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REVALORIZATION OF FOOD BY-PRODUCTS AS SOURCES OF BIOACTIVE COMPOUNDS

Memoria presentada por

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LIST OF CONTENTS

LIST OF CONTENTS

LIST OF ABREVIATIONS

ARS .	Alkylresorcinols
CI……	Chemical ionization
EI……	Electron ionization
ESI……	Electrospray ionization
FLD……..	Fluorescence detector
GC……...	Gas chromatography
GLC……	Gas liquid chromatography
GSC……	Gas–solid chromatography (GSC)
HAT……...	Hydrogen atom transfer
HPLC……..	High-performance liquid chromatography
IEC……..	Ion exchange chromatography
LDL-C……	Low-density lipoprotein cholesterol (LDL-C)
MAE……	Microwave-assisted extraction
NP-HPLC……	Normal phase chromatography
OLE……	Olive leaf extract
PEF…….	Pulsed electric field
PLE…….	Pressurized-liquid extraction
PLOT……..	Porous layer open tubular column
Q……	Quadrupole
RNS……	Reactive nitrogen species
ROS……...	Reactive oxygen species
RP-HPLC ……...	Reversed phase chromatography
SEC…….	Size exclusion chromatography
SET…….. .	Single-electron transfer
SFE…….	Supercritical-fluid extraction
TC……..	Cholesterol
TG……..	Triglycerides
TOF……	Time of flight
THF……..	Tetrahydrofuran

UAE…… Ultrasound-assisted extraction

UV-Vis……. Ultraviolet-visible

WCOT…… Wall coated open tubular column

LIST OF FIGURES

LIST OF TABLES

OBJECTIVES

Olive leaves can be found in large quantities as residues from olive oil industry, as well as by-products of pruning. Olive leaves are a rich source of bioactive compounds including phenolic compounds, which have shown certain beneficial properties such as antioxidant, antiinflammatory, anticancer, antimicrobial and antiviral activities. Specifically, there is evidence that certain phenolic compounds present in olive leaves modulate the activity of the AMPK pathway and the consequent inhibition of gerogenes such as mTOR, acting as metabolic reprogrammers. Therefore, this pathway is proposed as a therapeutic target in diseases associated with inflammation, such as obesity. Thus, bioactive compounds from olive leaves could be used to develop certain functional and/or nutraceutical foods, so their extraction can be an interesting way for the food industry to revalorize this by-product.

In addition, cereals and pseudocereals and their derivatives are also of great interest from a nutritional point of view because they represent an important source of energy in the diet. These foods are very rich in a wide variety of beneficial compounds for health such as fiber, vitamins, minerals, phytochemicals, etc. In fact, the consumption of whole grains is associated with a reduction in mortality and the risk of suffering chronic diseases such as obesity, cardiovascular diseases, type 2 diabetes, etc. Among these compounds, a series of minor compounds such as phenolic compounds with strong antioxidant activity are present. In cereals, phenolic compounds are found in their free and bound (linked to the cell walls) forms, both of which show strong physiological activities. Despite that, during the refining process of cereals, the outermost layers of the grain (bran) are removed, leading to a significant loss of these types of compounds. For this reason, in recent years different technologies have been developed to recover the richest fractions in phytochemicals.

Consequently, the main objective of this thesis is to search bioactive compounds of interest in food by-products extracts (olive leaves, cereal and pseudocereals by-products) that can be used to develop functional foods and/or nutraceuticals and to perform the subsequent qualitative and quantitative analysis of these bioactive

compounds using advanced chromatographic techniques that allow the development of fast, efficient and reliable methods for their determination.

This objective can be itemized into the following categories:

1. Selection of olive leaves, cereals, and pseudocereals by-products (buckwheat, wheat and barley) generated in industrial processes such as: olive oil production, the refining process of cereals and beer production, which can be used as sources of bioactive compounds, mainly phenolic compounds (including simple phenols, phenolic acids, secoiridoids, flavonoids, alkylresorcinols and proanthocyanidins).
2. Separation of different buckwheat and wheat flour fractions obtained through a dry fractionation process (sieving and air classification) for subsequent assessment of the distribution of phenolic compounds.
3. Use of pulsed electric field (PEF) as valuable pretreatment to improve phenolic compounds recovery in barley by-products (brewer's spent grain).
4. Use of efficient extraction techniques such as ultrasound assisted extraction (UAE), microwave assisted extraction (MAE) and pressurized liquid extraction (PLE) for obtaining olive leaves and cereals and pseudocereals extracts enriched in bioactive compounds..
5. Identification and quantification of phenolic compounds in the obtained extracts by using different separative analytical techniques such as high performance liquid chromatography (HPLC) and gas chromatography (GC) coupled to different detection systems: diode array detector (DAD), fluorimeter (FLD), mass spectrometry (MS) with analyzers as simple quadrupole (Q) and time of flight (TOF).

OBJETIVOS

Las hojas de olivo representan uno de los principales residuos procedentes de la industria del olivo, generándose tanto durante la obtención del aceite de oliva, así como durante el proceso de poda. Las hojas de olivo son una fuente rica de compuestos bioactivos, entre los cuales se encuentran los compuestos fenólicos, que han demostrado poseer propiedades beneficiosas para la salud como actividad antioxidante, antiinflamatoria, anticancerígena, antimicrobiana y antivírica, entre otras. En concreto, existen evidencias científicas que indican como determinados compuestos fenólicos presentes en las hojas de olivo son capaces de modular la actividad de la ruta metabólica de la AMPK, así como la consecuente inhibición de gerogenes como mTOR, actuando así como reprogramadores metabólicos. Debido a ello, esta vía se propone como diana terapéutica en enfermedades asociadas a la inflamación, como la obesidad. Por tanto, la hoja de olivo podría utilizarse para obtener extractos enriquecidos en compuestos fenólicos que puedan utilizarse para desarrollar nuevos alimentos funcionales y/o nutracéuticos. De esta forma, la obtención de extractos con alto valor añadido permitiría revalorizar este subproducto.

Asimismo, los cereales y pseudocereales y sus derivados son de gran interés desde el punto de vista nutricional porque representan una importante fuente de energía en la dieta. Estos alimentos son muy ricos en una amplia variedad de compuestos beneficiosos para la salud como fibra, vitaminas, minerales, fitoquímicos, etc. De hecho, el consumo de cereales integrales se asocia a una reducción de la mortalidad y del riesgo a padecer enfermedades crónicas como obesidad, enfermedades cardiovasculares, diabetes tipo 2, etc. Entre estos compuestos se encuentran presentes una serie de compuestos minoritarios como los compuestos fenólicos, con fuerte actividad antioxidante. Tanto en los cereales como en los pseudocereales, los compuestos fenólicos se encuentran en forma libre e hidrolizable (enlazados a las paredes celulares), y ambas han demostrado una importante actividad fisiológica. A pesar de ello, durante el proceso de refinado de los cereales, se eliminan las capas más externas del grano (salvado), lo que lleva a una pérdida significativa de este tipo de compuestos. Por ello, en los últimos años

se han desarrollado diferentes tecnologías con la finalidad de recuperar las fracciones más ricas en fitoquímicos.

En consecuencia, el objetivo principal de esta tesis es obtener extractos o fracciones ricos en compuestos bioactivos de interés a partir de subproductos alimentarios (hojas de olivo, subproductos de cereales y pseudocereales) que puedan ser utilizados para desarrollar alimentos funcionales y/o nutraceúticos. Así como, analizar cualitativa y cuantitativamente estos compuestos bioactivos utilizando técnicas separativas que permitan el desarrollo de métodos rápidos, eficientes y fiables para su determinación.

Este objetivo se puede desglosar en las siguientes categorías:

1. Selección de subproductos de hojas de olivo, cereales y pseudocereales (trigo sarraceno, trigo y cebada) generados en procesos industriales como: la producción de aceite de oliva, el proceso de refinado de cereales y la producción de cerveza, que puedan utilizarse como fuente de compuestos bioactivos como los compuestos fenólicos (comolos fenoles simples, ácidos fenólicos, secoiridoides, flavonoides, alquilresorcinoles y proantocianidinas).
2. Separación de las distintas fracciones de trigo sarraceno y harina de trigo mediante un proceso de fraccionamiento en seco (tamizado y clasificación al aire) para la posterior evaluación de la distribución de los compuestos fenólicos.
3. Empleo de la tecnología denominada campos eléctricos pulsados (CEP) como posible pretratamiento para la mejora de la extracción de los compuestos fenólicos en subproductos de cebada (bagazo).

4. Empleo de técnicas de extracción eficientes como la extracción asistida por ultrasonidos (EAU), la extracción asistida por microondas (EAM) y la extracción mediante líquidos presurizados (EPL) para la obtención de extractos de hoja de olivo, y subproductos de cereales y pseudocereales enriquecidos en compuestos fenólicos. Identificación y cuantificación de

compuestos fenólicos en los extractos obtenidos utilizando técnicas separativas como la cromatografía líquida de alta resolución (HPLC) y la cromatografía de gases (CG) acopladas a diferentes sistemas de detección: espectrofotometría ultravioleta-visible (UV-Vis), fluorimetría (FLD) y espectrometría de masas (MS) con analizadores de cuadrupolo (Q) y tiempo de vuelo (TOF).

SUMMARY

The current report encompasses all the results found during the work carried out for the PhD Thesis entitled: **"REVALORIZATION OF FOOD BY-PRODUCTS AS SOURCES OF BIOACTIVE COMPOUNDS".**

The present doctoral dissertation comprises the extraction, identification and quantification of phenolic compounds from olive leaves, cereals and pseudocereals fractions and/or by-products to revalorize these matrices as possible bioactive compounds sources to be used as bioactive ingredients against certain diseases. It is divided into two main parts; the first one is the INTRODUCTION, which reports a brief description of the classification of phenolic compounds and an overview of the phenolic composition of olive leaves, cereals and pseudocereals grains (brewer's spent grains, buckwheat and wheat), their beneficial properties, the sample pretreatment, the subsequent treatment and green technologies used for the extraction and the characterization by different analytical approaches. Firstly, phenolic compounds are described and classified according to their chemical structure. After that, the fractionation technologies used in cereal and pseudocereal grains in order to enrich cereal flour fractions are included. In addition, the four green extraction technologies (e.g. microwave assisted extraction, pressurized liquid extraction, ultrasound assisted extraction and pulsed electric field) used for the phenolic recovery in this thesis are reported.,. Finally, chromatographic analytical techniques used for separation, such as high-performance liquid chromatography (HPLC) and gas chromatography (GC), and the coupling to detection systems like UV-Vis and mass spectrometry (MS) for the identification and quantification of phenolic compounds are described.

The second part includes the "EXPERIMENTAL PART. RESULTS AND DISCUSSION" carried out in the current PhD thesis and it is subdivided into two sections. ***Section I*** provides the optimization of different extraction techniques to obtain the highest phenolic recovery in olive leaves. It is organized in three chapters:

Chapter 1 deals with the optimization of a pressurized liquid extraction procedure for the extraction of phenolic compounds from dried olive leaves. An experimental Box-Behnken design coupled to response-surface methodology was performed to optimize extraction parameters of temperature, percentage solvent (ethanol and water), and extraction time in order to maximize the recovery of simple phenols, secoiridoids, flavonoids, elenolic acids, total compounds and extraction yield. Phenolic compounds were determined by high-performance liquid chromatography coupled to electrospray ionization-time of flight mass spectrometry (HPLC-ESI-TOF-MS).

Chapter 2 focuses on an experimental Box-Behnken response surface design that was used to optimize the microwave assisted extraction parameters such as, temperature, solvent composition (ethanol and water) and extraction time to obtain extracts with high concentration in total phenolic compounds and extracts enriched in phenolic compounds with demonstrated capacity to activate AMP-activated protein kinase (AMPK) from olive leaves. The determination of phenolic compounds was carried out by high-performance liquid chromatography coupled to electrospray ionization-time of flight mass spectrometry (HPLC-ESI-TOF-MS).

Chapter 3 is based on ultrasound-assisted extraction via sonotrode to evaluate the recovery of phenolic compounds from olive leaves. For that purpose, a Box-Behnken design based on response surface methodology was used to optimize the effects of parameters such as solvent composition (ethanol and water), extraction time and amplitude in the total phenolic content. Qualitative and quantitative analyses of phenolic compounds were performed using HPLC coupled to DAD and mass spectrometer detectors. The optimal conditions selected for the sonotrode were compared with the results obtained by a conventional ultrasonic bath.

The ***Section II*** deals with the use of fractionation technologies (milling, sieving and air-classification) applied to obtain bioactive enriched cereal and pseudocereal flours fractions and the optimization of different treatments and

extraction techniques in cereal by-products. This section is structured in six chapters:

Chapter 4 studies the influence of the application of pulsed electric field (PEF) as a pretreatment in brewer's spent grains in order to increase the phenolic compounds recovery. For that reason, an experimental design was performed to optimize parameters that affect to the effectiveness of PEF including electric field strength, frequency and total time of treatment. Identification and quantification of phenolic compounds in PEF brewer's spent grain extracts was performed by HPLC-MS. In addition, it was carried out a comparison on the content of free and bound phenolic compounds in PEF extracts obtained at optimum conditions with those obtained without PEF treatment.

Chapter 5 evaluates the recovery of proanthocyanidins from brewer's spent grains by establishing a sonotrode ultrasonic-assisted extraction method. For that purpose, response surface methodology was performed to evaluate extraction parameters of solvent composition, time of extraction, and ultrasound power with an experimental Box-Behnken design. The characterization of proanthocyanidins was performed by using high performance-liquid chromatography coupled to fluorescence and mass spectrometry detectors (HPLC-FLD-MS).

Chapter 6 concerns the evaluation of the phenolic composition in dehulled buckwheat milling fractions (dehulled whole buckwheat flour, light flour, bran flour, and middling flour). Buckwheat is a rich source of phenolic compounds with high antioxidant power that have shown to possess beneficial effects to reduce different diseases. Phenolic compounds are present in the free and in the bound form (linked to the cell walls) that are mainly concentrated in the outer grain layers (hull and bran). Despite that, hull is removed before the milling of buckwheat to obtain flours. The determination of free and bound phenolic compounds in these buckwheat milling fractions was carried out by high-performance liquid chromatography coupled to mass spectrometry (HPLC-Q-MS).

Chapter 7 concerns the use of a physical separation as sieving applied to dehulled whole buckwheat flour to obtain flour fractions with a particle size of 215 µm, 160 µm, 85 µm, and 45 µm with the aim to obtain enriched flour fractions in phenolic compounds, especially in rutin. For that purpose, the determination of free and bound phenolic compounds was carried out by HPLC-MS. In addition, the content of protein, ash, and total starch content was determined in these sieved buckwheat flour fractions.

Chapter 8 focuses on air fractionation of dehulled buckwheat flour as a technological process to obtain flours enriched in phenolic compounds. Air-classification provided the following flours: 30% and 20% of coarse fraction and 70% and 80% of fine fraction. Free and bound phenolic compounds were identified and quantified by high-performance liquid chromatography coupled to electrospray ionization-time of flight mass spectrometry (HPLC-ESI-TOF-MS). In addition, the antioxidant capacity by ferric reducing antioxidant power (FRAP) and 2,2-difenil-1-picrylhydrazyl (DPPH) essays were carried out to compare the antioxidant activities of different buckwheat flour fractions.

Chapter 9 focuses on the production of two different wheat bran fractions enriched in aleurone (AF1, 55-70% aleurone and AF2, 75-90% aleurone) obtained by a patented dry fractionation technology based on air classification. Free and bound phenolic compounds were determined by HPLC-DAD-ESI-TOF-MS. In addition, alkylresorcinols (ARs), which are amphiphilic phenolic lipids were determined in bran fractions by GC-MS.

RESUMEN

La presente memoria recoge todos los resultados hallados durante el trabajo realizado en la Tesis Doctoral titulada: **"REVALORIZACIÓN DE SUBPRODUCTOS ALIMENTARIOS COMO FUENTES DE COMPUESTOS BIOACTIVOS".**

La presente tesis doctoral supone un nuevo aporte científico en el estudio de los compuestos fenólicos de las hojas de olivo y fracciones y/o subproductos de cereales y pseudocereales con el fin de revalorizar estas matrices como posibles fuentes de compuestos bioactivos para su posterior uso como ingredientes funcionales. La memoria se estructura en tres secciones principales: (1) Introducción, (2) Parte experimental, resultados y discusión y (3) Conclusiones.

La INTRODUCCIÓN recoge una breve descripción de la clasificación de los compuestos fenólicos y una visión general de la composición fenólica de las hojas de olivo y los granos de cereales y pseudocereales (trigo sarraceno, trigo y bagazo de cerveza), sus propiedades beneficiosas, el pretratamiento de muestra, su posterior extracción mediante tecnologías verdes y la caracterización mediante distintas plataformas analíticas. En primer lugar, se describen y se clasifican los compuestos fenólicos según su estructura química. Posteriormente, se incluyeron las tecnologías de fraccionamiento físico utilizadas en los granos de cereales y pseudocereales para obtener fracciones de harina enriquecidas en compuestos fenólicos. A continuación, se recogen las cuatro tecnologías de extracción verde utilizadas en esta tesis para la recuperación de los compuestos fenólicos (extracción asistida por microondas, extracción por líquidos presurizados, extracción asistida por ultrasonidos y campo eléctrico pulsado). Finalmente, se exponen las técnicas analíticas separativas empleadas para la determinación de los compuestos fenólicos: cromatografía líquida de alta resolución (HPLC) y cromatografía de gases (GC), y el acoplamiento a sistemas de detección como UV-Vis y espectrometría de masas (MS).

La segunda parte incluye la "PARTE EXPERIMENTAL. RESULTADOS Y DISCUSIÓN" y se subdivide en dos secciones. La sección I aborda la optimización de diferentes técnicas de extracción con el fin de obtener los mayores rendimientos de extracción posibles. Esta consta de tres capítulos:

El **capítulo 1** presenta la optimización de un procedimiento de extracción mediante líquidos presurizados para la recuperación de los compuestos fenólicos de hojas de olivo secas. Se realizó un diseño experimental Box-Behnken para optimizar los diferentes parámetros de extracción: temperatura, porcentaje de disolvente (etanol/agua) y tiempo de extracción, con el fin de maximizar la recuperación de fenoles simples, secoiridoides, flavonoides, ácido elenólico, compuestos fenólicos totales y rendimiento de extracción. Los compuestos fenólicos se determinaron mediante cromatografía líquida de alta resolución acoplada a espectrometría de masas de tiempo de vuelo con ionización por electrospray (HPLC-DAD-ESI-TOF-MS).

El **capítulo 2** se centra en la determinación de los compuestos fenólicos de hojas de olivo previamente extraídos mediante extracción por microondas. Para ello se llevó a cabo un diseño experimental de superficie de respuesta de Box-Behnken con el fin de optimizar los parámetros de extracción: temperatura, composición del disolvente (etanol/agua) y tiempo de extracción, para obtener extractos con alta concentración en compuestos fenólicos totales y extractos enriquecidos en compuestos fenólicos, con capacidad para activar la proteína quinasa activada por AMP (AMPK), previamente demostrada. La determinación de los compuestos fenólicos se realizó mediante cromatografía líquida de alta resolución acoplada a espectrometría de masas con analizador de tiempo de vuelo e ionización por electrospray (HPLC-ESI-TOF-MS).

El **capítulo 3** se basa en la evaluación del poder de extracción de los compuestos fenólicos de la hoja de olivo mediante extracción asistida por ultrasonidos con sonótrodo. Para ello se utilizó un diseño Box-Behnken para optimizar los efectos

de parámetros como la composición del disolvente (etanol/agua), el tiempo de extracción y la amplitud, en el contenido fenólico total. Se realizaron análisis cualitativos y cuantitativos de compuestos fenólicos usando HPLC acoplada a detectores de espectrómetría de masas y diodos en fila (DAD). La concentración de compuestos fenólicos alcanzada con las condiciones óptimas obtenidas con el sonótrodo se comparó con el resultado obtenido mediante la extracción con un baño de ultrasonidos convencional.

La Sección II recoge el uso de tecnologías de fraccionamiento (molienda, tamizado y clasificación por aire) aplicadas para la obtención de fracciones de harinas de cereales y pseudocereales enriquecidas en compuestos bioactivos y la optimización de diferentes tratamientos y técnicas de extracción en subproductos de cereales (bagazo). Esta sección está estructurada en seis capítulos:

El **capítulo 4** estudia la influencia del pretratamiento con campos eléctricos pulsados (PEF) en el bagazo de cerveza en la posterior recuperación de los compuestos fenólicos. Por esta razón, se llevó a cabo la optimización de los parámetros que afectan a la efectividad del PEF mediante un diseño experimental, entre ellos: la intensidad del campo eléctrico, la frecuencia y el tiempo total de tratamiento. La identificación y cuantificación de los compuestos fenólicos de los extractos de bagazo de cerveza con PEF se llevó a cabo mediante HPLC-MS. Además, se realizó una comparación del contenido de compuestos fenólicos libres e hidrolizables en extractos de PEF obtenidos aplicando las condiciones óptimas con los obtenidos sin tratamiento con PEF.

El **capítulo 5** evalúa la recuperación de las proantocianidinas del bagazo de cerveza mediante extraídas mediante extracción asistida por ultrasonidos con sonótrodo. Para ello, se aplicó una metodología de superficie de respuesta para la optimización de los parámetros de extracción: composición del disolvente, tiempo de extracción y potencia de ultrasonido, con un diseño experimental Box-Behnken. La caracterización de las proantocianidinas se realizó mediante cromatografía

líquida de alta resolución en fase normal acoplada a detectores de fluorescencia y espectrometría de masas (HPLC-FLD-MS).

El **capítulo 6** se ocupa de la evaluación de la composición fenólica en los productos de la molienda de trigo sarraceno descascarillado (harina de trigo sarraceno integral descascarillado, harina refinada, harina de salvado y harinilla). El trigo sarraceno es una fuente de compuestos fenólicos con alta capacidad antioxidante y efectos beneficiosos en la prevención de ciertas enfermedades. Estos compuestos fenólicos se presentan tanto en su forma libre como hidrolizable y están principalmente concentrados en las capas más externas del grano (cáscara y salvado). La determinación de compuestos fenólicos libres e hidrolizables en estas fracciones de molienda de trigo sarraceno se llevó a cabo mediante cromatografía líquida de alta resolución acoplada a espectrometría de masas (HPLC-Q-MS).

El **capítulo 7** describe el uso del tamizado de harina de trigo sarraceno integral descascarillada para la obtención de harinas con distintos tamaño de partícula: 215 µm, 160 µm, 85 µm y 45 µm. El objetivo de este pretratamiento es conseguir fracciones de harina enriquecidas en compuestos fenólicos, especialmente en rutina, que presenta numerosas propiedades como actividades antiinflamatorias, anticancerígenas y antioxidantes. La determinación de los compuestos fenólicos libres e hidrolizables se llevó a cabo mediante HPLC-MS. Además, en estas mismas fracciones se determinó el contenido de proteínas, cenizas y almidón total.

El **capítulo 8** se centra en la clasificación por aire del trigo sarraceno descascarillado como proceso tecnológico para la obtención de harinas enriquecidas en compuestos fenólicos. En este estudio, a partir de la harina integral, y dependiendo de la forma de llevar a cabo la clasificación por aire, se obtuvieron dos grupos de harinas: 30% de fracción gruesa y 70% de fracción fina y 20% de fracción gruesa y 80% de fracción fina. Los compuestos fenólicos libres e hidrolizables se identificaron y cuantificaron mediante cromatografía líquida de

alta resolución acoplada a espectrometría de masas con analizador a tiempo de vuelo e ionización poe electrospray (HPLC-ESI-TOF-MS). Además, se realizaron ensayos de capacidad antioxidante mediante la evaluación del poder de reducción antioxidante del ión férrico (FRAP) y el test radicalario del DPPH (2,2-difenil-1-picrilhidrazilo) para comparar las actividades antioxidantes de las diferentes fracciones de harina de trigo sarraceno.

El **capítulo 9** se centra en la obtención de dos fracciones diferentes de salvado de trigo enriquecidas en aleurona (AF1, 55-70% de aleurona y AF2, 75-90% de aleurona) mediante un fraccionamiento en seco patentado basado en una clasificación por aire. Los compuestos fenólicos libres e hidrolizables se determinaron mediante HPLC-DAD-ESI-TOF-MS. Además, en las mismas fracciones, se determinaron los alquilresorcinoles (AR) (lípidos fenólicos anfifílicos) mediante GC-MS.

INTRODUCTION

1. PHENOLIC COMPOUNDS IN VEGETABLES

In the last years, the consumption of vegetables has attracted the interest of consumers. This fact has been mainly due to the high number of epidemiological studies that have showed a significant association between the intake of these natural foods and the reduction of several diseases induced by oxidative stress, and prevention of certain cardiovascular diseases, neurodegenerative diseases and cancers[1]. This protection is attributed in part to their content in phenolic compounds that are the main phytochemicals with antioxidant properties found in plants. The health effects depend on the kind of phenolic compounds, the quantity consumed, and their bioavailability[2].

Phenolic compounds are the most abundant secondary metabolites distributed in all vascular plants, they are a class of chemical compounds consisting of one or more hydroxyl groups (OH) linked directly to at least one aromatic ring. Quideau et al. 2011 proposed that the term "plant phenolics" should be used to define plant secondary metabolites derived exclusively from the shikimate- and/or the acetate pathway(s), featuring more than one phenolic ring and being devoid of any nitrogen-based functional group in their most basic structural expression[3]. In addition, the products of the acetate pathway can react with the products of the shikimate pathway given rise to chalcones, from which flavonoids are derived. **Figure 1** shows the diagrammatic representation of the metabolic routes relative to phenolic compounds generated by plant metabolism[4].

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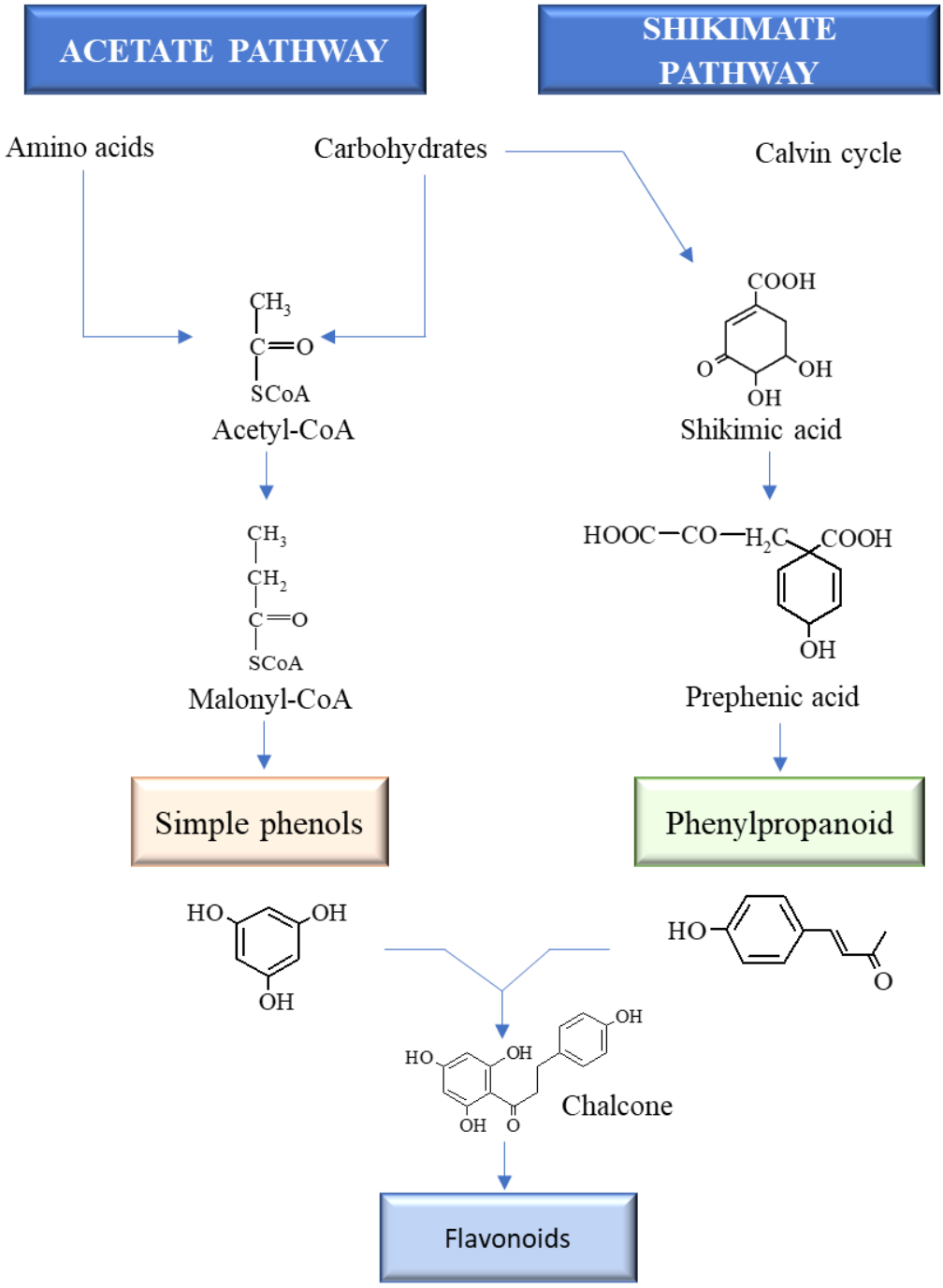

Figure 1. Scheme of the biosynthetic pathways of phenolic compounds. Adapted from[4].

Phenolic compounds are found mainly in plant-based foods, such as vegetables, fruits, cereals, whole grains, coffee, tea, legumes, cocoa, and wine. In fact, plant food processing industries generate lots of phenolics-rich by-products. Some of

these by-products have been the subject of investigations and have showed to be sources of phenolic compounds[5].

1.1. Classification of phenolic compounds

Phenolic compounds are one of the most numerous and extensively distributed groups of natural products in the vegetable kingdom. Currently, there are more than 8000 known phenolic structures and, among them, about 4000 flavonoids have been identified[6,7]. Fruits, vegetables, whole grains and other types of foods and beverages such as tea, chocolate and wine are rich sources of phenolic compounds. The diversity and broad distribution of phenolic compounds in plants have given rise to different forms of categorizing these naturally occurring compounds[7]. Phenolic compounds have been classified by their source of origin, biological function, and chemical structure. Figure 2 shows the most general classification phenolic compounds taking into account the main carbon skeleton that constitutes the fundamental axis for structural differentiation. Therefore, phenolic compounds can be classified in simple phenols, phenolic acids, naphthoquinones, coumarins, benzophenones and xanthones, stilbenes, lignans, flavonoids, alkylresorcynols, lignins and tannins[4] . In addition, most of phenolic compounds in plants exist as glycosides with different sugar units and acylated sugars at different positions of the phenolic skeletons[8].

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[5] Balasundram, N.; Sundram, K.; Samman, S. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem.* **2006**, *99*, 191–203.

[6] Cheynier, V. Polyphenols in foods are more complex than often thought. *Am J Clin Nutr* **2005**, *81*, 223–229.

[7] Tsao, R. Chemistry and biochemistry of dietary polyphenols. *Nutrients* **2010**, *2*, 1231–1246.

[8] Ramawat, K.G.; Mérillon, J.M. Phenolic compounds: Introduction. In *Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes*; 2013; pp. 1544–1573 ISBN 9783642221446.

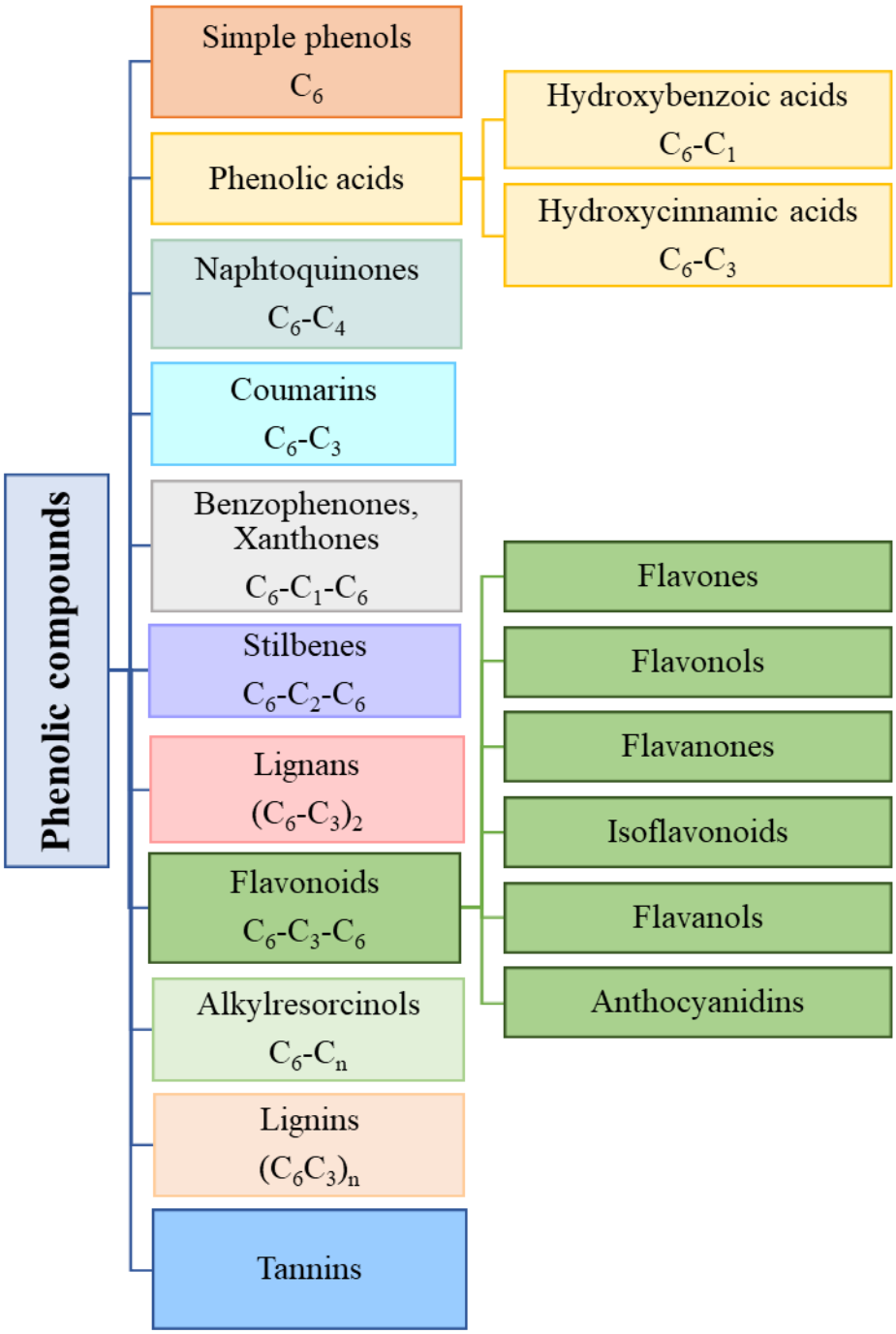


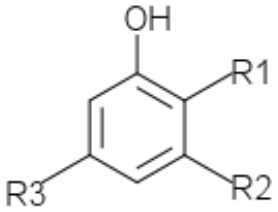
Figure 2. Classification of the main families of phenolic compounds in plants[9,10].

