix, the rejection percentages were 36% of HB, 32% of HC, and 40% of F for the 30 kDa membrane; the 50 kDa membrane rejected 25% of HB, 23% of HC, and 38% of F (see Table 2). On the other hand, for the orange matrix, these values were 0% of HB, 67% of HC, and 29% of F for the 30 kDa membrane; the 50 kDa membrane rejected 0% of HB, 44% of HC, and 58% of F (see Table 3). Therefore, the level of retention observed with UF membranes can be attributed to the molecular weight of hydroxycinnamic acids and flavonoids being between 164.15 and 960.88 Da and between 254.24 and 978.85 Da, respectively, and these values are lower than the nominal MWCO of the UF membranes (30,000 and 50,000 Da).

Nevertheless, focusing on the lower rejection of HC and F, the low MWCO 30 kDa membrane was selected for clarification of spinach and orange extracts. Hence, this membrane can be proposed for an integrated membrane design that represents an interesting alternative to conventional separation systems for the recovery of natural antioxidants from spinach and orange residues.

3.2.3. Nanofiltration

For the polyphenol pre-concentration from the spinach and orange matrices, five membranes were studied: NF270, NF90, TFC-HR, DURACID, and TFCS (see Table 1).

For spinach and orange extracts, J_v values increased depending on the feed-flow rate, this trend was observed in the five membranes for the spinach extracts.

For the spinach matrix, the initial J_v average was 3.04 ± 0.50 (average, 1 mL/min) and increased until 32.52 ± 1.90 L/h·m² (average, 10 mL/min). For the orange matrix, the permeate fluxes of NF270 and DURACID membranes were 81.53 ± 3.66 and 83.03 ± 2.54 L/h·m² at 10 mL/min, respectively, which were the highest J_v. Indeed, the DURACID membrane showed a higher permeate flux under the same operational conditions. In the case of the NF270 membrane, due to the dense polymeric structure of the rejection surface layers of this membrane, contaminants easily penetrated it, resulting in an increase in the hydraulic resistance and therefore a reduction in its permeate flux at the end of the process [55]. Regarding the DURACID membrane, the sulfonation of the hydrophilic surface of this membrane improves the biocompatibility of the polymers, favoring the permeability to water and inhibiting the adhesion of biomolecules such as proteins, and thus obtaining better permeate flux [56].

Figure 4a,b show the rejection of NF membranes as a function of the permeate flux. All the NF membranes investigated presented high rejections for total phenolic content from spinach and orange extracts (rejection values higher than 70%). For the spinach matrix, the TFCS, DURACID, and TFC-HR membranes reported higher rejection values than NF90 and NF270 membranes (see Figure 4a).

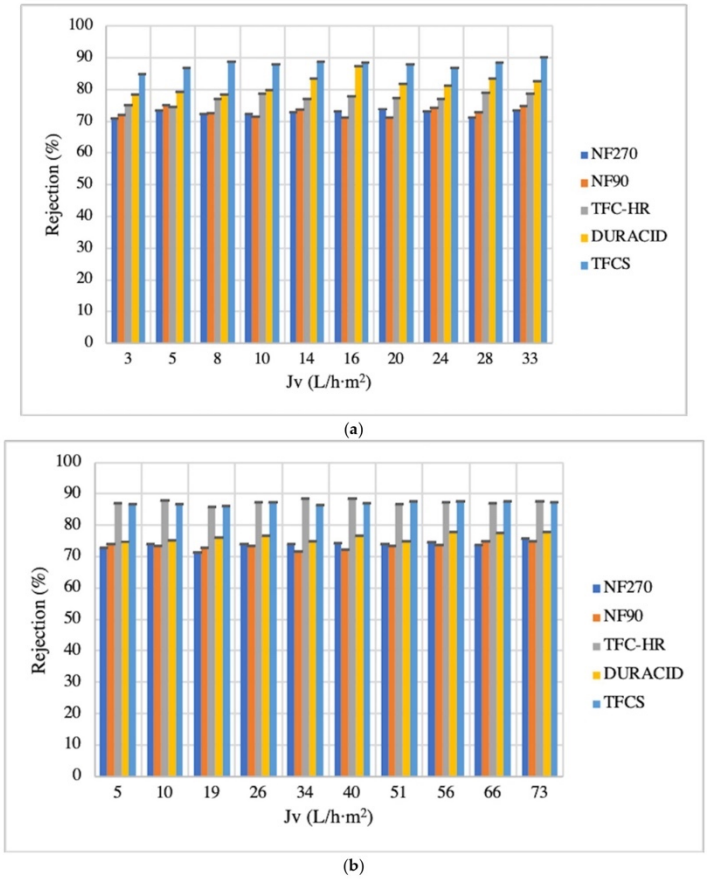


Figure 4. NF rejection evolution with permeate flux of spinach and orange matrices ((**a**,**b**), respectively).

For the orange extracts, TFCS (300 Da of MWCO) and TFC-HR (300–500 Da of MWCO) membranes showed the highest average rejection of 87% (see Figure 4b). Similarly, Conidi et al. [57] observed a higher rejection percentage of polyphenols (91% to 99%) by NF MWCO of 450 Da using a bergamot juice. Statistical results confirmed that significant differences (spinach matrix $p = 8.40 \times 10^{-48}$ and orange matrix. $p = 8.90 \times 10^{-67}$) were found between the studied NF membranes. Therefore, it is indispensable to consider the rejection results of the three families of polyphenols (HB, HC, and F). HB was completely rejected for all the studied membranes for both matrices. For the spinach matrix, the HC rejections were 100% for TFCS, 90% for DURACID, 83% for TFC-HR, 79% for NF270, and 78% for NF90. The F rejections were 81% for TFCS, 73% for DURACID, 71% for TFC-HR, 66% for NF90, and 63% for NF270 (see Table 2). On the other hand, for the orange matrix, the HC rejections were 89% for TFCS and TFC-HR, 79% for DURACID, 77% for NF90, and 76% for NF270. The F rejections were 72% for TFCS, 71% for TFC-HR, 66% for DURACID, 68% for NF270, and 55% for NF90 (see Table 3).

The higher rejections of polyphenols at acidic pH values (the pH values of the spinach and orange extracts were 6 and 4, respectively) can be explained by a cooperative effect of the increased interaction between the functional groups of the membrane itself, as well as the electrostatic repulsion established between the membrane-dissociated species and the charged surface of the membrane [58].

As shown in Table 1, the five NF membranes are made of polyamide and possess fixed dissociable carboxyl and amino groups on the surface [59]. Therefore, the pH can affect the dissociation of the membrane surface groups and the distribution charge (negative or positive) on the surface. In addition, the five membranes have similar IEPs, and also, at acidic pH, the membranes exhibit negative charge (zeta potential, Table 1).

Consequently, the electrostatic repulsion, independently of the MWCO of the selected NF membranes, contributes to the high rejection percentages of the membranes toward polyphenols from both matrices.

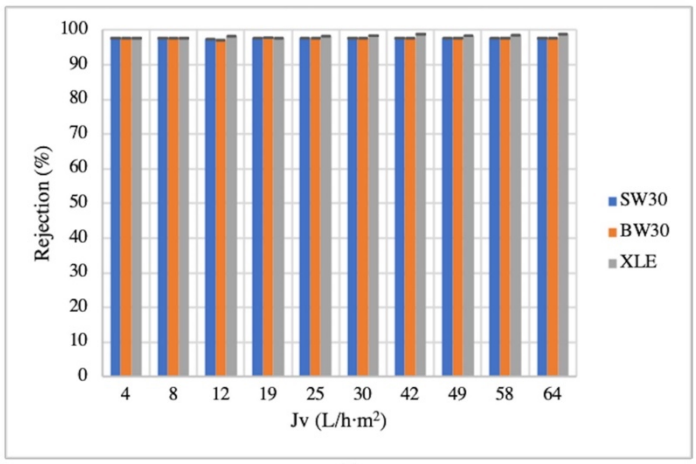
According to Kosmulski [60], the surface charge is determined by the existence of an isoelectric point or point of zero charge due to the presence of amino and carboxylic groups, which can dissociate in aqueous solution. The adsorption of organic compounds on the surface of the membrane can determine, in many cases, its functioning.

Another characteristic to consider is the hydrophobicity of membranes. Most high-pressure membranes are considered hydrophobic, a characteristic determined by the contact angle. The rejection and adsorption of organic solutes are not favored in membranes with higher hydrophobicity (higher contact angle) [58]. It can be seen in Table 1 that the contact angles of NF90, NF270, DURACID, TFCS, and TFC-HR membranes are 41.4–83.4°, 27–64.1°, 62.2°, 18.7°, and 35.7°, respectively. TFCS membrane has the smaller contact angle, so it has better hydrophilicity, which can more effectively prevent the membrane from being contaminated with other substances. According to Gao et al. [59], this hydrophilic behavior makes it more difficult for contaminants to be deposited, which can prolong the useful life of the membrane.

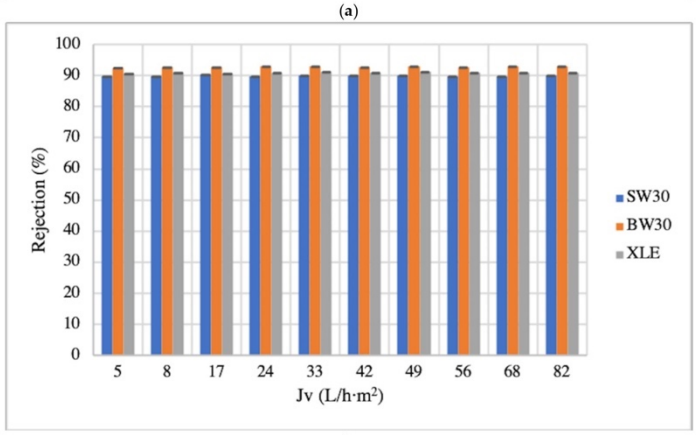
It is worth mentioning that the electrostatic repulsion also contributes to the high rejection percentages of NF membranes towards HB, HC, and F. Thus, TFCS was the membrane selected to pre-concentrate polyphenols for spinach and orange matrix extracts due to its full rejection of HB and higher rejection of HC and F. According to Montenegro-Landívar et al. [6] and Senit et al. [61], these polyphenol families report high antioxidant activity. Since spinach extracts are a suitable source of caffeic acid and ferulic acid, which are HC, and rutin, which is an F, they present values of 0.93 ± 0.26 mg/L, 0.67 ± 0.23 mg/L and 1.47 ± 0.23 mg/L amounts, respectively. Moreover, the orange extracts could be a rich source of hesperidin, an F, presenting a value of 48.63 ± 0.85 mg/L. These polyphenols could be of potential interest for cosmetic, pharmaceutical, and food applications [1,6,7].

3.2.4. Reverse Osmosis

For the reverse osmosis process, three membranes—SW30HR, BW30LE, and XLE—were evaluated for the concentration of polyphenols from spinach and orange extracts as shown in Figure 5.



(**a**)



(**b**)

Figure 5. RO rejection evolution with permeate flux of spinach and orange matrices ((**a**,**b**), respectively).