[9] Panche, A.N.; Diwan, A.D.; Chandra, S.R. Flavonoids: An overview. *J. Nutr. Sci.* **2016**, *5*, 1–15.

[10] Brglez Mojzer, E.; Knez Hrnčič, M.; Škerget, M.; Knez, Ž.; Bren, U. Polyphenols: Extraction Methods, Antioxidative Action, Bioavailability and Anticarcinogenic

1.1.1. Simple phenols

Simple phenols are the simplest phenolic compounds, which contain at least one hydroxyl group (OH) attached directly to an aromatic ring (C_6) (**Figure 3**). Phenol, catechol, resorcinol, and phloroglucinol are examples of simple phenols. These phenols are themselves uncommon plant constituents; however, phloroglucinol, resorcinol and catechol can be found in combination with cinnamic acids to form plant flavonoids[11].



Hydroxybenzoic acids	Substitution
Phenol	$R_1 = R_2 = R_3 = H$
Catechol	$R_1 = OH, R_2 = R_3 = H$
Resorcinol	$R_1 = R_3 = H, R_2 = OH$
Phloroglucinol	$R_1 = H, R_2 = R_3 = OH$

Figure 3. Chemical structure of the main simple phenols

1.1.2. Phenolic acids

Effects. *Molecules* **2016**, *21*, 1–38.

[11] Kougan, G.B.; Tabopda, T.; Kuete, V.; Verpoorte, R. *Simple Phenols, phenolic Acids, and related esters from the medicinal plants of Africa*; Elsevier Inc., 2013; ISBN 9780124059276.

Phenolic acids possess a carboxyl group linked to the benzene ring and they have been widely studied recently because their potential protective role[1]. Phenolic acids are produced in plants via shikimic acid through the phenylpropanoid pathway, as by-products of the monolignol pathway and as breakdown products of lignin and cell-wall polymers in vascular plant[12]. Additionally, some phenolic acids are of microbial origin. Phenolic acids can be divided into two categories: benzoic acid derivatives such as hydroxybenzoic acids C_6-C_1 (phenylpropanoid type) and cinnamic acid derivatives such as hydroxycinnamic acids C_6-C_3 (phenylmethyl type) based on the constitutive carbon frameworks[12].

Hydroxybenzoic acids have a structure C_6–C_1 type derived from benzoic acid. p-Hydroxybenzoic, vanillic, syringic, protocatechuic and gallic acids are the main phenolic acids in this family (**Figure 4**)[1,13]. The first three are constituents of lignin, from which they are released by alkaline hydrolysis. Gallic acid is one of the most common hydroxybenzoic acids which participate in the formation of hydrolyzable gallotannins[13]. Gallic acid is mainly found in strawberries, pineapples, bananas, lemons, red and white wines, gallnuts, sumac, witch hazel, tea, oak bark and apple peels[10,14]. Gallic acid possess a wide range of biological properties such as antioxidative, antiinflammatory, antibacterial, antiviral, antimelanogenic, antimutagenic and anticancer activities[10,15]. Moreover, its dimeric condensation product (hexahydrodiphenic acid) and the related dilactone (ellagic acid) are also another prevalent hydroxybenzoic acid in plants[13]. Ellagic acid is found mainly in the glucoside form or as a part of hydrolyzable tannins

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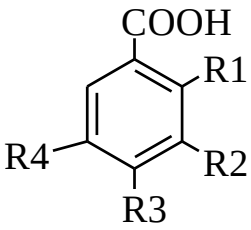
[12] Mandal, S.M.; Chakraborty, D.; Dey, S.; Mandal, S.M.; Chakraborty, D.; Dey, S. Phenolic acids act as signaling molecules in plant- microbe symbioses. *Plant Signal. Behav.* **2010**, *5*, 359–368.

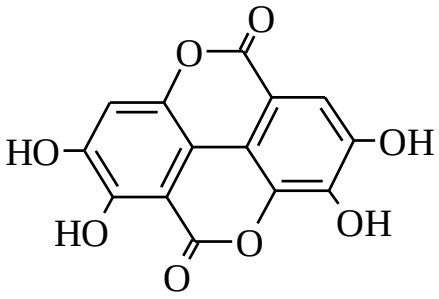
[13] Murkovic, M. Phenolic Compounds. In *Encyclopedia of Food Sciences and Nutrition (Second Edition)*; Caballero, B., Ed.; Academic Press, 2003; pp. 4507–4514 ISBN 9780122270550.

[14] Lall, R.K.; Syed, D.N.; Adhami, V.M.; Khan, M.I.; Mukhtar, H. Dietary polyphenols in prevention and treatment of prostate cancer. *Int. J. Mol. Sci.* **2015**, *16*, 3350–3376.

[15] Santos, I.S.; Ponte, B.M.; Boonme, P.; Silva, A.M.; Souto, E.B. Nanoencapsulation of polyphenols for protective effect against colon-rectal cancer. *Biotechnol. Adv.* **2013**, *31*, 514–523.

(glucose esters) called ellagitannins[15]. This compound is a dimeric derivative of the gallic acid that is present in blackberries, raspberries, strawberries, cranberries, pomegranate, walnuts and pecans[14]. Ellagic acid possesses anticarcinogenic, antioxidant, antiinflammatory, antibacterial, antiatherosclerosis, antihyperglycemic, antihypertensive, anti-fibrosis and cardioprotective effects[15].





Ellagic acid

Hydroxybenzoic acids	Substitution
p-hydroxybenzoic acid	$R_1 = R_2 = R_4 = H$; $R_3 = OH$
Vanillic acid	$R_1 = R_4 = H$; $R_2 = OCH_3$; $R_3 = OH$
Syringic acid	$R_1 = H$; $R_2 = R_4 = OCH_3$; $R_3 = OH$
Gallic acid	$R_1 = H$; $R_2 = R_3 = R_4 = OH$
Protocatechuic acid	$R_1 = R_4 = H$; $R_2 = R_3 = OH$

Figure 4. Chemical structure of the main hydroxybenzoic acids in plants

Hydroxycinnamic acids (C_6-C_3 derivatives), also known as phenylpropanoids, are more common than hydroxybenzoic acids. The four basic structures that can be found in their free natural state are coumaric, caffeic, ferulic, and sinapic acids (**Figure 4**)[4]. These phenolic acids are present in some vegetables like spinach, broccoli and kale; in berry fruits; apples; some beverages like coffee, tea, citrus juices, wine; in cereal bran and in olive oil[10]. These phenolic acids are usually present in plants in the combined forms as glycosylated rather than in their free form. Caffeic acid is one of the most common hydroxycinnamic acids present in many fruits and vegetables. It is usually found esterified with quinic acid as chlorogenic acid, which is the major phenolic compound in coffee (70-350 mg of chlorogenic acid derivatives)[16]. Ferulic acid is another common phenolic acid present in high concentrations in cereals and it is often esterified to hemicelluloses of the cell walls[16].

Hydroxycinnamic acids are distributed throughout all parts of the fruits, but they are found in the highest levels in the outer parts of ripe fruits. Fruits such as blueberries, kiwis, plums, cherries, and apples contain hydroxycinnamic acid derivatives in a range from 0.5 to 2 g/kg of fresh weight. Rice, oat, and wheat flours also have a high content of phenolic acids (0.8–2 g/kg of dry weight)[1].

Caffeic acid, ferulic acid, chlorogenic acid and curcumin possess effects anticancerogenic, antitumoral and antimetastatic properties[14,15]. Caffeic acid has also demonstrated *in vitro* antioxidant and antibacterial activities and possesses the capability to contribute to the prevention of atherosclerosis and other cardiovascular diseases[17].

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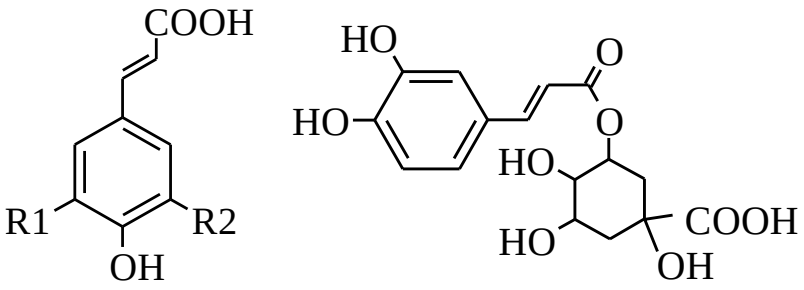
[16] Scalbert, A.; Manach, C.; Morand, C.; Em, C.R. Dietary polyphenols and the prevention of diseases. *Crit. Rev. FoodScience andNutrition* **2005**, *45*, 287–306.

[17] Magnani, C.; Isaac, V.L.B.; Correa, M.A.; Salgado, H.R.N. Caffeic acid: A review of its potential use in medications and cosmetics. *Anal. Methods* **2014**, *6*, 3203–3210.



Chlorogenic acid

Hydroxycinnamic acids	Substitution
p-coumaric acid	$R_1 = R_2 = H$
Caffeic acid	$R_1 = OH$; $R_2 = H$
Ferulic acid	$R_1 = OCH_3$; $R_2 = H$
Sinapic acid	$R_1 = R_2 = OCH_3$

Figure 5. Chemical structure of the main hydroxycinnamic acids in plants

1.1.3. Naphtoquinones

Quinones are derived from aromatic compounds by the conversion of an even number of –CH= groups into –C(=O)– groups with any necessary rearrangement of double bonds[1]. Quinones are found in bacteria, fungi, lichens, gymnosperms and angiosperms[18]. Their common basic structural pattern consists of an ortho or a para substituted dione conjugated either to an aromatic nucleus (benzoquinones) or to a condensed polycyclic aromatic system, such as naphtoquinones, anthraquinones and anthracyclinones (**Figure 6**)[19].

Naphthoquinones are structurally related to naphthalene and are characterized by their two carbonyl groups in the 1,4 position, which are named 1,4-naphthoquinones. Carbonyl groups may also be present in the 1,2 position but with

[18] Saibu, M.; Sagar, S.; Green, I.; Ameer, F.; Meyer, M. Evaluating the cytotoxic effects of novel quinone compounds. *Anticancer Res.* **2014**, *34*, 4077–4086.

[19] El-najjar, N.; Gali-muhtasib, H.; Ketola, R.A.; Vuorela, P.; Urtti, A.; Vuorela, H. The chemical and biological activities of quinones : Overview and implications in analytical detection. *Phytochem. Rev.* **2011**, *10*, 353–370.

minor incidence[20]. Quinones have antioxidant activity and improve general health conditions. Concretely, hydroxy-1,4-naphthoquinones and their derivatives have shown to possess beneficial activities including anti-fungal, anti-protozoal, anti-bacterial, and anti-cancer activity[18]. Nevertheless, quinones have also toxicological effects by their presence as photoproducts from air pollutants[19].

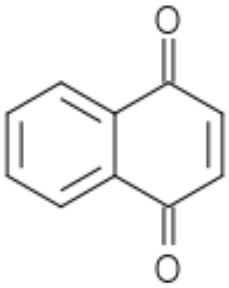


Figure 6. Chemical structure of 1,4-naphtoquinone.

1.1.4. Coumarins

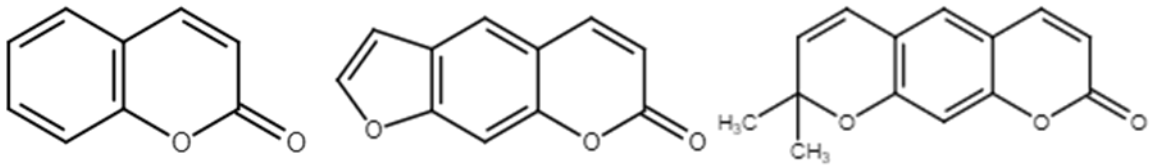
Coumarins are a very numerous class of C_6-C_3 derivatives belonging to the benzo-α-pyrone group, which consist of benzene ring linked to a pyrone ring[21]. Most of coumarins are isolated from chlorophyll containing plant materials, found in nature in combination with sugars, as glycosides[22]. Coumarins are classified in simple coumarins, furano-coumarins and pyrano-coumarins. Furanocoumarins are furan-derivatives of coumarin that contain a five-membered furan ring with the benzene ring of a coumarin, and they are classified into linear (psoralen) or angular (angelicin) types based on the skeleton structure. Pyrano coumarins are composed

[20] Lamoureux, G.; Perez, A.L.; Araya, M.; Agüero, C. Reactivity and structure of derivatives of 2-hydroxy-1, 4-naphthoquinone (lawsone). *J. Phys. Org. Chem.* **2008**, *21*, 1022–1028.

[21] Rohini K, S.P. Therapeutic role of coumarins and coumarin-related compounds. *J. Biofertilizers Biopestic.* **2014**, *05*, 10–12.

[22] Gopi, C.; Dhanaraju, MD. Synthesis, characterization and anti-microbial action of novel azo dye derivied from 4-Methyl 7- OH 8 Nitro coumarin. *J. Pharm. Res.* **2011**, *4*.

by six-membered pyran ring adhered to the benzene ring, divided into linear (xanthyletin) or angular (seselin) types[22].



Simple coumarins Furanocoumarins Pyranocoumarins

Figure 7. Structure of the coumarins

Coumarins such as esculetin (6,7-dihydroxycoumarin) and umbelliferone (3-hydroxycoumarin) are found in all the constitutive parts of the plant whose highest content is found in fruits, followed by the roots, stems, and leaves[1]. They possess beneficial properties including free radical scavenging activity, anticancer, antiinflammatory, antimicrobial, anticoagulant, anticonvulsant, antidepressant and antiHIV activities[23].

1.1.5. Benzophenones

Natural benzophenones have benzene-carbonyl-benzene skeleton (**Figure 8**). The A-ring, derived from the shikimic acid pathway, is a benzene ring that can have 0, 1, or 2 substituents. The B-ring, derived from the acetate-malonate pathway, undergoes prenylation and cyclization producing a wide range of structurally unique compounds with bi-, tri-, and/or tetra-cyclic ring systems[24]. Some studies have examined benzophenones in conjunction with other natural products provided by concretely plant genus. Specifically, polyprenylated benzophenones

[23] Srikrishna, D.; Godugu, C.; Dubey, P.K. A Review on Pharmacological Properties of Coumarins. *Mini-Reviews Med. Chem.* **2016**, *16*.

[24] Wu, S.B.; Long, C.; Kennelly, E.J. Structural diversity and bioactivities of natural benzophenones. *Nat. Prod. Rep.* **2014**, *31*, 1158–1174.

(PPBS), have been found in a large amount of plants and certain fungi. Some of these compounds such as pestalone (2-(3,5-dichloro-2-hydroxy-6-methoxy-4-methylbenzoyl)-4,6-dihydroxy-3-(3-methylbut-2-enyl)benzaldehyde) and moronone ((6Z)-2,2-bis[(2E)-3,7-dimethylocta-2,6-dienyl]-5-hydroxy-6-[hydroxy(phenyl)methylidene]cyclohex-4-ene-1,3-dione), which have unusual rearranged skeletons with strong antibacterial or anticancer effects[24,25].

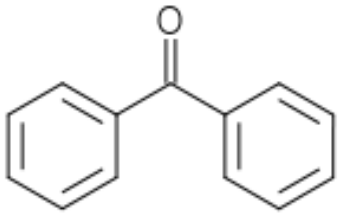


Figure 8. Basic structure of benzophenones

1.1.6. Xathones

Xanthones are present in a few higher plants, fungi, and lichens. The xanthone skeleton is a planar, conjugated ring system composed by 1-4 carbons (aromatic ring A) and carbons 5-8 (aromatic ring B), fused through a carbonyl group and an oxygen atom (**Figure 9**). 9H-xanthen-9-one is the simplest member of xantones, which is a symmetrical compound with a dibenzo-γ-pyrone skeleton[26]. These compounds possess diverse beneficial properties due to their oxygenation nature and diversity of functional groups[27]. Xanthones such as dihydroxy-3,5-dimethoxyxanthone (swerchirin) have shown antibacterial, antiviral, antioxidative, antiinflammatory, antiproliferative, antihypertensive properties, among others[26].

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[25] Wang, Z.Q.; Li, X.Y.; Hu, D.B.; Long, C.L. Cytotoxic garcimultiflorones K–Q, lavandulyl benzophenones from Garcinia multiflora branches. *Phytochemistry* **2018**, *152*, 82–90.

[26] Mazimba, O.; Nana, F.; Kuete, V.; Singh, G.S. *Xanthones and Anthranoids from the Medicinal Plants of Africa*; Elsevier Inc., 2013; ISBN 9780124059276.

[27] Masters, K.S.; Bräse, S. Xanthones from fungi, lichens, and bacteria: The natural products and their synthesis. *Chem. Rev.* **2012**, *112*, 3717–3776.

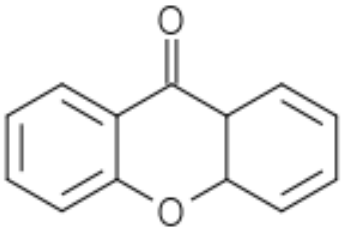


Figure 9. Structure of xanthone

1.1.7. Stilbenes

The stilbene structure consists of C_6–C_2–C_6 backbone, which contains two aromatic rings linked by an ethylene bridge[28]. Stilbenes are a small family of phenylpropanoids provided in a number of unrelated plant species, including dicotyledon angiosperms such as grapevine (*Vitis vinifera*), peanut (*Arachis hypogaea*), and Japanese knotweed (*Fallopia japonica*, formerly *Polygonum cuspidatum*), monocotyledons such as sorghum (*Sorghum bicolor*), and gymnosperms such as various *Pinus* and *Picea* species[28]. The most known is resveratrol (3,5,4'-trihydroxystilbene), which is found either as the aglycone or the 3-glucoside (piceid). Resveratrol has shown to slow the progression of a wide range of illnesses such as cancer and cardiovascular disease and increase the life spans of various organisms[29].

[28] Parage, C.; Tavares, R.; Réty, S.; Baltenweck-Guyot, R.; Poutaraud, A.; Renault, L.; Heintz, D.; Lugan, R.; Marais, G.A.B.; Aubourg, S.; et al. Structural, functional, and evolutionary analysis of the unusually large stilbene synthase gene family in grapevine. *Plant Physiol.* **2012**, *160*, 1407–1419.

[29] Baur, J.A.; Sinclair, D.A. Therapeutic potential of resveratrol: The in vivo evidence. *Nat. Rev. Drug Discov.* **2006**, *5*, 493–506.

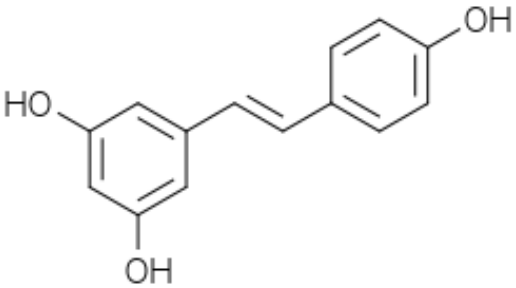


Figure 10. Chemical structure of resveratrol

1.1.8. Lignans

Lignans belong to the group of diphenolic compounds derived from the combination of two phenylpropanoid C6–C3 units at the β and β' carbon. The main lignans are secoisolariciresinol, lariciresinol, matairesinol, pinoresinol, medioresinol, and syringaresinol[30]. **Figure 11** shows the structure of secoisolariciresinol. Secoisolariciresinol content in flaxseed is about 3.7 g/kg dry weight, whereas matairesinol is found in a low quantity of 10.9 mg/kg dry weight. Other foods such as cereals, fruits, legumes, vegetables, berries, and tea also contain lignans but in low quantities from 0.1 to 81.9 mg/kg dry weight[1]. Concretely, lignans in cereals and cereal-based products are ranged between 23-401 µg/100 g dry weight[30].

[30] Durazzo, A.; Lucarini, M.; Camilli, E.; Marconi, S.; Gabrielli, P.; Lisciani, S.; Gambelli, L.; Aguzzi, A.; Novellino, E.; Santini, A.; et al. Dietary lignans: Definition, description and research trends in databases development. *Molecules* **2018**, *23*, 1–14.

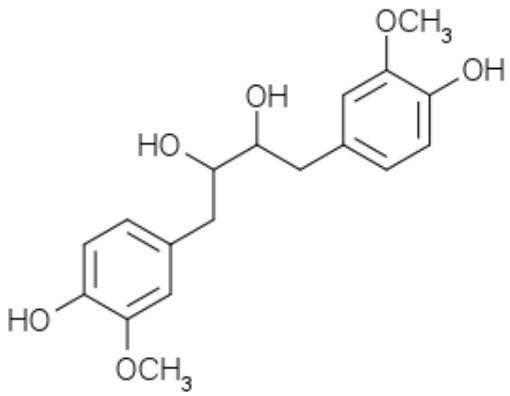


Figure 11. Structure of secoisolariciresinol.

1.1.9. Flavonoids

Flavonoids are an important class of natural products that belong to a class of plant secondary metabolites having a phenolic structure[9]. Flavonoids contain two aromatic rings connected by a three-carbon bridge (C_6-C_3-C_6), being normally associated with sugar molecules (glycoside derivatives)[31,32]. Flavonoids are structurally classified according to the carbon of the C ring to which the B ring is attached. For instance, an isoflavone is formed if the B ring is linked to C_3, whereas, if the B ring is linked to C_4, the formed compound is a neoflavonoid; otherwise, if the B ring is attached to the C_2, different subclasses of compounds are produced, which depend on the structural characteristics of the C ring such as number of double bonds or level of oxidation. These subgroups are classified in flavones, flavonols, flavanones, flavanonols, flavanols and anthocyanins (**Figure**

[31] Lewandowska, H.; Kalinowska, M.; Lewandowski, W.; Stepkowski, T.M.; Brzóska, K. The role of natural polyphenols in cell signaling and cytoprotection against cancer development. *J. Nutr. Biochem.* **2016**, *32*, 1–19.

[32] Del Rio, D.; Rodriguez-Mateos, A.; Spencer, J.P.E.; Tognolini, M.; Borges, G.; Crozier, A. Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxidants Redox Signal.* **2013**, *18*, 1818–1892.

12)[9]. Flavonoids exhibit antioxidant, anticancerogenic, atherosclerosis, antiinflammatory properties[33-35]

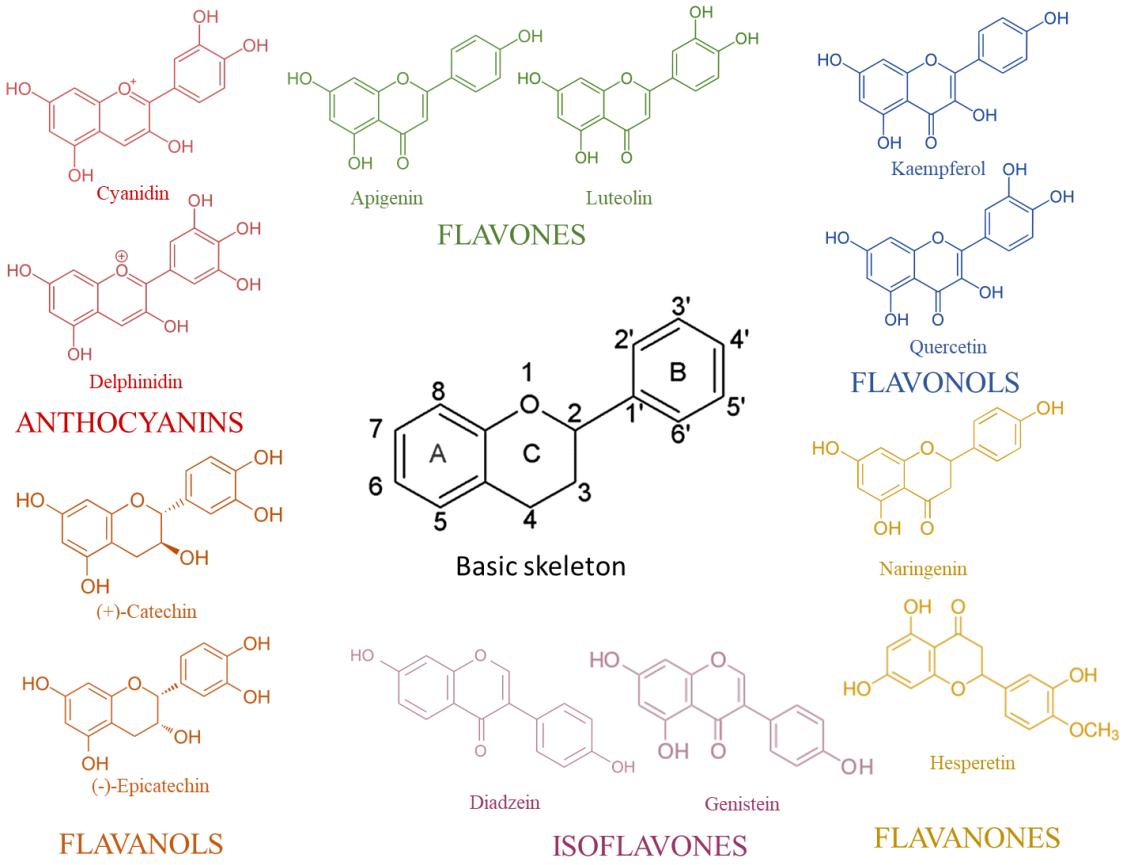


Figure 12. Structure of the main types of flavonoids. Adapted from[9].

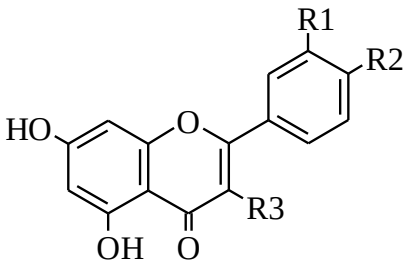
[33] Rathee, P.; Chaudhary, H.; Rathee, S.; Rathee, D.; Kumar, V.; Kohli, K. Mechanism of action of flavonoids as anti-inflammatory agents: A review. *Inflamm. Allergy - Drug Targets* **2009**, *8*, 229–235.

[34] Abotaleb, M.; Samuel, S.M.; Varghese, E.; Varghese, S.; Kubatka, P.; Liskova, A.; Büsselberg, D. Flavonoids in cancer and apoptosis. *Cancers (Basel).* **2019**, *11*,*28*.

[35] Grassi, D.; Desideri, G.; Ferri, C. Flavonoids: Antioxidants against atherosclerosis. *Nutrients* **2010**, *2*, 890–902.

1.1.9.1. Flavones

Flavones have a double bond in the C ring between positions 2 and 3 and a ketone in position 4. Flavones, such as apigenin, luteolin are structurally very similar to flavonols, except for their lack of a hydroxyl group at the C_3 position (**Figure 13**)[32]. A great range of substitutions is possible with flavones including hydroxylation, methylation, O- and C-glycosylation and alkylation. Most flavones in vegetables and fruits contain a hydroxyl group in position 5 of the A ring, whereas hydroxylation in other positions, mainly in position 7 of the A ring or 3′ and 4′ of the B ring, may vary according to the taxonomic classification of each vegetable or fruit[9]. Flavones are commonly found in leaves, flowers and fruits as glucosides.



Flavones	Substitution
Apigenin	$R_1 = R_3 = H$; $R_2 = OH$
Luteolin	$R_1 = R_2 = OH$; $R_3 = H$
Luteolin glucoside	$R_1 = OH$; $R_3 = H$; $R_2 = Glu$

Figure 13. Chemical structure of the main flavones.

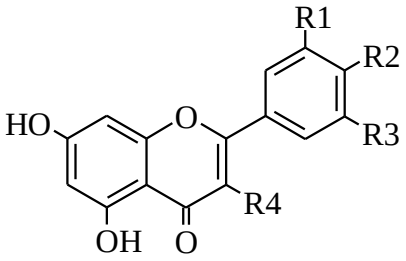
1.1.9.2. Flavonols

In comparison with flavones, flavonols have a hydroxyl group in position C_3 of the C ring, which can also be glycosylated. Flavonols are the largest subgroup of flavonoids due to their very diverse patterns in methylation and hydroxylation, as well and, the different glycosylation patterns. For instance, the most common flavonols in fruit and vegetables are kaempferol, quercetin, myricetin, fisetin and rutin (**Figure 14**)[36]. Onions, kale, lettuce, tomatoes, apples, grapes and berries are rich sources of flavonols. There is a remarkable variety of flavonols with almost 380 flavonol glycosides and, among them, 200 types of quercetin and kaempferol glycosides[37]. Glycosides of quercetin are more efficiently absorbed than quercetin itself[10].

Biosynthesis of flavonols is stimulated by light. Therefore, the concentration of flavonols is usually higher in the outer parts (skins and leaves) of the plants and fruits than in the other parts[1]. In addition, the consumption of flavonols is associated with a wide range of health benefits including antioxidant activities and the reduction of the risk of vascular diseases[36].

[36] Iwashina, T. Flavonoid Properties of five Families newly Incorporated into the Order Caryophyllales (Review). *Bull. Natl. Mus. Nat. Sci., Ser. B* **2013**, *39*, 25–51.

[37] Cherniack, E.P. Polyphenols and Aging. *Mol. Basis Nutr. Aging A Vol. Mol. Nutr. Ser.* **2016**, *3*, 649–657.



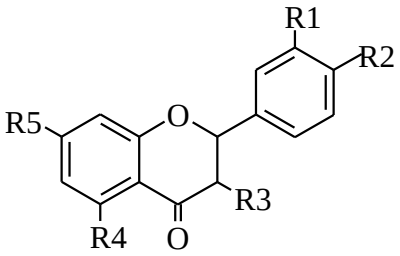
Flavonols	Substitution
Kaempferol	R_1 = H; R_2 = OH; R_3 = H; R_4 = OH
Quercetin	R_1 = OH; R2 = OH; R_3 = H; R_4 = OH
Myricetin	R_1 = OH; R_2 = OH; R_3 = OH; R_4 = OH
Fisetin	R_1 = H; R_2 = OH; R_3 = OH; R_4 = OH
Rutin	R_1 = OH; R_2 = OH; R_3 = H; R_4 = Rut

Figure 14. Chemical structures of the main flavonols.

1.1.9.3. Flavanones

Flavanones, also called dihydroflavones, have the C ring saturated; thus, the double bond between positions 2 and 3 is missing, being the only structural difference between the two subgroups of flavones and flavonols[36]. Flavanones are a small group of compounds mainly present in all citrus fruits and grapes. Hesperidin, naringenin and eriodyctiol are examples of flavanones (**Figure 15**)[9]. The most important form is the aglycone one, because this form is rapidly absorbed; however, these are commonly found in their glycosidic forms in the nature and are classified into two groups: neohesperidosides and rutinosides[10,38]. Flavanones are associated with a large amount of health benefits because of their free radical-scavenging properties. Citrus flavonoids exert interesting pharmacological effects as antioxidant, antiinflammatory, blood lipid-lowering and cholesterol-lowering agents[36].

[38] Manach, C.; Williamson, G.; Morand, C.; Scalbert, A. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. **2005**, *81*, 230–242.



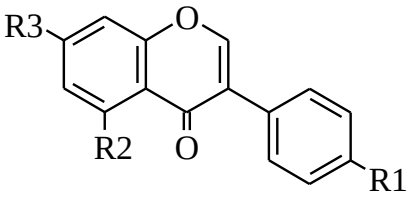
Flavanones	Substitution
Hesperidin	$R_3 = R_4 = R_1 = OH$; $R_2 = OCH_3$; R_5 = Rut
Naringenin	$R_2 = R_4 = R_5 = OH$; $R_1 = R_3 = H$
Eriodyctiol	$R_1 = R_2 = R_4 = R_5 = OH$; $R_3 = H$

Figure 15. Chemical structure of the main flavanones

1.1.9.4. Isoflavonoids

Isoflavonoids have their ring B linked with the C_3 position of ring C. Isoflavonoids are mainly found in soybeans and other leguminous plants[9]. Genistein, daidzein, biochanin A, and formononetin are the main isoflavones in soy (**Figure 16**). These compounds are found in four chemical forms, unconjugated (aglycone, IFA), sugar-conjugated (isoflavone glucoside, IFG), acetylglucosides, and malonylglucosides. All isoflavone aglycones are largely found as 7-O-glucosides and 6"-O-malonyl-7-O-glucosides[7]. Isoflavonoids have a tremendous potential to fight several diseases. E.g. genistein and daidzein have shown estrogenic activity because of that they are considered natural phytoestrogens. Several clinical studies have also reported benefits of genistein and daidzein in chemoprevention of breast and prostate cancer, cardiovascular disease, osteoporosis and in the alleviation of postmenopausal symptoms[39].

[39] Vitale, D.C.; Piazza, C.; Melilli, B.; Drago, F. Isoflavones: Estrogenic activity, biological effect and bioavailability. *Eur. J. Drug Metab. Pharmacokinet.* **2012**, *38*, 15–25.



Isoflavonoids	Substitution
Genistein	$R_1 = R_2 = R_3 = OH$
Daidzein	$R_1 = R_3 = OH$; $R_2 = H$
Biochanin A	$R_1 = OCH_3$; $R_2 = R_3 = OH$
Formononetin	$R_1 = OCH_3$; $R_2 = H$; $R_3 = OH$

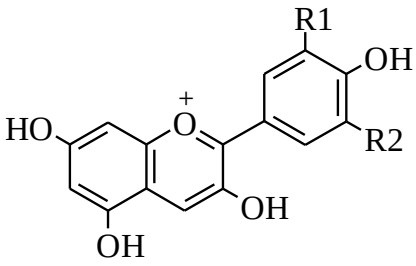
Figure 16. Chemical structures of the main isoflavones.