The highest flux was obtained using the SW30HR membrane with a permeate flux of 72.67 ± 0.81 L/h·m² at 10 mL/min, and the lowest permeate flux of 57.83 ± 0.73 L/h·m² was obtained using the BW30LE membrane at 10 mL/min for the spinach matrix. For the orange matrix, the highest J_v value of 86.30 ± 1.81 L/h·m² at 10 mL/min was obtained with the BW30LE membrane, followed by the permeate flux, obtaining 81.15 ± 5.48 L/h·m² at 10 mL/min with the SW30HR membrane; the lowest permeate flux of 77.39 ± 0.98 L/h·m² was obtained at 10 mL/min with the XLE membrane. No decline in flux was observed throughout the experiments on the selected membranes; this behavior shows that the membranes were resistant to fouling [14].

The rejection results for the three studied membranes used to treat spinach extracts (see Figure 5a) showed that the three membranes had performances ca. 100% (at 10 mL/min) of polyphenol rejection. In addition, Figure 5b shows the results of the rejection values for the orange extracts, where the BW30LE membrane displayed a higher rejection

of 93% (at 10 mL/min). ANOVA results confirmed that there were significant differences between the three RO membranes for spinach and orange matrices ($p = 2.36 \times 10^{-9}$ and $p = 1.82 \times 10^{-9}$, respectively) on the polyphenol recovery.

Regarding HB, HC, and F families for the spinach matrix, the rejection results were 100% of HB, 97% of HC, and 100% of F for the XLE membrane, and 100% of HB, 93% of HC, and 100% of F for the SW30HR and BW30LE membranes (see Table 2). For the orange matrix HB, HC, and F families, HB was rejected at 100% for the three membranes; HC was rejected at 94% for BW30LE, 91% for SW30HR, and 92% for XLE; and F was rejected at 80% for BW30LE, SW30HR, and XLE (see Table 3).

The hydrophobicity of the active layer is one of the most common mechanisms used to determine if the membrane is susceptible to fouling [13]. In this case, the contact angle of the RO polyamide membranes was very similar, as can be seen in Table 1. Furthermore, the SW30HR membrane has the lowest contact angle (50–52.8º) compared to the BW30LE (50–72.2°) and XLE (55–70.9°) membranes (see Table 1). Thus, the membranes' properties suggest that BW30LE has preferable hydrophilicity compared to the other membranes; therefore, due to its higher hydrophilicity (higher contact angle), organic solute (e.g., polyphenols) rejections were favored [58]. The XLE membrane showed higher water permeability (allowing the free passage of water to the permeate stream and concentrating polyphenols in the retentate stream [33,58]) than SW30HR and BW30LE membranes (see Table 1), since the reported pore size of the XLE (0.89 nm) membrane was higher than that of BW30LE (0.32 nm). Supplier data and the literature do not contain much information on the pore size of SW30HR membrane. According to Leo et al. [33], the surface energy, membrane roughness, and porous structure affect the water contact angle on the membrane surface.

The selected membrane for spinach extracts was XLE, due to its higher rejection of HC acids and F compared to the SW30HR and BW30LE counterparts. This is because, as has been mentioned throughout the manuscript, these polyphenol families are present in the spinach and orange wastes extracts. Thus, the selected membrane for the orange matrix was BW30LE due to the higher rejection of flavonoids. According to Gunathlikae et al. [62], the BW30 membrane can be applied to concentrate flavonoids of apple, blueberry, and cranberry juices. In addition, the antioxidant capacity of the fruit juices increased between 30% and 40%, so the BW30 membrane can be applied to enhance the bioactive concentration of fruit juices with enhanced antioxidant activity.

3.3. A Proposal for an Integrated Membrane Process for Polyphenol Separation and Concentration from Spinach and Orange Extracts

An integrated treatment train is proposed for spinach and orange extracts at the selected separation conditions resulting from the membrane tests. Figures 6 and 7 show the proposed schemes for the polyphenol recovery for each matrix. In these figures, the membrane type was selected based on the maximum rejection results for the three polyphenol families studied (HB, HB, and F), and the proposed operational conditions are also depicted (the feed flow rate was based on the maximum rejection results by the three polyphenol families).

It is worth mentioning that for both matrices, MF (0.22 μm membrane) is a good approach to remove suspended solids of spinach and orange extracts (cleanup stage). About the UF membranes, it can be considered the next stage that allows a complete separation of proteins, sugars, and polyphenols contained in the permeate stream. As can be seen in Figures 6 and 7, 30 kDa (UF) membrane would be selected to clarify the extracts in which the three families of polyphenols are found: hydroxybenzoic acids (HB), hydroxycinnamic acids (HC), and flavonoids (F). For spinach extracts, the most abundant values are HC and F; and for orange extracts F [6], it is a priority to recover these polyphenol families.

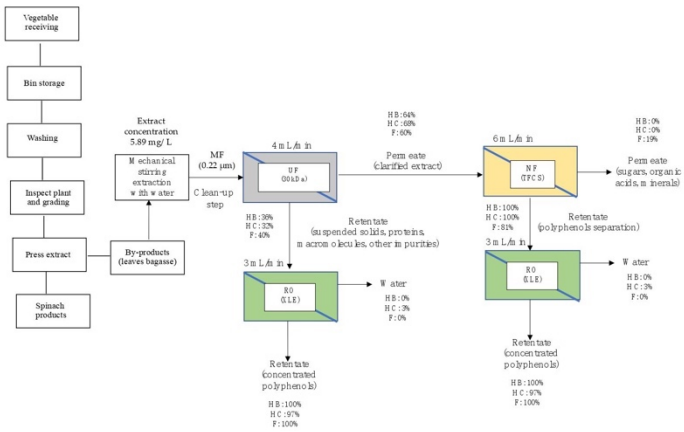


Figure 6. Scheme of the proposed integrated membrane process for the recovery of polyphenols from spinach extracts.

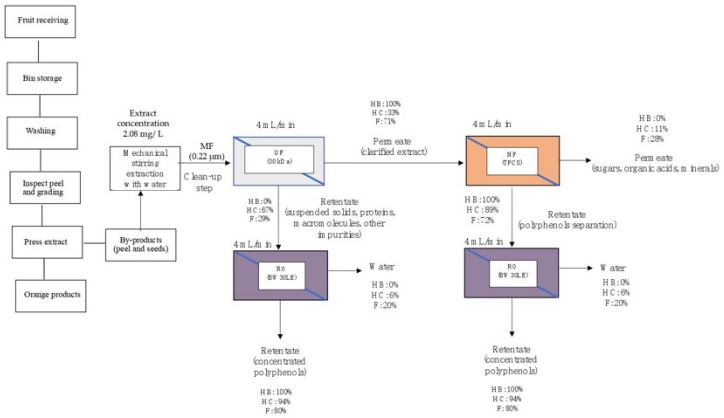


Figure 7. Scheme of the proposed integrated membrane process for the recovery of polyphenols from orange extracts.

Then, the UF permeate would be subjected to a NF process with a MWCO membrane between 150 and 300 Da. TFCS (300 Da) is proposed for spinach and orange extracts as a pre-concentration stage. Most polyphenols would be rejected by these membranes, especially HB, HC, and F. On the other hand, a permeate stream with a low polyphenol concentration would be obtained. In particular, for spinach, the NF membrane allows a permeate stream to be obtained with only F (19%), which is interesting for this matrix; for the orange matrix, the permeate stream does not contain HB, and the majority would be F (28%). Finally, the UF and NF retentate would be subjected to RO process (XLE for spinach matrix and BW30LE for the orange matrix) to concentrate polyphenols in the retentate stream. The permeate stream would be composed of water with low salinity, low COD, and reduced phytotoxicity due to the lowest contents of phenolic compounds, which are retained and concentrated by RO membranes [63]. An optional treatment for the obtained permeate could be the use of a high rejection hermetic RO technology as a

second stage to polish the permeate from the first RO stage and recover pure water and a concentrated antioxidant solution with very low fouling effects. According to Konstantinos et al. [64], the results obtained with this treatment were that the proposed RO stages can be applied on a commercial scale as liquid antioxidants and water suitable for reuse purposes (e.g., beverage formulation).

For the spinach matrix, it would be possible to separate HC (3%) from HB and F in this stream, whereas for the orange extract, the permeate stream will not contain HB, and HC (6%) would be present in a low concentration, since F is the major polyphenol group (20%), which would be interesting for the orange matrix.

As a result, the three polyphenol families are concentrated in the rejection streams, making the spinach and orange extracts rich in HB, HC, and F. Additionally, the RO concentration percentages obtained with XLE were 100% for HB, 97% for HC, and 100% for F for the spinach matrix. For the orange matrix, the values for the BW30LE were HB 100%, HC 94%, and F 80%.

Conidi et al. [65] proposed a similar integrated membrane process for the fractionation and recovery of phenolic compounds. In particular, UF and NF were a valid approach for the clarification and separation, in which UF can be used as the first step to remove suspended solids from artichoke wastewater. The UF permeate can then be treated with the NP030 nanofiltration membrane (400 Da MWCO) to separate sugars from phenolic compounds, yielding a polyphenol-enriched retentate stream of interest for food, pharmaceutical, and cosmetic applications. The permeate stream is enriched in sugars of interest for food applications; this stream can be treated with the Desal DL nanofiltration membrane (MWCO 150–300 Da) to obtain water in the permeate stream, which can be reused in the artichoke industry.

Similarly, Cassano et al. [12] suggested an integrated membrane process for the recovery and concentration of flavonoids from orange press liquor, in which UF would be used as a preliminary step to remove suspended solids and obtain a flavonoid-enriched permeate stream. The UF permeate stream could be treated with NF membranes to pre-concentrate flavonoids (such as flavanones and anthocyanins), obtaining a permeate stream enriched in sugars and minerals. Finally, the NF retentate stream could be treated by OD (osmotic distillation), producing a concentrated phenolic solution of great interest for cosmetic, food, and pharmaceutical applications.

According to Conidi et al. and Cassano et al. [65,12], the proposed process allows the traditional flow chart of the spinach and orange processing industry to be redesigned with important advantages—including the reduction in decontamination costs and environmental impact, the reduction in water and energy consumption, and, above all, the recovery and reuse of high-added-value compounds such as polyphenols—compared to conventional techniques such as electrodialysis, adsorption, and desorption on macroporous resin and chromatographic techniques [65].

Galanakis et al. [66] reported that electrodialysis and NF are assumed to be safe, while adsorption and chromatographic techniques depend on the toxicity of the materials involved in the process. The cost of electrodialysis is usually higher than that of NF. However, depending on the frequency of the membrane sheet discharge, the operating cost could be very high. In addition, the efficiency of adsorbent regeneration and chromatographic column cleaning affect the cost proportionally.

Considering that pre-concentrated and concentrated extracts were produced without thermal damage to phenolic compounds, these results offer interesting prospects for the use of these products as natural colorants and/or for nutraceutical applications and a clear permeate that is reusable as process water or for membrane cleaning. For example, lignans can be used in fats and oils to increase their stability during heating and storage [67]. Plant extracts (containing polyphenols) are effectively used in water- and tea-based functional beverages [68]. Regarding pharmaceutical applications, it should be taken into account that the suggested daily intake of polyphenols is 1 g/day to provide high concentrations of metabolites in the blood [69]. Taking this suggestion into account, Rodrigo et al. [70]

studied the behavior of ingested quercetin. They obtained the result that after 2 or 3 h of ingestion, quercetin was absorbed, reaching its point of maximum concentration in plasma. They also considered that the antioxidant activity was responsible for its defensive benefits by reducing the level of free radicals in the body. Additionally, in cosmetics, quercetin is used to formulate skin creams due to its high antioxidant activity [71].

4. Conclusions

The investigated pressure-driven membrane processes presented good potential for recovering polyphenols from spinach and orange residues for different applications such as natural antioxidants to be used as functional ingredients in the food, pharmaceutical, and cosmetic industries.

Results of this work demonstrate the suitability of MF, UF, NF, and RO to perform a purification (clean-up stage, clarification, pre-concentration, and concentration, respectively) of natural polyphenols from spinach and orange residues.

On the basis of the behavior of the transmembrane flux, these results depend on the feed flow rates of the MF, UF, NF, and RO membranes. The selected feed flow rate for both extracts was 10 mL/min for the MF, UF, NF, and RO membranes.

The 0.22 μm (MF) membrane was used to remove suspended solids from both matrices.

Concerning the UF process, the 30 kDa membrane was selected for a clarification stage for the two extracts, with rejections of 36% for hydroxybenzoic acids, 32% for hydroxycinnamic acids, and 40% for flavonoids for spinach extracts. For the orange matrix, 0% for hydroxybenzoic acids, 67% for hydroxycinnamic acids, and 29% of flavonoids were rejected.

Furthermore, NF membranes were selected for the pre-concentration stage. The best rejection percentages were obtained by using the TFCS membrane, which had a higher rejection of hydroxybenzoic acids, hydroxycinnamic acids, and flavonoids (100%, 100%, and 81%, respectively) for the spinach extracts; and for orange extracts, TFCS rejected 100% of hydroxybenzoic acids, 89% of hydroxycinnamic acids, and 72% of flavonoids.

Finally, the XLE and BW30LE (RO) membranes were selected to concentrate polyphenols from spinach and orange matrices, respectively. The rejection percentages were 100% for hydroxybenzoic acids, 97% for hydroxycinnamic acids, and 100% for flavonoids for the spinach matrix, and 100% of hydroxybenzoic acids, 94% of hydroxycinnamic acids, and 80% of flavonoids were rejected for the orange matrix.

Overall, a conceptual process design is proposed in which the MF and UF membranes could be used to obtain clean extracts from spinach and orange extracts and to remove impurities (e.g., suspended solids and microorganisms). NF membranes could be used for the pre-concentration of polyphenols from low-molecular-weight compounds. Additionally, RO membranes could be used to concentrate them and could facilitate the removal of the solvent. Concentrated polyphenols extracts (as solution and/or powder) with RO can be used as food additives for the formulation of nutraceutical products, natural colorants in cosmetics, etc.

The bottleneck of membrane processes can be the fouling, scale-up, and/or the lifetime of the membranes. However, the proposed membrane processes allow for significant advantages in comparison with other separation technologies such as the reduction in depollution costs, the environmental impact, water consumption, and the recovery of value-added compounds such as polyphenols. Overall, the valorization of agri-food wastes allows one to minimize the environmental concerns derived from its treatment and improve its use as a source of polyphenols that could be recovered by using membrane technologies in the scope of the circular economy.

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review and editing, P.T.-Q., X.V., M.R., A.F., C.V., M.G., J.L.C. and J.S.; supervision, X.V., C.V. and J.S.; project administration, J.L.C.; funding acquisition, J.L.C. All authors have read and agreed to the published version of the manuscript.

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CHAPTER 7

Publication 4

"Polyphenols and their potential role to fight viral diseases: An overview"

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Review

Polyphenols and their potential role to fight viral diseases: An overview

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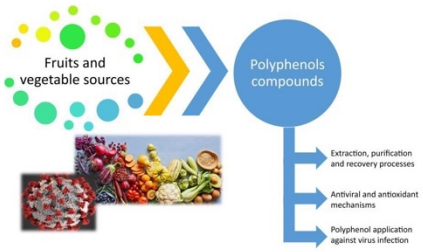
HIGHLIGHTS

- Fruits and vegetables residues are a natural source of polyphenols.
- Polyphenols, from agri-food industry, present antiviral and antioxidant activities.
- Natural polyphenols could be used as a treatment/prevention against virus infection.
- Especially flavonoids are used for prevention/cure of diseases caused by viruses.
- Polyphenols with antiviral activity could be a novel strategy against SARS-CoV-2.

GRAPHICAL ABSTRACT



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ABSTRACT

Fruits, vegetables, spices, and herbs are a potential source of phenolic acids and polyphenols. These compounds are known as natural by-products or secondary metabolites of plants, which are present in the daily diet and provide important benefits to the human body such as antioxidant, anti-inflammatory, anticancer, anti-allergic, antihypertensive and antiviral properties, among others. Plentiful evidence has been provided on the great potential of polyphenols against different viruses that cause widespread health problems. As a result, this review focuses on the potential antiviral properties of some polyphenols and their action mechanism against various types of viruses such as coronaviruses, influenza, herpes simplex, dengue fever, and rotavirus, among others. Also, it is important to highlight the relationship between antiviral and antioxidant activities that can contribute to the protection of cells and tissues of the human body. The wide variety of action mechanisms of antiviral agents, such as polyphenols, against viral infections could be applied as a treatment or prevention strategy; but at the same time, antiviral polyphenols could be used to produce natural antiviral drugs. A recent example of an antiviral polyphenol application deals with the use of hesperidin extracted from *Citrus sinensis*. The action mechanism of hesperidin relies on its binding to the key entry or spike protein of SARS-CoV-2. Finally, the extraction, purification and recovery of polyphenols with potential antiviral activity, which are essential for virus replication and infection without side-effects, have been critically reviewed.

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Contents

1. Introduction

Since ancient times, plants have played an important role for humanity, for example as food, clothing, perfumes and/or medicines (e.g., drugs in traditional medicine). Already in 1978, the World Health Organization (WHO) highlighted the need of scientific research in traditional medicine. Since then it has begun to put in value this type of medicine, as well as to investigate the efficacy of the mechanism of action and chemical bases of traditional herbal medicine for the development of new drugs, and also within the antiviral property that some plants possess (Ruwali et al., 2013; World Health Organization, 1978). Additionally, it is known that plants produce secondary metabolites such as polyphenols to protect themselves from the plethora of biotic and abiotic stresses. Biotic stress can be weeds, insect pests, fungi, and other microorganisms, whereas abiotic stresses can be physical and environmental conditions like salinity, drought, UV radiation, extreme temperatures, and toxic metals (Tuladhar et al., 2021). Polyphenols are not only involved in the defense mechanism of the plant system, but also, they have been found, for example, in the cell division, photosynthetic activity, reproduction, hormonal regulation, and nutrient mineralization mechanisms (Sharma et al., 2019). Thus, for instance, coumarins and tannins reduce stress on plants by repelling herbivores (Lattanzio, 2013).

Infections caused by viruses in humans are a critical and vitally important issue, as has been demonstrated during the last year with the 2019-nCovid caused by SARS-CoV-2 (severe acute respiratory syndrome corona virus-2) (Gorbalenya et al., 2020), in particular to safeguard the health of the population and mitigate its impact on economic and social vectors. Therefore, antiviral agents (e.g., vaccines containing specific virus, or specific antibody with protective therapeutic effect, or plants with antiviral activity) are reported to fight viral diseases, and some cases have been eradicated such as: i) smallpox, poliomyelitis (>99%); and ii) endemic measles, rubella and congenital rubella syndrome practically eliminated from America since 2010 thanks to vaccination (Plotkin et al., 2012). But although immunisation and drug development have been progressing for decades, this has not been enough, as many viruses do not have preventive vaccines and, worse still, traditional antiviral treatments such as drugs with unnatural components (e.g., acyclovir for herpes) may lead to the generation of viral mutants (Lin et al., 2014).

Besides, some drugs used to combat viral diseases can cause human health hazards due to their viral resistance or, in some cases, low efficiency. In addition, viruses are made up of DNA or RNA enclosed in protein capsules (Kamboj et al., 2012), which can invade human body cells and use components from those cells for their replication. This process often damages or destroys infected cells, causing a viral disease (Rouse and Sehrawat, 2010).

On this basis, it is necessary to find alternatives to the traditional treatments for viral diseases (El-Toumy et al., 2018). For example, recently, the use of bioactive compounds like polyphenols has been proposed as an alternative treatment due to their potential health benefits. These substances can be found in vegetables and fruits and can also be recovered from secondary sources, such as agri-food residues (Montenegro-Landívar et al., 2021; Tapia-Quirós et al., 2020). Scientific publications have detailed more than 500 different polyphenols in more than 400 food components (Galanakis, 2018). Additionally, based on epidemiological research and associated meta-analyses, the long-term consumption of diets rich in polyphenols from plants are considered as tools that could offer protection against for example to cardiovascular diseases, neurodegenerative diseases, diabetes development, osteoporosis, and cancers development among others (Pandey and Rizvi, 2009). Therefore, polyphenols are widely promoted to be used as functional foods or in drugs preparations accepted for human consumption around the world (Cheynier, 2012; Shahidi and Ambigaipalan, 2015; Cory et al., 2018).

Recently, Russo et al. (2020), have proposed that alternative treatments to antiviral drugs may also represent a valid approach for the 2019-nCovid disease. It is also stressed that polyphenols, specifically flavonoids, can act satisfactorily in various stages of the coronavirus entry and replication stages. Another example is catechin epigallocatechin-3-gallate (EGCG), present in green tea leaves, which inhibits replication of DNA viruses such as herpes simplex (oral dose of 800 mg), and HIV-1 (concentration ranging from 25 to 250 μmol/L), among others (Steinmann et al., 2013). Moreover, the half-maximal inhibitory concentration (IC_{50}) of 47–73 μM of luteolin, hesperetin, and quercetin, among others flavonoids, can inhibit key proteins (PL^{pro}, $3CL^{pro}$) involved in the infectious cycle of SARS coronavirus (Nguyen et al., 2012; Soukhova et al., 2004). More examples can be found as described below, where a list of some types of viruses could be inhibited by phytochemicals such as polyphenols. Of the diseases caused by harmful viruses where the use of polyphenols has been critically reviewed and shown an antiviral activity against them, kaempferol and quercetin, extracted from *Broussonetia papyrifera*, have shown activity against MERS-CoV and SARS-CoV-1 viruses; and hesperetin and naringenin block the replication of Sindbis virus. Thus, these are examples which, with future developments, could be approved as alternative treatments. In view of above, the main objective of this comprehensive review is to compile and evaluate the studies that have demonstrated the antiviral activity of polyphenols, the postulated mechanisms of action of polyphenols to defeat viruses as well as the synergy effect of antiviral and antioxidant

Table 1
Different families and type of viruses, their specific virus and the role of polyphenols as a possible alternative to treat virus.