1.1.9.5. Anthocyanidins

Anthocyanidins are the main components of the red, blue and purple pigments of flower petals, fruits and vegetables, and some special varieties of grains. Their structure, flavylium cation, contain two benzene rings linked by an unsaturated cationic oxygenated heterocycle, derived from the 2-phenyl-benzopyrylium nucleus[40]. Anthocyanidins such as pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin are the most studied (**Figure 17**). Anthocyanidins in plants are mainly found as glycosidic forms. In fact, 90% of anthocyanins are based on cyanidin, delphinidin and pelargonidin and their methylated derivatives[7]. A total of more than 500 anthocyanins are known, which depend on their hydroxylation and methoxylation patterns on the B ring; the nature, position, and number of conjugated sugar units; the nature and number of conjugated aliphatic or aromatic acid groups; and the presence or absence of an acyl aromatic group in the molecule.

[40] Lorrain, B.; Ky, I.; Pechamat, L.; Teissedre, P.L. Evolution of analysis of polyhenols from grapes, wines, and extracts. *Molecules* **2013**, *18*, 1076–1100.

The color of anthocyanins is pH-dependent, i.e., red in acidic and blue in basic conditions[1].



Anthocyanidins	Substitution
Cyanidin	R_1 = H; R_2 = OH
Delphinidin	R_1 = OH; R_2 = OH
Pelargonidin	R_1 = H; R_2 = H
Peonidin	R_1 = OCH_3; R_2 = H
Petunidin	R_1 = OCH_3; R_2 = OH
Malvidin	R_1 = OCH_3; R_2 = OCH_3

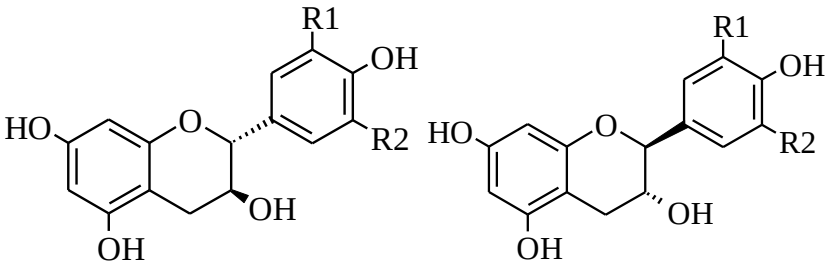
Figure 17. Chemical structures of the main anthocyanidins

1.1.1.9.6. Flavanols

Flavanols or flavan-3-ols, which are also called as dihydroflavonols or catechins, are the 3-hydroxy derivatives of flavanones. Catechin and epicatechin are the most abundant flavanols found in fruits, whereas the main flavanols found in grapes, in certain seeds of leguminous plants, and in tea are gallocatechin, epigallocatechin, and epigallocatechin gallate[41]. Catechin is composed by two benzene rings and a dihydropyran heterocycle with a hydroxyl group in the C_3 position. The hydroxylation at C_3 allows flavanols to have two chiral centers on the molecule on C_2 and C_3. Therefore, four diastereoisomers can be formed, being catechin the

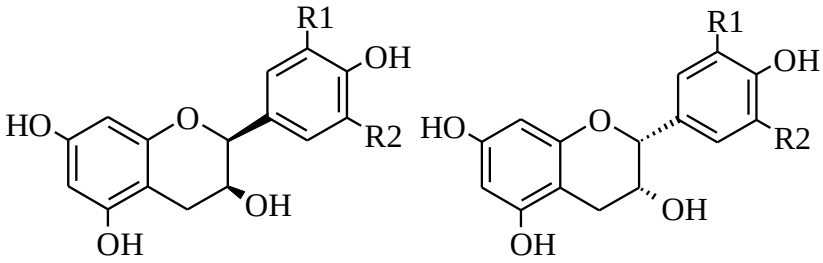
[41] Bernatoniene, J.; Kopustinskiene, D.M. The Role of Catechins in Cellular Responses to oxidative stress. *Molecules* **2018**, *23*, 1–11.

isomer with trans configuration and epicatechin the one with cis configuration. Each of these two configurations has two steroisomers, i.e., (+)-catechin, (−)-catechin, (+)-epicatechin and (−)-epicatechin (**Figure 18**). Flavanols can form gallic acid conjugates such as epicatechin gallate, epigallocatechin and epigallocatechin gallate by means of the esterification with gallate groups[41].



(+) – catechin .. (-) - catechin

Flavanols	Substitution
Catechin	R_1 = OH; R_2 = H
Gallocatechin	R_1 = OH; R_2 = OH



(-) - Epicatechin.....(+) – Epicatechin

Flavanols	Substitution
Epicatechin	R_1 = OH; R_2 = H
Epigallocatechin	R_1 = OH; R_2 = OH

Figure 18.Chemical structures of the main flavanols.

1.1.10. Alkylresorcinols

The chemical structure of 5-n-ARs is characterized by a benzene ring with two hydroxyl groups in the positions 1 and 3 (the hydrophilic head) and an odd-numbered alkyl chain at position 5 of the benzene ring (the hydrophobic tail) (**Figure 19**)[42]. Orcinol is the simplest homologue of the 1,3-dihydroxy-5-n-alk(en)ylbenzene series. Their homologues differ in the alkyl chain according to a combination of tail length and the degree of unsaturation. These compounds have a strong amphiphilic character due to their hydrophilic and hydrophobic regions. The ARs are found in a number of plants, algae, mosses, fungi and bacteria[43]. In plants, ARs are found in angiosperm species, including *Anacardiaceae*, *Gramineae*, *Proteaceae*, *Myrsinaceae*, *Primulaceae*, *Myristinaceae*, *Iridaceae*, *Araceae*, *Asteraceae* and *Fabaceae*. In addition, ARs have been found in high levels (>500 µg/g) in wheat, rye, and triticale and in low amounts in barley, millet, and maize. ARs are found mainly in the outer layers (bran fraction) of cereal grains, which mean that they are largely missing in refined cereal flour and conventional cereal products[42].

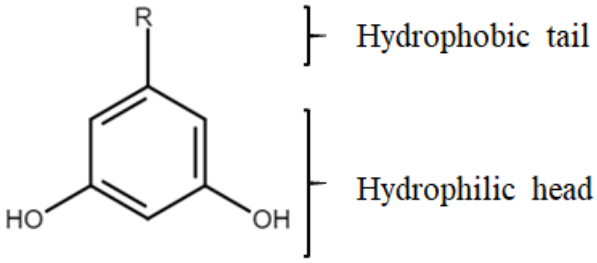


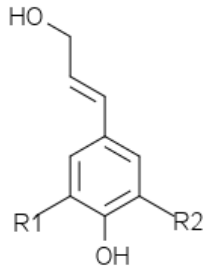
Figure 19. General structure of alkylresorcinols. R is a linear alkyl chain that can be saturated, unsaturated and/or have different degrees of oxygenation. Adapted from[43].

[42] Ross, A.B.; Shepherd, M.J.; Schüpphaus, M.; Sinclair, V.; Alfaro, B.; Kamal-Eldin, A.; Åman, P. Alkylresorcinols in cereals and cereal products. *J. Agric. Food Chem.* **2003**, *51*, 4111–4118.

[43] Sampietro, D.A.; Belizán, M.M.E.; Vattuone, M.A.; Catalán, C.A.N. Alkylresorcinols: Chemical properties, methods of analysis and potential uses in food, industry and plant protection. In *Natural Antioxidants and Biocides from Wild Medicinal Plants*; Cespedes, C.L., Sampietro, D.A., Seigler, D.S., Rai, M., Eds.; CAB International: Wallingford, 2013; pp. 148–166.

1.1.11. Lignins

Lignin is a secondary metabolite produced by the phenylalanine/tyrosine metabolic pathway in plant cells. Lignin biosynthesis is a very complex network that consists of three processes: biosynthesis of lignin monomers, transport and polymerization[44]. Finally, lignin is generally polymerized with three main types of phenylpropanoids alcohols (sinapyl alcohol, coniferyl alcohol, and p-coumaroyl alcohol) by peroxidase and laccase in secondary cell wall[45]. The complex of these compounds is cross-linked through carbon-carbon, ester and ether linkages. Lignin is a natural biopolymer making up 10-25% of plant biomass[46]. Lignin is an important organic polymer which is abundant in cell walls of some types of cells. It has many biological functions such as water transport, mechanical support and resistance to some stresses[44].



Phenylpropanoids alcohols	Substitution
Coumaryl alcohol	R_1. R_2= H
Coniferyl alcohol	R1= OCH_3, R_2= H
Syringyl alcohol	R_1, R_2= OCH3

[44] Liu, Q.; Luo, L.; Zheng, L. Lignins: Biosynthesis and biological functions in plants. *Int. J. Mol. Sci.* **2018**, *19*, 1–16.

[45] Mahmood, Z.; Yameen, M.; Jahangeer, M.; Riaz, M.; Ghaffar, A.; Javid, I. Lignin as natural antioxidant capacity. In *Lignin-Trends and Applications*; IntechOpen: London, UK, 2018; pp. 181–205.

[46] Vanholme, R.; Demedts, B.; Morreel, K.; Ralph, J.; Boerjan, W. Lignin biosynthesis and structure. *Plant Physiol.* **2010**, *153*, 895–905.

Figure 20. Chemical structures of the phenylpropanoid alcohols used to construct the lignin polymer.

1.1.1. Tannins

The term tannin refers to a group of phenolic compounds with given properties, whose fundamental characteristic is high molecular weight. These structures have the capacity to associate with proteins and carbohydrates. Tannins are found in the plant kingdom in two large metabolic modalities: the hydrolysable and condensed tannins. Hydrolyzable tannins are simpler structures constituted from units of free or esterified gallic acid, which are also known as gallotannins[4] (**Figure 21**).

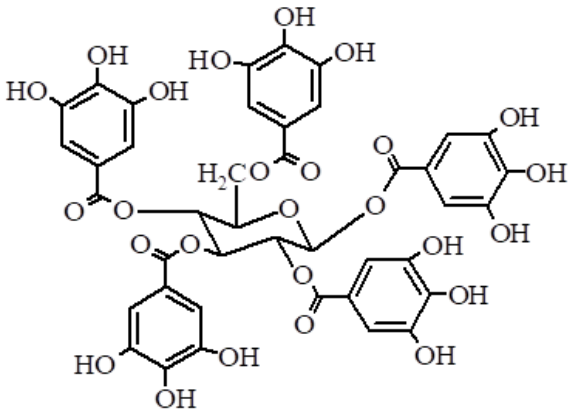
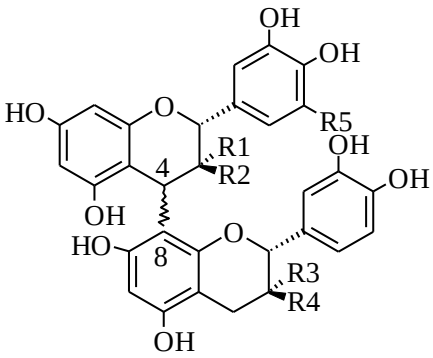


Figure 21. Structure of a hydrolyzable tannin (tannic acid).

The condensed tannins, usually called proanthocyanins, are natural polymers composed of flavan-3-ol units[4]. The most recurrent of these structures are proanthocyanins that are based on (+) catechin and (-) epicatechin. Oligomers contain 2-6 monomeric units and polymers contain more than 7 monomer units. Proanthocyanidins are divided into procyanidins and prodelphinidins: procyanidins consist of oligomers of (+) - catechin, (-) – epicatechin whereas; prodelphinidins have as monomeric units (+) - gallocatechin, (-) - epigallocatechin[47]. Depending on the bonds between monomers, oligomers can be

[47] Zuiter, A.S.; Zarqa, J. *Proanthocyanidin: Chemistry and Biology: From Phenolic*

A-type structure in which monomers are linked through C_2–O–C_7 or C_2–O–C_5, or B-type with C_4–C_6 or C_4–C_8 bonding[7] (**Figure 22**).



Substitution	Proanthocyanidins
Procyanidin B1	$R_1 = R_4 = OH$; $R_2 = R_3 = R_5 = H$
Procyanidin B2	$R_1 = R_3 = OH$; $R_2 = R_4 = R_5 = H$
Procyanidia B3	$R_1 = R_3 = R_5 = H$; $R_2 = R_4 = OH$
Procyanidin B4	$R_1 = R_4 = R_5 = H$; $R_2 = R_3 = OH$
Prodelphinidin	$R_1 = R_3 = H$; $R_2 = R_4 = R_5 = OH$

Figure 22. Chemical structures of the main proanthocyanidins

1.2. Bioactivity of phenolic compounds

The health benefits from the intake of phenolic compounds depend on their absorption and metabolism, which are determined by their structure including their conjugation with other phenolics, degree of glycosilation/acylation, molecular size and solubility[48]. Phenolic compounds passage through the wall of the small intestine into the circulatory system and later transport to the liver in the portal vein. Nevertheless, these metabolites of phenolic compounds are quickly

Compounds to Proanthocyanidins; Elsevier Inc., 2014; ISBN 9780124095472.

[48] Ozcan, T.; Akpinar-Bayizit, A.; Yilmaz-Ersan, L.; Delikanli, B. Phenolics in Human Health. *Int. J. Chem. Eng. Appl.* **2014**, *5*, 393–396.

eliminated from plasma. Therefore, daily consumption of plant products is essential to supply high metabolite concentrations in blood[49] [49]. However, the most abundant phenolic compounds in daily diet are not essentially those that have the best bioavailability. The bioavailability of phenolic compounds can also be affected by differences in cell wall structures, location of glycosides in cells and joining of phenolic compounds in the food matrix[49]. Epidemiological studies have shown that phenolic compounds have important functions, like inhibition of pathogens and decay microorganisms, antideposition of triglycerides, reduce the incidence of certain diseases such as cardiovascular diseases, diabetes, cancer and stroke, antiinflammation and antiallergic effect[48]. These protective effects are partly due to their antioxidant properties that allow lowering the levels of free radicals present in the body[50].

Antioxidant, antiinflammatory, anticarcinogenic, antimicrobial and antiviral are the most common properties of phenolic compounds, which are detailed below.

1.2.1. Antioxidant activity

Phenolic compounds produced naturally by plants possess a high antioxidant activity. Oxygen is a very important element for every living organism. However, reactive oxygen species (ROS) can be toxic and mutagenic[51]. The reactive oxygen forms include all those reactive molecules, free radicals or not, with chemical reactivity centered in an atom of oxygen and also comprise chemical species with nitrogen and chlorine (**Table 1**). Excessive production of ROS can lead to oxidative stress, providing damage in cell structures such as lipids, proteins and DNA. This damage can cause many diseases such as cancer, inflammation,

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[49] Crozier, A.; Jaganath, I.B.; Clifford, M.N. Dietary phenolics: Chemistry, bioavailability and effects on health. *Nat. Prod. Rep.* **2009**, *26*, 1001–1043.

[50] Cory, H.; Passarelli, S.; Szeto, J.; Tamez, M.; Mattei, J. The Role of Polyphenols in Human Health and Food Systems: A Mini-Review. *Front. Nutr.* **2018**, *5*, 1–9.

[51] Działo, M.; Mierziak, J.; Korzun, U.; Preisner, M.; Szopa, J.; Kulma, A. The potential of plant phenolics in prevention and therapy of skin disorders. *Int. J. Mol. Sci.* **2016**, *17*, 1–41.

cataract, hypertension, diabetes, cardiovascular disease, Parkinson and Alzheimer [7,52]. Oxidative stress is known as the disturbance of the homeostasis between reactive oxygen forms and the antioxidative defense system in the organism[53].

Table 1. Free radicals and reactive oxygen, nitrogen, and chlorine species[54].

Free radicals	**Non radical reactive species**
Superoxide O_2^-	Hydrogen peroxide H_2O_2
Hydroxyl OH^-	Hydroperoxide ROOH
Alkoxy RO^-	Hypochlorite ClO^-
Peroxy ^-OOH	Singlet oxygen $^1O_2$
Nitric oxide NO	Ozone O_3
Nitric dioxide NO_2	Peroxynitrile NO O_2^-

Phenolic compounds provide antioxidant activity through various mechanisms of action: inhibition of ROS formation, ROS trapping and extinction of singlet oxygen; and reducing the chelated metal ions, which are the catalysts of the reactions that produce the formation of ROS, impeding the cascade of free radical reactions in lipid peroxidation[51].

The two main mechanisms of action of phenolic compounds are: single-electron transfer (SET) and hydrogen atom transfer (HAT)[54].

In the first mechanism, the phenolic compound (ArO:H) has the capacity to transfer a single electron (SET) to the free radical (R·) and reduce certain compounds, including carbonyls, metals, and radicals, becoming itself a radical

[52] Sies, H. Polyphenols and health: Update and perspectives. *Arch. Biochem. Biophys.* **2010**, *501*, 2–5.

[53] Yoo, H.G.; Lee, B.H.; Kim, W.; Lee, J.S.; Kim, G.H.; Chun, O.K.; Koo, S.I.; Kim, D.O. Lithospermum erythrorhizon extract protects keratinocytes and fibroblasts against oxidative stress. *J. Med. Food* **2014**, *17*, 1189–1196.

[54] Miguel-Chávez, R.S. Phenolic Antioxidant Capacity: a Review of the state of the art. In *Phenolic Compounds - Biological Activity*; Soto-Hernandez, M; Palma-Tenango, M; García-Mateos, R.M.d. Ed.; InTech: Rijeka, 2017; pp. 60–74.

cation ($ArO{\cdot}H^+$) (**eq.1**)[54,55]. The lower of the ionization potential (IP), the easier will be the electron abstraction[55].

$$R{\cdot}+ArO{:}H \rightarrow R{:}^- + ArO{\cdot}H^+ \quad (\textbf{eq.1})$$

In the second mechanism (HAT), phenolic compounds (ArO:H) act as scavengers of free radicals (R·) by proton donation, becoming itself a radical ($ArO{\cdot}$)[54,55]. In this mechanism, the bond dissociation energy (BDE) of the O–H bonds is an important factor that allows evaluating the antioxidant activity, because the weaker the OH bond the easier will be the reaction of free radical inactivation[55].

$$R{\cdot}+ArO{:}H \rightarrow R{:}H + ArO \quad (\textbf{eq.2})$$

1.2.2. Antiinflammatory activity

During the inflammation process, an excess of reactive species is provided. The production of reactive nitrogen species (RNS) and ROS is related to biological responses against the activation of the transcription factor AP-1 and nuclear transcription factor kappa B (NF-κB). These factors regulate secretion of signaling molecules, such as pro-inflammatory cytokines. The overproduction of these pro-inflammatory cytokines such as interleukin (IL)-1b, IL-6, and tumor necrosis factor alpha (TNF-α) cause several illnesses such as arthritis, allergy, atherosclerosis, and cancer[56]. Phenolic compounds can inhibit the pro-inflammatory mediators, neutralize the free radicals, ROS, RNS and, thus, inhibit lipid peroxidation[51,57].

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[55] Bendary, E.; Francis, R.R.; Ali, H.M.G.; Sarwat, M.I.; El Hady, S. Antioxidant and structure–activity relationships (SARs) of some phenolic and anilines compounds. *Ann. Agric. Sci.* **2013**, *58*, 173–181.

[56] Shahidi, F.; Yeo, J.D. Bioactivities of phenolics by focusing on suppression of chronic diseases: A review. *Int. J. Mol. Sci.* **2018**, *19*, 1–16.

[57] Nagula, R.L.; Wairkar, S. Recent advances in topical delivery of flavonoids: A review. *J. Control. Release* **2019**, *296*, 190–201.

As an example, during the inflammation process arachidonic acid is liberated from the cell membrane phospholipids. The enzyme implicated in this reaction is phospholipase A2 (PLA2), which is stimulated by oxidative stress. The release of arachidonic acid is converted by either the cyclooxygenase or lipoxygenase pathway. Phenolic compounds have the capacity to inhibit both reactions, mainly due to the break of substrate binding to the enzyme by disruption of the hydrogen bonding system or due to chelation ions in the active center of the enzyme[58].

1.2.3. Anticarcinogenic activity

Phenolic compounds from fruit and vegetables possess the capacity of scavenging free superoxide radicals, preventing the risk of cancer and protecting biological systems against the injurious effects of oxidative processes on macromolecules, such as carbohydrates, proteins, lipids and DNA[59]. In addition, their anticarcinogenic effects are related to their capacity to inhibit cell proliferation (extracellular signal-regulated kinase (Erk)1/2, D-type cyclins, and cyclin-dependent kinases (CDKs)), angiogenic factors (vascular endothelial growth factor (VEGF) and cytokine-1 MIC-1), oncogenic signaling cascades (phosphoinositide 3-kinase (PI3K) and protein kinase B (Akt)), inducing apoptosis, and preventing cellular migration and metastasis[60]. Hydroxybenzoic and hydroxycinnamic acids such as vanillic acid, protocatechuic acid, gallic acid, syringic acid, caffeic acid, ferulic acid, *p*-coumaric acid and sinapic acid have proven to induce apoptosis and inhibit the proliferation and metastasis in colon, leukemia, gastric, prostate,

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[58] Arct, J.; Bielenda, B.; Oborska, A.; Pytkowska, K. The tea and its cosmetic application. *J. Appl. Cosmetol.* **2003**, *21*, 117–127.

[59] Basli, A.; Belkacem, N.; Amrani, I. Health Benefits of Phenolic Compounds Against Cancers. In *Phenolic Compounds - Biological Activity*; intechOpen, 2017; pp. 193–210.

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cervical, lung, breast, endothelial, thyroid and breast cancers[61]. Besides, flavonols have shown a direct cellular proliferation inhibitory activity in several cancers[62]. Specifically, quercetin inhibits cancer metastasis, inhibits MAPK phosphorylation, induces differentiation of HL-60 cells into granulocytes and monocyte in gastric cancer and lung cancer (SK-LU1, SW900, H441, H661, haGo-K-1, A549 cells)[63]. Flavones such as luteolin inhibit proliferation and induce apoptosis in colon cancer (Caco-2, HT-29, IEC-6, HCT-15 cells)[62]. Moreover, apigenin inhibits cancer metastasis, inhibits MAPK phosphorylation, induces apoptosis and induces differentiation of HL-60 cells into granulocytes and monocytes in leukemia (HL-60, K562, Jurkat cells)[64]. Isoflavonoids such as daidzein and genistein inhibit proliferation and induces apoptosis in breast cancer (MCF-7 cells) and prostate cancer lines (LNCaP, PC3, DU145 cells)[64].

1.2.4. Antibacterial and antiviral activity

Antibacterial and antiviral agents can kill or slow down the action of bacteria and viruses without causing any damage to the surrounding cells and tissues. In this sense, many phenolic compounds have shown to be potent antibacterial and anti-viral agents[56]. For instance, phenolic compounds (e,g. gallic acid, catechin, epicatechin, rutin, quercetin, apigenin, luteolin, etc.) have a potential activity against various virus types. Concretely, against retroviridae, hepadnaviridae, hespervirides, HIV (Human immune deficiency virus) virus, influenza virus,

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herpes simplex virus, dengue virus, polio virus, diarrhea virus, etc[65]. In addition, phenolic compounds such as stilbenes, tannins, and isoflavones have demonstrated to inhibit the growth of fungi, yeasts, and viruses as well as bacteria such as *Salmonella*, *Clostridium*, *Bacillus*, and *E. coli* [56]. Specifically, it has been reported that gallic acid and its derivatives mitigate the growth of cariogenic and periodontopathic bacteria[66]. Moreover, apigenin has shown antibacterial activity against five pathogenic bacterial strains like *Pseudomonas aeruginosa*, *Salmonella Typhimurium*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Enterobacter aerogenes*[67].

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2. OLIVE LEAVES

2.1. Introduction

Olive trees (*Olea europaea* L.) belongs to the plant family *Oleaceae,* which includes 30 genera and 600 species, is an evergreen tree that produces the olive fruit[68,69]. The genus *Olea* acquired its name from the Greek “elaia” and the Latin “oleum”. The genus *Olea* is made up of more than 30 species, but *Olea europaea* L. is the most popular member of the genus *Olea* [70,71]**.**

Figure 23. *Olea europaea* (Olive tree).

Olive is an important crop in the Mediterranean basin (MB), economically, culturally, and historically. The history of olives continues to be enigmatic. Archeological and genetic investigations prove that the crop was likely

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domesticated from its wild progenitor, the oleaster (*O. europaea* ssp. *europaea* var. *sylvestris*), c. 6000 years ago in the Middle East, possibly in a geographic location close to the border between Turkey and Syria[72]. It is thought that the Phoenicians, Greeks and Romans spread the olive cultivation from East to West across both the Northern and Southern coasts of the MB[73]. It is commonly thought that the Phoenicians took olives to Spain and North Africa over 1000 BCE, and the Greeks imported the trees into Italy[74]. Archeological discoveries show that olive, oil, and leaves had nutritional, medicinal, and ceremonial uses[75]. Many rituals implicated the use of olive oil, including the anointing of royalty, warriors, and the general public for religious purposes[74]. Ancient documents show that Greeks used its oils for the body health[70]. The Quran praises the olive as a 'precious fruit'[73]. Olive leaf was used medicinally for the first time in ancient Egypt and was the symbol of heavenly power. The oil extracted from leaf was used in mummification ceremonies. Olive leaf has also been used in the treatment for conditions of influenza, common cold, malaria, dengue, severe diarrhoea, and dental, ear, urinary tract, and surgical infections, concretely in Mediterranean cultures. In the middle of the 19th century, olive leaf tea was employed to counter malaria. Olive leaf tea was real popular in England and was very used to cure sick sailors and colonists returning from tropical climates in colonial times[76,77].

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[72] Kaniewski, D.; Van Campo, E.; Boiy, T.; Terral, J.F.; Khadari, B.; Besnard, G. Primary domestication and early uses of the emblematic olive tree: Palaeobotanical, historical and molecular evidence from the Middle East. *Biol. Rev.* **2012**, *87*, 885–899.

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Nowadays, olive trees spread over in continents such as Asia-Pacific, and North and South America (**Figure 24**). Although, Mediterranean countries account for 98% of the world's olive cultivation (approximately ten million hectares)[78] . The annual olive production in 2018 was of 21066062 tonnes being Spain, Italy and Greece the main producers representing 46.6, 8.9 and 5.1% of total olives production, respectively[79]..

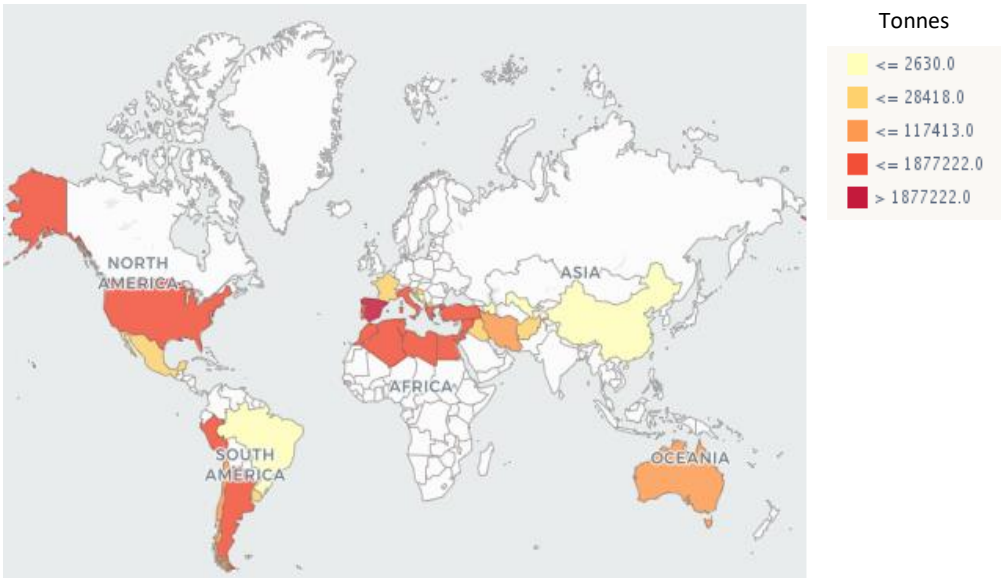

Figure 24. Production of olives in 2018 by continent (FAOSTAT)

The main products from olive cultivation are edible olives and olive oil. During olive oil processing, lots of wastes and by-products are produced including olive pomace, olive mill waste waters, olive leaves, and olive stones and seeds[80]. Olive leaves are obtained in large amounts as a residue from olive oil industries, pruning of olive trees and harvesting and cleaning of olives[81]. They represent 10% of the

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total weight of olives collected for olive oil extraction. These by-products are used as animal feed or are burned with excess branches gathered from pruning, generating an environmental impact[78]. Moreover, olive leaves contain bioactive compounds such as tocopherols, triterpenoids, pigments, and phenolic compounds that have shown benefits to the human health. Therefore, the recovery of bioactive compounds from olive leaves is an interesting way of olive leaves revalorization that could be beneficial to the Food Industry, because olive leaf extracts can be used as food additives or for nutraceutical and cosmeceutical scopes. Several studies have shown that olive leaves extracts possess a wide range of *in vitro* and *in vivo* properties, including antioxidant activity, radioprotective, antiproliferative, anticancer, anti-HIV, antifungal, gastroprotective and antidiabetic effects[82].

2.2. Phenolic compounds in olive leaves

The main phenolic compounds in olive leaves can be classified in simple phenols (phenyl alcohols), phenolic acids, flavonoids and secoiridoids (**Table 2**)[82,83].
Among simple phenols, hydroxytyrosol is the most abundant in olive leaves, which is also found in olive oil[84]. Phenolic acids in olive leaves include hydroxycinnamic acids such as *p*-coumaric acid, caffeic acid and ferulic acid and and

europaea L.) leaves. *Eur. Food Res. Technol.* **2017**, *243*, 89–99.

[82] Talhaoui, N.; Taamalli, A.; Gómez-Caravaca, A.M.; Fernández-Gutiérrez, A.; Segura-Carretero, A. Phenolic compounds in olive leaves: Analytical determination, biotic and abiotic influence, and health benefits. *Food Res. Int.* **2015**, *77*, 92–108.

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[84] Gómez-Caravaca, A.M.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Fernández-Gutiérrez, A. A simple and rapid electrophoretic method to characterize simple phenols , lignans, complex phenols, phenolic acids, and flavonoids in extra- virgin olive oil. *J. Sep. Sci.* **2006**, *29*, 2221–2233.

hydroxybenzoic acids like *p*-hydroxybenzoic acid and gallic acid[85,86]. Verbascoside was also identified in olive leaves; it is a caffeoyl phenylethanoid glycoside formed by a caffeic acid and a hydroxytyrosol linked to sugar moieties via an ester and an ether bond, respectively. It is defined as the main hydroxycinnamic derivative in olives extracts and it was also identified in olive leaves[87,88].

Flavonoids contained in olive leaves are subdivided into flavonols (rutin and quercetin) and flavones (diosmetin-7-glucoside, luteolin-7-glucoside, luteolin and apigenin-7-glucoside). Their structural variation is in part due to modifications produced by hydroxylation, methoxylation, prenylation, or glycosylation[89].

Secoiridoids, a subclass of iridoids (monoterpene derivatives with an iridane ring), come from the secondary metabolism of terpenes and their carbon skeleton is derived from mevalonic acid. These compounds are restricted to the *Oleaceae* family and are the main family of compounds contained in olive leaves[82]. The secoiridoid structure is comprised by a phenyl ethyl alcohol (hydroxytyrosol or tyrosol), elenolic acid and, eventually, a glucosidic residue[90]. The main secoiridoid is oleuropein, followed by ligstroside. Oleuropein is an ester of hydroxytyrosol and

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an elenolic acid glucoside, whereas ligstroside is an ester of tyrosol and an elenolic acid glucoside[91-94].

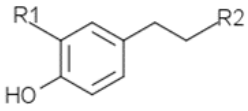
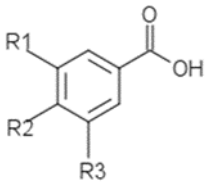
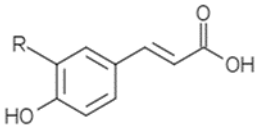
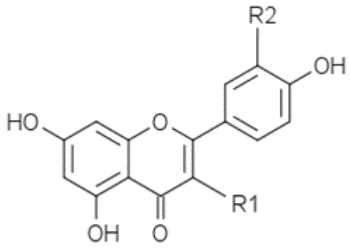
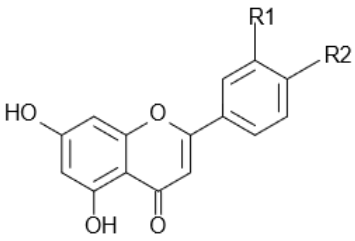
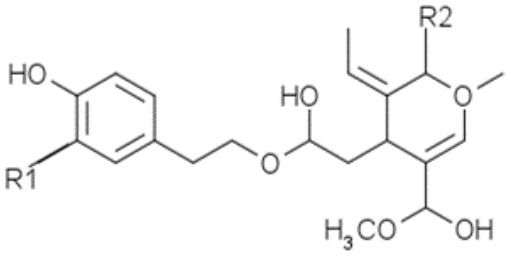
[91] Pérez-Trujillo, M.; Gómez-Caravaca, A.M.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Parella, T. Separation and identification of phenolic compounds of extra virgin Olive Oil from Olea Europaea L. by HPLC-DAD-SPE-NMR/MS. Identification of a new diastereoisomer of the aldehydic form of oleuropein aglycone. *J. Agric. Food Chem.* **2010**, *58*, 9129–9136.

[92] Talhaoui, N.; Gómez-Caravaca, A.M.; Roldan, C.; León, L.; De la Rosa, R.; Fernández-Gutiérrez, A.; Segura-Carretero, A. Chemometric analysis for the evaluation of phenolic patterns in olive leaves from six cultivars at different growth stages. *J. Agric. Food Chem.* **2015**, *63*, 1722–1729.

[93] Ricciutelli, M.; Marconi, S.; Chiara, M.; Caprioli, G.; Sagratini, G.; Ballini, R.; Fiorini, D. Olive oil polyphenols : A quantitative method by high-performance liquid-chromatography-diode-array detection for their determination and the assessment of the related health claim. *J. Chromatogr. A* **2017**, *1481*, 53–63.

[94] Tylewicz, U.; Nowacka, M.; Martín-García, B.; Wiktor, A.; Gómez Caravaca, A.M. *Target sources of polyphenols in different food products and their processing by-products*; Elsevier, 2018; ISBN 9780128135723.