Type of virus	Specific virus	Disease characteristics	Conventional treatment	Alternative treatment with polyphenols	Reference
Respiratory infections	Influenza virus (A, B and C)	Annually responsible for high mortality in both humans and animals worldwide	NA inhibitors and M2 protein channel blockers after infection, while vaccination is the most effective therapy	1,2,3,4,6-Penta-*O*-galloyl-ß-D-glucose (IC_{50} of 2.36 μg/mL) purified from *Echinacea purpurea* against *Paramyxovirus avidae* Linn inhibits virus replication	(Fox and Christenson, 2014; Liu et al., 2011; Moscona, 2008)
	Coronavirus (HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) and the novel SARS-CoV-2)	Respiratory tract infections in humans with outbreaks around the world, especially in winter	Currently there are no specific treatments for the CoV infection and preventive vaccines are being developed	Polyphenol extract from *Echinacea purpurea* against SARS-CoV-2 provided 50% of inhibition Kaempferol and quercetin from *Broussonetia papyrifera* against MERS-CoV and SARS-CoV effectively inhibited with an IC_{50} of 27.9 μM and 30.2 μM, respectively	(Chiow et al., 2016; Liu et al., 2020; Park et al., 2017; Signer et al., 2020)
	Rhinovirus	The main cause of the common cold, among other respiratory diseases, also responsible for otitis media in asthmatic people, acute otitis and bronchiolitis	There are no vaccines or antiviral agents for the prevention or treatment of this virus	Resveratrol showed a therapeutic approach to reduce infection when the IC_{50} was 50 μM (the IC_{50} of ribavirin was 100 μM)	(Choi et al., 2010; Mastromarino et al., 2015; Rauhadmand et al., 2013)
	Syncytial virus	Causes infections in infants and the elderly, causing not only acute morbidity but also recurrent breathing problems	There is no safe and effective treatment. Corticosteroids was treated children of normal health who had early bronchitis, but the results were not satisfactory and failed to reduce the infection or breathing problems	*Woodfordia fruticose* flowers, reported 55% virus inhibition Resveratrol (IC_{50} 189 pg/mL) inhibits 40% virus replication and down-regulates the TIR-domain-containing adapter-inducing interferon-β (TRIF) complex, which controls signals for the activation of innate immune cells	(Beigelman et al., 2014; Tagarro et al., 2014; Xie et al., 2012)
Gastrointestinal infections	Rotavirus	Causes dehydrating gastroenteritis, especially in children under five years of age	There is a vaccine against rotavirus but access to the vaccine is limited. 200,000 deaths worldwide. The treatment focuses on dehydration and not on the use of antiviral agents	Polyphenols (licocoumarone, glycyrrin, glycyrrhizic acid) extracted from *Glycyrrhiza uralensis* root (IC_{50} 18.7–60.5 μM) can inhibit 50% virus absorption and replication after the cell's entry	(Crawford et al., 2017; Gastaminza et al., 2010; Kwon et al., 2010)
Hepatic infections	Hepatitis virus (A, B and C)	Cause high morbidity and mortality around the world	Anti-hepatitis virus drugs are members of nucleosides or nucleoside analogs, which inhibit the activity of polymerase or reverse transcriptase, but the prolonged use giving rise to the existence of mutant viruses	Curcumin (150 μM) inhibits hepatitis B virus	(Moafer Rechtman et al., 2010; Sultovati et al., 2016; Yapp et al., 2016)
	Epstein–Barr virus	Infects human epithelial and lymphoid cells. Infection is associated with a number of human cancers, such as Hodgkin's disease	A vaccine is not yet approved	(−)-Epigallocatechin gallate (EGCG) extracted from green tea (50 μM) blocked the EBV lytic cycle, inhibiting the expression of immediate-early genes in a range of 40–50%	(Abba et al., 2015; Chang et al., 2003; Cohen, 2018)
	Human cytomegalovirus	Not present obvious symptoms, but infection causes morbidity and mortality in transplant recipients or patients with acquired immunodeficiency syndrome (AIDS)	Drugs such as cidofovir, valganciclovir and ganciclovir, which target viral DNA polymerase, but their side-effects include bone marrow toxicity, low bioavailability, plus drug resistance to the virus	Curcumin, using a low dose of 0.2 μg/mL, inhibits virus protein expression	(Ahmed, 2012; Evers et al., 2005; Li et al., 2014)
	Herpes simplex virus (HSV-1 and HSV-2)	Responsible for orolabial and genital diseases producing, in general, benign lesions but, in some cases, putting the life of carriers at risk if the infections are recurrent	There is no vaccine and existing drugs (e.g., acyclovir) do not eradicate the virus infection and cause resistance to drugs	Ent-epiafzelechin-(4 → → 8)-epiafzelechin extracted from *Cassia javanica* leaves (250 μM of) inhibits more than 90% of HSV-2 penetration to the host cell	(Cheng et al., 2006; Morfin and Thou, 2003; Piret and Boivin, 2011)
Exanthematous infections	Varicella-zoster virus	Causes fever and vesicular rash. Once the disease has disappeared, the virus enters into a state of latency, but it can be reactivated due to stress, causing herpes	Generally, uses drugs such as acyclovir, valaciclovir, etc., which are often combined with analgesics for pain and corticosteroids for inflammation	Resveratrol (219 μM of) inhibits 100% virus replication	(Docherty et al., 2006; Johnson and Whitton, 2004)

(*continued on next page*)

Table 1 (*continued*)

Type of virus	Specific virus	Disease characteristics	Conventional treatment	Alternative treatment with polyphenols	Reference
		zoster and acute pain in latently infected lymph nodes			
Neurologic infections	Rabies virus	Causes an acute and fatal neurological infection in humans and mammals	Disease can be prevented by vaccination	Tannin pentagalloylglucose (PGG) (10 μM) for 24 h possess significant anti-RABV activity; PGG can reverse the expression of miR-455-5p (a microRNA whose excess production regulates host cell signalling pathways and innate immune responses)	(Fisher et al., 2018; Riedel et al., 2019; Tu et al., 2019)
	Polio virus	The virus drains into the cervical and mesenteric lymph nodes and then into the blood, causing a transient viremia	The incidence has been largely reduced especially by the use of a vaccine, but the disease is still endemic in Africa and Asia	Extract of *Avicennia marina* leaf, the IC_{50} was 145.7 μg/mL before and 314.3 μg/mL after attachment stages of virus replication, with a cytopathic effect of 50% in both stages	(Felipe et al., 2006; Racaniello, 2006; Zandi et al., 2009)
Haemorrhagic fevers	Dengue virus	Causing from a mild fever to haemorrhagic fever, nausea, joint pains, etc.	There are no effective vaccines, and the prevention options available for the control of the virus infection are very limited	Baicalein (IC_{50} was 7.14 μg/mL) potent antiviral agent against adsorption in the host and after entry viral replication, and IC_{50} of 1.55 μg/mL presents a virucidal effect	(Zandi et al., 2011, 2012)
	Sindbis virus	Cause of disease outbreaks in humans in South Africa and Northern Europe	There are no vaccines or therapeutic means	Hesperidin and naringenin with a 50% inhibitory dose (ID_{50}) of 20.5 μg/mL and 14.9 μg/mL respectively, reaching 50% for hesperidin and up to 80% for naringenin of virus replication inhibition	(Ling et al., 2019; Paredes et al., 2003)
Immune system infection	Human immunodeficiency virus (HIV-1 and HIV-2)	Spreads through certain body fluids and attacks the immune system, destroying T lymphocytes. Thus, the body loses its ability to fight infections and diseases	Since HIV was discovered, there has been no preventive vaccine for virus infection, and the applied treatment is antiretroviral therapy drugs which help control the multiplication of HIV in infected patients	Tricyclic coumarin compound from *Calophyllum brasiliense* stem bark (IC_{50} was 8.44 μM) inhibits virus replication by suppressing nuclear factor-kappa B (a protein complex that controls DNA transcription) activation	(Bhatti et al., 2016; Häggblom et al., 2016; Kudo et al., 2013; Lin et al., 2014; Sundquist and Kräusslich, 2012)
Multisystem diseases	Coxsackie virus	Causes muscle injury, paralysis and death	There is no specific treatment or vaccine available	Apigenin (IEC_{50} of 9.7 mg/L) and ursolic acid (IEC_{50} of 6.6 mg/L) extracted from *Ocimum basilicum* interfere with virus replication after infection	(Bedard and Semler, 2004; Chiang et al., 2005; Wong et al., 2013)

properties against viral diseases. Additionally, the extraction, purification and recovery technologies to produce polyphenols from natural products and/or agri-food waste are also evaluated, since they could be limiting steps for the application of polyphenol molecules as an alternative against viruses on a large scale.

1.1. Therapeutic tools against viral diseases

The type of virus diseases and the specific virus considered in the review are summarized in Table 1, and includes: i) respiratory infections: influenza virus, coronavirus, rhinovirus, and syncytial virus; ii) gastrointestinal infections: rotavirus; iii) hepatic infections: hepatitis virus, Epstein-Barr virus, human cytomegalovirus, and herpes virus; iv) exanthematous infections: varicella-zoster virus; v) neurologic infections: rabies virus and poliovirus; vi) haemorrhagic fevers: dengue virus and Sindbis virus; vii) immune system infections: human immunodeficiency virus; and viii) multisystem disease: coxsackie virus.

In view of the examples described in Table 1, there are a large number of viruses, which can affect human health to different extent. For this reason, it is important to identify new therapeutic and functional strategies using natural sources through bioactive compounds and, more specifically, polyphenols. Furthermore, it is worth noting that polyphenols extracted from plants can efficiently inhibit the different stages of replication of various viruses in a dose-dependent matter.

2. The potential of phenolic acids and polyphenols as antiviral agents

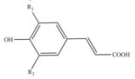
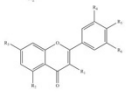
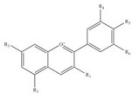
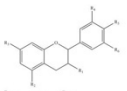
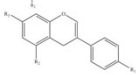
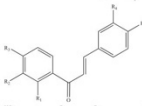
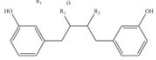
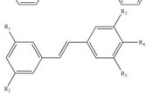
Plants not only have the function of feeding human beings, but have also been used since ancient times as a source of therapeutic agents. According to Naithani et al. (2008), up to 80% of the world population uses plants as alternative medicine for various reasons such as their well-known antiviral features. This claimed activity is due to a wide variety of bioactive compounds present, such as polyphenols, proteins, and terpenoids, among others (Kamboj et al., 2012). Although, polyphenols are common components of the human diet, it has been reported that polyphenols are also toxic due to their biocidal activity at intake concentrations between 1 and 5% of the total daily diet (Galanakis, 2018). Considering the significant amounts of compounds that a person must be consumed, being approximately between 0.025 and 1 g per day (Scalbert and Williamson, 2000), and their multitude activities, it should be noted that they could play an important role in the prevention of numerous diseases, including antivirals. However, it is necessary to consider that despite many promising results obtained in vitro or animal experiments, there is still not enough convincing evidence from human studies, especially with large populations. More research is needed to better understand the value of therapeutic polyphenols, dietary polyphenols, and in the context of their ability to prevent the progression of diseases caused by viruses (Koch, 2019; Martin, 2009; Yang et al., 2020).

The focus of this review is on phenolic compounds and polyphenols, which have a common structural feature consisting of the presence of one or more hydroxyl groups attached to a benzene ring. Polyphenols can be classified into different classes based on their chemical structure, ranging from simple to highly polymerized compounds. Their fundamental physiological functions deal with the growth and reproduction of plants, as well as protection against pathogenic organisms and ultraviolet radiation. In addition, polyphenols strongly influence the organoleptic characteristics of food products, such as color and flavour (Ignat et al., 2011).

Polyphenols are often classified into four main families, namely: phenolic acids, flavonoids, stilbenes and lignans (Saurina and Sentellas, 2015). The basic chemical structure and examples of these polyphenol families are collected in Table 2.

As shown in Table 2, polyphenols have a great structural diversity as a function of the number of phenol rings that they contain and the elements that bind these rings. Flavonoids are the largest and most studied group.

Table 2
List of relevant polyphenol classified according to their structure (adapted from Saurina and Sentellas, 2015).