Table 2. Classification of the main phenolic compounds in olive leaves

PHENYL ALCOHOLS	HYDROXYCINNAMIC ACIDS	HYDROXYBENZOIC ACIDS
		
Tyrosol (R_1=H, R_2=OH) Hydroxytyrosol (R_1, R_2=OH)	p-hydroxybenzoic acid (R_1, R_3=H, R_2=OH) Gallic acid (R_1, R_2, R_3=OH)	p-coumaric acid (R=H) Caffeic acid (R=OH) Ferulic acid (R=OCH3)
FLAVONOIDS		
FLAVONOLS	**FLAVONES**	
		
Quercetin (R_1=OH, R_2=OH) Rutin (R_1=Rut, R_2=OH)	Apigenin (R_1=H, R_2=OH) Luteolin (R_1, R_2=OH)	
SECOIRIDOIDS		
	Oleuropein (R_1=OH, R_2=Glucose) Ligstroside aglycone (R_1=H, R_2=OH) Oleuropein Aglycone (R_1=OH, R_2=OH)	

The total concentration of phenolic compounds in olive leaves ranges from 14.27 to 83.30 x10^3 mg/kg dry leaf, which is higher than the reported in olive oil (40–

1000 mg/kg olive oil)[87,92,94,95]. Secoiridoids are the main phenolic family present in olive leaves; oleuropein is the most abundant secoiridoid with a concentration between 9.87-66.44x10^3 mg/kg dry leaf [87,92,95]. In addition, some flavonoids are present in a high content in olive leaves such as luteolin glucoside (380-5724 mg/Kg d.w.), rutin (205-967 mg/Kg d.w.) and luteolin rutinoside (199-491 mg/Kg d.w.), whereas the most concentrated simple phenols are tyrosol glucoside (41-1278 mg/Kg d.w.) and hydroxytyrosol-hexose (179-1284 mg/Kg d.w.)[87,92]. Phenolic acids such as *p*-coumaric acid (63-237 mg/Kg d.w.), ferulic acid (26.4-129.1 mg/Kg), *p*-hydroxybenzoic acid (0.6-26.8 mg/Kg d.w.) and gallic acid acid (7.4-55.8 mg/Kg d.w.) were found in low quantities in olive leaves[96] . Verbascoside was found in some olive leaves in quantities of 1127-4069 mg/Kg d.w[87].

2.3. Bioactivity of phenolic compounds in olive leaves

The large number of phenolic compounds present in olive leaves aroused the interest of researchers around the world. In fact, studies with animals and humans have reported a high antioxidant capacity and several beneficial health effects related to these compounds, such as neuroprotective, antihypertensive, cholesterol lowering, cardioprotective, antiinflammatory, anticancerogenic and as coadjuvant in the treatment of obesity. **Table 3** summarizes some bioactive properties *in vivo* and *in vitro* of phenolic compounds present in olive leaf extracts (OLE) with their attributed mechanisms.

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Table 3. Bioactivity of phenolic compounds in olive leaves extracts

Phenolic content in OLE	Therapeutic properties	Mechanisms/Effects	Ref
	In vitro		
Fractions from OLE including hydroxyoleuropein, rutin and luteolin rutinoside	Anti-obesity	Decreased intracellular lipid accumulation through AMPK-dependent mechanisms in a hypertrophic and insulin resistant adipocyte model	97
Oleuropein-19.8% Luteolin-7-O-glucoside- 0.04%) Apigenin-7-O-glucoside- 0.07% Quercetin- 0.04% Caffeic acid- 0.02%	Protective activity on peripheral blood leukocytes against adrenaline-induced DNA damage	The protective effect of OLE is due to the synergistic activation of several molecular mechanisms such as reactive oxygen species (ROS) scavenging and increasing the antioxidant capacity of cells	98
Oleuropein Hydroxytyrosol	Anticarcinogenic	Decrease of the number of MCF-7 cells by inhibiting the rate of cell proliferation and inducing cell apoptosis. Besides, it provides a significant block of the transition from G1 to S phase manifested by the increase in the number of cells in the G0 / G1 phase.	99

[97] Jimenez-Sanchez, C.; Olivares-Vicente, M.; Rodríguez-Pérez, C.; Herranz-López, M.; Lozano-Sanchez, J.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Encinar, J.A.; Micol, V. AMPK modulatory activity of olive – tree leaves phenolic compounds : Bioassay-guided isolation on adipocyte model and in silico approach. *PLoS One* **2017**, *12*, 1–22.

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Oleuropein, hydroxytyrosol, hydroxytyrosol acetate, luteolin, luteolin-7-O-glucoside y luteolin-O-glucoside	Antiproliferative activity against cancer	Strong antioxidant potency and inhibited cancer and endothelial cell proliferation	100
Rutin- 0.34% Verbascoside- 0.38% Luteolin-7-glucoside- 0.68% Apigenin-7-glucoside- 0.18% Oleuropein- 12.8% Oleuroside- 0.51%	Anti-HIV	OLE inhibits acute infection and cell-to-cell transmission of HIV-1 as assayed by syncytia formation using uninfected MT2 cells co-cultured with HIV-1-infected H9 T lymphocytes	101
	In vitro		
Total phenolic content- 120 mg/g Oleuropein-100 mg/g d.w. Ligstroside- 2.31 mg/g d.w.	Anti-obesity	OLE administration reduced body weight gain, basal glycaemia and insulin resistance, and showed improvement in plasma lipid profile when compared with high fat diet-fed mice. The extract significantly ameliorated the high fat diet-induced altered expression of key adipogenic genes, like perosyxomel proliferator activated receptors	102

[100] Goulas, V.; Exarchou, V.; Troganis, A.N.; Psomiadou, E.; Fotsis, T.; Briasoulis, E.; Gerothanassis, I.P. Phytochemicals in olive-leaf extracts and their antiproliferative activity against cancer and endothelial cells. *Mol. Nutr. Food Res.* **2009**, *53*, 600–608.

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[102] Vezza, T.; Rodríguez-Nogales, A.; Algieri, F.; Garrido-Mesa, J.; Romero, M.; Sánchez, M.; Toral, M.; Martín-García, B.; Gómez-Caravaca, A.M.; Arráez-Román, D.; et al. The metabolic and vascular protective effects of olive (Olea europaea L.) leaf extract in diet-induced obesity in mice are related to the amelioration of gut microbiota dysbiosis and to its immunomodulatory properties. *Pharmacol. Res.* **2019**, *150*, 104487.

		(PPARs), adiponectin and leptin receptor, in adipose tissue.	
OLE	Hypocholesterolemic	OLE possess antiobesity effects in high cholesterol diet rats by activating AMPK and suppressing PPAR γ (Peroxisome proliferator-activated receptor γ) expression in adipose tissues	103
Oleuropein- 94 %	Antioxidant	OLE attenuates ethanol-induced oxidative stress and lipid peroxidation by two mechanisms: a rapid conversion of H_2O_2 to H_2O and by quenching the hydroxyl radicals whereby trapping of HO° induces the oxidative break- down of the phenolic compounds	104
Oleuropein- 356 mg/g dry extract Tyrosol- 3.73 mg/g dry extract Hydroxytyrosol - 4.89 mg/g dry extract Caffeic acid- 49.41 mg/g dry extract	Inhibitory effect of OLE on gentamicin-induced nephrotoxicity	OLE ameliorates gentamicin nephrotoxicity via antioxidant activity, increase of renal glutathione content, and increase of renal antioxidant enzymes activity, except for glutathione peroxidase.	105
Oleuropein	Antiinflamatory	Attenuated tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β),	106

[103] Hadrich, F.; Mahmoudi, A.; Bouallagui, Z.; Feki, I.; Isoda, H.; Feve, B.; Sayadi, S. Evaluation of hypocholesterolemic effect of oleuropein in cholesterol-fed rats. *Chem. Biol. Interact.* **2016**, *252*, 54–60.

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		nitrotyrosine, inducible nitric-oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and poly(ADP-ribose) polymerase (PARP) expression in rats	
Oleuropein-356 mg/g d.w. Tyrosol -3.73 mg/g d.w. Hydroxytyrosol - 4.89 mg/g d.w. Caffeic acid- 49.41 mg/g d.w.	Neuroprotective and anticholesterolemic	OLE slows down the lipid peroxidation process and increases the antioxidant enzymes activity. In addition, OLE influences in brain water content and brain water homeostasis by rising blood–brain barrier (BBB) integrity modulating the cell volume of neurons and astrocytes directly.	107
Oleuropein- 19.8% Luteolin-7-O-glucoside -0.04% Apigenin-7-O-glucoside -0.07% Quercetin 0.04% Caffeic acid- 0.02%	OLE decreases lipid peroxidation	OLE synchronizes antioxidant enzymes and inhibites lipid peroxidation in the liver of rats exposed to cold restraint stress	108
OLE enriched in hydroxytyrosol - 22%	Antiatherosclerotic	The antiatherosclerotic effect of OLE is related to the suppressed inflammatory response	109

[107] Mohagheghi, F.; Bigdeli, M.R.; Rasoulian, B.; Hashemi, P.; Pour, M.R. The neuroprotective effect of olive leaf extract is related to improved blood-brain barrier permeability and brain edema in rat with experimental focal cerebral ischemia. *Phytomedicine* **2011**, *18*, 170–175.

[108] Dekanski, D.; Ristić, S.; Radonjić, N. V.; Petronijević, N.D.; Dekanski, A.; Mitrović, D.M. Olive leaf extract modulates cold restraint stress-induced oxidative changes in rat liver. *J. Serbian Chem. Soc.* **2011**, *76*, 1207–1218.

[109] Wang, L.; Geng, C.; Jiang, L.; Gong, D.; Liu, D.; Yoshimura, H.; Zhong, L. The anti-atherosclerotic effect of olive leaf extract is related to suppressed inflammatory response in rabbits with experimental atherosclerosis. *Eur. J. Nutr.* **2008**, *47*, 235–243.

Hydroxytyrosol and its triacetylated derivative	Lipid-lowering and antioxidant effects	Hypolipidemic effect of triacetylated hydroxytyrosol and hydroxytyrosol, which can lower serum of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) levels decreasing the atherosclerotic index and prevent the lipid peroxidation process:	110

[110] Jemai, H.; Fki, I.; Bouaziz, M.; Bouallagui, Z.; El Feki, A.; Isoda, H.; Sayadi, S. Lipid-lowering and antioxidant effects of hydroxytyrosol and its triacetylated derivative recovered from olive tree leaves in cholesterol-fed rats. *J. Agric. Food Chem.* **2008**, *56*, 2630–2636.

3. CEREALS AND PSEUDOCEREALS

3.1. Introduction

Cereals are defined as a grain or edible seed of the grass family *Gramineae*[111]. Among the major cereals, wheat, rye, rice, oats or barley are the most consumed for humans since old times[112]. Pseudocereals grains are edible seeds that belong to dicotyledonous species; they are known as pseudocereals due to their similar physical appearance and high starch content to true cereals (monocotyledonous of the *Gramineae* family). The most important pseudocereals include quinoa, amaranth and buckwheat. Pseudocereals are a current trend in human diets as they are gluten-free grains[113]. Cereals and pseudocereals are a major source of energy, protein, vitamins and minerals for the world population[114]. The total production of cereals and pseudocereals was 2963 million of tons in the world in 2018. Among them, maize was the most produced cereals in 2018 followed by rice, wheat, barley, sorghum, millet, and oat (**Figure 25**)[115]. Besides, other cereals and pseudocereals are produced in low quantities such as triticale, rye, buckwheat and quinoa. Europe represents 9.97% of the world cereal and pseudocereal production. France is the main producer in Europe and it represented 21% of total European cereal production with 63 million of tons in 2018, whereas Spain represented 8% of cereals and pseudocereals production in Europe with 24 million of tons (**Figure 26)**[115].

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[111] AE, B.D.& B. *Benders' Dictionary of Nutrition and Food Technology*; 7th edn.; Woodhead Publishing: Abington, 1999.

[112] Călinoiu, L.F.; Vodnar, D.C. Whole grains and phenolic Acids : A review on bioactivity, functionality, health benefits and bioavailability. *Nutrients* **2018**, *10*, 1615.

[113] Martínez-Villaluenga, C.; Peñas, E.; Hernández-Ledesma, B. Pseudocereal grains: Nutritional value, health benefits and current applications for the development of gluten-free foods. *Food Chem. Toxicol.* **2020**, *137*, 111178.

[114] Mckevith, B. Nutritional aspects of cereals. *Nutr. Food Sci.* **2004**, *34*, 5–10.

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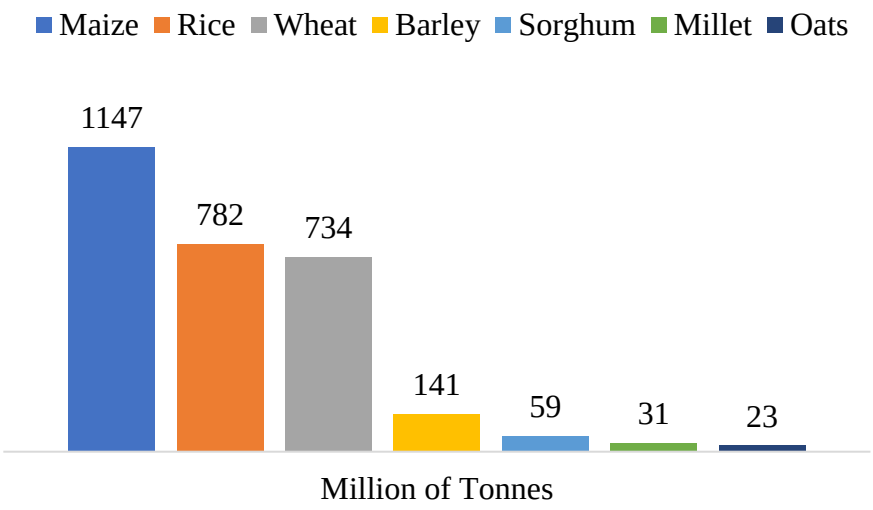

Figure 25. Cereals production in 2018.

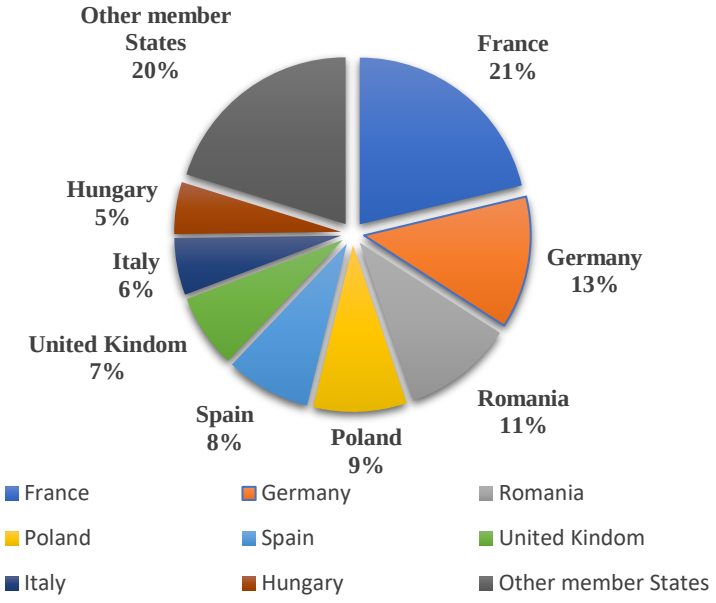

Figure 26. Total cereals and pseudocereals production in Europe by country in 2018[115]

In this section, only the cereals and pseudocereals studied in this thesis have been reported.

3.2. Buckwheat

3.2.1. Introduction

Buckwheat is a pseudocereal belonging to the *Polygonaceae* family that is usually grouped with cereals due to its use. Two main species of buckwheat are produced and consumed around the world: common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tartaricum* L.). Buckwheat is originated from Central and Northeastern Asia and it was cultivated in China during the fifth and sixth centuries. However, it was introduced into Europe through Turkey and Russia in the fourteenth and fifteenth centuries and into North America in the seventeenth century[116].

Figure 27. Buckwheat plant[117].

For many years, its cultivation had decreased, but in recent times it has increased due to its high nutritional value. Buckwheat production in 2018 was 2905294 tons in the world. China is the main producer of buckwheat and it represents 39% of its

[116] Mazza, G.; Oomah, B.D. Buckwheat. In *Encyclopedia of Food Sciences and Nutrition*; Elsevier Science: Summerland, Canada, 2003; pp. 692–699.
[117] https://www.printsofjapan.com/toyokuni_i.htm.

world production. Other major producing countries are Russia (32%), France (6.5%), Ukraine (4.7%), Poland (3.2%), United States of America (2.91%), Kazakhstan (2.8%), Brazil (2.3%), Lithuania (1.8%) and Japan (1%)[115].

Buckwheat grain is composed of a hard-outer layer named hull (pericarp), which has a hard-fibrous structure and surrounds the seed coat (bran), endosperm, and embryo with axis (germ), and two cotyledons (**Figure 28**).

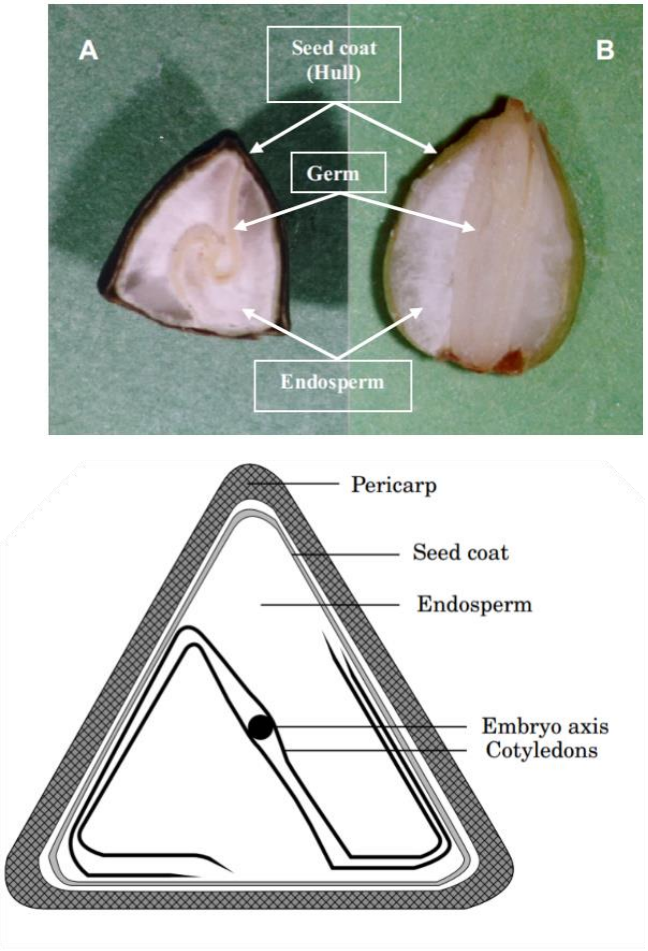

Figure 28. Structure of buckwheat grain[118.119].

[118] Steadman, K.J.; Burgoon, M.S.; Lewis, B.A.; Edwardson, S.E.; Obendorf, R.L. Buckwheat seed milling fractions: Description, macronutrient composition and dietary fibre. *J. Cereal Sci.* **2001**, *33*, 271–278.

[119] Van Hung, P.; Maeda, T.; Morita, N. Buckwheat starch: structure and characteristics–a review. *Eur. J. Plant Sci. Biotechnol.* **2009**, *3*, 23–28.

The nutritional composition of buckwheat depends on the parts of the grain[119]. It is a rich source of nutrients including proteins, polysaccharides, dietary fiber, lipids and micronutrients (minerals and vitamins)[120]. Whole buckwheat groats (dehulled seeds) contain 12% of proteins, 55% starch, 2% ash, 4% lipids, 2% soluble carbohydrates and 7% of total fiber. Buckwheat flour is composed by the endosperm consisting of 6% protein, 75% starch, 1% ash, 1% lipids, 1% soluble carbohydrates and 3% total fiber. Buckwheat bran contains 36% protein, 18% starch, 7% ash, 11% lipids, 6% soluble carbohydrates and 15% total fiber[121]. Therefore, bran contains the highest concentration of protein, half of which is constituted by globulin, whereas its lysine level is higher than in all other cereals[120,121]. Bran also contains the highest quantity of dietary fiber, its soluble fraction (77–92 mg/g) is higher than in wheat bran (43 mg/g), or even in oat bran (72 mg/g)[120,122]. Buckwheat lipids are divided into neutral (81–85%), phospholipids (8–11%), and glycolipids (3–5%). Palmitic (16:0), oleic (18:1), and linoleic (18:2) are the most concentrated (87.3–88%) fatty acids[123]. In buckwheat, the neutral lipids constitute 810–850 mg/g of the total lipids, compared with 350 mg/g in wheat and rye. Generally, lipids are concentrated in the embryo (70–140 mg/g) and their lowest quantity is found in the hull (4–9 mg/g)[122] . Flour obtained from buckwheat is a good source of essential minerals, where microelements of phosphorus (P), potassium (K), magnesium (Mg), and calcium (Ca) are present in concentrations higher than 100 mg/kg dry weight[124]. In addition, buckwheat flour

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[120] Qin, P.; Wang, Q.; Shan, F.; Hou, Z.; Ren, G. Nutritional composition and flavonoids content of flour from different buckwheat cultivars. *Int. J. Food Sci. Technol.* **2010**, *45*, 951–958.

[121] Kinet, J.-M.; Cawoy, V.; Quinet, M.; Jacquemart, A.-L.; Ledent, J.-F. Is Buckwheat (Fagopyrum esculentum Moench) still a valuable crop today? *Eur. J. Plant Sci. Biotechnol.* **2012**, *6*, 1–10.

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[123] Bobkov, S. *Biochemical and Technological Properties of Buckwheat Grains*; Elsevier Inc., 2016; ISBN 9780128037140.

[124] Steadman, K.J.; Burgoon, M.S.; Lewis, B.A.; Edwardson, S.E.; Obendorf, R.L. Minerals, phytic acid, tannin and rutin in buckwheat seed milling fractions. *J. Sci. Food Agric.* **2001**, *81*, 1094–1100.

contains higher levels of copper, manganese, magnesium, potassium, and phosphorus in comparison with wheat, rice and maize[121,125]. The absence of the proteins that form gluten makes buckwheat flour a good raw material for the production of gluten free products. Nevertheless, this flour is not suitable for making bread; thus, it needs to be used mixed with other flours[119]. For instance, buckwheat flour is usually mixed with wheat, maize and/or rice flours to make noodles, pancakes, girdle cakes, cakes, biscuits, cracknels, etc…[126].

3.2.2. Phenolic compounds in buckwheat

Whole buckwheat contains 2 times more phenolic compounds than barley[127]. They are distributed throughout the entire grain, however, the highest total content is found in the outer layers. Phenolic compounds in buckwheat can be classified in phenolic acids and flavonoids, being flavonoids the most abundant phenolic compounds in buckwheat (**Table 4**). The main phenolic acids are hydroxycinnamic acids and its derivatives such as caffeic acid hexose, *p*-coumaric acid and swertiamacroside (1-O-caffeoyl-O-rutinose ester) and hydroxybenzoic acids such as 2-hydroxy-3-O-β-D-glucopyranosylbenzoic acid, protocatechuic-4-O-glucoside acid and syringic acid[128,129]. Flavonoids present in buckwheat are: flavanols such as catechin and its derivatives, epicatechin, propelargonidins

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[128] Verardo, V.; Arráez-Román, D.; Segura-carretero, A.; Marconi, E.; Fernández-Gutiérrez, A.; Caboni, M.F. Determination of free and bound phenolic compounds in buckwheat spaghetti by RP-HPLC-ESI-TOF-MS : Effect of thermal processing from farm to fork. *J. Agric. Food Chem.* **2011**, *59*, 7700–7707.

[129] Inglett, G.E.; Chen, D.; Berhow, M.; Lee, S. Antioxidant activity of commercial buckwheat flours and their free and bound phenolic compositions. *Food Chem.* **2011**, *125*, 923–929.

(epiafzelechin-(4–6)-epicatechin, epiafzelechin-(4-8) -epiafzelechin-(4–8)-epicatechin-O-(3,4-dimethyl)-gallate etc.), proanthocyanidins (procyanidin A and procyanidin B2, etc.); flavonols (rutin, quercetin, etc.) and flavones (orientin (luteolin-8-C-glucoside), isorientin (luteolin-6-C-glucoside), vitexin (apigenin-8-C-glucoside), etc)[128,129,130].

Regarding the content of buckwheat phenolic compounds, phenolic acids such as 2-hydroxy-3-O-β-D-glucopyranosylbenzoic acid, swertimacroside, caffeic acid hexose and protocatechuic-4-O-glucoside acid are found in higher quantities in the free form, whereas syringic acid is found in a higher concentration in its bound form. Swertimacroside is the most concentrated phenolic acid in buckwheat, which total content is 278.2 mg/Kg d.w[128]. (+)-Catechin and (-)-epicatechin are the most concentrated bound flavonoids, which total content represents around 8.4% and 7% of its total phenolic content[128]. In addition, rutin (quercetin-3-rutinoside) is the main free flavonoid in whole buckwheat grain, which is found mostly in its free form. It has been reported that rutin content in the free and bound forms was 331 mg/Kg and 43 mg/Kg d.w., respectively, that represents 23.1% of its total phenolic content[128]. Taking into account the distribution of phenolic compounds in the different parts of buckwheat grain, it has been reported that the seed coat is enriched in epicatechin (257.60 mg/kg), procyanidin B2 (118.6 mg/kg) and epicatechin gallate (61.27 mg/kg), whereas the embryo axis with the cotyledons is rich in rutin (283.37 mg/kg) and catechin (161.41 mg/kg)[131].

Table 4. Classification of the main phenolic compounds in buckwheat.

PHENOLIC ACIDS

[130] Olschläger, C.; Regos, I.; Zeller, F.J.; Treutter, D. Identification of galloylated propelargonidins and procyanidins in buckwheat grain and quantification of rutin and flavanols from homostylous hybrids originating from F . esculentum Â F . homotropicum. *Phytochemistry* **2008**, *69*, 1389–1397.

[131] Kalinová, J.P.; Vrchotová, N.; Tříska, J. Phenolics levels in different parts of common buckwheat (Fagopyrum esculentum) achenes. *J. Cereal Sci.* **2019**, *85*, 243–248.

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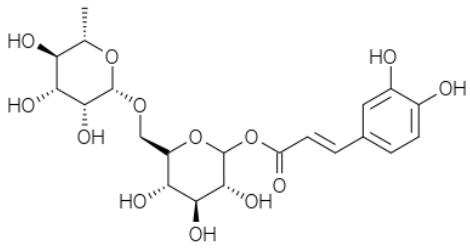
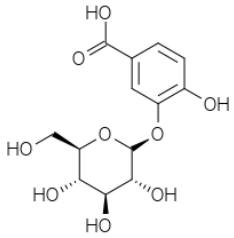
<tr><th colspan="2">HYDROXYCINNAMIC ACIDS</th><th colspan="2">HYDROXYBENZOIC ACIDS</th></tr>
<tr><td colspan="2"> 
Swertiamacroside</td><td colspan="2"> 
Protocatechuic-4-O-glucoside</td></tr>
<tr><th colspan="4">FLAVONOIDS</th></tr>
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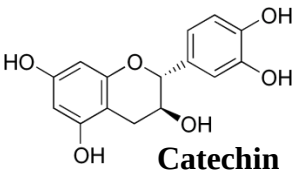
<tr><td> 
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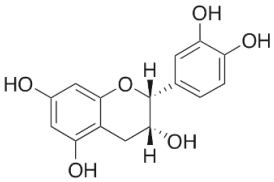
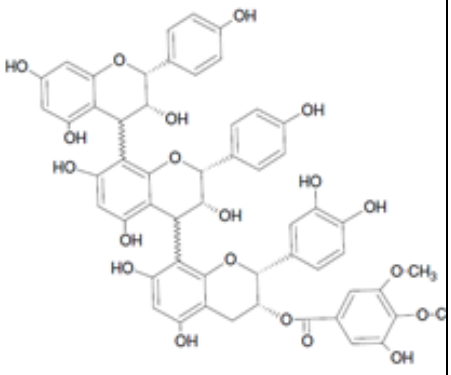
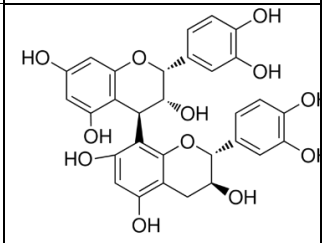
 
Epicatechin</td><td> 
Epiafzelechin-(4.8) epiafzelechin-(4–8)-epicatechin-O-(3,4-dimethyl)-gallate</td><td colspan="2"> 
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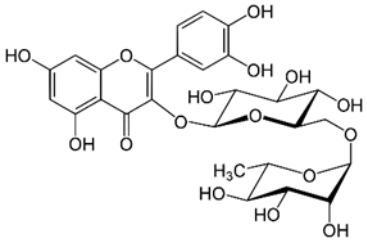
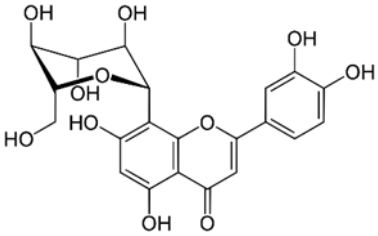
<tr><td colspan="2"> 
Rutin</td><td colspan="2"> 
Orientin</td></tr>
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3.2.3. Bioactivity of phenolic compounds in buckwheat

Phenolic compounds in buckwheat have showed beneficial activities. Concretely, rutin, which is the major compound in buckwheat, possesses antioxidant, anti-inflammatory and anticancer properties and also reduces the fragility of blood vessels related to hemorrhagic diseases and hypertension[122]. **Table 5** shows some

therapeutic properties of phenolic compounds in buckwheat and their mechanisms or effects.

Table 5. Bioactivity of phenolic compounds in buckwheat.

Phenolic compounds in buckwheat	Therapeutic properties	Mechanisms/Effects	Ref
	In vitro		
Rutin	Anticoagulant	Rutin administration is likely to reduce the anticoagulant effect of racemic warfarin, reflecting a significant decrease in the elimination half-life of the more potent S-enantiomer Rutin administration reduce the anticoagulant effect of racemic warfarin, reflecting a significant decrease in the elimination half-life of the more potent S-enantiomer	132
Rutin	Antidiabetic	Reduce glucolipotoxic effects through activating AMP-activated protein kinase signaling to inhibit the activities of lipogenic enzymes and ameliorating mitochondrial function	133
Rutin	Anticancerogenic acid	Rutin showed the highest cytotoxic effects against SW480 cells	134
Buckwheat extract enriched in free caffeic acid and rutin	Anticancerogenic	Inhibition on the proliferation of MDA-MB-231 cells through the p38 MAPK signaling pathway	135

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[135] Li, F.; Zhang, X.; Li, Y.; Lu, K.; Yin, R.; Ming, J. Phenolics extracted from tartary (Fagopyrum tartaricum L. Gaerth) buckwheat bran exhibit antioxidant activity, and an antiproliferative effect on human breast cancer MDA-MB-231 cells through the

		by regulating the cell cycle and/or inducting cell apoptosis	
Quercetin and pelargonidin	Anti-inflammatory	Inhibition of nuclear factor-κB (NF-κB)	136
Proanthocyanidins	Improve the activity of stomach helping the digestion of ingested foods	Proanthocyanidins reduce nitrous acid producing nitric oxide (NO) when the flour was suspended in acidified saliva or in acidic buffer solution in the presence of nitrite	137.
	In vivo		
Rutin	Anti-cancerogenic	Reduction of tumor weight and volume when compared with the control groups	138
Rutin	Anti-inflamatory	Improve inflammatory status and thereby to reduce medical disorders associated with high fat diet-induced obesity	139

p38/MAP kinase pathway. *Food Funct.* **2017**, *8*, 177–188.

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[139] Guo, X.; Tang, R.; Yang, S.; Lu, Y.; Luo, J.; Liu, Z. Rutin and its combination with inulin attenuate gut dysbiosis, the inflammatory status and endoplasmic reticulum stress in Paneth cells of obese mice induced by high-fat diet. *Front. Microbiol.* **2018**, *9*, 1–11.

3.3. Wheat

3.3.1. Introduction

The first cultivation of wheat was around 10000 years ago, as part of the 'Neolithic Revolution', when there was a transition from hunting and gathering of food to settled agriculture. The first cultivated forms were diploid and tetraploid wheats and they were originated from the Southeastern part of Turkey. Wheat cultivation extended to the Near East by about 9000 years ago when hexaploid wheat appeared[140]. It is a rich source of proteins, starch, minerals, B vitamins and dietary fiber and, currently, it is the third most consumed cereal for human food after maize and rice[141].

Figure 29. Common wheat (*Triticum aestivum*)[142].

Wheat world production was 734 million of tons in 2018. Around 95% of the wheat produced is *Triticum aestivum*, which is a hexaploid specie usually named as "common", "bread" or "soft" wheat (**Figure 29**)[143]. The main wheat producing

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[143] Dinu, M.; Whittaker, A.; Pagliai, G.; Benedettelli, S.; Sofi, F. Ancient wheat species and human health: Biochemical and clinical implications. *J. Nutr. Biochem.* **2018**,

countries are China, India, Russia, USA, France, Canada, Pakistan, Ukraine, Australia and Germany. China is the first wheat producer with 17.8% of the total world production followed by India with 13.6%[115]. Grain comprises of 13-17% bran, 2-3% germ or embryo and 80-85% endosperm rich in starch. Bran is composed by aleurone, the intermediate layer (hyaline), testa and inner and outer pericarp. **Figure 30** shows the chemical composition of the different parts of wheat grain**.** Briefly, wheat bran contains 33.4-63% of dietary fiber, 9.60-18.6% of proteins, 9.10-38.9% of starch, 3.9-8.10% ash and 60.0-75% of total carbohydrates[144]. Aleurone layer represents until the 50% of the wheat bran and it is particularly rich in nutrients such as protein (30%), minerals (12%), phytates, B vitamins such as niacin and folates, lipids (9%) and bioactive compounds such as phenolic compounds[145].

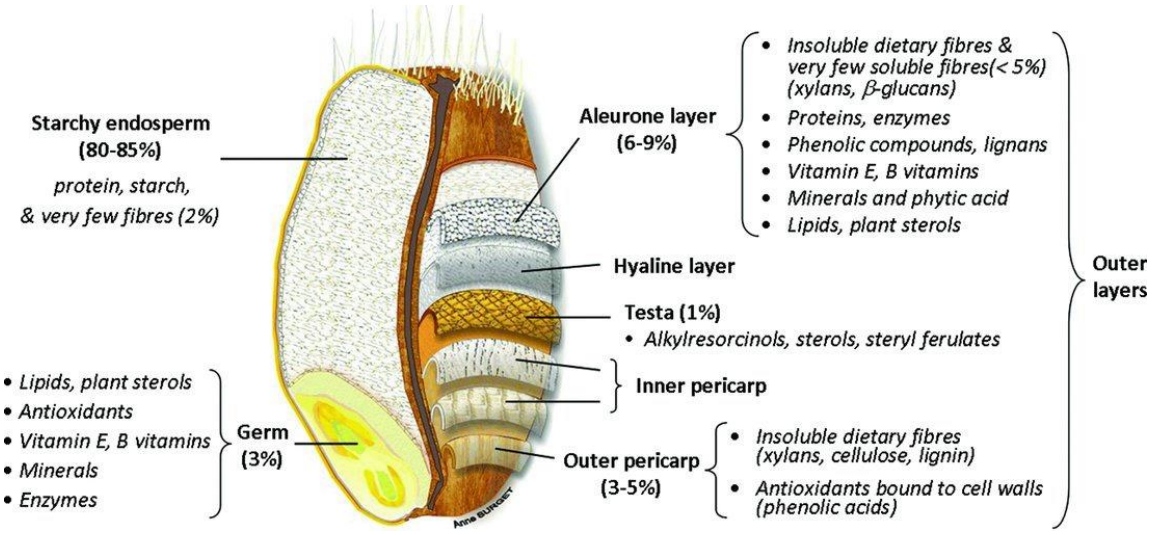

Figure 30. Structure of wheat grain[144]

52, 1–9.

[144] Onipe, O.O.; Jideani, A.I.O.; Beswa, D. Composition and functionality of wheat bran and its application in some cereal food products. *J. Food Sci. Technol.* **2015**, *50*, 2509–2518.

[145] Brouns, F.; Hemery, Y.; Price, R.; Anson, N.M. Wheat Aleurone: Separation, Composition, Health Aspects, and Potential Food Use. *Crit. Rev. Food Sci. Nutr.* **2012**, *52*, 553–568.

3.3.2. Phenolic compounds in wheat

Phenolic compounds mainly present in wheat grain are phenolic acids, alkylresorcinols and flavonoids (**Table 6**). Most phenolic acids are found in three forms: soluble free acids, soluble conjugated moieties esterified to sugars and other low molecular mass compounds, and insoluble bound moieties esterified to the arabinoxylans and other cell wall structural components. Ferulic acid, *p*-coumaric acid and syringic acid are mainly found in the bound form, whereas sinapic and vanillic acids are mainly found in their conjugated form[146]. Ferulic acid is the most concentrated hydroxycinnamic acid in wheat and it can be found free, dimerized or esterified with polysaccharides and proteins in the cell walls[147]. Ferulic acid dimers are found in various forms such as 5-5-, 8-O-4-, 8-5-, and 8-8-diferulic acids[148]. Concentration of ferulic acid in wheat grain ranges from 400 to 870 µg/g d.w., whereas dimers of ferulic range from 19 to 280 µg/g d.w.[145,148,149,150]. The greatest concentration of phenolic acids is found in bran; in this wheat layer the content of ferulic acid and its dimers ranges between 1376-5600 µg/g and 780-1550 µg/ g d.w., respectively[144,145,150,151-153]. However, aleurone is the most concentrated layer in ferulic acid, which shows 70% of total ferulic content in wheat. The outer pericarp and inner pericarp are enriched in ferulic acid (FA)

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[146] Li, L.; Shewry, PR.; Ward, JL.; Phenolic acids in wheat varieties in the HEALTHGRAIN diversity screen. *J. Agric. Food Chem.* **2008**, *56*, 9732–9739.

[147] Fazary, A.E.; Ju, Y.H. Feruloyl esterases as biotechnological tools: Current and future perspectives. *Acta Biochim. Biophys. Sin. (Shanghai).* **2007**, *39*, 811–828.

[148] Boz, H. Ferulic acid in cereals - A review. *Czech J. Food Sci.* **2015**, *33*, 1–7.

[149]Zhao, Z.; Moghadasian, M.H. Chemistry, natural sources, dietary intake and pharmacokinetic properties of ferulic acid: A review. *Food Chem.* **2008**, *109*, 691–702.

[150] Mattila, P.; Pihlava, J.M.; Hellström, J. Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. *J. Agric. Food Chem.* **2005**, *53*, 8290–8295.

[151] Laddomada, B.; Caretto, S.; Mita, G. Wheat bran phenolic acids: Bioavailability and stability in whole wheat-based foods. *Molecules* **2015**, *20*, 15666–15685.

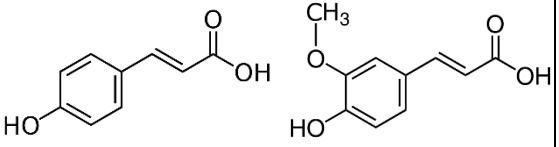
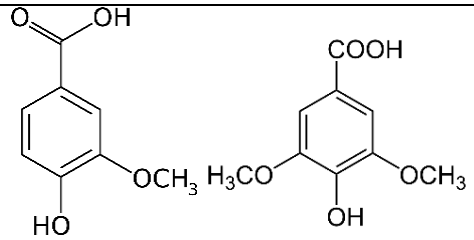
[152] Pazo-Cepeda, V.; Benito-Román, Ó.; Navarrete, A.; Alonso, E. Valorization of Wheat Bran: Ferulic Acid Recovery Using Pressurized Aqueous Ethanol Solutions. *Waste and Biomass Valorization* **2019**.

[153] Zhao, Z.; Egashira, Y.; Sanada, H. Phenolic antioxidants richly contained in corn bran are slightly bioavailable in rats. *J. Agric. Food Chem.* **2005**, *53*, 5030–5035.

oligomers. Wheat bran also contains a high content of sinapic and *p*-coumaric acid (90-280 µg/g d.w. and 130-250 µg/g)[144,145,150-153].

Alkylresorcinols (ARs) are phenolic lipids that are commonly present in wheat. The alkyl side chain may contain between 17 and 25 carbon atoms. Around 5–10% of alkylresorcinols in wheat present unsaturated, keto- or hydroxyl-substituted alkyl chains [154,155]. ARs concentrations in whole wheat range from 280 to 1429 µg/g d.w.[42,156]. However, these compounds are mainly located in the testa[145]. In addition, wheat contains flavonoids in low quantity, which are mainly located in the pericarp such as 5,7,4'-trihydroxyflavone (apigenin) which is in the range of 190-365 µg/g d.w[157].

Table 6. Classification of the main phenolic compounds found in wheat.

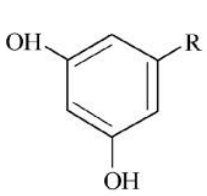
PHENOLIC ACIDS	
HYDROXYCINNAMIC ACIDS	HYDROXYBENZOIC ACIDS
 p-coumaric acid . ….. Ferulic acid	 Vanillic acid.……Syringic acid
ALKYLRESORCINOLS	**FLAVONES**

[154] Luthria, D.L.; Lu, Y.; John, K.M.M. Bioactive phytochemicals in wheat : Extraction , analysis , processing , and functional properties. *J. Funct. Foods* **2015**, *18*, 910–925.

[155] Chen, Y.; Ross, A.B.; Åman, P.; Kamal-Eldin, A. Alkylresorcinols as markers of whole grain wheat and rye in cereal products. *J. Agric. Food Chem.* **2004**, *52*, 8242–8246.

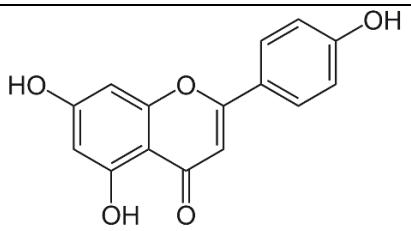
[156] Landberg, R.; Kamal-Eldin, A.; Salmenkallio-Marttila, M.; Rouau, X.; Aman, P. Localization of alkylresorcinols in wheat, rye and barley kernels. *J. Cereal Sci.* **2008**, *48*, 401–406.

[157] Hernández, L.; Afonso, D.; Rodríguez, E.M. Phenolic compounds in wheat grain cultivars. *Plant Foods Hum. Nutr.* **2011**, *66*, 408–415.



$R = C_nH_{2n+1}$

n = 15
n = 17
n = 19
n = 21
n = 23
n = 25



Apigenin

3.3.3. Bioactivity of phenolic compounds in wheat

Ferulic acid is the most concentrated phenolic compound in wheat and possess a wide range of bioactivities against chronic human diseases and oxidative damage due to its high antioxidant activity[158,159]. Its potent antioxidant activity is associated with its phenolic hydroxyl group, which promptly terminates radical chain reactions by a radical scavenging mechanism. A reactive radical (R•) abstracts a radical hydrogen from ferulic acid generating a stable compound (R-H) and a phenoxyl radical which is stabilized by resonance along both the entire aromatic ring and the unsaturated side chain. The resultant stabilized phenoxyl radical is practically unreactive, not initiating any further radical chain reaction[160].. **Table 7** shows some properties of the main phenolic compounds in wheat.

[158] Gani, A.; Sm, W.; FA, M.; Hameed, G. Whole-grain cereal bioactive compounds and their health benefits: A review. *Food Process. Technol.* **2012**, *3*, 1–10.

[159] de Oliveira Silva, E.; Batista, R. Ferulic Acid and Naturally Occurring Compounds Bearing a Feruloyl Moiety: A Review on Their Structures, Occurrence, and Potential Health Benefits. *Compr. Rev. Food Sci. Food Saf.* **2017**, *16*, 580–616.

[160] Batista, R. Uses and potential applications of ferulic acid. *Ferulic Acid Antioxid. Prop. Uses Potential Heal. Benefits* **2014**, 39–70.

Table 7. Bioactivity of phenolic compounds in wheat.

Phenolic compounds in wheat	Therapeutic properties	Mechanisms/Effects	Ref
	In vitro		
Ferulic acid	Photo protection	Ferulic acid exhibits beneficial effects against UVB-induced inflammatory responses through down-regulating COX-2 and TNF-α expressions and activating PPARα/γ agonists.	161
Ferulic acid	Anticarcinogenic	Ferulic acid decreases cell viability and colony formation while inhibiting migration of MIA PaCa-2 human pancreatic cancer cells in vitro	162
Ferulic acid	Antibactereological effect against the pathogenic Gram-negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Staphylococcus aureus*	The antibacterial mechanism of ferulic acid was found to be related to the inhibition of arylamine N-acetyltransferase, a specific enzyme that catalyzes acetylation of arylamines in the bacteria	163
Procyanidin B3	Antiarteriosclerosis	Procyanidin B3 has the capacity to inhibit the binding of oxidized LDL to the lectin-like oxidized LDL receptor (lectin-like oxidized LDL receptor-1	164

[161] Kanagalakshmi, A.; Agilan, B.; Mohana, S.; Ananthakrishnan, D.; Velmurugan, D.; Karthikeyan, R.; Ganesan, M.; Srithar, G. Ferulic acid modulates ultraviolet-B radiation mediated inflammatory signaling in human dermal fibroblasts. *J. Res. Biol.* **2014**, *4*, 1505–1515.

[162] Fahrioğlu, U.; Dodurga, Y.; Elmas, L.; Seçme, M. Ferulic acid decreases cell viability and colony formation while inhibiting migration of MIA PaCa-2 human pancreatic cancer cells in vitro. *Gene* **2016**, *576*, 476–482.

[163] Saavedra, M.; Borges, A.; Dias, C.; Aires, A.; Bennett, R.; Rosa, E.; Simões, M. Antimicrobial Activity of Phenolics and Glucosinolate Hydrolysis Products and their Synergy with Streptomycin against Pathogenic Bacteria. *Med. Chem. (Los. Angeles).* **2012**, *6*, 174–183.

[164] Mizuno, M.; Nakanishi, I.; Matsubayashi, S.; Imai, K.; Arai, T.; Matsumoto, K. ichiro; Fukuhara, K. Synthesis and antioxidant activity of a procyanidin B3 analogue. *Bioorganic Med. Chem. Lett.* **2017**, *27*, 1041–1044.

		(LOX-1), which is involved in the pathogenesis of arteriosclerosis	
Wheat extract (Alkylresorcynols such as 5-(16-heneicosenyl) resorcinol (trans), 5-(14-nonadecenyl) resorcinol (trans) and 5-(2-oxotricosanyl) resorcinol)	Anticarcinogenic	High inhibitory effect against human prostrate adenocarcinoma cells	165
Wheat extract with a purity >95% of alkylresorcynols (C17:0, 1.5%; C19:0, 20.8%; C21:0, 58.2%; C23:0, 9% and C25:0, 10.5%)	Protective activity	Protect human retinal pigment epithelial cells against H_2O_2-induced oxidative damage through Akt-dependent Nrf2/HO-1 signaling	166
In vivo			
Ferulic acid	Antidiabetic	Ferulic acid encapsulated chitosan nanoparticles caused an enhancement in body weight, decrease in blood glucose level along with a regulatory effect on blood lipid profile of diabetic rats.	167
Alkylresorcinol C21	Anti-cancerogenic	the combination of alkylresorcinol C21 and butyrate inhibited the growth of human colon cancer cells and induced apoptosis	168

[165] Liu, L.; Winter, K.M.; Stevenson, L.; Morris, C.; Leach, D.N. Wheat bran lipophilic compounds with in vitro anticancer effects. *Food Chem.* **2012**, *130*, 156–164.

[166] Wang, Z.; Hao, Y.; Wang, Y.; Liu, J.; Yuan, X.; Sun, B.; Wang, J. Wheat alkylresorcinols protect human retinal pigment epithelial cells against H_2O_2-induced oxidative damage through Akt-dependent Nrf2/HO-1 signaling. *Food Funct.* **2019**, *10*, 2797–2804.

[167] Panwar, R.; Raghuwanshi, N.; Srivastava, A.K.; Sharma, A.K.; Pruthi, V. In-vivo sustained release of nanoencapsulated ferulic acid and its impact in induced diabetes. *Mater. Sci. Eng. C* **2018**, *92*, 381–392.

[168] Zhao, Y.; Shi, L.; Hu, C.; Sang, S. Wheat Bran for Colon Cancer Prevention: The Synergy between Phytochemical Alkylresorcinol C21 and Intestinal Microbial Metabolite Butyrate. *J. Agric. Food Chem.* **2019**, *67*, 12761–12769.

3.4. Barley

3.4.1. Introduction

Barley (*Hordeum vulgare* L.) is one of the most ancient cereal crops grown in the world today. Archeological evidences have shown the existence of barley along the River Nile in Egypt around 17,000 years ago[169].

Figure 31. Barley (*Hordeum vulgare)*[170].

It is one of the most cultivated crops globally (12% of total cereal cultivated). Around 65% of barley is used for animal feed and 33% as a source of malt for alcoholic beverages, especially beer, although only 2% is used directly for human consumption[171]. The production of barley has increased in the last 5 years and the annual production reached over 141 million tons in 2018[115]. The main barley producers are Russia, France, Germany, Australia, Spain, Canada, Ukraine,

[169] Badr, A.; M, K.; Sch, R.; Rabey, H. El; Effgen, S.; Ibrahim, H.H.; Pozzi, C.; Rohde, W.; Salamin, F. On the origin and domestication history of barley (Hordeum vulgare). *Mol. Biol. Evol.* **2000**, *17*, 499–510.

[170] Barley. In *2020*; Encyclopedia Britannica, inc.

[171] Sullivan, P.; Arendt, E.; Gallagher, E. The increasing use of barley and barley by-products in the production of healthier baked goods. *Trends Food Sci. Technol.* **2013**, *29*, 124–134.

Turkey, United Kingdom and Argentina. Russia is the main barley producer and it represents 12% of its total world production. Spain was the fifth largest barley producer with 6.5% of its global production in 2018[115]. Barley is composed by approximately 70% starch, 10-20% protein, 5-10% β-glucan, 2-3% free lipids and 2.5% minerals and its total dietary fiber and soluble dietary is ranged from 11 to 34% and 3-20% respectively. Concretely, barley is rich in the polysaccharide (1 → 3)(1 → 4)-β-D-glucan (beta-glucan)[171]. Barley grain can be divided into four main parts: hull, bran, endosperm and germ (**Figure 32**). During the germination/brewing process, the starchy endosperm of malted barley is subjected to enzymatic degradation, providing a liberation of fermentable (maltose and maltotriose) and non-fermentable (dextrins) carbohydrates, soluble proteins, polypeptides and amino acids. The wort is the resulting medium, which will be fermented into beer by the yeast. The insoluble grain components, which are composed mainly by bran, are the brewers spent grains (BSG)[172]. BSG is the main by-product of the beer-brewing process with an annual global production of around 39 million tonnes. During the beer production, about 20 kg of wet BSG per 100 L of brewed beer are generated, which is partly used for animal feed and discarded[173]. Nevertheless, BSG are a rich source of proteins (19-30%) and also contain cellulose (12-25%), hemicellulose (20-25%), lignin (12-28%), lipid (10%), ash (2-5%) and low quantities of phenolic compounds (0.7-2%). Therefore, their reuse would be advantageous for the food industry[172].

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[172] Lynch, K.M.; Steffen, E.J.; Arendt, E.K. Brewers' spent grain: a review with an emphasis on food and health. *J. Inst. Brew.* **2016**, *122*, 553–568.

[173] Mussatto, S.I.; Dragone, G.; Roberto, I.C. Brewers' spent grain: Generation, characteristics and potential applications. *J. Cereal Sci.* **2006**, *43*, 1–14.

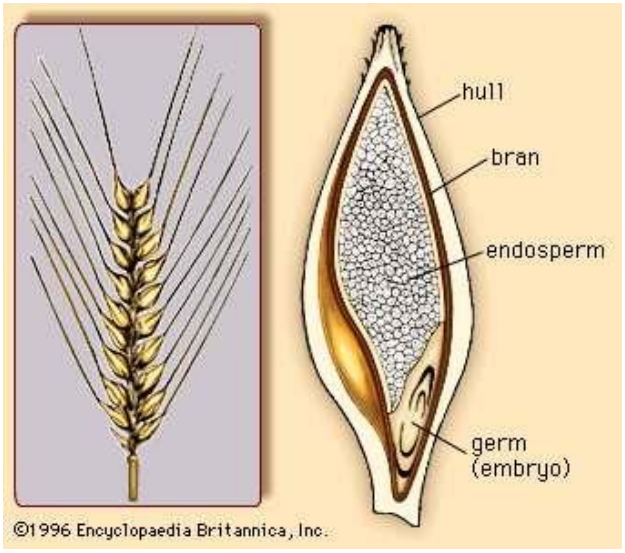

Figure 32. Structure of barley grain[174].

3.4.2. Phenolic compounds in barley

Phenolic compounds in barley can be classified in hydroxycinnamic acid, hydroxybenzoic acids, flavonols, anthocyanins and proanthocyanidins (**Table 8**). Phenolic acids are primarily located in the outer layers of the kernel[175]. They can be found in a free, free-esterified or in an insoluble bound form and they are distributed through the different layers of the grains[176,177]. These phenolic acids are found in a high quantity in their bound form. Ferulic acid is the most abundant bound phenolic acid and it is present in its two isomeric forms: *trans* and *cis* ferulic acids. Total bound ferulic acid content represents approximately 51.2-74.2% of total bound phenolic compounds[178]. *trans*-Ferulic acid is the most abundant isomer

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[174] Kent-Jones, D.W.; Singh, R.P. Cereal processing. *Encycl. Br. inc* **2010**.

[175] Dykes, L.; Rooney, L.W. Phenolic compounds in cereal grains and their health benefits. *Cereal Foods World* **2007**, *52*, 105–111.

[176] Dv, M.; Guido, L.F.; Dostálek, P.; Skulilová, Z.; Moreira, M.M.; Barros, A.A.; Brew, J.I. Antioxidant properties of free, soluble ester and insoluble-bound phenolic compounds in different barley varieties and corresponding malts. *J. Inst. Brew.* **2008**, *114*, 27–33.

[177] Carvalho, D.O.; Curto, A.F.; Guido, L.F. Determination of phenolic content in different barley varieties and corresponding malts by liquid chromatography-diode array detection-electrospray ionization tandem mass spectrometry. *Antioxidants.* **2015**, 4, 563-576.

[178] Gómez-Caravaca, A.M.; Verardo, V.; Berardinelli, A.; Marconi, E.; Caboni, M.F. A

with a concentration that ranges from 241.6 to 761.8 µg/g d.w[148-150,178,179]. The second bound phenolic acid in terms of concentration is *p*-coumaric acid; its content ranges between 10.1 and 32.9% of total bound phenolic compounds[178,180]. Other minor bound phenolic acids found in barley are vanillic, syringic, sinapic, *p*-hydroxybenzoic, synapoyl hexose, caffeoyl hexose and caffeic acid[176].

Regarding flavonoids, flavan-3-ols such as (+)-catechin and (−)-epicatechin and proanthocyanidins dimers (prodelphinidin B3 and procyanidin B3) and trimers (procyanidin C2) are the most concentrated phenolic compounds in barley, which are mainly found in their free form[181,182]. Catechin in barley ranges between 5.1 and 17.1% of total free phenolic compounds[178]. Prodelphinidin B3 (gallocatechin-(4α→8)-catechin) and procyanidin B3 (catechin-(4-alpha-->8)-catechin) are the two major phenolic compounds, which represent around 13–25% and 18–26% of total free phenolic compounds in barley, respectively[178,180,183]. In addition, anthocyanins are mainly located in the pericarp of barley colored grains where they exist mostly as glycoside derivatives, including cyanidin-3-glucoside, penidin-3-glucoside, and delphinidin-3-glucoside[184]. Changes and degradation of

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chemometric approach to determine the phenolic compounds in different barley samples by two different stationary phases: A comparison between C18 and pentafluorophenyl core shell columns. *J. Chromatogr. A* **2014**, *1355*, 134–142.

[179] Jilek, M.L.; Bunzel, M. Dehydrotriferulic and dehydrodiferulic acid profiles of cereal and pseudocereal flours. *Cereal Chem.* **2013**, *90*, 507–514.

[180] Holtekjolen, A.K.; Kinitz, C.; Knutsen, S.H. Flavanol and Bound Phenolic Acid Contents in Different Barley Varieties. *J. Agric. Food Chem.* **2006**, *54*, 2253–2260.

[181] Verardo, V.; Bonoli, M.; Marconi, E.; Caboni, M.F.; . Determination of free flavan-3-ol content in barley (*Hordeum vulgare* L.) air-classified flours : Comparative study of HPLC-DAD / MS and spectrophotometric determinations. *J. Agric. Food Chem.* **2008**, *56*, 6944–6948.

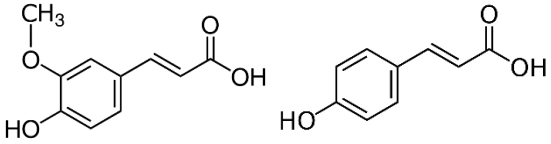
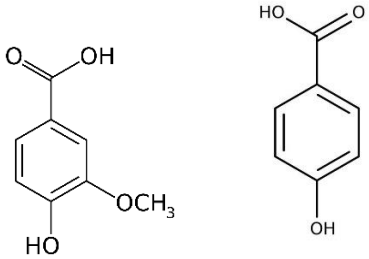
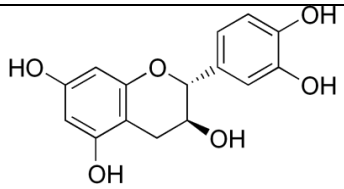
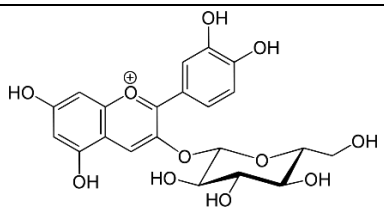
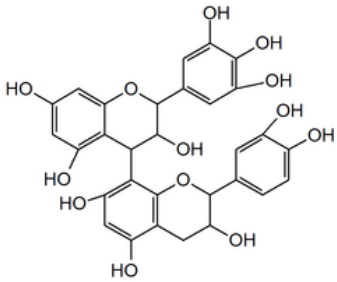
[182] Gómez-Caravaca, A.M.; Maggio, R.M.; Cerretani, L. Chemometric applications to assess quality and critical parameters of virgin and extra-virgin olive oil. A review. *Anal. Chim. Acta* **2016**, *913*, 1–21.

[183] Dvorakova, M.; Moreira, M.M.; Dostalek, P.; Skulilova, Z.; Guido, F.; Barros, A.A. Characterization of monomeric and oligomeric flavan-3-ols from barley and malt by liquid chromatography – ultraviolet detection – electrospray ionization mass spectrometry. *J. Chromatogr. A* **2008**, *1189*, 398–405.

[184] Idehen, E.; Tang, Y.; Sang, S. Bioactive phytochemicals in barley. *J. Food Drug Anal.* **2016**, *25*, 148–161.

endogenous phenolic compounds could occur during the malting process causing modifications in the composition of barley[177].

Table 8. Classification of the main phenolic compounds of barley.

<table>
<tr><th colspan="3">PHENOLIC ACIDS</th></tr>
<tr><td colspan="2">HYDROXYCINNAMIC ACIDS</td><td>HYDROXYBENZOIC ACIDS</td></tr>
<tr><td colspan="2"> 
Ferulic acid……. P-coumaric acid</td><td> 
…Vanillic acid…… p-hydroxybenzoic acid</td></tr>
<tr><th colspan="3">FLAVONOIDS</th></tr>
<tr><td>FLAVANOLS</td><td>ANTHOCYANINS</td><td>PROANTHOCYANIDINS</td></tr>
<tr><td> 
Catechin</td><td> 
Cyanidin-3-glucoside</td><td> 
Prodelphinidin B3</td></tr>
</table>

3.4.3. Bioactivity of phenolic compounds in barley

Previous studies have reported that barley extracts enriched in phenolic acids such as ferulic and *p*-coumaric acids, have shown to possess antiinflammatory and anticholesterolemic properties[185]. In addition, proanthocyanidins which are found in high quantities in barley have shown beneficial properties including antioxidant,

[185] Hole, A.S.; Grimmer, S.; Jensen, M.R.; Sahlstrøm, S. Synergistic and suppressive effects of dietary phenolic acids and other phytochemicals from cereal extracts on nuclear factor kappa B activity. *Food Chem.* **2012**, *133*, 969–977.

anticancer, antidiabetic, neuroprotective, and antimicrobial[186,187]. **Table 9** shows some beneficial properties of the main phenolic compounds in barley.

Table 9. Bioactivity studies of barley phenolic compounds

Phenolic compounds in barley	Therapeutic properties	Mechanisms/Effects	Ref
	In vitro		
Barley extract enriched in free and bound phenolic acids (ferulic, caffeic, ***p***-coumaric and sinapic acids)	Anti-inflammatory	Modulate Nuclear factor kappa B (NF-kB) activity. NF-kB is a transcription factor that regulates pro-inflammatory genes by controlling the processes of innate immunity, apoptosis, cell proliferation, and cell survival.	185
Procyanidin B3	Antiarteriosclerosis	Inhibit the binding of oxidized LDL to the lectin-like oxidized LDL receptor (lectin-like oxidized LDL receptor-1 (LOX-1), which is involved in the pathogenesis of arteriosclerosis	164
	In vitro* and *in vivo		
Black highland barley extract (ferulic acid- 9.14 mg/g, p-coumaric acid-14.59 mg/g, catechin-4.78 mg/g)	Anticholesterolemic	Barley extract showed significant decreases in total cholesterol, low-density lipoprotein cholesterol and the atherosclerosis index and increased high-density lipoprotein cholesterol levels.	187

[186]Rauf, A.; Imran, M.; Abu-izneid, T.; Patel, S. Proanthocyanidins : A comprehensive review. *Biomed. Pharmacother. J.* **2019**, *116*.

[187] Shen, Y.; Zhang, H.; Cheng, L.; Wang, L.; Qian, H.; Qi, X. In vitro and in vivo antioxidant activity of polyphenols extracted from black highland barley. *Food Chem.* **2016**, *194*, 1003–1012.

Among phenolic acids, *p*-coumaric acid is found in most cereals and possesses antitumor activities and antibacterial activities[188,189]. In addition, *p*-coumaric acid has shown antiinflammatory effect in synovial tissue of adjuvant-induced arthritic rats by lowering the expression of inflammatory mediator TNF-α. Caffeic acid has shown a decrease in the expression of inflammatory mediators such as IL-6, IL-1-β, tumor necrosis factor (TNF)-α [190]. Moreover, other minor phenolic acids in cereals such as syringic acid has shown antioxidant, antibacterial and hepatoprotective activities[191,192]. Another phenolic acid with beneficial activities is vanillic acid that has showed antisicking and anthelmintic activities, and it can also suppress hepatic fibrosis in chronic liver injury[191]. In addition, protocatechuic acid possesses numerous beneficial properties including antioxidant, antimicrobial, cytotoxic, chemopreventive, apoptotic, neuroprotective properties, and also a LDL oxidation inhibitor[193-196].

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[188] Heleno, S.A.; Ferreira, I.C.F.R.; Calhelha, R.C.; Esteves, A.P.; Martins, A.; Queiroz, M.J.R.P. Cytotoxicity of Coprinopsis atramentaria extract, organic acids and their synthesized methylated and glucuronate derivatives. *Food Res. Int.* **2014**, *55*, 170–175.

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[194] Alves, M.J.; Ferreira, I.C.F.R.; Froufe, H.J.C.; Abreu, R.M.V.; Martins, A.; Pintado, M. Antimicrobial activity of phenolic compounds identified in wild mushrooms, SAR analysis and docking studies. *J. Appl. Microbiol.* **2013**, *115*, 346–357.

[195] Yin, M.C.; Lin, C.C.; Wu, H.C.; Tsao, S.M.; Hsu, C.K. Apoptotic effects of protocatechuic acid in human breast, lung, liver, cervix, and prostate cancer cells: Potential mechanisms of action. *J. Agric. Food Chem.* **2009**, *57*, 6468–6473.

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4. SAMPLE PRETREATMENT

Different sample pretreatment processes can be applied in plants before the extraction of phenolic compounds depending on the nature of the sample matrix[197]. Drying is a very useful technique to extend the shelf-life of plants with a high phenolic content and a potent antioxidant activity[198]. Commonly, plants are dried by air-drying or oven-drying[199]. Higher quantities of phenolics can be extracted from air-dried than from oven-dried samples due to the degradation of phenolic compounds related to thermal process[200]. Dried samples are usually milled or grounded to obtain a certain particle size. Higher extraction yields of phenolics are obtained by milling the sample into smaller particle sizes. The smaller the particle size of the sample, the shorter the path that the solvent has to travel, which leads in shorter extraction times for maximum phenolic recovery. Indeed, smaller particles also have a much larger surface area providing a greater mass transfer rate[201]. In general, milling into small particle size in combination with air-drying is advised as an useful plant pretreatment prior to the extraction[199]. For that reason, these pretreatment techniques were applied in olive leaves.

Besides, dry processing technologies have been applied in cereals and pseudocereals to obtain flours fractions enriched in bioactive components. The dry technologies often involve particle size reduction of groats (de-hulled grains) by dry milling to produce meal/flour followed by separation of the particulates based

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Chem. Toxicol. **2006**, *44*, 436–443.

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[201] Yeop, A.; Sandanasam, J.; Pan, S.F.; Abdulla, S.; Yusoff, M.M.; Gimbun, J. The effect of particle size and solvent type on the gallic acid yield obtained from Labisia pumila by ultrasonic extraction. *MATEC Web Conf.* **2017**, *111*, 1–5.

on size and density in order to achieve flour fractions. These dry fractionation techniques are explained below.

4.1. Fractionation techniques of cereal grains

During cereal grain processing, bran and germ are separated from the starchy endosperm to produce white flours (refined flour), which are used in making pasta and bakery products. Therefore, outer layers are removed from the flour fraction. However, these tissues contain most of the micronutrients such as minerals, vitamins, fibers, and phytochemicals that could contribute largely to increase the nutritional quality of human food if these were included in flours or used as food ingredients. Consequently, dry fractionation technologies have been developed to provide new flours enriched in healthy compounds[202]. Dry fractionation is relatively energy efficient and it does not require solvent removal. Some grain pretreatments such as dehulling, peeling and pearling are applied before dry fractionation in cereals. Dry technologies often start with a particle size reduction of groats (dehulled grains) by dry milling (i.e. roller, impact, hammer, etc.) to obtain a meal/flour. This process is followed by the separation of the obtained particles based on size and/or density to achieve flour fractions enriched in phenolic compounds (sieving and air-classification) (**Figure 33**)[202,203].

[202] Hemery, Y.; Rouau, X.; Lullien-Pellerin, V.; Barron, C.; Abecassis, J. Dry processes to develop wheat fractions and products with enhanced nutritional quality. *J. Cereal Sci.* **2007**, *46*, 327–347.

[203] Kołodziejczyk, P.; Makowska, A.; Pospieszna, B.; Michniewicz, J.; Paschke, H. Chemical and nutritional characteristics of high-fibre rye milling fractions. *Acta Sci. Pol. Technol. Aliment.* **2018**, *17*, 149–157.

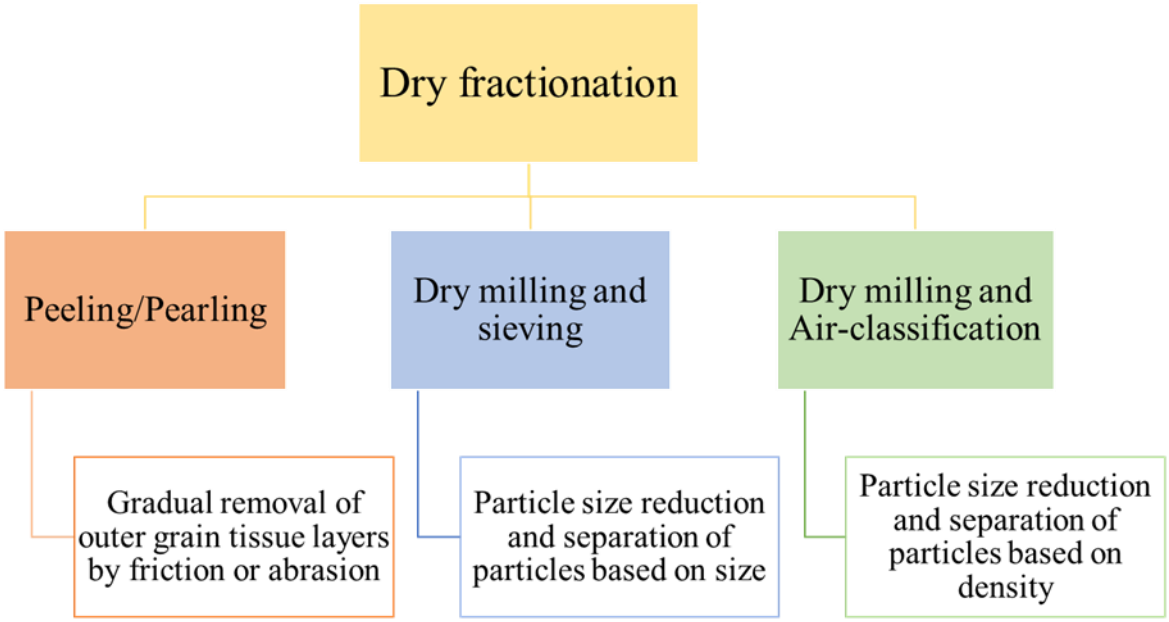

Figure 33. Dry fractionation technologies for concentration of phenolic compounds. Adapted from[204].