Class	Structure	Substitutions	Examples
Phenolic acids *Hydroxybenzoic acids*		R1: H, OH, OCH_3 R2: H, OH, OCH_3	Gallic acid Vanillic acid Procyanidin B1 Theogallin
Hydroxycinnamic acids		R1: H, OH, OCH_3 R2: H, OH, OCH_3	Caffeic acid Ferulic acid *p*-Coumaric acid Rosmarinic acid
Flavonoids *Flavonols* *Flavones* *Flavanones*		R1: H, OH R2: H, OH R3: H, OH R4: H, OH R5: OH, OCH_3 R6: H, OH	Hesperidin Naringenin Quercetin Kaempferol Luteolin
Anthocyanidins		R1: H, OH R2: OH, OCH_3 R3: OH R4: H, OH R5: OH R6: H, OH	Cyanidin Pelargonidin
Catechins		R1-R3: OH R4: H, OH R5: OH R6: H, OH	Catechin Epicatechin Epigallocatechin
Isoflavones		R1: OH R2-R3: H, OH	Genistein Daidzein
Chalcones		R1-R5: H, OH	Xanthohumol Phloretin Isosalipurpurin
Lignans		R1-R2: H, OH	Enterodiol Matairesinol
Stilbenes		R1-R4: H, OH, OCH_3 R5: H, OH	Resveratrol Piceatannol

Phenolic acids possess a high antioxidant capacity and their medical properties, such as vasodilatory, antibacterial, antiviral, anticarcinogenic and anti-inflammatory, have been reported elsewhere (Oroian and Escriche, 2015).

An important derivation from hydroxybenzoic acids are the so-called hydrolysable tannins, which are mostly present as phenolic polymers with different molecular weights, from 500 to 3000 Da (Andronescu and Grumezescu, 2017). As their principal characteristic, tannins precipitate proteins, thus contributing to regenerate, for example, a burn tissue, besides their antimicrobial, antioxidant and antiviral properties (Haminiuk et al., 2012). Their antiviral activity against Epstein–Barr virus DNA polymerase has been demonstrated, and especially those tannins extracted from mouse-tail plant (*Phyllanthus myrtifolius*) and chamber bitter (*Phyllanthus urinaria*) (Naithani et al., 2008).

Regarding flavonoids, they represent the largest amount of polyphenols (up to 60%) consumed in the human diet (Brglez Mojzer et al., 2016). Actually, more than 9000 different flavonoids have been reported, having important benefits on human health because of their antiviral, anti-inflammatory and antidiabetic attributes (Zhang et al., 2015; Krych and Gebicka, 2013; Tian et al., 2013; Ragab et al., 2014).

Table 3
Summary of relevant polyphenols present in plants with antiviral activity according to the reviewed publications.

Plant source	Polyphenol	Type of virus	Reference
Berries, tea, almond, beans, tomato, *Ficus carica* L., capers, caraway, cloves, cumin, Cambuci	Kaempferol	Coronavirus, rotavirus, human cytomegalovirus, HSV-1 and HSV-2, coxsackie B virus	(Naithani et al., 2008; Kamboj et al., 2012; Russo et al., 2020; Watson et al., 2013; Haminiuk et al., 2012)
Propolis, *Oroxylum indicum*	Chrysin	Coronavirus, rotavirus, human cytomegalovirus, HSV-1 and HSV-2, coxsackie B virus	(Cheng and Wong, 1996; Kumar and Pandey, 2013; Cushnie and Lamb, 2005)
Euphorbia cooperi, *Morus alba*, *Rhus succedanea*	Catechin	HIV, HSV-1	(Kamboj et al., 2012; Cushnie and Lamb, 2005; El-Toumy et al., 2018)
Citrus spp., cocoa, fish mint (*H. cordata*), *Spondias mombin*, *Spondias tuberosa*	Quercetin	Rabies virus, poliovirus, syncytial virus, HSV-2, respiratory syncytial virus, dengue virus, coronavirus	(Suárez et al., 2010; Silva et al., 2011; Zandi et al., 2011; El-Toumy et al., 2018; Chiow et al., 2016)
Betula pendula, apple	Quercitrin	Rabies virus, HSV-1, influenza virus	(Kumar and Pandey, 2013; Suárez et al., 2010)
Spondias spp., *Pavetta owariensis* (bark)	Rutin	Rabies virus, influenza virus, dengue virus	(Kamboj et al., 2012; Cushnie and Lamb, 2005)
Citrus spp., peppermint, grapefruit	Hesperidin	Influenza virus, HSV, poliovirus, syncytial virus, SARS-CoV-2	(Mhatre et al., 2020; Bellavite and Donzelli, 2020)
Chamomile, parsley, oregano, thyme, grapefruit, orange, onion, mango	Apigenin	HSV-1, HIV	(Kumar and Pandey, 2013; Kamboj et al., 2012)
Citrus spp., tomato, aromatic plants	Naringin	Respiratory syncytial virus	(Kumar and Pandey, 2013)
Broadleaf plantain (*Plantago major*), papaya, peach, avocado	Caffeic acid	HIV, HSV	(Pommier et al., 2005; Sytar et al., 2021)
Broccoli, rosemary, pistachio, lentils, olive, artichoke, lemon, *Aloe vera*	Luteolin	HSV-1 and HSV-2	(Naithani et al., 2008; Lopez-Lazaro, 2008)
Berries, pomegranate, walnuts, pecans	Ellagic acid	Dengue virus, hepatitis A and B	(Kang et al., 2006; Kamboj et al., 2012)
Grape, berries, peanuts	Resveratrol	Influenza A, hepatitis C virus, respiratory syncytial virus, varicella-zoster virus, Epstein–Barr virus, HSV, HIV	(Docherty et al., 2006; Mastromarino et al., 2015)

Flavonoids have been studied against the type-1 and type-2 herpes simplex virus, and against the human immunodeficiency virus (HIV-1 and HIV-2) (Naithani et al., 2008).

Finally, stilbenes (including curcuminoids) and lignans have been extensively studied because of their antioxidant properties, but their antiviral activity is not far behind. These compounds and their derivates have been studied against viruses such as herpes simplex (type-1 and type-2), HIV, influenza, and human papilloma, among others (Naithani et al., 2008; Abba et al., 2015).

A list of polyphenols with antiviral activity, the type of virus against which they act, and their plant source is collected in Table 3.

As shown in Table 3, polyphenols with antiviral activity such as quercetin, rutin, hesperidin, apigenin, catechin, and morin are present in abundance in plants like fruits (e.g., berries, citrus fruits, tropical fruits), popular beverages (e.g., green tea, coffee), vegetables (e.g., spinach, beans, onions, olives), spices and herbs (e.g., turmeric, rosemary, ginger), which are consumed in the daily human diet (Brglez Mojzer et al., 2016). Currently, the antiviral activity of various polyphenols makes their study more attractive; for example, 3 mg/kg body weight of curcumin, extracted and purified from turmeric, is sufficient to inhibit HIV (Praditya et al., 2019; Barthelemy et al., 1998; Haslberger et al., 2020).

2.1. Polyphenol recovery from secondary sources

Phenolic compounds can also be obtained from by-products of plant processing, being cheap and easily available to recover them following a circular economy strategy (Tapia-Quirós et al., 2020; Montenegro-Landívar et al., 2021). In addition, the growing interest in polyphenols recovery has led to the study of different technologies that can allow their extraction without losing their antiviral properties (Dzah et al., 2020). Successful cases of extraction and recovery of polyphenols with antiviral activity is summarized in Table 4.

Table 4 lists not only the application of polyphenols extracted from different plant sources against numerous target viruses, but also the different extraction techniques integrated, being maceration the most used. Additionally, most of the trials were carried out on a pilot scale.

The polyphenols extraction from plants or from their processing wastes can be achieved by conventional (e.g., mechanical stirring) and enhanced (e.g., ultrasound and microwave assisted) solid-liquid extractions or by combination of both extraction approaches by means of organic and/or aqueous solvents. For their subsequent purification implies a preliminary stage of clean-up and concentration by using sorption on resins, or pressure-driven membrane processes such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) followed by a final purification by using extraction chromatography (Bottino et al., 2020; Charcosset, 2016).

As mentioned, the commonest technique for polyphenols extraction is maceration (e.g., a solid-liquid process). For example, Edziri et al. (2012) used maceration for polyphenol extraction from *Marrubium deserti*. The dried product (250 g) was extracted with methanol, butanol, chloroform and ethyl acetate (using a feed to solvent ratio of 1:10) for an extraction time of five days. Results from antiviral activity tests concluded that the extracts with methanol and ethyl acetate showed significant antiviral activity against coxsackie B3 virus with IC_{50} of 100 and 135 μg/mL, respectively.

Magnetic agitation at 20 °C has been applied to recover active components from apple pomace (10 g) using 100 mL of 70% acetone and 80% methanol in darkness. The results showed that acetonic and methanolic extracts could inhibit the replication of HSV-1 and HSV-2 by more than 50% (Suárez et al., 2010).

The purification of plant extracts is, in general, a complex process and no single method is complete enough. It requires a combination and integration of them to achieve the highest separation and purification factors. A successful example is the study by Zahoor et al. (2020), who compared the separation efficiency of quercetin extracted from *Rubus fruticosus* by using RO and NF membranes. Quercetin is used against rabies virus, poliovirus, syncytial virus, and HSV-2, among other viruses (Suárez et al., 2010; Silva et al., 2011; Zandi et al., 2011; El-Toumy et al., 2018; Chiow et al., 2016). The results obtained indicated that the RO membrane accomplished a quantitative recovery (e.g., >99%) of the drainage pipe, while NF membranes achieved a 95% of polyphenol recovery. The study shown that the cost of use the RO membranes is higher, due to the higher energy consumption than the use of NF membrane stage followed by a sorption stage of the remaining 5% of the permeate stream by using magnetic carbon nanocomposite.

It should be noted that the phenolic compounds recovered from extracts, such as kaempferol, luteolin, chrysin, gallic acid, ferulic acid, catechin, anthocyanins among others, could be used as a treatment and/or prevention against virus infection (Singh et al., 2020; Kumar and Goel, 2019; Watson et al., 2013; Marín et al., 2015).

Table 4
Applications of extracted polyphenols from different plant sources to treat several target viruses.