The first objective of dry fractionation is the production of flours with high levels of certain parts of the outer layers to achieve the enrichment of cereal products with bioactive compounds. First of all, a gradual removal of the outermost grain layers is carried out thanks to the debranning of grains by friction (peeling) or abrasion (pearling) [204]. These technologies are combined with milling to provide flours with determined tissue composition; thus, their content in some bioactive compounds can be monitored[202,205]. For example, a dry fractionation process in wheat consists in a first pearling to eliminate the outermost layers and a second pearling containing the aleurone layer and the pearled grain obtained is milled to eliminate the bran crease material, and finally white flour is re-mixed with the

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[205] Delcour, J.A.; Rouau, X.; Courtin, C.M.; Poutanen, K.; Ranieri, R. Technologies for enhanced exploitation of the health-promoting potential of cereals. *Trends Food Sci. Technol.* **2012**, *25*, 78–86.

second pearling fraction to incorporate the aleurone layer material into the flour [205].

Milling disintegrates grains into fine particles. Roller milling is the most used in cereals that comprises two steps: the break system which splits the kernel and crushes the outer layers, and the reduction of particle size of the kernel through rollers and sifters[206]. There are three objectives of roller milling: Separation of endosperm from the bran and germ, gradual size reduction of flour and getting a high flour yield from grain. The percentage of flour obtained from a given unit of whole wheat kernel is the flour yield or the extraction rate, averaging from 72 to 80%[207].

After milling, a complex particulate material is obtained (grain meal), where each particle has a chemical composition according to their extent of size reduction (i.e. starch, protein, beta-glucan, hemi-cellulose, cellulose, lipids, minerals, etc.)[204]. Sieving provides a separation of ground grain into particle sized-classification using size sieves or screens[207].

Air-classification of the meal after dry milling or flour after dry milling and sieving can be used to separate particles based on their differences in density (**Figure 34**). The procedure applies an air flow in a confined space and segregates the heterogeneous particle mixture in two groups, fine and coarse fraction[206] . This procedure provides an enrichment of selected components such as starch, protein and fiber. Air-classification parameters such as feed rate, air flow rate, and classifier wheel speed can be optimized to obtain flours with a high concentration of phenolic compounds, among other components[204]. Coarse fraction that is mostly composed of outer layers is enriched in phenolic compounds and fiber, whereas fine fraction is enriched in starch and proteins[206].

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[206] Wang, Y.; Bamdad, F.; Chen, L. New technologies in the proccesing of functional and nutraceutical cereals and extruded products. In *Nutraceutical and Functional Food Processing Technology*; Sons, J.W.&, Ed.; 2015; pp. 235–267.

[207] Baik, B.-K.; Newman, C.W.; Newman, R.K. Food uses of Barley. In *Barley: Production, Improvement, and Uses*; Ullrich, S.E., Ed.; Wiley Blackwell, 2011; pp. 548–555.

Figure 34. Air Classifier

Previous studies have reported the effect of dry fractionation in the phenolic content in buckwheat, wheat and barley flour fractions (**Table 10**). The content of phenolic compounds in fractions depends on the layers of grains. In general, flour grain fractions enriched in the outer layers contain the highest phenolic content. In buckwheat the highest content of rutin was found in embryo axis with cotyledons and in farinetta, which is a flour fraction composed by aleurone layer with embryo, whereas the highest content in epicatechin and procyanidin B2 is mainly found in the seed coat[129,131].

In wheat milling, fractions enriched in bran contained the highest phenolic content. One study reported the milling of wheat to obtain different fractions with different particle size, where the fine wheat with particle size of 194.9 µm was enriched in phenolic compounds[208]. This fact is attributed due to the higher rupture of components of the grain during milling process to reduce the particle size.

Air classification in barley has showed to be a good technique to obtain enriched fractions in phenolic compounds. One study reported an increase of free flavan-3-

[208] Bolea, C.-A.; Vizireanu, C. Polyphenolic content and antioxidant properties of black rice flour. *Food Technol.* **2017**, *41*, 75–85.

ols and bound phenolic compounds in coarse barley fractions of 157-173% and 160-236%, respectively, in comparison with whole flour[209]. In addition, other study has reported that barley coarse fraction showed high amounts of β-glucans (until two-fold higher than in whole meal) and free and bound phenolic compounds around 1.2–1.3 times higher than in whole meal[210].

[209] Verardo, V.; Gómez-Caravaca, A.M.; Marconi, E.; Caboni, M.F. Air classification of barley flours to produce phenolic enriched ingredients: Comparative study among MEKC-UV , RP-HPLC-DAD-MS and spectrophotometric determinations. *LWT - Food Sci. Technol.* **2011**, *44*, 1555–1561.

[210] Gómez-Caravaca, A.M.; Verardo, V.; Candigliota, T.; Marconi, E.; Segura-Carretero, A.; Fernandez-Gutierrez, A.; Caboni, F.M. Use of air classification technology as green process to produce functional barley flours naturally enriched of alkylresorcinols, β-glucans and phenolic compounds. *Food Res. Int.* **2015**, *73*, 88–96.

Table 10. Effects of dry fractionation on total phenolic content in cereal grains fractions

Cereals	Pre-treatment	Dry fractionation	Fractions	Phenolic content in fractions	Ref
Buckwheat	Dehulling	Milling	Seed coat, endosperm and embryo axis with cotyledons	The seed coat was rich in epicatechin (257.60 mg/kg), procyanidin B2 (118.6 mg/kg), and epicatechin gallate (61.27 mg/kg). The embryo axis with the cotyledons is rich in rutin (283.37 mg/kg) and catechin (161.41 mg/kg).	131
Buckwheat		Milling	-Fancy: inner endosperm, aleurone, and embryo -Farinetta: aleurone layer along with embryo -Supreme: Certain percentage of hull and endosperm -Whole	Farinetta flour contained the highest free and bound phenolic contents, followed by supreme, whole buckwheat, and fancy flour.	129
Buckwheat	Dehulling apparatus with disks	Milling	17 fractions from the innermost part to the outermost part of grain	The amounts of ferulic acid and rutin increased from 2.5 and 2.5 µg/g flour, respectively, in the fraction with the inner part of grains to 609.5 and 389.9	211

[211] Hung, P. Van; Morita, N. Distribution of phenolic compounds in the graded flours milled from whole buckwheat grains and their antioxidant capacities. *Food Chem.* **2008**, *109*, 325–331.

				µg/g, respectively, in the fraction with the outer layers of grains.	
Wheat		Milling	Sieved whole wheat fractions: 82.67µm, 194.98µm, 608.44µm and 830 µm	Fraction with particle size of 194.98 µm is enriched in phenolic compounds due to the higher rupture of components of the grain during the milling process.	212
Wheat	Pearling	Roller milling	15 fractions	The flours milled from the outer parts of grain contained significantly higher amount of phenolics and exhibited significantly higher antioxidant capacity than the whole grain.	213
Wheat		Milling and sieving	Wheat fraction: Whole grain, bran Flour, shorts, feed flour and semolina	The highest total phenolic content was obtained in the bran.	214
Barley	Pearling	Air classification	-Coarse fraction: Yield:40%, particle size:120-477µm -Fine fraction:	Coarse fraction was enriched in β-glucan, free flavan-3-ols and bound phenolic compounds.	209,210

[212] Bressiani, J.; Oro, T.; Santetti, G.S.; Almeida, J.L.; Bertolin, T.E.; Gómez, M.; Gutkoski, L.C. Properties of whole grain wheat flour and performance in bakery products as a function of particle size. *J. Cereal Sci.* **2017**, *75*, 269–277.

[213] Beta, T.; Nam, S.; Dexter, J.E.; Sapirstein, H.D. Phenolic content and antioxidant activity of pearled wheat and roller-milled fractions. *Cereal Chem.* **2005**, *82*, 390–393.

[214] Liyana-Pathirana, C.M.; Shahidi, F. Antioxidant and free radical scavenging activities of whole wheat and milling fractions. *Food Chem.* **2007**, *101*, 1151–1157.

	Yield: 60%, particle size: 45-120µm

5. SAMPLE TREATMENT FOR THE EXTRACTION OF PHENOLIC COMPOUNDS FROM CEREALS, PSEUDOCEREALS AND OLIVE LEAVES

Solid-liquid extraction is the most important treatment to recover phenolic compounds from the studied matrices before analysis. The extraction of phenolic compounds from vegetables could be carried out by using conventional solid-liquid extraction processes. The main conventional extraction techniques in relation to phenolic compounds are Soxhlet, maceration, and hydrodistillation. The Soxhlet technique involves a small quantity of dry sample, which is placed on the equipment where the solvent passes through[215]. This process is repeated until the extraction is completed. Nevertheless, this technique requires extensive extraction time and large amounts of solvent[216]. Maceration consists of putting the solid sample in contact with the extraction solvent in a closed container for a certain time with possibility of agitation. The agitation in the maceration process favours the extraction by increasing the diffusion and by removing the concentrated solution from the surface of the sample[217]. Hydrodistillation is used to extract the volatile fraction in foods; this method takes around 6–8 h and organic solvents are not involved. This technique comprises three main physicochemical processes: hydrodiffusion, hydrolysis, and decomposition by heat. However, the use of this technique is limited due to high temperatures that can degrade phenolic compounds[215].

Conventional techniques have been used for a long time. However, they require high volume of solvents, long extraction times and possess a low selectivity and

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[215] Soquetta, M.B.; Terra, L. de M.; Bastos, C.P. Green technologies for the extraction of bioactive compounds in fruits and vegetables. *CYTA - J. Food* **2018**, *16*, 400–412.

[216] Heleno, S.A.; Diz, P.; Prieto, M.A.; Barros, L.; Rodrigues, A.; Barreiro, M.F.; Ferreira, I.C.F.R. Optimization of ultrasound-assisted extraction to obtain mycosterols from Agaricus bisporus L. by response surface methodology and comparison with conventional Soxhlet extraction. *Food Chem.* **2016**, *197*, 1054–1063.

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

reproducibility[218]. Therefore, in recent years there have been growing demands for new extraction techniques. These advanced techniques avoid the use of toxic solvent (when extracts are produced for food scope) and reduce the energy and solvent consumption, waste generation, extraction time and operator effort[219]. In the following sections have been described the four advanced solid-liquid extraction methods used as sample treatment in this PhD thesis: ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), pressurized-liquid extraction (PLE), and pulsed electric field (PEF).

5.1. Ultrasonic assisted extraction

Ultrasound-assisted extraction has been widely used in recent years due to their numerous advantages in the recovery of phenolic compounds from different matrices in comparison with conventional extraction methods. The main profits are shorter and more effective extractions that imply reducing energy consumption, and using moderate temperatures, which is beneficial for phenolic compounds that are heat sensitive. Ultrasounds (US) are mechanical waves with frequencies from 20 kHz to 10 MHz. In a solid-liquid extraction assisted by US, the sample is immersed in the solvent and subjected to ultrasound using an US probe or US bath (**Figure 34**)[220]. When the waves are transmitted across the liquid medium, they induce a longitudinal displacement of particles, whereas the source of the sound wave acts as a piston, providing a succession of compression and rarefaction phases on the medium[221]. If the rarefaction cycle is strong enough, the

[218] Plaza, M.; Domínguez-Rodríguez, G.; Castro-Puyana, M.; Marina, M.L. Polyphenols analysis and related challenges. In *Polyphenols: Properties, Recovery, and Applications*; Galanakis, C.M., Ed.; Elsevier, 2018; pp. 177–220.

[219] Armenta, S.; Garrigues, S.; de la Guardia, M. The role of green extraction techniques in Green Analytical Chemistry. *TrAC - Trends Anal. Chem.* **2015**, *71*, 2–8.

[220] Carciochi, R.A.; D'Alessandro, L.G.; Vauchel, P.; Rodriguez, M.M.; Nolasco, S.M.; Dimitrov, K. Valorization of agrifood by-products by extracting valuable bioactive compounds using freen processes. In *Ingredients Extraction by Physicochemical Methods in Food*; Elsevier, 2017; pp. 191–228 ISBN 9780128115213.

[221] Chemat, F.; Zill-E-Huma; Khan, M.K. Applications of ultrasound in food technology: Processing, preservation and extraction. *Ultrason. Sonochem.* **2011**, *18*, 813–835.

distance between contiguous molecules of the liquid can reach or even overrun the critical molecular distance[220]. The holes generated into the medium are cavitation bubbles. These incipient bubbles can grow during rarefaction phases and reduce in size during compression cycles. When the size of the bubbles achieves a critical point, they collapse during the subsequent compression[221] (**Figure 35**). Because of this collapse, a considerable amount of energy is produced; this massive energy release provides extreme changes in temperature (up to 5000 K) and pressure (100 MPa)[222]. In vegetal matrix, when the bubbles collapse over the surface of the solid material cause shockwave-induced damages and microjet impacts providing the breakdown of the cell walls, this improves the solvent penetration into the plant matrix causing the release of its content into the medium[223,224]. Therefore, the use of US improves the extraction process by increasing the mass transfer of phenolic compounds from the plant to the solvent[225].

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[222] Ameer, K.; Shahbaz, H.M.; Kwon, J.H. Green extraction methods for polyphenols from plant matrices and their byproducts: A review. *Compr. Rev. Food Sci. Food Saf.* **2017**, *16*, 295–315.

[223] Toma, M.; Vinatoru, M.; Paniwnyk, L.; Mason, T.J. Investigation of the effects of ultrasound on vegetal tissues during solvent extraction. *Ultrason. Sonochem.* **2001**, *8*, 137–142.

[224] Esclapez, M.D.; García-Pérez, J. V.; Mulet, A.; Cárcel, J.A. Ultrasound-Assisted Extraction of Natural Products. *Food Eng. Rev.* **2011**, *3*, 108–120.

[225] Roselló-Soto, E.; Koubaa, M.; Moubarik, A.; Lopes, R.P.; Saraiva, J.A.; Boussetta, N.; Grimi, N.; Barba, F.J. Emerging opportunities for the effective valorization of wastes and by-products generated during olive oil production process: Non-conventional methods for the recovery of high-added value compounds. *Trends Food Sci. Technol.* **2015**, *45*, 296–310.

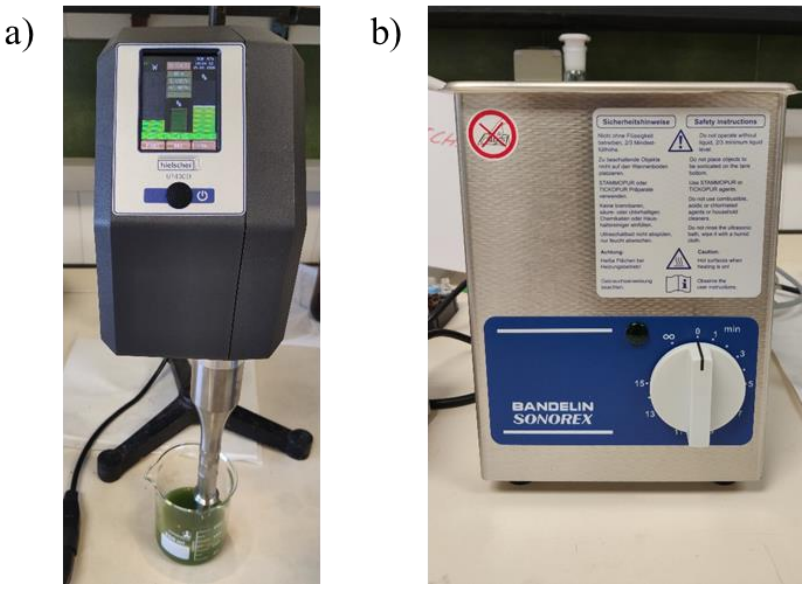

Figure 35. Ultrasound probe (US probe) and bath (US bath) used in laboratory.

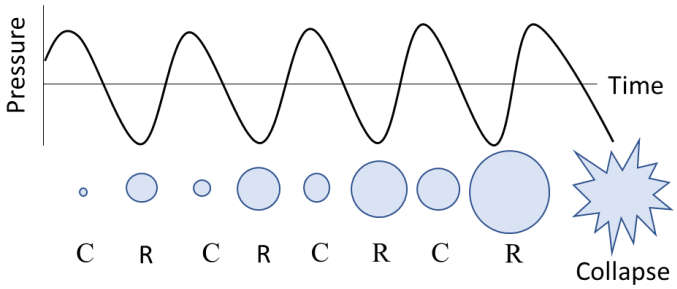

Figure 36. Acoustic cavitation phenomenon, showing formation, grown and implosion of a bubble: C: Compression; R: Rarefaction. Adapted fom[220].

UAE effectivity depends on some factors such as non-ultrasonic factors: temperature, particle size and solid/solvent ratio. Low extraction temperatures avoid degradation of the phenolic compounds keeping their stability and their bioactivity. In addition, small particle size and low solid/solvent ratios improve the extraction. Ultrasonic factors are power and frequency and time. In this regard, extraction is favorable at middle-low frequencies and power and short extraction times[226].

[226] Carciochi, R.A.; Dimitrov, K. Optimization of antioxidant phenolic compounds extraction from quinoa (Chenopodium quinoa) seeds. *J. Food Sci. Technol.* **2014**,

Ultrasonic assisted extraction has been very used in the phenolic recovery from olive leaves and cereals (**Table 11**). According to previous studies, this technique requires short extraction times and low ratio solid to solvent to obtain a high phenolic recovery and requires low energy consumption. Regarding the extraction solvents, mixtures of ethanol or methanol with water are the most efficient in the phenolic extractions, whereas other mixtures such as acetone with water are used to extract high molecular weight phenolic compounds such as oligomeric and polymeric flavan-3-ols.

Table 11. Usual UAE conditions to extract phenolic compounds from olive leaves and cereals

Sample	Phenolic content	Type of US	UAE conditions	Ref
Olive leaves	52.13-60.64 mg/g d.w.	US bath	Solvent= 80%MeOH Time= 10 min Ratio= 1/20 g/mL (w/v)	87
Olive leaves	46.04-83.30 mg/g d.w.	US bath	Solvent = 80% MeOH Time =10 min Ratio= 1/20 g/mL	95
Olive leaves	14.27-54.81 mg/g d.w.	US bath	Solvent= 80% MeOH Time =10 min Ratio= 1/20 g/mL (w/v)	92
Olive leaves	Oleuropein- 22610 ± 632 mg/Kg d.w. Verbascoside- 488 ± 21 mg/Kg d.w. Apigenin-7-glucoside- 1072 ± 38 mg/Kg d.w. Luteolin-7-glucoside- 970 ± 43 mg/kg d.w.	US probe	Temperature= 40°C Solvent = 59% EtOH Time=25 min Amplitude =30% Power = 450 power	227

52, 4396–4404.

[227] Japón-Luján, R.; Luque-Rodríguez, J.M.; Luque De Castro, M.D. Dynamic ultrasound-assisted extraction of oleuropein and related biophenols from olive leaves. *J. Chromatogr. A* **2006**, *1108*, 76–82.

Olive leaves	Oleuropein- 69.91 g/Kg Luteolin-7-glucoside- 1.82 g/Kg d.w. Total phenolic content- 210.25 mmol GAE/Kg	US probe	Temperature= 40ºC Solvent= 60% EtOH Time= 17.91 min Ratio= 1/13 g/mL (w/v) Amplitude= 30%	228
Olive leaves	Flavonoid content- 74.95 mg/g dry extract	US probe	Temperature= 50ºC Solvent= MeOH Time= 50 min Ratio= 1/41 g/mL (w/v) Power= 270W	229
Buckwheat	1008.91 mg/Kg d.w.	US bath	Solvent= 80% EtOH Time = 10 min Ratio = 1/20 g/mL (w/v)	128
Barley	Flavan-3-ols content- Coarse fraction-138-172.1mg/100 g d.w. Whole flour-85.4-109.4 mg/100g d.w. Fine fraction-49.5-72.8 mg/100g d.w.	US bath	Solvent= 80% Acetone Time= 10 min Ratio = 1/8 g/mL (w/v)	209
Wheat bran	3.12 ± 0.03 mg gallic acid equivalent/ g bran	US bath	Temperature= 60 C Solvent= 64% EtOH Time = 25 min Ratio = 1/20 g/mL (w/v)	230
Tartary buckwheat	Flavonoid yield- 3.94 ± 0.062%	US bath	Temperature= 60ºC	231

[228] Lama-Muñoz, A.; Contreras, M.D.M.; Espínola, F.; Moya, M.; Romero, I.; Castro, E. Optimization of oleuropein and luteolin-7-o-glucoside extraction from olive leaves by ultrasound-assisted technology. *Energies* **2019**, *12*.

[229] Wang, B.; Qu, J.; Luo, S.; Feng, S.; Li, T.; Yuan, M.; Huang, Y.; Liao, J.; Yang, R.; Ding, C. Optimization of ultrasound-assisted extraction of flavonoids from olive (olea europaea) leaves, and evaluation of their antioxidant and anticancer activities. *Molecules* **2018**, *23*.

[230] Wang, J.; Sun, B.; Cao, Y.; Tian, Y.; Li, X. Optimisation of ultrasound-assisted extraction of phenolic compounds from wheat bran. *Food Chem.* **2008**, *106*, 804–810.

[231] Peng, L.X.; Zou, L.; Zhao, J.L.; Xiang, D.B.; Zhu, P.; Zhao, G. Response surface modeling and optimization of ultrasound-assisted extraction of three flavonoids

	Solvent= 72% MeOH Time = 21 min Ratio =1/250 (w/v)

5.2. Microwave assisted extraction

Microwave assisted extraction has been used in different vegetable matrices to extract phenolic compounds. Energy transfer is the central characteristic of microwave heating[232]. Microwave is an electromagnetic wave; it comprises both electric and magnetic field, which oscillates perpendicularly among them in frequency between 0.3 to 300 GHz. Microwave can penetrate some materials and interacts with the polar components generating heat.

The heating of microwave energy acts on the molecules by ionic conduction and dipole rotation[233,234]. Ionic conduction refers to the electrophoretic migration of ions when an electromagnetic field is applied. Besides, the friction between molecules/ions and the solution results in the heating of the solvent. Dipole rotation is due to dipolar molecules that are sensitive to the alternating electric field caused by microwaves, which, constantly changing its direction, provides the molecules to rotate quickly to try to line up their own dipole with that of the electric field[235]. As the field declines, thermal disorder is restored and this results in the release of thermal energy[236]. Heat improves the diffusivity of phenolic compounds from the matrix to the solvent. The ability of a solvent to absorb microwave energy and provide heat depends on the dissipation factor (tan δ) (**eq.3**). where ε' is the

from tartary buckwheat (Fagopyrum tataricum). *Pharmacogn. Mag.* **2013**, *9*, 210–215.

[232] Al-mamoori, F.; Al-Janabi, R. Recent advances in microwave assisted extraction (MAE) of medicinal plants : A review. *Int. Res. J. Pharm.* **2018**, *9*.

[233] Chan, C.; Yusoff, R.; Ngoh, G.; Kung, F.W. Microwave-assisted extractions of active ingredients from plants. *J. Chromatogr. A* **2011**, *1218*, 6213–6225.

[234] Routray, W.; Orsat, V. Microwave-assisted extraction of flavonoids: A review. *Food Bioprocess Technol.* **2012**, *5*, 409–424.

[235] Moret, S.; Conchione, C.; Srbinovska, A.; Lucci, P. Microwave-based technique for fast and reliable extraction of organic contaminants from food, with a special focus on hydrocarbon contaminants. *Foods* **2019**, *8*, 1–20.

[236] Destandau, E.; Michel, T.; Elfakir, C. Microwave-Assisted Extraction. In *Natural Product Extraction: Principles and Applications*; Prado, M.A.R. and J.M., Ed.; The Royal Society of Chemistry, 2013; pp. 113–156 ISBN 9781849737579.

dielectric constant, which refers to the capacity of a molecule to be polarized by an electric field, and ε'' is the dielectric loss factor, which refers to the efficiency of transformation of electromagnetic energy into heat[236].

$$\tan \delta = \varepsilon' / \varepsilon'' \quad \textbf{(eq.3)}$$

The greater the dielectric constant, the greater the absorption capacity of this energy and, therefore, the heating of the solvent[237]. Polar solvents possess a high dielectric constant such as water, methanol, ethanol, etc. and can absorb the microwave energy, whereas nonpolar solvents (e.g., hexane) do not heat when exposed to microwave radiation[220]. Dielectric constants of common solvents in MAE are shown in **Table 12.** The improvement of phenolic compounds recovery by MAE is commonly attributed to its quickly heating effect on solvent, which increases the solubility of these phenolic compounds[238].

Table 12. Dielectric constants of solvent used in MAE*236*.

Solvent	**ε'**
Water	78.3
Acetonitrile	37.5
Methanol	32.6
Ethanol	24.3
Acetone	20.7
2-propanol	19.9
Hexane	1.89

[237] Misra, H.; Mehta, D.; Mehta, B.K.; Jain, D.C. Microwave-Assisted Extraction Studies of Target Analyte Artemisinin from Dried Leaves of Artemisia annua L. . *Org. Chem. Int.* **2013**, *2013*, 1–6.

[238] Ahmad, J.; Langrish, T.A.G. Optimisation of total phenolic acids extraction from mandarin peels using microwave energy: The importance of the Maillard reaction. *J. Food Eng.* **2012**, *109*, 162–174.

There are two types of MAE: Open microwave systems at atmospheric pressure and closed extraction vessels.

Figure 37. Closed MAE system[239].

The closed MAE system allows the control of temperature and pressure (**Figure 37**). The increase of the temperature up to the boiling point of solvent rising the extraction efficiency due to the improvement of the mass transfer[233].

Several parameters affect MAE effectivity: solvent type, solvent volume, composition of solvent, solid-to-solvent ratio, microwave power, exposure time, and temperature[218]. The parameters time and volume of the solvent depend on the matrix and the type of phenolic compounds[240]. The major advantages of using MAE include shorter extraction times and lower volumes of solvent than conventional extraction techniques[241]. Previous studies have used MAE for the phenolic recovery from olive leaves (**Table 13**). According to these previous

[239] Paar, A. Microwave Reaction System for Sample Preparation. **2019**.

[240] Belwal, T.; Bhatt, I.D.; Rawal, R.S.; Pande, V. Microwave-assisted extraction (MAE) conditions using polynomial design for improving antioxidant phytochemicals in Berberis asiatica Roxb. ex DC. leaves. *Ind. Crops Prod.* **2017**, *95*, 393–403.

[241] Dahmoune, F.; Spigno, G.; Moussi, K.; Remini, H.; Cherbal, A.; Madani, K. Pistacia lentiscus leaves as a source of phenolic compounds: Microwave-assisted extraction optimized and compared with ultrasound-assisted and conventional solvent extraction. *Ind. Crops Prod.* **2014**, *61*, 31–40.

studies, methanol, water and ethanol were the most used solvents in the phenolic recovery by MAE. However, it is not possible to compare absolute final recoveries because the extraction times differ from one to another study.

Table 13. Usual MAE conditions to extract phenolic compounds from olive leaves.

Sample	Phenolic content	MAE conditions	Ref
"El Hor"	Oleuropein - 12.51 ± 0.07 % of the total peak area	Temperature= 80ºC Solvent= 80% MeOH Time = 6 min Ratio =1/8 g/mL (w/v)	83
"Arbequina"	104.22 ± 0.61 mg GAE/g d.w.	Temperature= 86°C Solvent= water Time = 3 min Ratio= 1/50 g/mL (w/v) Power= 1000 W	242
Olive leaves	Oleuropein-2.32±0.85% Verbascoside- 631±43 mg/kg Apigenin-7-glucoside- 1076±65 mg/kg Luteolin-7-glucoside- 1016±60 mg/kg	Solvent= 80% EtOH Time= 8 min Ratio= 1/8 g/mL (w/v) Power= 200 W	243
"Koroneiki" "Roghani" "Mission"	68.833-88.298 mg tannic acid eq /g d.w.	Solvent= 50% EtOH Time= 15 min Ratio= 1/50 g/mL (w/v)	244

[242] Da Rosa, G.S.; Vanga, S.K.; Gariepy, Y.; Raghavan, V. Comparison of microwave, ultrasonic and conventional techniques for extraction of bioactive compounds from olive leaves (Olea europaea L.). *Innov. Food Sci. Emerg. Technol.* **2019**, *58*, 102234.

[243] Japón-Luján, R.; Luque-Rodríguez, J.M.; Luque De Castro, M.D. Multivariate optimisation of the microwave-assisted extraction of oleuropein and related biophenols from olive leaves. *Anal. Bioanal. Chem.* **2006**, *385*, 753–759.

[244] Rafiee, Z.; Jafari, S.M.; Alami, M.; Khomeiri, M. Microwave-assisted extraction of phenolic compounds from olive leaves; a comparison with maceration. *J. Anim. Plant Sci.* **2011**, *21*, 738–745.

5.3. Pressurized liquid extraction

Pressurized liquid extraction is a technique that works at high temperature and pressure. The fact that PLE operates further the atmospheric boiling point improves solubility and mass transfer properties and reduces the viscosity and surface tension, thus, accelerated extraction rate is provided[217]. PLE has various advantages in comparison with conventional extraction methods such as shorter extraction times, lower solvent consumption and the filtration process in the extraction cell[245].

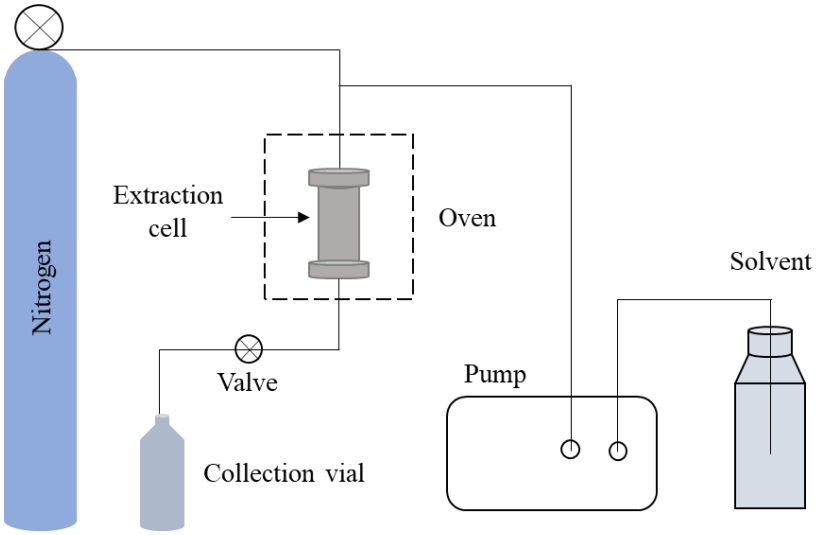

Figure 38. Pressurized liquid extraction system. Adapted from [241].

PLE is a green extraction process, especially when a nontoxic solvent (water and/or water-ethanol mixtures) is used. Briefly, to perform a PLE, sample matrix is put into a steel extraction cell and there are two main modes of working for PLE: static and dynamic modes[246].

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[245] Alam, M.A.; Sarker, M.Z.I.; Ghafoor, K.; Happy, R.A. Bioactive Compounds and Extraction Techniques. In *Recovering Bioactive Compounds from Agricultural Wastes*; Van Tang Nguyen, J.W.& S., Ed.; 2017; pp. 33–53.

[246] Garcia-Salas, P.; Morales-Soto, A.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Phenolic-compound-extraction systems for fruit and vegetable samples. *Molecules*

In static mode PLE, the solvent is pressurized at the extractor, the outlet valve handle is closed. After extraction, this valve is opened to collect the sample extract obtained. This batch process could be carried out using multiple cycles with the addition of fresh solvent in each cycle. Whereas in dynamic mode, the process is continuously pumped through the extractor that contains the vegetable sample, both inlet and outlet valves are open during the extraction[247]. Among them, static PLE is the most efficient for phenolic compounds recovery due to the higher penetration of solvent into the pores of the vegetable sample. A schematic diagram of a PLE system appears in **Figure 38**.

Table 14 shows different PLE extractions at static mode that have been applied in olive leaves for their phenolic recovery. Generally, PLE uses short times (5-20 min), high temperatures (80-200ºC) and high pressures of 10.34 MPa, this value of pressure ensures the solvent is maintained in a liquid state and can penetrate easily the pores of the matrix[220].

Table 14. Usual PLE conditions to extract phenolic compounds from olive leaves

Sample	Phenolic content	PLE conditions	Ref
Olive leaves	Oleuropein 26.1% ± 3.47%	Temperature= 190°C Solvent= 57% EtOH Static time=5-25min Cycles= 1 cycle Pressure= 10.34MPa	[248]
"Hojiblanca"	13.42 mg/g extract	Temperature= 200ºC Solvent= 100% H_2O Time= 20 min	[249]

2010, *15*, 8813–8826.

[247] Carabias-Martínez, R.; Rodríguez-Gonzalo, E.; Revilla-Ruiz, P.; Hernández-Méndez, J. Pressurized liquid extraction in the analysis of food and biological samples. *J. Chromatogr. A* **2005**, *1089*, 1–17.

[248] Xynos, N.; Papaefstathiou, G.; Gikas, E.; Argyropoulou, A.; Aligiannis, N.; Skaltsounis, A.-L. Design optimization study of the extraction of olive leaves performed with pressurized liquid extraction using response surface methodology. *Sep. Purif. Technol.* **2014**, *122*, 323–330.

[249] Herrero, M.; Temirzoda, T.N.; Segura-Carretero, A.; Quirantes, R.; Plaza, M.; Ibañez, E. New possibilities for the valorization of olive oil by-products. *J. Chromatogr. A* **2011**, *1218*, 7511–7520.