Antiviral polyphenol	Plant source	Target virus	Extraction technique	Purification technique	Study effectiveness against virus	Results	Scaling-up	Reference
1,2,3,4,6-Penta-O-galloyl-ß-D-glucose (PGG)	Pomegranate	Influenza A (H1N1)	Maceration	Sephadex LH-20 column	EC_{50} 2.36 ± 0.29 μg/mL of PGG 5 or 8 h upon infection	Significant inhibition virus release	Lab scale	(Liu et al., 2011)
Extract rich in polyphenols	*Magnolia officinalis* bark	Influenza A (H1N1)	PLE	–	In vivo oral administration (10 and 20 mg/kg) for 5 days	Infected mice reduce the production of nitric oxide, pro-inflammatory cytokines, TNF-α and IL-6	Pilot scale	(Wu et al., 2011)
Isoquercetin (quercetin glucoside form)	*Hypericum perforatum*, *Equisetum arvense L.*	Influenza A (H1N1)	Maceration	–	In vivo administrated intraperitoneal	Reduce virus titres and pathological changes in lungs of mice infected with influenza A (H1N1) by up to 20-fold at 1:500 (*Equisetum arvense L.*) or 1:1000 dilutions (*Hypericum perforatum*) at 24 h post-inoculation	Pilot scale	(Kim et al., 2010)
Baicalein	*Scutellaria baicalensis* root	Influenza H1N1	Maceration	–	In vivo oral administration	Infected mice showed significant therapeutic activities, including death prevention and lung virus titre reduction	Pilot scale	(Xu et al., 2010)
Quercetin, kaempferol, myricetin, quercetin-3-*O*-galactoside, morin, apigenin, catechin, epicatechin, caffeic acid and rimantadine	*Geranium sanguineum* aerial roots	Influenza (H3N2)	Maceration	–	Administered in aerosol way (dose 5.4 mg/mL)	Around 70% was the protective index and the survival time was in a range of 2.9–4.9 days, the animal lung infectious virus titre was reduced in comparison with control	Pilot scale	(Serkedjieva et al., 2008)
Epigallocatechingallate (EGCG), epigallocatechin (EGC), epicatechingallate (ECG), epicatechin (EC) and catechin gallate	Tea	Influenza	Maceration	–	76 adult persons around 65 years old gargling 200 mg/mL 3 times daily for 3 months	The catechin-treated group have lower incidence of influenza infection than the control group	Pilot scale	(Yamada et al., 2006)

2.2. Economic prospects of polyphenol recovery

Taking into account the different antiviral applications of polyphenol extracts, as well as the need to investigate innovative extraction and purification procedures, it is interesting to mention some examples of the economic evaluation of the recovery of polyphenols, which is also applicable to antiviral polyphenols.

The manufacturing cost (COM), expressed as €/kg per year, of the polyphenols extraction from raw material could be estimated using the methodology described by Turton et al. (2009), where five main costs must be taken into account: (i) fixed capital investment (FCI), (ii) cost of operating labour (COL), (iii) cost of utilities (CUT), (iv) cost of waste treatment (CWT), and (v) cost of raw material (CRM). Following this methodology, Vieira et al. (2013, 2017) compared the COM of extraction of jussara pulp (*Euterpe edulis* Martius) with strong antioxidant activity by ultrasonic assited extraction (UAE) and agitated bed extraction (ABE) at lab scale. The UAE extracts presented a higher cost (75.2–137.2 €/kg) than the ABE extracts (72.5–139 €/kg). It was reported that the ABE and UAE extracts contained polyphenols such as kaempferol, luteolin, apigenin, quercetin and rutin, which may have medical applications (e.g., antivirals) for human health; and anthocyanidins as natural pigments for feed applications (Favaro et al., 2018; Vieira et al., 2017). Moreover, Osorio-Tobón et al. (2014) also used the COM methodology to evaluate the extraction process of curcumin from turmeric (*Curcuma longa* Linneo), which possesses a remarkable antiviral effect against dengue virus (Ichsyani et al., 2017). When advanced solid-liquid extractions techniques were evaluated, pressurized liquid extraction (PLE), Soxhlet extraction and low-pressure solvent extraction (LPSE), COM values of 79, 204 and 161 €/kg, respectively were estimated. The results showed that the PLE extraction technique was less expensive due to the short extraction time required (e.g., 30 min) compared with 6 h for Soxhlet and 3 h for LPSE. However, the extraction yields of the three techniques were similar (PLE 11 ± 1, Soxhlet 12 ± 1 and LPSE 12 ± 1).

On the other hand, Ioannou-Ttofa et al. (2017) determined that the total cost of the treatment of olive mill waste water through an integrated process using UF and NF membranes (UFZW-10/NF270) was 9.94 €/m^3. The high-added value polyphenols present in this stream, like hydroxytyrosol, can be purified, and the cost can reach 14,900 to 20,900 €/kg.

Besides, it is worth noting that the approximately market value, according to the Sigma-Aldrich website, of 10 mg of kaempferol (≥90% purity) is 113 €, 10 mg of luteolin (≥98%) is 169 €, 10 mg of apigenin (≥97%) is 92 €, 10 g of quercetin (≥95%) is 44 €, 10 g of rutin (≥94%) is 24 € and 10 mg of hydroxytyrosol is 191 € (≥90%), but their purity can make they more expensive.

2.3. Stability, reactivity, synergism and bioavailability of polyphenols

Technically, polyphenols are extracted, purified and concentrated from plants using different techniques as described above, which can be processed into tablets or capsules for human consumption. However, polyphenols are unstable, prone to degrade and/or react with some elements (e.g., oxygen and metal ions during their processing and storage stages), resulting in changing structures and decreasing activities (e.g., antiviral activity) (Galanakis, 2018). Therefore, the stability as well as their reactivity, synergism and bioavailability of polyphenols are the main aspects that must be taken into account in the recovery, processing, storage and consumption of phenolic compounds for their market applications.

2.3.1. Stability

Instability generally due to many polyphenols are sensitive to chemical, enzymatic and physical treatments, which are used in food

processing. Chemical and enzymatic instability leads to changes such as oxidation or polymerization, among others, causing alterations on their nutritional and physical-chemical attributes. Physical instability leads to changes like phase separation, flocculation, etc. that can also alter their attributes (Joye and McClements, 2014; Zhang et al., 2020).

2.3.2. Reactivity

Another factor that influences polyphenols is their reactivity, as they can be enzymatically degraded and polymerized during food processing stages. One of the most notable enzymatic reactions is on color, taste and nutritional value of polyphenols, which can even cause significant economic problems due to their impact on quality and shelf life of the products (Galanakis, 2018).

2.3.3. Synergism

The synergism of polyphenols in plant extracts means that a combination of two or more of compounds creates a higher biological activity than when the extracts are analyzed relative to individual polyphenols isolated from the same extracts (Yao et al., 2012; Zhang et al., 2020). However, the commercial application of polyphenols is currently limited due to instability when exposed to light, heat or oxygen as well as low bioavailability. A solution of the limitations mentioned, it could be the encapsulation (Zhang et al., 2020). Long et al. (2015) showed that bioactive food compounds can produce synergistic effects, as they have been reported in traditional Chinese medicine research. Therefore, the synergism between polyphenols must be taken into account for the development of functional foods and thus promote human well-being and prevent diseases such as viral ones.

2.3.4. Bioavailability

Bioavailability plays an important role in terms of the biological properties of polyphenols, which makes it possible to understand the proportion of their absorption, digestion and metabolism after their entry into the circulatory system (Carbonell-Capella et al., 2014). Several epidemiological and experimental studies describe the protective role of polyphenols in diseases such as viral diseases, diabetes, inflammation, among others (Kumar and Goel, 2019). Scalbert and Williamson (2000) reported few human bioavailability studies showing that the amounts of intact polyphenols in urine vary from one to another polyphenols. For example, for quercetin glycoside the percentage found in excretion urine was between 0.3 and 1.4%, while in the case of hesperidin, it was 24.4%. Similar variations were also observed for naringin consumed with grapefruit juice depending on the individual (5 to 57%).

Hong et al. (2014) and Liang et al. (2017) demonstrated that EGCG loading in nanoparticles constructed with zein a protein as zein or with a polysaccharide as chitosan, improved the stability of said polyphenols at the gastrointestinal level. Another study by Xue et al. (2014) using glycosylated casein nanoparticles to encapsulate EGC demonstrated the improvement of its physical stability during storage. Xue et al. (2018) encapsulated curcumin in zein-caseinate nanoparticles, and reported improved stability against UV radiation and heat treatments.

On the other hand, there has also been an interest in encapsulating combinations of polyphenols and taking advantage of their synergistic effects. For example, curcumin and resveratrol have been encapsulated within hyaluronic-coated lipid droplets, as they have similar mechanisms of action to inhibit tumor cell growth and antioxidant and antiviral effects (Nasr, 2016). Encapsulated polyphenols have been shown to have better chemical stability that non-encapsulated ones. However, after encapsulation no improvement in the bioavailability of polyphenols has been observed, as polyphenols can become indigestible. Therefore, the most appropriate administration system for the polyphenols and the food matrix used should be thoroughly evaluated case by case (Dueik and Bouchon, 2016).

3. Antiviral activity of polyphenols

The antiviral activity of different polyphenols has the target of interacting directly with viral particles, but this binding will depend on the nature of the virus (DNA or RNA virus) (Sundararajan et al., 2010; Palamara et al., 2005; Liu et al., 2011). Another characteristic of antiviral polyphenols is that they can exert the activity during intracellular replication, which may be attributed to antioxidant features of phenolic groups, thus inhibiting the oxidation of cells by the replication of some viruses (Sundararajan et al., 2010; Fraternale et al., 2009).

Many natural polyphenols have provided research results and are becoming an important target in the development of some drugs to combat viruses, thanks to their wide availability, inexpensive production and, above all, their low side-effects (Kumar and Goel, 2019; El-Toumy et al., 2018). This is the case with the virus that is currently attacking the entire world, the novel severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), which causes the 2019-nCoV disease transmitted person-to-person (Kampf et al., 2020).

As of June 2021, a total of nearly 171 million confirmed cases have been reported, including almost 4 million deaths worldwide since the start of the outbreak (World Health Organization, 2020). Due to the high infectivity and mortality rate of SARS-CoV-2, there is an opportunity to use the great amount of information on plants used in the Traditional Chinese Medicine (TCM) to be used to treat symptoms related to SARS (homology of SARS-CoV and SARS-CoV-2), considering that natural polyphenols could inhibit SARS-CoV-2 (Mehany et al., 2021). According to Wang et al. (2020) and Chojnacka et al. (2020), the entry of SARS-CoV-2 into the host cells (lung epithelium) is facilitated by a trimeric glycoprotein, called the spike protein (protein S), located in the capsid of the virus (outer envelope). SARS-CoV-2 uses angiotensin converting protein II (ACE2) as a receptor for binding to host cells. The protein S is hydrolysed by endosomal proteases, such as cathepsin or transmembrane cellular serine protease 2 (TMPRSS2), which results in membrane fusion. After the virus enters the host cell, it produces new RNA and the proteins that form its envelope. The binding of SARS-CoV-2 to the receptor ACE2 may depend on several factors, such as variants in the virus protein S that promote the efficiency of their interaction. In the replication and transcription, the main protease $3CL^{pro}$ and papain-like protease (PL^{pro}) are involved. The therapeutic targets to protect the human body from the entry, replication and transcription of the SARS-CoV-2 virus are the receptor with the proteases cutting spike protein and the proteases. Polyphenols with antiviral activity (e.g., flavonoids such as kaempferol, quercetin, and naringenin, see Table 3) have been developed as protease inhibitors, helping to stop virus infection (e.g., HIV, MERS and SARS) (Paraiso et al., 2020). Current studies show that some extracted polyphenols have antiviral activity, specifically as protease inhibitors. Park et al. (2016) used 95% ethanol to extract chalcones from *Angelica keiskei* that showed inhibition of protease $3CL^{pro}$ as well as non-competitive inhibition of protease PL^{pro} of SARS-CoV with IC_{50} values of 11.4 and 1.2 μM, respectively. Also Park et al. (2017) extracted polyphenols with ethanol from *Broussonetia papyrifera* with potential anti-coronaviral agents, which inhibit 100% PL^{pro} (the IC_{50} was 3.7 μM) protease. Recent studies, such as Yudi Utomo and Meiyanto (2020), reported that hesperidin and naringenin, among other citrus flavonoids, and polyphenols from *Curcuma* spp., such as curcumin, bind strongly to the $3CL^{pro}$ substrate, the binding domain of SARS-CoV-2, while interacting with the receptor ACE2 and protein S. Khalifa et al. (2020a) demonstrated that anthocyanidins, such as cyanodelphine, phacelianin, techofilin and gentiodelphin, authentically interact with the receptor binding site of SARS-CoV-2-$3CL^{pro}$. Khalifa et al. (2020b) found that pedunculagin, castalin and tercatain, which are tannins, strongly interact with the SARS-CoV-2-$3CL^{pro}$ receptor binding site. Other polyphenols such as sinigrin, with IC_{50} of 217 μM, and hesperidin, with IC_{50} of 8.3 μM, contained in the water extract of the root of *Isatis indigotica*, have also been shown to be anti-SARS-CoV-2-$3CL^{pro}$ (Xu et al., 2020). These studies triggered

Table 5
Selected polyphenols and their role against SARS-CoV-2.