		Ratio = 2/11 g/mL (w/v)	
"Oblica"	53.15 mg GAE/g	Temperature= 80ºC Solvent= 50%EtOH Static time= 5 min Ratio= 1/34 g/mL (w/v) Cycles= 2 cycles Pressure= 10.34MPa	[250]
"Picual"	Oleuropein-63.35 g/Kg d.w. Luteolin-7-O-glucoside- 2.71 g/Kg d.w.	Temperature= 190ºC Solvent= 80% EtOH Time= 5 min Ratio= 3/22 g/mL (w/v)	[251]
"Arbequina", "Picual", "Royal" and three wild cultivars	Oleuropein- 43.4-115 g/Kg d.w. Luteolin-7-glucoside- 0.94-3.80 g/Kg d.w.	Temperature= 190ºC Solvent= 60% EtOH Time= 5 min Ratio= 3g/22mL	[252]

5.4. Pulsed electric field

Pulsed electric field-assisted extraction consists of an application of duration pulses (μs to ms) of electric voltage of around 0.5–20 kV/cm to a sample which is placed between two electrodes[253]. Low to mild PEF treatment intensities have shown to be an effective pretreatment method for improvement of phenolic compounds extraction yields in plant samples[254,255]. The basic principle of PEF-

[250] Putnik, P.; Barba, F.J.; Španić, I.; Zorić, Z.; Dragović-Uzelac, V.; Bursać Kovačević, D. Green extraction approach for the recovery of polyphenols from Croatian olive leaves (Olea europea). *Food Bioprod. Process.* **2017**, *106*, 19–28.

[251] Lama-Muñoz, A.; Contreras, M. del M.; Espínola, F.; Moya, M.; de Torres, A.; Romero, I.; Castro, E. Extraction of oleuropein and luteolin-7-O-glucoside from olive leaves: Optimization of technique and operating conditions. *Food Chem.* **2019**, *293*, 161–168.

[252] Lama-muñoz, A.; Contreras, M. del M.; Espínola, F.; Moya, M.; Romero, I.; Castro, E. Content of phenolic compounds and mannitol in olive leaves extracts from six Spanish cultivars: Extraction with the Soxhlet method and pressurized liquids. *Food Chem.* **2020**, *320*, 126626.

[253] Yang, N.; Huang, K.; Lyu, C.; Wang, J. Pulsed electric field technology in the manufacturing processes of wine, beer, and rice wine: A review. *Food Control* **2016**, *61*, 28–38.

[254] López, N.; Puértolas, E.; Condón, S.; Raso, J.; Alvarez, I. Enhancement of the extraction of betanine from red beetroot by pulsed electric fields. *J. Food Eng.* **2009**, *90*, 60–66.

[255] Fincan, M.; Dejmek, P. In situ visualization of the effect of a pulsed electric field on plant tissue. *J. Food Eng.* **2002**, *55*, 223–230.

assisted extraction is electroporation due to dielectric disruption of cell membrane (**Figure 39**)[256]. Cell membranes act as a capacitor with low dielectric constant possessing natural trans-membrane potential because of the presence of free charges of opposite polarities through the membrane[257]. The application of an external electric field provides an increase in the trans-membrane potential due to the accumulation of charges across the membrane. The later exposure to electric field further increases the potential generating an electrostatic attraction between opposite charges across the membrane producing thinning of membrane. Breakdown of the membrane is caused when the critical breakdown voltage is reached by an additional increase in the external field strength that results in trans-membrane pore formation[257]. PEF has been applied in grapes, grape pomace, onion, orange peel, sorghum flour and apple pomace[253,258-260] to improve the recovery of phenolic compounds[217]. The effectiveness of PEF treatment depends on parameters including electric field strength (E), pulse shape, pulse width, number of pulses (n), pulse specific energy, and frequency[261, 262].

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[256] Raso, J.; Frey, W.; Ferrari, G.; Pataro, G.; Knorr, D.; Teissie, J.; Miklavčič, D. Recommendations guidelines on the key information to be reported in studies of application of PEF technology in food and biotechnological processes. *Innov. Food Sci. Emerg. Technol.* **2016**, *37*, 312–321.

[257] Kumari, B.; Tiwari, B.K.; Hossain, M.B.; Brunton, N.P.; Rai, D.K. Recent Advances on Application of Ultrasound and Pulsed Electric Field Technologies in the Extraction of Bioactives from Agro-Industrial By-products. *Food Bioprocess Technol.* **2018**, *11*, 223–241.

[258] Liu, Z.; Zeng, X.; Ngadi, M. Enhanced extraction of phenolic compounds from onion by pulsed electric field (PEF). *J. Food Process. Preserv.* **2018**, *42*, e13755.

[259] Esteve, M.J. Bioaccessibility of Bioactive Compounds and Antioxidant Capacityfrom Orange Peel after Pulsed Electric Fields and High Voltage Electrical Discharges. *MOJ Food Process. Technol.* **2015**, *1*, 77–83.

[260] Lohani, U.C.; Muthukumarappan, K. Application of the pulsed electric field to release bound phenolics in sorghum flour and apple pomace. *Innov. Food Sci. Emerg. Technol.* **2016**, *35*, 29–35.

[261] Heinz, V.; Toepfl, S.; Knorr, D. Impact of temperature on lethality and energy efficiency of apple juice pasteurization by pulsed electric fields treatment. *Innov. Food Sci. Emerg. Technol.* **2003**, *4*, 167–175.

[262] Puértolas, E.; Luengo, E.; I.Alvarez; Raso, J. Improving mass transfer to soften tissues by pulsed electric fields : Fundamentals and applications. *Annu. Rev. Food Technol.* **2012**, *3*, 263–282.

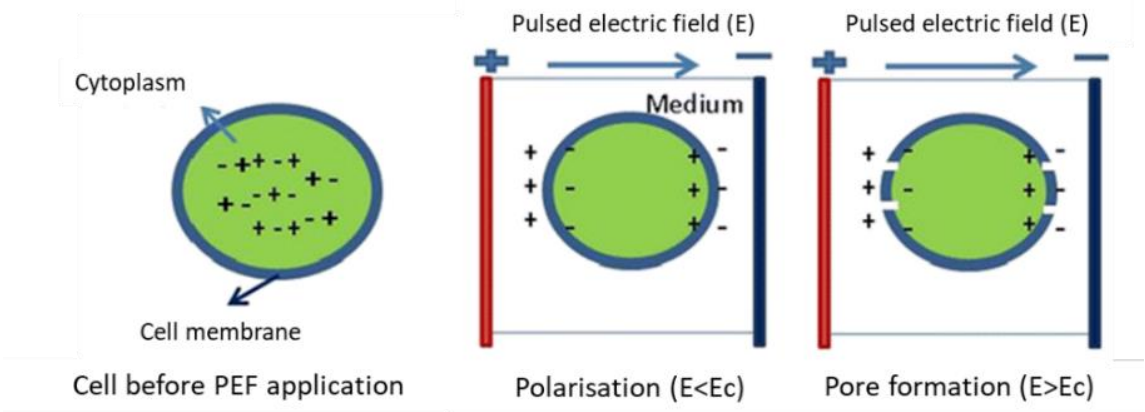

Figure 39. Schematic representation of electroporation mechanism in cell membrane by PEF[257].

A recent study reported the effect of PEF treatment with an electric field strength of 2.8 kV/cm, frequency of 10 Hz and a total of 3000 pulses with a pulse width of 20 μs in the phenolic content in dark and light brewers spent grain extracts and also in their antioxidant, antimicrobial and immunomodulatory properties. Light brewers spent grain extracts after PEF treatment showed a higher antimicrobial activity in comparison with the control. Nevertheless, PEF treated extracts did not show an improvement in their phenolic content in comparison with untreated extracts[263]. This fact can be due to electric field strength used in PEF is the most influence in the phenolic recovery and lower values of electric field strength (0.5 - 2 kV/cm) are commonly used in fresh samples[263].

[263] Kumari, B.; Tiwari, B.K.; Walsh, D.; Griffin, T.P.; Islam, N.; Lyng, J.G.; Brunton, N.P.; Rai, D.K. Impact of pulsed electric field pre-treatment on nutritional and polyphenolic contents and bioactivities of light and dark brewer's spent grains. *Innov. Food Sci. Emerg. Technol.* **2019**, *54*, 200–210.

Figure 40. Pulse generator S-P7500 60A 8kV (Alintel srl., Bologna)

6. ANALYTICAL TECHNIQUES USED FOR THE DETERMINATION OF PHENOLIC COMPOUNDS

Despite there are a very large number of published methods to quantify phenolic compounds in vegetables, high performance liquid chromatography coupled to mass spectrometry is the most commonly used technique for the determination of phenolic compounds in olive leaves and cereal grains[87,92178,249,264,265]. Nevertheless, alkylresorcinols are also analyzed by gas chromatography (GC)[42,155,266,267], besides that high-performance liquid chromatography (HPLC)[150,268]. However, gas chromatography (GC) provides better resolutions than HPLC and, for that reason, gas chromatography has been chosen to analyze alkylresorcinols in wheat aleurone samples.

6.1. Gas Chromatography

Gas chromatography (GC) is a separative technique in which the components are distributed between two phases, one of them is the stationary phase (placed in the column) and the other is the mobile phase (carrier gas)[269]. The sample is vaporized

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[264] Verardo, V.; Gómez-Caravaca, A.M.; Messia, M.C.; Marconi, E.; Caboni, M.F. Development of functional spaghetti enriched in bioactive compounds using barley coarse fraction obtained by air classification. *J. Agric. Food Chem.* **2011**, *59*, 9127–9134.

[265] Barros Santos, M.C.; Ribeiro da Silva Lima, L.; Ramos Nascimento, F.; Pimenta do Nascimento, T.; Cameron, L.C.; Simões Larraz Ferreira, M. Metabolomic approach for characterization of phenolic compounds in different wheat genotypes during grain development. *Food Res. Int.* **2019**, *124*, 118–128.

[266] Wang, J.; Gao, X.; Wang, Z. Non-destructive determination of alkylresorcinol (ARs) content on wheat seed surfaces and prediction of ARs content in whole-grain flour. *Molecules* **2019**, *24*, 21–23.

[267] Giambanelli, E.; Ferioli, F.; D'Antuono, L.F. Retention of alkylresorcinols, antioxidant activity and fatty acids following traditional hulled wheat processing. *J. Cereal Sci.* **2018**, *79*, 98–105

[268] Ross, A.B.; Kochhar, S. Rapid and sensitive analysis of alkylresorcinols from cereal grains and products using HPLC - Coularray-based electrochemical detection. *J. Agric. Food Chem.* **2009**, *57*, 5187–5193.

[269] Harold M. Mcnair, J.M.M. *Basic Gas Chromatography*; 2ª ed.; John Wiley & Sons, Inc. All: New York, 1997; Vol. 53; ISBN 9788578110796.

and carried by the carrier gas through the column, where analyte separation can be based on adsorption or solubility with the stationary phase. In case of solid stationary phases, adsorption chromatography takes place (gas-solid chromatography (GSC)). In case of liquid stationary phases solution is provided, this partition chromatography is called gas-liquid chromatography (GLC). However, also mixed retention mechanisms can take place. The basic elements in a gas chromatography are the following: carrier gas supply, injector, column, oven, detector and data processor (**Figure 41**)[270].

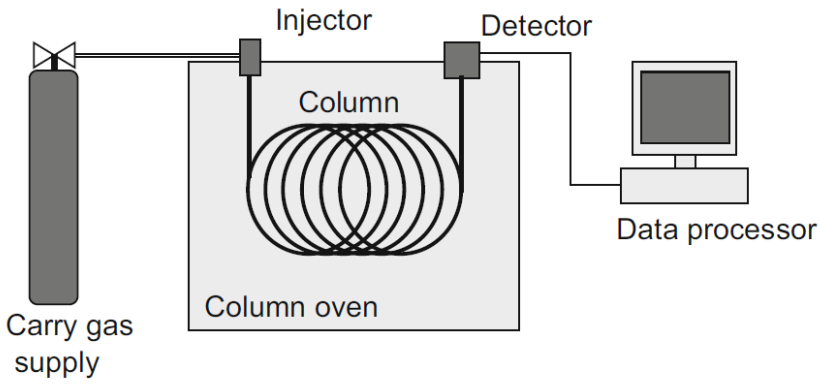

Figure 41. Basic scheme of a gas chromatography system[270].

Carrier gas

The function of the carrier gas is to transport the sample through the stationary phase to the detector; it must be an inert, high purity gas such as He, N_2 or H_2. Besides, to obtain the highest reproducibility of the method, the flow and pressure of the carrier gas must be controlled[271].

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[270] Katja Dettmer-Wilde, W.E. *Practical Gas Chromatography: A Comprehensive Reference*; Springer, 2014; ISBN 9783642546396.

[271] Nascimento, R.F. do; Lopes, A.F.; Júnior, F.S.G.; Oliveira, F.F. de; Lima, G.C.; Sobrinho, N.N.; Barbosa, P.G.A.; Silva, V.P.A. da; Longhinotti, E.; Becker, H.; et al. The Use of GC- BID in the Validation of Analytical Methodology for Pesticides Determination in Vegetables. In *Advances in Chromatographic Analysis*; Nascimento, R., Ed.; AvidScience, 2017; pp. 1–64.

Injector

The injector is a device to introduce gaseous or liquid samples onto the column head. Liquid samples are commonly injected using a microliter syringe while gases are applied by a gastight syringe or gas valves[270]. The most common of sample injection is liquid injection through a self-sealing septum into a heated split/splitless injection port. The liquid sample solution is vaporized in the heated port. In the split injection, only a small part of the vapor enters the column, with the remainder being vented to waste, whereas, in the splitless injection, the entire sample injected is directed towards the column[270]. Split injection is usually used for concentrated liquid samples. The primary advantage of split injection is that narrow injection bands are introduced to the capillary GC column and provides a high-resolution and fast-GC separation. By sending most of the sample to waste, split injection is not suitable for trace analysis[272]. Modern instruments are equipped with an autosampler to automatically inject the samples[270]. The basic scheme of an injector is shown in **Figure 42**.

[272] Shellie, R.A. Gas Chromatography. *Encycl. Forensic Sci. Second Ed.* **2013**, *1*, 579–585.

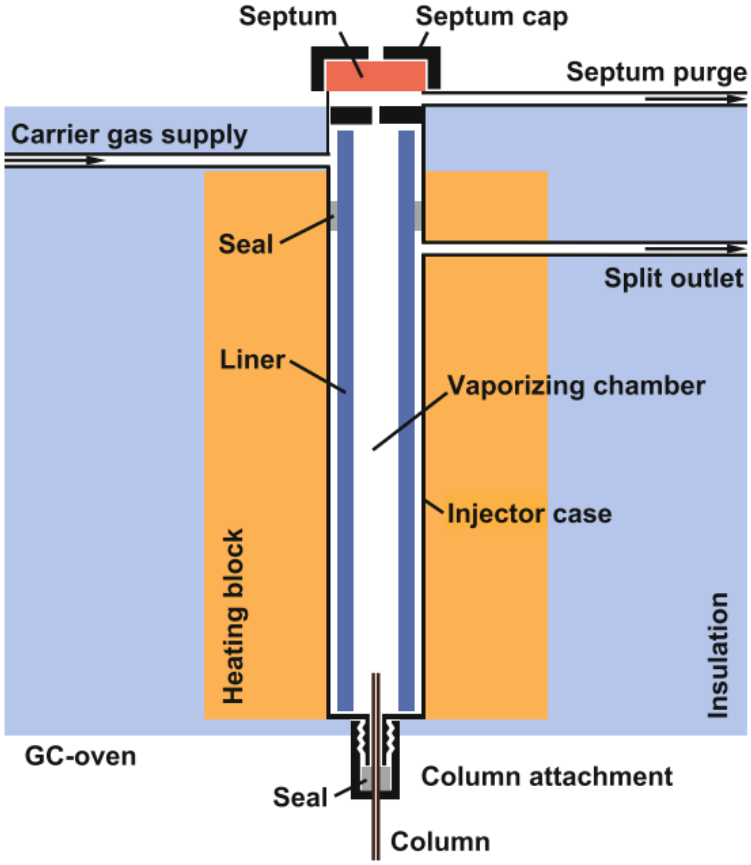

Figure 42. Schematic of a split/splitless injector[270].

Columns

The column consists of a tube that can be made of various materials (preferably inert), inside which is the stationary phase where the separation of sample components takes place. The stationary phase could be gas-solid chromatography (GSC) and gas-liquid chromatography (GLC). The column is considered packed if the glass or metal column tubing is filled with small spherical inert supports. In capillary columns, the stationary phase is coated with the inner wall of the column. Capillary column can be classified in porous layer open tubular (PLOT), wall-coated open tubular (WCOT) and support coated open tubular (SCOT). PLOT columns contain a porous layer of a solid adsorbent as stationary phase such as alumina, molecular sieves, or Porapak. In WCOT columns, the wall is directly coated with the liquid stationary-phase layer at a film thickness. Finally, SCOT columns contain a layer of small support particles coated with a liquid stationary

phase on the inner column wall (GLC)[273]. WCOT columns tend to be more efficient than SCOT columns but also possess a smaller sample capacity. The main characteristics of packed and capillary columns WCOT and PLOT are summarized in the **Table 15.**

Table 15. Column types and their characteristics in GC. Adapted from270.

	Packed columns	**Capillary columns**	
			
		Wall coated open tubular column (WCOT)	**Porous layer open tubular column (PLOT)**
Stationary phase	-Small spherical inert supports impregnated with a liquid (GLC) -Adsorbent particles (GSC)	Liquid stationary phase as a thin film on the inner wall	Inner wall is coated with a porous layer of a solid adsorbent
Retention mechanisms	-Partition -Absorption	Partition (solubility)	Absorption
Lenght	0.5-6 m	5-100 m	2-30 m
Inner diameter	2-4 mm	0.1-0.6 mm	0.2-0.6 mm
Particle size	100-300µm		5-50 µm
Film thickness		0.1-10 µm	
Column material	Copper, stainless steal, glass and quartz	Glass (fragile), fused silica (quartz) made from ultra pure SiO_2 with an outer protective coating of polyimide (flexible), fused silica coated stainless steal (high temperature resistant)	

The permeability of the capillary columns towards the gases is much greater than that of the packed columns (of the order of 100 times greater), because of this, these columns can have a much larger length (they are frequent columns of 100 m). The great use of capillary columns is due to the high efficiency they offer

[273] Rahman, M.M.; El-aty, A.M.A.; Choi, J.-H.; Shin, H.-C.; Shin, S.C.; Shim, J.-H. Basic overview on gas chromatography columns. In *Analytical Separation Science*; Jared L. Anderson, Berthod, A., Estévez, V.P., Stalcup, A.M., Eds.; Wiley-VCH Verlag GmbH & Co. KGaA, 2015; pp. 823–834.

(values of 30000-50000 theoretical plates in capillary columns are frequent compared to 2000-4000 of a packed column). Moreover, the high efficiency of capillary columns allows obtaining good resolutions without resorting to stationary phases of high selectivity, which simplifies the problem of choosing the stationary phase. The main drawback of the capillary columns is their small load capacity, which forces the use of special injection systems to introduce small amounts of sample and very high sensitivity detectors[270].

The main adsorbent solids used in gas chromatography are silica, alumina, graphitized carbon and molecular sieves. Liquid phases must have the following characteristics: not being volatile in a temperature range of 100-300ºC, good thermal stability, low viscosity, chemical stability, dissolve the solutes in the mixture to a different extent and to be chemically inert to the solutes at working temperature[273]. The choice of stationary phase will depend on the composition of the analytes. Stationary phases are classified based on their polarity as reported in

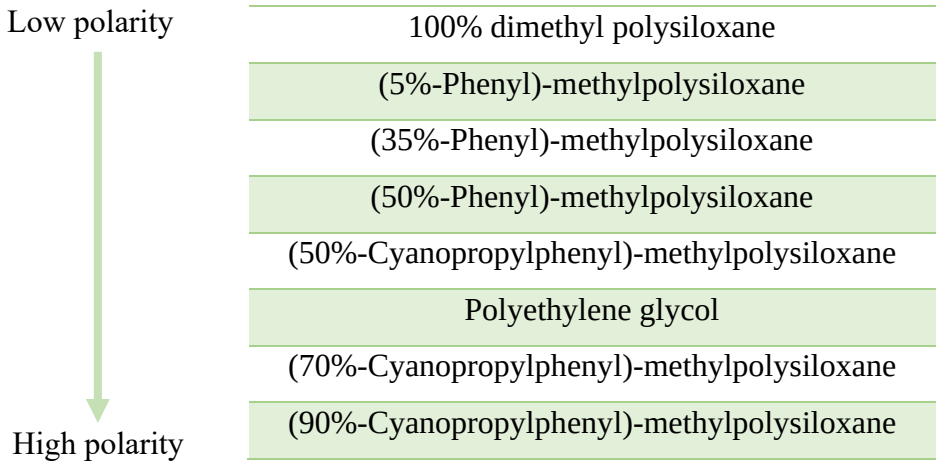

Figure 43. Classification of GC stationary phases with regard to polarity. Adapted from[273].

The alkylresorcinols are usually separated on non-polar stationary phases, being 5% phenyl-methylpolysiloxane the most used in previous studies [42,155,266,267].

Oven

The column is inserted into a thermostatically controlled oven. The optimal temperature to obtain a good separation depends on the boiling point of the analytes and the degree of separation required. The temperature must be equal to or slightly higher than the average boiling point of the sample. For samples with a high boiling range, it is often appropriate to use a programmed temperature, increasing the column temperature either continuously or in stages at the same time as separation takes place. A constant (isothermal) temperature is used for samples with analytes that present similar boiling points[274].

Detector

Mass spectrometry is one of the types of detection that give the highest quantity of information with a low concentration of sample. Gas chromatography coupled to mass spectrometry allows the qualitative identification of unknown compounds as well as quantitative analysis of samples. When GC is coupled to a mass spectrometer, the compounds that elute from the GC column are ionized by using electrons (EI, electron ionization also known as electron impact ionization) or a chemical reagent (CI, chemical ionization).

Electron impact ionization is the most used ionization system in GC. **Figure 44** shows a scheme with the different parts of this system.

[274] Douglas A Skoog, F James Holler, S.R.C. *Principles of Instrumental Analysis*; 6ª ed.; 2007;

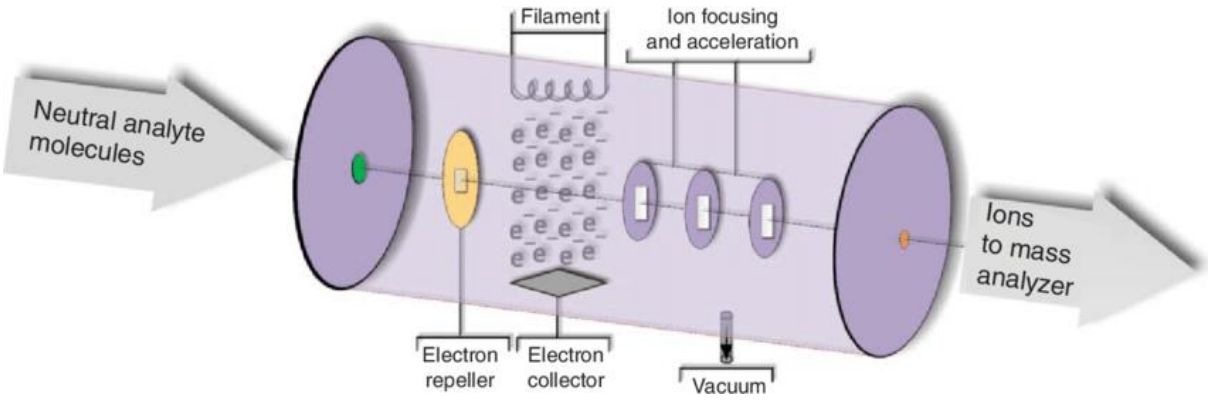

Figure 44. Scheme of an electron impact ionization system.

Briefly, electrons produced by a filament "react" with neutral analytes producing charged species. These charged species are targeted and accelerated into a mass analyzer: typically, a quadrupole mass analyzer. Fragments with different mass to charge ratios provide different signals[275].

Table 16 summarizes previous studies that have used gas chromatography coupled to mass spectrometry to analyze alkylresorcinols in wheat samples.

[275] Piatanida, A.G.; Barron, A.R. *Principles of Gas Chromatography*; OpenStax-CNX, 2014;

Table 16. Conditions used for the analysis of alkylresorcinols in wheat by G

Phenolic compounds	Injector temperature	Transfer line temperature	Temperature Program	Column	Flow colun rate
C15, C17, C19, C21, C23 and C25			50 °C for 2 min, ramp 20 °C/min to 220 °C, hold for 2 min, ramp 1.6 °C/min to 310 °C, and hold for 18 min at 310 °C	Capillary GC column (length 30 m, 0.32 mm i.d., film thickness 0.25 μm)	1.5 m min
C17, C19, C21, C23, C25, C19:1, C21:1, C23:1a, C23:1b and C25:1	325ºC	350ºC	120°C (0min), 200°C (5min), 320°C (20min) and 320°C (35min)	Zebron ZB-5 column (30 m × 0.25 mm i.d., film thickness 0.25 μm) coated with diphenyl-dimethyl-polysiloxane	1.5 mL/m
C17, C19, C21, C23 and C25	250ºC	330ºC	250 °C (0 min), 320 °C (20 min), 320 °C (22 min) and 330 °C (30 min)	BP-5 fused silica capillary column (5% phenyl-methylpolysiloxane; length 25m, 330μm i.d. , film thickness 0.25μm)	1.8 mL/m

[276] Andersson, A.A.M.; Kamal-Eldin, A.; Fraś, A.; Boros, D.; Åman, P. Alkylresorcinols in wheat varietie diversity screen. *J. Agric. Food Chem.* **2008**, *56*, 9722–9725.

6.2. High performance liquid chromatography

Liquid chromatography is an analytical technique that consists of a separation due to different interactions of the analyte in two phases: a stationary phase and a mobile phase. When the mobile phase passes through the stationary phase, the components of the mixture are distributed between the two phases and each component is selectively retained by the stationary phase, providing differential migrations[277].

High performance liquid chromatography (HPLC) was developed in 1960s due to the improvements in columns technology and certain components (pump, injection valves and detectors). Initially the term of HPLC was referred to high pressure generated by early columns. After 1970, however, HPLC term was emphasized to the effectiveness of separation accomplished. Besides, newer columns and packing materials provide performance at moderate pressure. HPLC has higher advantages in comparison with column chromatography: speed (many analysis in shorter times), a wide range of stationary phases, higher resolution and better sensitivity (different detectors can be used)[278] .

[277] Milhome, M. AL; Castro, R.C.; Silva, R. de O.; Nobre, C. de A.; Nascimento, R.F. do Advances in liquid chromatography coupled to mass spectrometry for determination of pesticide residues in food. In *Advances in Chromatographic Analysis*; Nascimento, R.F. do, Ed.; AvidScience, 2017; pp. 1–49 ISBN 9789386337511.

[278] Reuhs, B.L. High performance liquid chromatography. In *Food Analysis*; Nielsen, S.S., Ed.; Springer: Mason, Ohio, USA, 2017; pp. 315–331 ISBN 978-3-319-45776-5.

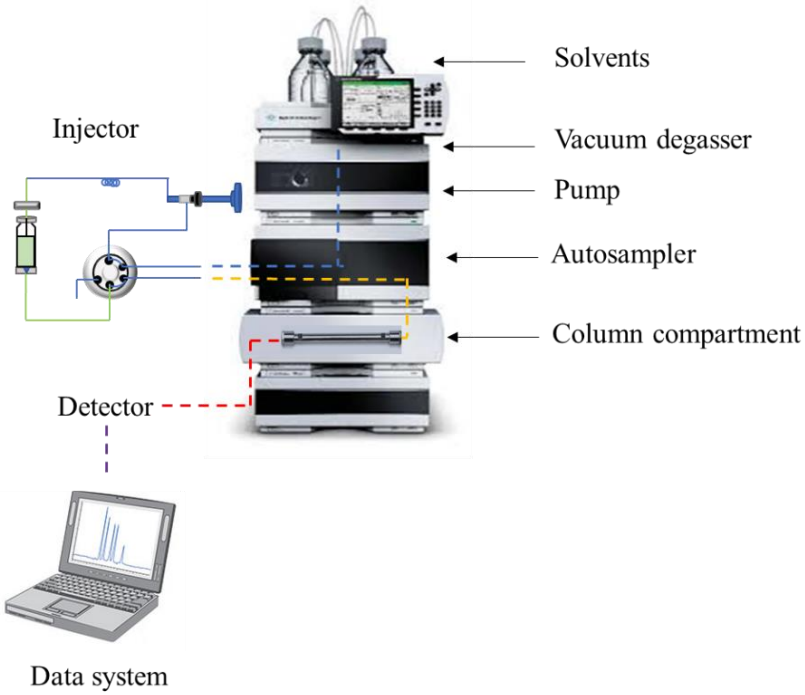

Figure 45. Scheme of a high-performance liquid chromatography. Source: Adapted from Agilent.

A schematic diagram of a basic HPLC system appears in **Figure 45.** The system is mainly composed by: pump, injector, column, detector, and data system[279]. The HPLC pump impulse the mobile phase through the system, commonly at a flow rate of 0.3−1.6 mL/min, in a controlled, accurate, and precise manner. Most pumps currently used in HPLC (>90 %) are reciprocating, piston-type pumps[218,278]. The injector role is to place the sample into the flowing mobile phase for introduction onto the column. The most common injection volumes are ranged from 1 to 100 µL [218]. Analytes are separated travelling at different speed according to the chemical structure of the analyte and the mobile phase composition. The time at which analyte elutes (rises out of the column) is called as retention time. After elution, compounds are detected generating an analytical signal[280]. An HPLC

[279] Malviya R, B. V; O.P., P.; P.K, S. High performance liquid chromatography: A short review. *J. Glob. Pharma Technol.* **2014**, 22–26.

[280] Thammana, M. A Review on High Performance Liquid Chromatography (HPLC). *J. Pharm. Anal.* **2016**, *5*, 22–28.

column is commonly built of stainless-steel tubing with terminators that allow connecting with the injector and detector systems.

There are different types HPLC separations depending on the stationary and mobile phases used in the process: **Normal phase chromatography** (NP-HPLC) that uses a polar stationary phase and a non-polar mobile phase, **reversed phase chromatography:** (RP-HPLC or RPC) that uses a non-polar stationary phase and polar mobile phase, **size exclusion chromatography** (SEC) that separates particles based on size and **ion exchange chromatography** (IEC), where the retention is due to the attraction among solute ions and charged sites bound to the stationary phase[279].

The separation efficiency depends on the properties and packing material that is composed the stationary phase and the composition of the mobile phase[281]. The HPLC mode used to determine phenolic compounds is mainly reversed-phase mode. The most used packing materials in reversed-phase are octadecylsilyl (ODS) bonded phases, with an octadecyl (C18) chain [-$(CH_2)_{17}CH_3$][280]. These columns range from 3 to 30 cm in length, 1 to 4.6 mm of internal diameter and 1.7 -10 μm of particle size[282] . The reduction in particle size and column length allows very fast separations with greater resolution[283]. The main advantages of small internal diameter comprises a decreased use of mobile phase, an increased peak concentration, improved resolution, and the ability to couple HPLC to MS[284]. Regarding the mobile phases, gradient elution is generally carried out with a binary solvent system for the separation of phenolic compounds. Acetonitrile, methanol and tetrahydrofuran (THF) are the most used organic modifiers[197]. Besides, aqueous acidified solvents are used to avoid the ionization of analytes during the

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[281] Mcpolin, O. An Introduction to HPLC for pharmaceutical analysis. In; Mourne Training Services, 2009; p. 148 ISBN 9780956152800.

[282] Kumar, N.; Goel, N. Phenolic acids: Natural versatile molecules with promising therapeutic applications. *Biotechnol. Reports* **2019**, *24*, e00370.

[283] Magwaza, L.S.; Opara, U.L.; Cronje, P.J.R.; Landahl, S.; Ortiz, J.O.; Terry, L.A. Rapid methods for extracting and quantifying phenolic compounds in citrus rinds. *Food Sci. Nutr.* **2016**, *4*, 4–10.

[284] B.L., R. High-Performance Liquid Chromatography. In *Food Analysis. Food Science Text Series*; S., N., Ed.; Springer, Cham, 2017; pp. 315–331.

analyses, the most used are acetic acid and formic acid in percentages of 0.1 to 5% (v/v)[218,285] .

Nevertheless, RP-HPLC is unable to separate larger chains of phenolic compounds such as oligomers of proanthocyanidins (higher than trimers) due to these compounds elute as a large unresolved peak[286]. Normal phase (NP)-HPLC can solve these problems and allows the separation of proanthocyanidins according to their degree of polymerization using a silica gel column. However, the main problem of silica-based stationary phases is the high and irreversible adsorption of longer-chain of proanthocyanidins, leading to a diminished capacity of the separation system. These problems can be overcome using diol phases, which are prepared by chemically modification of the silica gel surface[287]. Therefore, a high polarity and hydrogen bonding affinities are showed; they can be used in hydrophilic interaction liquid chromatography (HILIC). HILIC uses polar stationary phases (such as those for NP-HPLC) eluted with mobile phases similar to those for RP-HPLC. Therefore, some important advantages are obtained: solubility issues (often encountered in NP-HPLC) are easily overcome as aqueous mobile phases are used: RP-HPLC unresolved peaks are separated with a better resolution[287]. In contrast to RP-HPLC, gradient elution in HILIC begins with a low-polarity organic solvent and polar analytes elute by increasing the polar aqueous content. The most common solvents used with diol columns acidic acetonitrile and acidic aqueous methanol[288].

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[285] Cazes, J. *Encyclopedia of Chromatography*; CRC Press, 2004;

[286] Karonen, M.; Ossipov, V.; Sinkkonen, J.; Loponen, J.; Haukioja, E.; Pihlaja, K. Quantitative analysis of polymeric proanthocyanidins in birch leaves with normal-phase HPLC. *Phytochem. Anal.* **2006**, *17*, 149–156.

[287] Luca, S.V.; Bujor, A.; Miron, A.; Aprotosoaie, A.C.; Skalicka-Woźniak, K.; Trifan, A. *Preparative separation and bioactivity of oligomeric proanthocyanidins*; 2019; Vol. 5; ISBN 0123456789.

[288] Robbins, R.J.; Leonczak, J.; Johnson, J.C.; Li, J.; Kwik-Uribe, C.; Prior, R.L.; Gu, L. Method performance and multi-laboratory assessment of a normal phase high pressure liquid chromatography-fluorescence detection method for the quantitation of flavanols and procyanidins in cocoa and chocolate containing samples. *J. Chromatogr. A* **2009**, *1216*, 4831–4840.