Polyphenol	Source	Mechanism of action	Analysis study	Reference
Kaempferol, quercetin, luteolin-7-glucoside, demethoxycurcumin, naringenin, apigenin-7-glucoside, oleuropein, curcumin, catechin, epicatechingallate, zingerol, gingerol, and allicin	Medicinal plants	Block the enzymatic activity of SARS-CoV-3CL^{pro}	In silico	(Khaerunnisa et al., 2020)
Malvidin, peonidin, petunidin, pelargonidin, cyanidin and malvidin	*Pimpinella anisum* L.	Binding affinities to 3C-like protease of SARS-CoV-2 (virus replication)	In silico	(Hasan et al., 2020)
Hesperetin, myricetin, caflanone, linebacker	Medicinal plants	High affinity to protein S, helicase and protease sites on the CE2 receptor (in silico analysis); in vitro analysis shows potential caflanone to inhibit virus entry	In silico and in vitro	(Ngwa et al., 2020)
Baicalein and baicalin	*Scutellaria baicalensis* and *Oroxylum indicum*	Down-regulators of the TMPRSS-2 expression. Baicalein (IC_{50} of 0.94 μM) and baicalin (6.41 μM) promising results to 3Cl^{pro}	In silico and in vitro	(Da Silva Antonio et al., 2020)
Polyphenol extract	*Echinacea purpurea*	Virus inactivated upon treatment with 50 μg/mL	In vitro	(Signer et al., 2020)

that some polyphenols could be used as effective and above all natural anti-2019-nCovid components. Even, the Chinese Health Commission officially confirmed that natural medicine (e.g., TCM) should be used in combination with conventional medicine for the treatment of 2019-nCovid patients, and currently experimental research is focused on the therapeutic potential of polyphenols against SARS-CoV-2 (Yang et al., 2020). Table 5 collects a summary of the antiviral activities of recent studies of natural polyphenols and their mechanism of action against SARS-CoV-2.

Although Table 5 present more in silico experiments that predict promising results, more in vitro and in vivo studies are needed to evaluate the mechanism of action of polyphenols against SARS-CoV-2. Most of the studies were carried out in silico, current technology to predict drug behavior, accelerating the detection rate, since it allows screening many drugs and reduction of the cost of laboratory work, limiting clinical trials to the best candidates.

Recently has been discovered that hesperidin (which is a flavonoid) easily binds to key proteins of the SARS-CoV-2, due to its physicochemical structure (see Table 2) (Adem et al., 2020; Chen et al., 2020; Das et al., 2021; Joshi et al., 2020; Wu et al., 2020; Yudi Utomo and Meiyanto, 2020). These authors investigated if hesperidin is able to bind with a low binding energy. The lower energy required, the stronger and more specific the binding will be in therapeutic terms. Wu et al. (2020) tested hesperidin as a potent antiviral agent. The binding of hesperidin to the spike protein was effective in superimposing the ACE2-receptor binding domain (RBD) on the hesperidin-RBD complex, where a clear overlap of hesperidin with the ACE2 interface was observed. Accordingly, it was concluded that hesperidin can interrupt the ACE2 with RBD. Another low-energy binding site for hesperidin against SARS-CoV-2 is the main protease. This enzyme is called 3CL^{pro} or M^{pro} and is the target of many chemical antiviral drugs. Das et al. (2021) studied the molecular coupling of the interaction between hesperidin and M^{pro}. The binding energy of hesperidin with hydrogen bonds to various amino acids (e.g., THR24, THR45, HIS4, SER46, etc.) was estimated as −37.7 kJ/mol. Finally Joshi et al. (2020) identified that hesperidin binds strongly to the main SARS-CoV-2 protease, and also to the ACE2-receptor.

Regarding vitro analysis, Suru et al. (2021) confirmed that pomegranate peel extract and its main polyphenols, such as punicalin and punicalagin, have a great capacity to attenuate the binding of the SARS-CoV-2 glycoprotein S to the ACE2 receptor. The most pronounced in vitro activity was observed in pomegranate peel extract, suggesting a possible synergistic effect of polyphenols, allowing their possible therapeutic application for 2019-nCovid.

On the other hand, there are few in vivo studies investigating the antiviral effect of polyphenols against this novel virus. Deng et al. (2020), studied Pudilan Xiaoyan Oral Liquid (EC_{50} of 1.078 mg/mL), a traditional Chinese medicine containing four herbs: Indigowoad root (*Isatis indigotica*), Bunge Corydalis (*Corydalis bungeana*), Mongolian Dandelion (*Taraxacum mongolicum*), Scutellaria Amoena (*Scutellaria baicalensis*) as well as more than 180 compounds (e.g., polyphenols

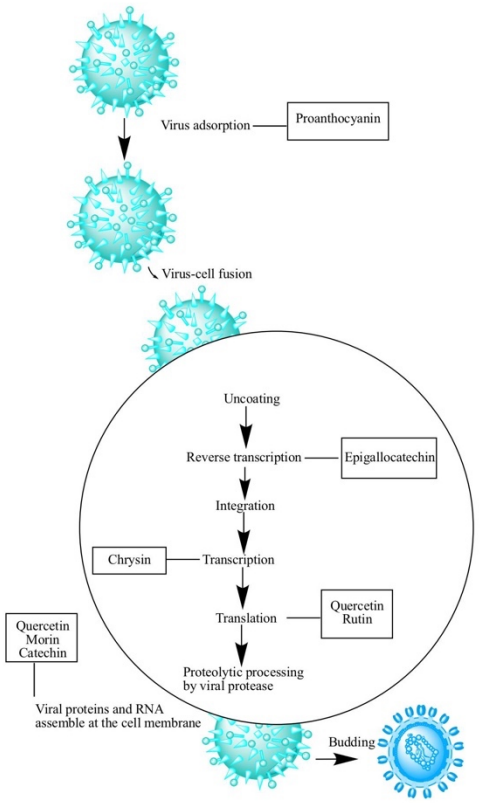


Fig. 1. Virus replication and polyphenol targets (adapted from Pommier et al., 2005; Kamboj et al., 2012).

Table 6
Examples of antiviral mechanism of polyphenols (adapted from Naithani et al., 2008; Haslberger et al., 2020).

Antiviral polyphenol	Plant source	Study	Virus type	Main mechanism
4′,5-Dihydroxy 3,3′,7-trimethoxy flavone	*Agastache rugosa* (Kuntze)	Effect on the replication virus	Rhinovirus coxsackie virus	Replication inhibition, selective inhibition of viral RNA synthesis in the cell culture
Quercetin Luteolin	*Achyrocline satureioides*	Effect on the viral replication cycle of HSV-1	HSV-1	Interferes with the events occurring between the third and ninth hour of HSV-1 replication cycle, which includes transcription and translation of viral proteins
Salvin	*Salvia officinalis*	Viral inhibitory before absorption stage	HSV-1, HIV, SARS-CoV	Efficacy before absorption stage, but not in the replication stage
Morin Coumarin Quercetin	*Rhus succedanea*, *Garcinia multiflora*, *Alnus firma*	Effect on viral replication	HIV	Blockage of RNA synthesis, exhibited HIV-inhibitory activity

such as chrysin, apigenin, rutin among others), which exhibited potent anti-SARS-CoV-2 activity in infected hACE2 mice. In another study, Schettig et al. (2020) reported that a nebulized formulation of quercetin (20 mg/mL) and *N*-acetylcysteine (100 mg/mL) greatly alleviated the respiratory symptoms of SARS-CoV-2 in a patient treated with hydroxychloroquine and antibiotics. This demonstrates the importance of conducting further clinical (in vivo) studies to evaluate the potential of polyphenols as an adjuvant or primary therapy for 2019-nCovid.

If polyphenols are analyzed in depth as traditional anti-2019-nCovid therapeutics on humans, they could be innovative and effective, or even against other lethal viral diseases. The use of medicinal plants containing antiviral polyphenols, still has some risks and needs massive and additional experiments.

3.1. Antiviral mechanism

The mechanism of polyphenols deals with the prevention of the entry of the virus into the host cell. This was the case of the proanthocyanidins extracted from *Rumex acetosa*, which inhibited the entry of the influenza type A virus in its first critical phase (Daglia, 2012). Fig. 1 shows the scheme of the target sites of the antiviral mechanism of some polyphenols (e.g., quercetin, morin, chrysin).

The general interest in polyphenols, as antiviral agents, is increasing because of the great advantages of using nature-derived compounds with almost no side-effects on human health (Naithani et al., 2008). Several studies have been done for discovering the antiviral mechanism of different polyphenols; Table 6 gives an overview of some of them.

As shown in Table 6, the polyphenols with antiviral properties may have different mechanisms of action, such as inhibiting the entry of the virus, an effect on replication, etc. (Haslberger et al., 2020). New trends in biotechnology and medicine, as well as new processing technologies, could help to optimise the solubility, administration and therapeutic activities to prevent infection by viruses (Patra et al., 2018; Thomford et al., 2018; Lin et al., 2014).

3.2. Relationship between antiviral activity and antioxidant properties

The study of the relationship between antiviral and antioxidant activities has not been explored in depth. Only a few studies report a comparative evaluation. It is worth mentioning that a high formation of free radicals leads to an imbalance in the oxidative metabolism at the mitochondrial level and, as a result, the vitality of the cells of each tissue is affected (Delgado-Roche and Mesta, 2020). This imbalance can be caused by various viruses (e.g., SARS-CoV-2, HIV, influenza virus) and leads to oxidative stress and helps the virus life cycle and eventually causes cell death (Bellavite and Donzelli, 2020).

Viral infection disrupts the defensive antioxidant mechanism of the human body, bringing inflammation and oxidative damage. Experimental animal models have achieved high levels of reactive oxygen species (ROS) and an alteration of innate antioxidant defenses during, for example, a SARS-CoV infection (van den Brand et al., 2014).

Therefore, the use of polyphenols with antiviral and antioxidant activities could be an alternative to prevent the onset of infection or development of viral disease. For example, Lin et al. (2002) reported that HSV-2 infection increases the amount of free radicals, and consequently causes immune response pathology. They used an ethyl acetate extract from *Euphorbia thymifolia* with antiviral and antioxidant activities (the IC_{50} was 7.72 ± 0.15 μg/mL) to inhibit HSV-2 growth in the kidney cell line.

As mentioned, many of the pathological effects of the viruses are not only directly related to viral replication, but also to the host response to infection (e.g., inflammation, oxidative stress, etc.) (Mateos-Martín et al., 2014; Bellavite and Donzelli, 2020). Therefore, the combination of antiviral therapy with the antioxidant properties could help favourably to combat the virus infection, reducing toxicity and preventing antiviral resistance.

4. Conclusions

Keeping in mind that diseases caused by viruses remain among the leading causes of morbidity and mortality, in both developed and developing countries, despite having conventional medicine, it is of interest to looking for alternative treatments more biocompatible for humans. As an example of alternative treatments, polyphenols are interesting and promising molecules that could be applied in the pharmaceutical sector. Polyphenols are secondary metabolites from plants which can also be extracted from agri-food residues. The bioactivities of polyphenols, like antioxidant capacity, as well as their mechanisms, such as forming stable radicals, delay and/or prevent oxidative stress-induced cellular damage and disease, are well defined and studied. However, in this comprehensive review, it has specifically shown the potential role of polyphenols with potential targets, such as antiviral activity, in the prevention of diseases caused by viruses. The state of the art indicates that there is not a single mechanism of action of polyphenols against viruses. Indeed, the antiviral mechanism of these bioactive compounds could be by antioxidant activities, viral entry or inhibition of viral reproduction, DNA inhibition among others.

Additionally, due to the complex polyphenol structure, new extraction, purification, formulation and processing technologies could help to improve the stability and bioavailability of antiviral polyphenols, as well as the administration protocols and the therapeutic effects as antiviral treatments. Thus, the use of plant extracts, as polyphenols, is postulated as useful for health, due to their synergistic effects, such as antioxidant, antiviral, anti-inflammatory, among others that must be considered and studied intensively. Therefore, future and more comprehensive studies of the antiviral activity of polyphenols against the SARS-CoV-2 coronavirus could provide an additional strategy, for example as a curative treatment in vaccines, to combat this pandemic that is causing the deadly disease 2019-nCovid.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER 8

Results

8. Results and Discussion

In this chapter, the most relevant findings of this PhD thesis are briefly summarized, for the sake of understanding the results are presented following the structure of the publications.

8.1 Polyphenol extraction from orange and spinach wastes by UAE, MAE, PLE and MSE

The improvement of the total phenolic content (TPC) yield was studied by three innovative techniques of extraction, UAE, MAE and PLE. The use of acidified ethanol:water (ethanol 0-80 %, HCl 0-0.5%) mixtures, different temperatures (25-120 °C), extraction times (0-30 min) and cycles (1 and 2, in PLE) were studied. The best TPC yield was obtained, for the orange matrix, with PLE using 60/39.9/0.1 ethanol/water/HCl (v/v/v) solvent at 80 °C for 15 min, providing 3 mg GAE/g fw. For the spinach matrix, UAE was the technique selected using the following conditions, ethanol/water/HCl in the ratio 80/19.9/0.1 (v/v/v) as the solvent at 25 °C for an extraction time of 30 min, providing 0.82 mg GAE/g fw. Comparing the three techniques considering the energy consumption and the cost related, it can be concluded that UAE was the most suitable technique for the polyphenol extraction from both residues.

The optimization of MSE extraction was performed by studying different factors such as the effect of temperature (25, 50, 70, and 90 °C) and extraction time (5, 15, and 30 min), the effect of solid to solvent ratios (1:10, 1:30, 1:50, 1:100 and 1:200), and the effect of pH (3, unadjusted and 10), using water as extractant. The highest TPC of 1 mg GAE/g fw was obtained using 1:100 (w/v) solid to solvent ratio, 15 min of extraction time at 70 °C and pH 4 for orange matrix. For the spinach matrix, a TPC of 0.8 mg GAE/g fw using

1:50 (w/v) solid to solvent ratio, 5 min of extraction time at 50 °C and pH 6 was recommended.

However, the comparison of MSE and UAE (using the optimal conditions for a previous research) resulted in a similar performance for the orange matrix and in an increase of the TPC yields 2-fold for spinach; in comparison with the TPC obtained by UAE.

8.1.1 Antioxidant evaluation of orange and spinach extracts obtained by UAE, MAE, PLE and MSE

After selecting the best conditions for each residue, the antioxidant activity was tested and the characterization of the extracts was carried out.

FRAP, DPPH, and ABTS assays were used to measure the antioxidant activity of the extracts from each matrix after UAE. Other fruit and vegetables were also evaluated applying the UAE optimal conditions for orange and spinach. Although, they may differ slightly from the optimal ones required for the other fruit and vegetables, they could be reasonably extended to other waste matrices to explore their valorization potential.

HPLC was used to characterize the obtained orange and spinach extracts, obtained compounds such as 4-hydroxybenzoic acid, vanillic acid, syringic acid, etc. are presented in **Table 6**.

Table 6. Identified phenolic compounds in the extracts from orange and spinach matrices, their concentration under optima conditions.

Polyphenol	**Residue**	**Concentration (mg/kg fw)**
4-hydroxybenzoic acid	Orange	104.51
Vanillic acid		394.37
Syringic acid		37.49
p-coumaric acid		27.75
Ferulic acid		25.45
Rutin		86.12
Hesperidin		676.68
4-hydroxybenzoic acid	Spinach	25.35
Vanillic acid		23.12
p-coumaric acid		10.51
Hesperidin		63.94

The antioxidant activity of the extracts after MSE was also evaluated by DPPH, FRAP and ABTS. FRAP generally provides higher values of antioxidant capacity than the other methods. Orange and spinach have high antioxidant capacity, but to visualize and compare easily in a more comprehensive way the antioxidant activity extracts PCA was applied.

The characterization of the orange and spinach extracts was evaluated by HPLC. It was found that the orange waste could be a rich source of 4-hydroxybenzoic acid (0.71 mg/g)

and hesperidin (4.86 mg/g). On the other hand, the spinach waste could be also a good source of caffeic acid (0.04 mg/g), ferulic acid (0.04 mg/g) and rutin (0.08 mg/g).

Then, it could be concluded that MSE was a viable technique to extract polyphenols from orange and spinach by-products with antioxidant activity (2.27 mg TE/g and 0.04 mg TE/g, respectively) which could be used in the food, pharmaceutical, and cosmetic industries.

8.2 Polyphenol purification from orange and spinach wastes by membrane technology

The separation and concentration efficiency were evaluated in terms of the content of total polyphenols, and by polyphenols families (hydroxybenzoic acids (HB), hydroxycinnamic acids (HC), and flavonoids (F)) using HPLC.

Results showed that for the orange and spinach matrices, MF (0.22 μm for both residues) could be used to remove suspended solids and colloids. UF (30 kDa for both residues) could be used for clarification, with rejections of 0% for hydroxybenzoic acids, 67% for hydroxycinnamic acids, and 29% of flavonoids for the orange matrix, and and 36% for hydroxybenzoic acids, 32% for hydroxycinnamic acids, 40% for flavonoids for spinach extracts. NF (TFCS) could be used for pre-concentration; and RO membranes such as, BW30LE and XLE, for orange and spinach matrices, respectively, could be used for concentration of some components.

Concerning NF membranes, the TFCS membrane was selected for polyphenol pre-concentration for orange and spinach extracts, due to it full rejection of hydroxybenzoic acids, and higher rejection of hydroxycinnamic acids and flavonoids (100%, 89%, and 72%, respectively for orange extracts; and 100%, 100%, and 81%, respectively for spinach extracts).

RO membranes, BW30LE and XLE concentrate polyphenols with higher rejection percentages, of hydroxybenzoic acids 100%, hydroxycinnamic acids 94% and flavonoids 80% for orange extracts; and 100% of hydroxybenzoic acids, 97% of hydroxycinnamic acids and 100% of flavonoids for spinach extracts.

Therefore, integrated membrane sequential designs for the treatment of orange and spinach extracts were proposed to recover polyphenols, using the MF and UF

membranes to obtain clean extracts and to remove impurities from orange and spinach MSE extracts. NF membranes are be used for the pre-concentration of polyphenols from low-molecular-weight compounds. Additionally, RO membranes are used to concentrate polyphenols and can facilitate the removal of the solvent. Concentrated polyphenols extracts (as solution and/or dry powder) with RO can be used as food additives for the formulation of nutraceutical products, natural colorants in cosmetics, etc. This raises a need for future research and development, by implementing at higher technology readiness levels (TRLs) the production of streams rich in polyphenols and at the same time water for reuse purposes, in a circular economy framework.

8.3 Pharmaceutical application of polyphenols

A review about the antioxidant and antiviral activity that different polyphenols possesses and their application as alternative treatments against various viral diseases was presented. A detailed description of the mechanism of action of these antiviral agents was also made, with the main idea to understand how polyphenols work as antiviral agents against different viral diseases, such as quercetin which blocks the RNA synthesis of the HIV virus. Is worth mentioning that the review also described the application of polyphenols with antiviral activity recovered from secondary sources.

Therefore, it was identified that polyphenols have different mechanisms of action against viruses. Then, polyphenols could be used as a treatment or to prevent diseases caused by viruses.

CHAPTER 9

Conclusions

9. Conclusions

The following are the main conclusions drawn from the research developed in this Thesis.

Related to the extraction of polyphenols from orange and spinach residues by using conventional and innovative techniques

The comparison of the extraction yields indicated that UAE was a convenient extraction technique for spinach matrix while PLE provided higher performance for the orange matrix, in which ca. a 5-fold increase was obtained compared with UAE. However, considering simplicity and operational cost UAE was eventually recommended for industrial waste processing, especially for dealing with labile sample components.

MSE can be postulated as a cost-effective technique to recover phenolic compounds from agri-food residues including orange and spinach wastes, using water as a solvent. When comparing the MSE with the UAE, the yield of phenolic compounds was similar for the orange matrix, while for the spinach matrix the yield of phenolic compounds increased substantially.

The evaluated orange and spinach residues provided extracts with higher values of antioxidant activity as determined by FRAP assay compared to the DPPH and ABTS assays.

Related to the purification of polyphenols from orange and spinach residues by means of membrane technology

MF, UF, NF, and RO membranes can be integrated into a purification train (clarification, pre-concentration, and concentration) for polyphenols recovery. The 0.22 μm of pore size (MF) membrane was identified to remove suspended solids from both matrix extracts. The 30 kDa (UF) was selected as suitable membrane in clarification stage for the two extracts. NF membranes were selected for the pre-concentration stage; TFCS reported higher rejection of hydroxybenzoic acids, hydroxycinnamic acids and flavonoids for the spinach and orange extracts. Finally, XLE and BW30LE (RO) membranes were selected to concentrate polyphenols from spinach and orange matrices, respectively, due to their higher rejection percentages of hydroxycinnamic acids and flavonoids for the spinach and orange matrices.

Related to the antiviral application of polyphenols recovered from orange and spinach residues

Alternative treatments using polyphenols, which are interesting and promising molecules, could be applied to the pharmaceutical sector against diseases caused by viruses (e.g., HIV, SARS-CoV-2, MERS).

The bioactivities of polyphenols like antioxidant capacity and antiviral activity, as well as their action mechanisms, such as forming stable radicals, delaying and/or preventing the oxidative stress induced by cellular damage and diseases. It is worth mentioning that there is not a single mechanism of action of polyphenols against viruses. Indeed, the antiviral mechanism of these bioactive compounds could rely on the antioxidant activity, the inhibition of viral entry or viral reproduction, and inhibition of DNA replication, among others.

Due to the complex polyphenol structure, new extraction, purification, formulation, and processing technologies could help to improve the stability and bioavailability of antiviral polyphenols, as well as the administration protocols and the therapeutic effects as antiviral drugs. Thus, the use of plant extracts rich in polyphenols could be useful for health, due to their synergistic effects, such as antioxidant, antiviral, and anti-inflammatory properties that must be studied intensively.