6.3. Detection systems

6.3.1. UV-Vis detector

The UV-Visible absorbance detector is one of the most used HPLC detectors because phenolic compounds have the capacity to absorb in the UV (or visible) region (from 190–600 nm)[289]. Sample concentration is related to absorbance, is determined by the fraction of the light transmitted through the detector cell by Beer's Law. Phenolic compounds contain π conjugated systems with hydroxyl-phenolic groups. UV absorbance occurs as a result of orbitals electronic transitions[290]. Depending on the structure, phenolic compounds absorb at different wavelengths. Most hydroxybenzoic acid derivatives present their maxima in the range of 250-300 nm[291]. Hydroxycinnamic acids absorb in two UV regions, one maximum being in the range of 225–235 nm and the other in the range of 290–330 nm[292]. Besides, most of flavonoids possess two major absorption bands (**Table 17**)[40,293].

Table 17. Absorption bands characteristics of the phenolic compounds 291-293

Phenolic compound class	ʎ Absorption

[289] Swartz, M. HPLC detectors: A brief review. *J. Liq. Chromatogr. Relat. Technol.* **2010**, *33*, 1130–1150.

[290] Aleixandre-Tudo, J.L.; Wessel du Toit The Role of UV-Visible Spectroscopy for Phenolic Compounds Quantification in Winemaking. In *Frontiers and New Trends in the Science of Fermented Food and Beverages diseases*; IntechOpen, 2016.

[291] Lin, L.-Z.; Harnly, J.M. Quantitation of Flavanols, Proanthocyanidins, Isoflavones, Flavanones, Dihydrochalcones, Stilbenes, Benzoic Acid Derivatives Using Ultraviolet Absorbance after Identification by Liquid Chromatography–Mass Spectrometry. *J Agric Food Chem* **2012**, *60*, 5832–5840.

[292] Goleniowski, M.; Bonfill, M.; Cusido, R.; Palazón, J. Phenolic acids. In *Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes*; 2013; pp. 1951–1973 ISBN 9783642221446.

[293] Awouafack, M.D.; Tane, P.; Morita, H. Isolation and Structure Characterization of Flavonoids. In *Flavonoids - From Biosynthesis to Human Health*; 2017; pp. 46–59.

Hydroxybenzoic acids and their derivatives	250-300 nm	
Hydoxycinnamic acids	225-235	290-330
Flavones	250–280	310–350
Flavonols (3-OH substituted)	250–280	330–360
Flavonols (3-OH free)	250–280	350–385
Isoflavones	245–275	310–330
Flavonones	270–295	
Chalcones	230–270	340–390
Aurones	230–270	380–430
Anthocyanidins and anthocyanins	270–280	465–560

UV detectors are classified as fixed wavelength detectors (UV detectors), and variable and diode array detectors (DAD) that rely on one or more wavelengths generated from a broad-spectrum lamp. Diode array detection is the most used detector for phenolic compounds because it provides UV-Vis spectra of compounds at various weight lengths that could furnish additional information due to the creation of an absorption spectrum[294].

Nevertheless, the identification of each phenolic compound is carried out by comparison of its spectral data and retention time with the data obtained from commercial standards (which are limited)[218].

6.3.2. Fluorescence detector

[294] Wolfender, J.L. HPLC in natural product analysis: The detection issue. *Planta Med.* **2009**, *75*, 719–734.

Fluorescence detection (FLD) provides a high sensitivity and selectivity. In fluorescence, the molecular absorption of a photon causes the emission of another photon with a longer wavelength[294].

Fluorimetric detection has showed to be suitable in molecules which exhibit native fluorescence. Phenolic compounds show low or moderate native fluorescence; therefore, there are few applications of this detection system for the analysis of phenolic compounds. However, the main applications of fluorescence detection have been found on the determination of flavanols and their oligomers[295]. FLD affords two main benefits in comparison with UV-Vis detection: higher sensitivity due to detection limits are around an order of magnitude lower and more selectivity due to the difference in wavelengths between absorption vs. emission. The fluorescent light is measured against a very low-light background, thus enhance the S/N ratio[294].

6.3.3. Mass spectrometry

Mass spectrometry is considered a powerful detection technique coupled to chromatography because it presents important characteristics: high sensitivity (detection at trace level), high selectivity (it has the capacity to distinguish different substances in the sample) and it offers qualitative and quantitative information on the compounds eluted from the column[277].

The basic components of a mass spectrometer are: sample insertion system, ion source, mass analyzer, detector and data system. Chromatographic platforms are used as sample injection system and they are coupled to the ion source. In the ion source, the components of the sample are charged (positive or negative),

[295] de Rijke, E.; Out, P.; Niessen, W.M.A.; Ariese, F.; Gooijer, C.; Brinkman, U.A.T. Analytical separation and detection methods for flavonoids. *J. Chromatogr. A* **2006**, *1112*, 31–63.

transferred into the gas phase and accelerated towards the mass analyzer. The mass analyzer separates these ions depending on their mass to charge ratio, *m/z* [296].

The main ion sources are electron impact (EI), chemical ionization (CI), fast atom bombardment (FAB,) atmospheric pressure ionization (API) including atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo-ionization (APPI), electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI)[297] [295]. ESI is a soft ionization technique widely used for phenolic compounds[298] [296]. The advantage of ESI is that provides stable ions and low spontaneous fragmentation and operates both in positive (molecular species $[M + H]^+$) and negative ion modes (molecular species $[M-H]^-$)[296].

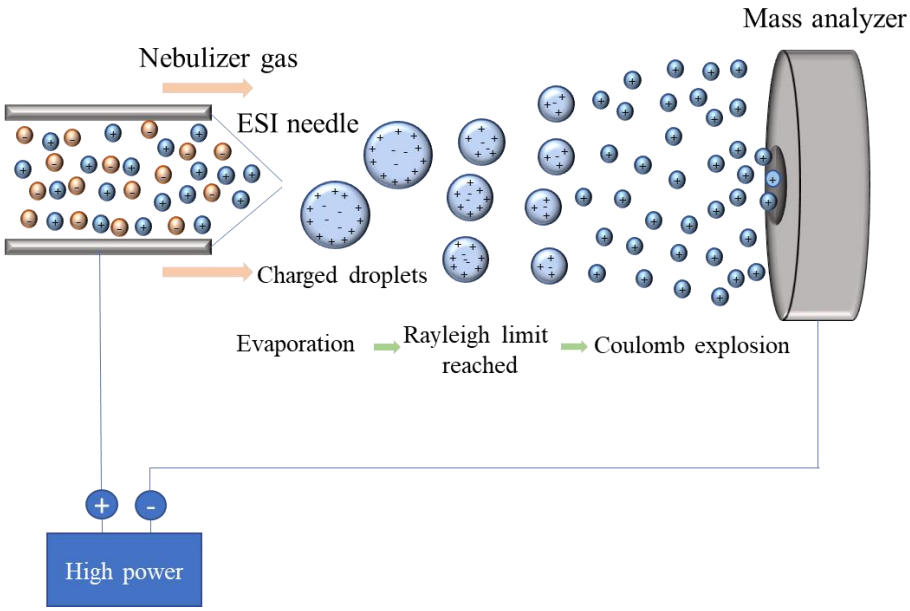

Figure 46. Schematic representation of ESI source. Adapted from[299].

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[296] Ignat, I.; Volf, I.; Popa, V.I. *Analytical Methods of Phenolic Compounds*; 2013; ISBN 9783642221446.

[297] Fulcrand, H.; Mané, C.; Preys, S.; Mazerolles, G.; Bouchut, C.; Mazauric, J.P.; Souquet, J.M.; Meudec, E.; Li, Y.; Cole, R.B.; et al. Direct mass spectrometry approaches to characterize polyphenol composition of complex samples. *Phytochemistry* **2008**, *69*, 3131–3138.

[298] Ignat, I.; Volf, I.; Popa, V.I. A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chem.* **2011**, *126*, 1821–1835.

[299] Alymatiri, C.M.; Kouskoura, M.G.; Markopoulou, C.K. Decoding the signal response of steroids in electrospray ionization mode (ESI-MS). *Anal. Methods*

In ESI (**Figure 46**), a high-voltage (3–5 kV) is applied to the solution passing through an extremely fine metal capillary tube (ESI needle) providing the nebulization resulting in charged droplets, the flow generally ranges from 1 to 10 μL min^{-1}. Nebulization of the solution is facilitated by a continuous flow of a dry gas, usually nitrogen (N_2). As the solvent evaporates, the superficial area of droplets decreases, and the ions of the surface are approximated among them. At a certain point, the repulsive forces between the same charges exceed the surface tensions, thus, reaching the Rayleigh limit that causes the droplets become unstable, so they explode (Coulomb explosion) causing smaller droplets. Then individual ions emerge in gas phase and then these ions are then separated by the MS system [277,294,299].

The analyzers used in this thesis and that were coupled to the liquid chromatography were: quadrupole (Q) and time-of-flight (TOF). Quadrupole (Q)-analyzer was used to determine alkylresorcinols in wheat aleurone thanks to a GC-MS system.

6.3.3.1. Quadrupole analyzer

Quadrupole analyzer is considered an universal analyzer because of its simplicity, relatively low price, good linearity in quantitative analyzes and ease of operation. The capacity to filter ions according to *m/z* gives this analyzer a great application. Its mass accuracy is commonly range from 0.1 to 0.2 atomic mass units (Da), whereas its mass range is compressed between 10 and 4000 Da [277].

2015, *7*, 10433–10444.

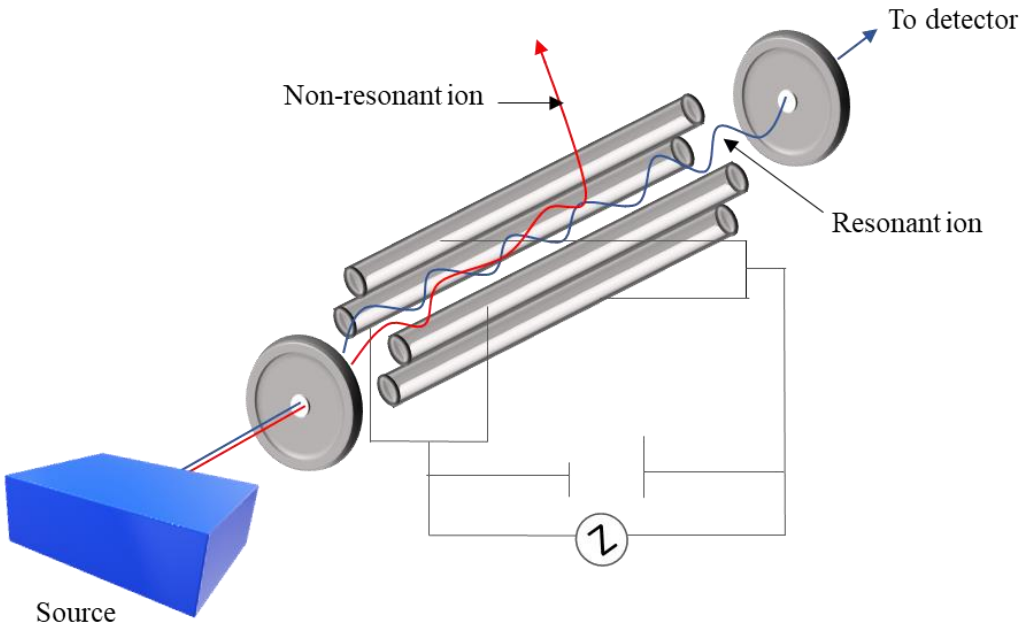

Figure 47. Scheme of a quadrupole analyzer. Adapted from[300].

Quadrupole comprises four parallel rods, normally made of metal, disposed in two pairs according to **Figure 47**[301]. A combination of direct current (DC) and radiofrequency (Rf) are applied to the quadrupole rods with one pair of rods having the opposite polarity to the other pair. These voltages create a fluctuating electric field between the rods, resulting in having equal but opposite charges. Quadrupoles work at a constant resolution (ratio Rf/DC constant). Considering a specific amplitude form the Rf and DC voltages, only the ions with a determined *m/z* (resonant ions) can pass across the quadrupole bars and could be detected. However, the other ions with unstable trajectories (non-resonant ions) will be eliminated by the vacuum pump after a collision with the quadrupole bars [302].

[300] Lanças, F.M. A cromatografia líquida moderna e a espectrometria de massas: Finalmente "compatíveis"? II. A escolha do analisador de massas. *Sci. Chromatogr.* **2013**, *5*, 27–46.

[301] Kinter, M. *Mass spectrometry/Principles and Instrumentation*; Elsevier, 2003;

[302] Thakur, R.A. Mass Spectrometry. In *Food Analyses*; S. Suzanne Nielsen, Ed.; Springer: West Lafayette, IN, USA, 2017; pp. 165–181.

6.3.3.2. Time of flight

Time of flight (TOF) analyzer has a high accuracy and resolution in phenolic compounds analysis in foods[298]. TOF was introduced around 1960s in the area of mass spectrometry and has highlighted for its high efficiency[277].

Simply, ions are pulsed into the TOF drift tube known as flight tube, and the time taken for the ions to traverse the flight tube to reach to detector is a measure of their *m/z* (**Figure 48**). TOF comprises three zones: the first one is the acceleration zone where ions with a mass m and total charge q=ze are accelerated by a potential V generated between an electrode and the extraction grid. The ions acquired kinetic energy (E_{kin})[303] and when leaving the acceleration region, the ions travel in the tube flight to reach the second zone, the reflectron of TOF that corrects the dispersion of kinetic energy of ions. Then, the ions are repelled and finally reach to the last component, the detector[303,304]. [301,302]:

$$E_{kin}= mv^2/2=zeV \quad (\textbf{eq.4})$$

where v is the velocity of the ion, z the number of charges on the ion and e the charge of an electron. The time *t* needed to cover the distance d of the flight tube to reach the detector is given by[303]:

$$t=d/v \quad (\textbf{eq.5})$$

Replacing v by its value in the previous equation gives[303]:

$$t^2 = m/z\ (d^2/2eV) \quad (\textbf{eq.6})$$

---

[303] Hoffmann, E. de; Stroobant, V. *Mass Spectrometry: Principles and Applications*; John Wiley & Sons: England, 2007; ISBN 9780470033104.

[304] Wang, Y.; Griffiths, W.J. Mass Spectrometry for Metabolite Identification. In *Metabolomics, Metabonomics and Metabolite Profiling*; Griffiths, W.J., Ed.; The Royal Society of Chemistry: Cambridge, UK, 2008; pp. 1–43.

Therefore, time is proportional to the mass-to-charge ratio (*m/z*).

TOF analyzers have the following advantages: a reasonably good resolution (10^4 FWMH) can be reached, a wide mass range (up to around *m/z* 2000000) is accessible with special detectors, fast work cycles (10–5000 scans/spectra/s), mass accuracy (2-50ppm), its high transmission generates great sensitivity (e.g., at the 1–10 fmol level) and provides lower errors than 2 mDa[305,306]. Mass resolution is proportional to the flight time and the flight path. Therefore, the resolution of these analyzers is improved when the length of the flight tube is increased[303].

[305] Telekes, A. Mass spectrometry instrumentation and technqiues. In *Medical Application of Mass Spectrometry*; K. Vékey, A.T. and A.V., Ed.; Elsevier, 2008; pp. 93–140.

[306] Petrovic, M.; Barceló, D. Application of liquid chromatography / quadrupole time-of-flight mass spectrometry (LC-QqTOF-MS). *J. mass Spectrom.* **2006**, *41*, 1259–1267.

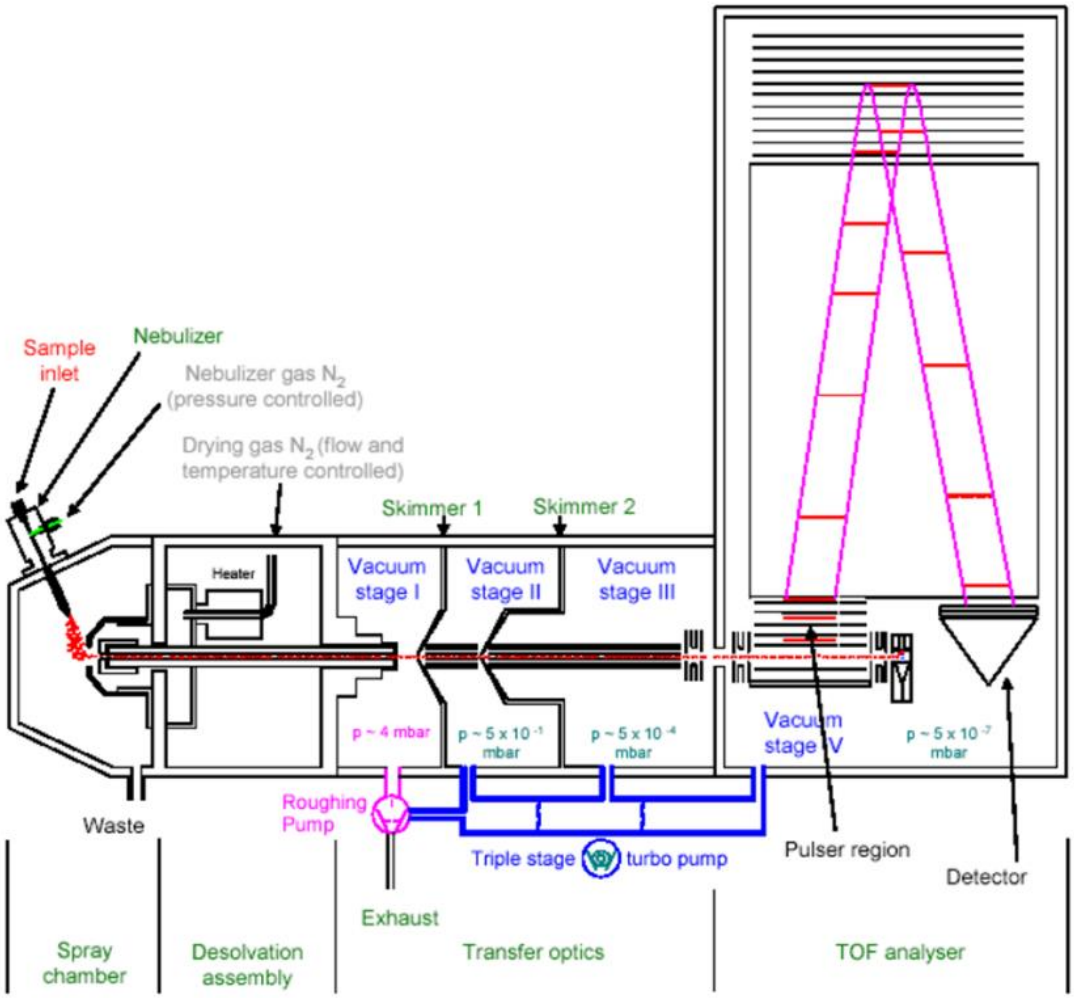

Figure 48. TOF analyzer[307]

6.3.3.3. Quadrupole-time of flight (QTOF)

QTOF analyzer is an hybrid of triple quadrupole analyzer and a time-of-flight analyzer. It is analogous to a triple quadrupole system but with the exception that the last quadrupole is replaced by a time-of-flight analyzer (**Figure 49**)[308]. In a

[307] *micrOTOFcontrol 1.1. Operator Manual*;
[308] Haag, A.M. Mass analyzers and mass spectrometers. In *Modern proteomics-sample preparation analysis and practical applications*; Mirzaei, H., Carrasco, M., Eds.;

QTOF, the sample is introduced via the interface and ions are focused using the hexapole ion bridge into the quadrupole MS. QTOF has the capacity of both MS and MS/MS operation modes. For MS, the first quadrupole is operated in band pass mode and the analysis is carried out in the TOF analyzer[309].For MS/MS, a precursor ion is selected by the first quadrupole, whereas the collision cell provides a collision-induced dissociation and the mass analysis of the fragment ions is carried out in the TOF analyzer[310].

QTOF-MS is a very attractive tool because its high sensitivity, high resolution and high mass accuracy for both precursor and fragment ions[310]. Therefore, this tool is very used for detection and quantification of phenolic compounds in plants [311].

Springer: Switzerland, 2016; Vol. 919, pp. 157–169 ISBN 9783319414461.

[309] Van Bocxlaer, J.F.; Vande Casteele, S.R.; Van Poucke, C.J.; Van Peteghem, C.H. Confirmation of the identity of residues using quadrupole time-of-flight mass spectrometry. *Anal. Chim. Acta* **2005**, *529*, 65–73.

[310] Lacorte, S.; R., A.; Fernandez-Alba Time of flight mass spectromety applied to the lquid chromatogrphic analysis of pesticides in water and food. *Mass Spectrom. Rev.* **2006**, *25*, 866–880.

[311] Rodríguez-Pérez, C.; Gilbert-López, B.; Mendiola, J.A.; Quirantes-Piné, R.; Segura-Carretero, A.; Ibáñez, E. Optimization of microwave-assisted extraction and pressurized liquid extraction of phenolic compounds from moringa oleifera leaves by multiresponse surface methodology. *Electrophoresis* **2016**, *37*, 1938–1946.

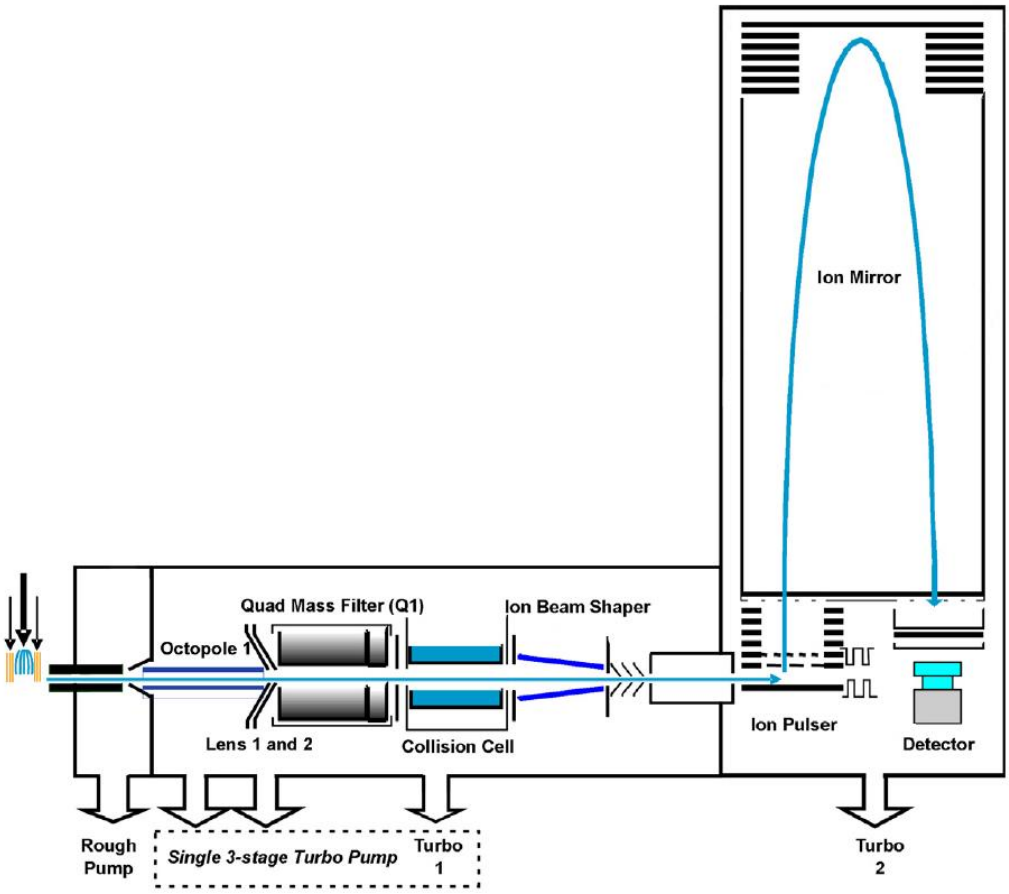

Figure 49. Scheme of the components of QTOF analyzer. Source: Agilent guide[312].

Table 18 shows usual HPLC conditions used for the analysis of phenolic compounds in olive leaves and cereals.

[312] Technologies, A. Agilent 7250 GC / MS System.

Table 18. Conditions used for the analysis of phenolic compounds in cereals and olive

Phenolic compounds	**Sample**	**Mode**	**Elution conditions**	**Column**	**Mobile phase**	**Injec volu**
Proanthocyanidins	Barley	Normal phase	3 min, 7%B 60 min, 37.6% B 63 min, 100% B 70 min, 100% B 76 min, 7%B	Develosil Diol 100 Å column, 5 µm, 250 × 4.6 mm i.d.	Phase A: CH3CN:HOAc, 98:2; v/v Phase B: acidic aqueous methanol (CH3OH: H2O:HOAc, 95:3:2; v/v/v).	5 µL
Phenolic compounds	Barley	Reverse phase	0 min, 5% B 12.5 min, 30% B 17.5 min, 60% B 22 min, 5% B	C18 (100 mm × 4.6 mm, 2.6 µm)	Phase A: acidified water (1% acetic acid) Phase B acetonitrile	2.5 µ
Phenolic compounds	Buckwheat	Reverse phase	14 min, 2% B 16 min, 6% B 20 min, 10% B 24 min, 17% B 38 min, 36% B 40 min, 38.5% B 53 min, 60% B 58 min, 100% B 78 min, 5% B 85 min, 2% B	C18 (5 µm, 250 × 3.0 mm i.d.)	Phase A: water/acetic acid (99:1, v/v) Phase B: mobile phase A/acetonitrile (60:40, v/v)	10µL
Phenolic compounds	Buckwheat	Reverse phase	From 2% B to 6% B in 16 min, from 6% to 10% in 4	C18 (4.6 µm, 150	Phase A: water/acetic acid (99:1, v/v)	10 µ

[313] Verardo, V.; Cevoli, C.; Pasini, F.; Gómez-Caravaca, A.M.; Marconi, E.; Fabbri, A.; Caboni, M.F. Analy in different barley genotypes using High-Performance Liquid Chromatography-Fluorescence Detect Infrared Methodologies. *J. Agric. Food Chem.* **2015**, *63*, 4130–4137.

[314] Verardo, V.; Arráez-Román, D.; Segura-Carretero, A.; Marconi, E.; Fernández-Gutiérrez, A.; Caboni, phenolic compounds by reverse phase high performance liquid chromatography-electrospray spectrometry (RP-HPLC e ESI-TOF-MS). *J. Cereal Sci.* **2010**, *52*, 170–176.

			min, from 10% to 17% in 4 min, from 17% to 36% in 14 min, from 36% to 38.5% in 2 min, from 38.5% to 60% in 13 min, from 60% to 100% in 5 min and from 100% to 2% in 2 min	mm, 1.8 mm)	Phase B: mobile phase A/acetonitrile (60:40, v/v)	
Phenolic compounds	Wheat	Reverse phase	0 min, 5% B; 10 min, 15% B; 30 min, 25% B; 35 min, 30% B; 50 min, 55% B; 55 min, 90% B; 57 min, 100% B	C18 (150 mm × 4.6 mm, particle size 2.7 nm)	Phase A: water/formic acid (97.5:2.5, v/v) Phase B: Acetonitrile	
Phenolic compounds	Olive leaves	Reverse phase	0 min, 10% B 10 min, 20% B 35 min, 0% B 40 min, 100% B 45 min, 100% B	C18 (5-µm, 150 x 4.6mm i.d.)	Phase A: water/ acetic acid (97.5:2.5, v/v) Phase B: acetonitrile	
Phenolic compounds	Olive leaves	Reverse phase	1% B at 0 min, 7% B over 5.50 min, 14% B over 5.5 min, 24% over 6.5 min, 40% B over 5 min, 100% B over 5 min, which was maintained 1 min, and then 1% B over 1 min, which was maintained until 35 min.	C18 (2.7 µm, 50 x 2.1 mm i.d.)	Phase A: water/ acetic acid (99.9:0.1, v/v) Phase B: acetonitrile	10 µ

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Phenolic compounds	Olive leaves	Reverse phase	From 2 to 25%, C over 40 min; a linear increase to 30% B, C over 5 min; another linear increase to 50%B, C over 15 min; and isocratic at 50% B, C for 8 min followed by alinear decrease to 2% B and C over 4 mi	C18 (5µm, 250 mm × 4.6 mm)	Phase A:water/orthophosphoric acid (99.8:0.2, v/v) Phase B: methanol Phase C: acetonitrile	20 µ
Phenolic compounds	Olive leaves	Reverse phase	0 min, 5% B 4 min, 9% B 7 min,12% B 8 min,15% B 9min,16% B 14 min, 20% B 15 min, 22% B 8 min, 28% B 19 min, 30% B 20 min, 31% B 21.50 min, 32% B 23 min, 34% B 24 min, 35% B 25.5 min, 40% B 27 min, 50% B 30 min, 100% B 35 min, 100% B 37 min, 5% B	C18 (4.6 µm, 100 mm, 2.7 mm)	Phase A: water/ acetic acid (99:1, v/v) Phase B: acetonitrile	2.5 µ
Phenolic compounds	Olive leaves	Reverse phase	0 min, 5% B 5 min, 15% B 25 min, 30% B 35 min, 95% B 40 min, 5% B	C18 (3 mm, 150 mm, 2mm)	Phase A: water/ acetic acid (99.5:0.5, v/v) Phase B: acetonitrile	1 µL

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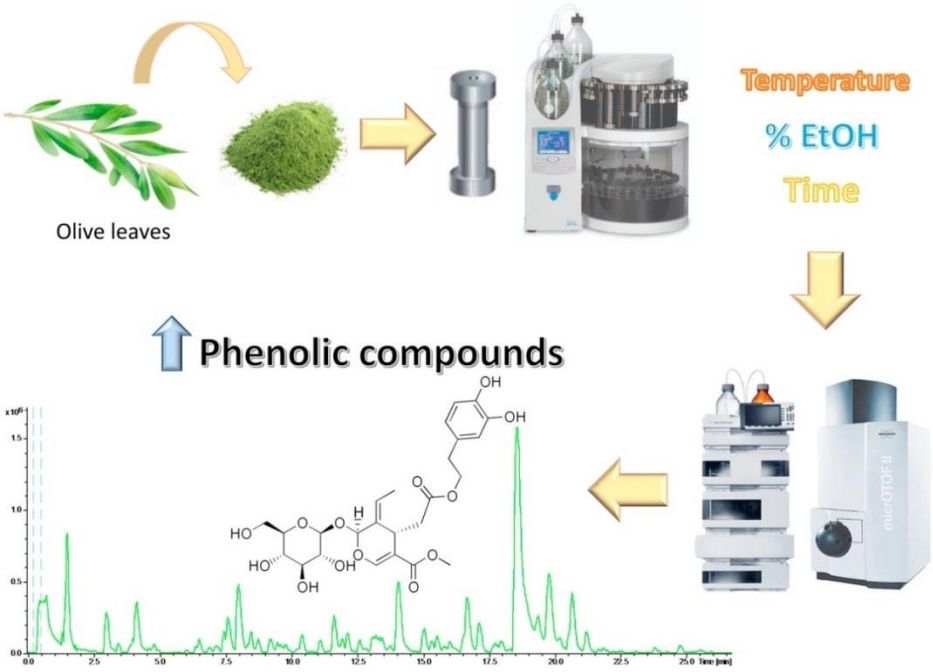
Phenolic compounds	Olive leaves	Reverse phase	0 min, 5% B 10 min, 30% B 12 min, 33% B 17 min, 38% B 20 min, 50% B 23 min, 95% B 25 min, 5% B 35 min, 5% (B)	C18 (4.6 × 150 mm, 1.8 μm)	Phase A: water/ acetic acid (99.5:0.5, v/v) Phase B: acetonitrile	10 μ

SECTION I

Optimization of different extraction techniques to obtain the highest phenolic recovery in olive leaves

Chapter 1

Box-Behnken experimental design for a green extraction method of phenolic compounds from olive leaves



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Box-Behnken experimental design for a green extraction method of phenolic compounds from olive leaves

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Abstract

Olive leaves are important olive by-products due to their high content of phenolic compounds and elenolic acids,which possess antioxidant, antimicrobial, anti-atherogenic and anti-inflammatory properties, among others.Thus, pressurized liquid extraction was used to obtain a high recovery of these compounds from olive leaves. ABox-Behnken design was performed to optimize the PLE conditions of temperature (50−200 °C), % ethanol-water (0–100 %) and extraction time (5−20 min) in order to obtain the highest content of simple phenols,secoiridois,flavonoids, elenolic acids, total compounds and extraction yield from olive leaves. Olive leaf extractswere analyzed by high performance liquid chromatography coupled to mass spectrometry (HPLC-MS). Thehighest content of total compounds was 158.7 ± 0.4 mg g-1d.w obtained at 105 °C, 100 % ethanol and 5 min,whereas the highest extraction yield was 43 ± 3% obtained at 198 °C, 100 % ethanol and 5 min. Therefore,multi-response analysis was carried out using desirability function to optimize the PLE conditions for both totalcompounds and yield. These optimal conditions were 138 °C, 100 % EtOH and 5 min obtaining 144 mg g-1d.w. oftotal compounds and 42.2 % of yield. The optimal temperature of 138 °C has shown a great phenolic recoveryand 100 % ethanol has been shown to be a safe solvent to use in the food industry. In addition, short extractiontimes (5 min) mean lower energy consumption and lower costs.

Therefore, these PLE optimum conditions couldbe implemented on an industrial scale.

Keywords: Pressurized liquid extraction, Phenolic compounds, HPLC-ESI-TOF-MS, olive leaves, Box-Behnken design, response surfaces.

1. Introduction

Olive (*Olea Europaea* L.) is one of the most abundant crops in the world, with an annual production in 2018 of 21.07 million tons. Specifically, Europe produces around 65% of all olives, with Spain being the largest olive producer producing 9.82 million tons in 2018[1]. The primary and best-known product of the olive tree is olive oil, but there are several by-products derived from olive harvesting or olive oil extraction, which are discarded such as olive leaves, stones, olive pomace, olive mill waste water, etc. Among them, olive leaves are the most important by-product because of their high content in phenolic compounds, which possess beneficial properties to human health[2]. Thus, the reutilization of olive leaves is of great interest to obtain extracts enriched in bioactive compounds such as phenolic compounds that can be used as supplements to produce food additives, functional food or pharmaceutical formulations[3]. It has been reported that oils with phenols from the olive leaf extract have been used in the enrichment of olive, sunflower and soy oils[4]. Olive leaf extract has also been marketed as dietary products[5]. Commercial products in the form of teas or food supplements are available around the world, such as dried leaves, powder, extracts or tablets[6]. Olive leaf tea is one of the most traditional herbal teas used by people across the Mediterranean to treat some diseases. For this reason, interest in the potential health benefits of olive leaves in various fields has increased[7]. Fractionation of extracts and purification of bioactive compounds for use in food and food supplements were also the objectives of previous studies[8,9]. These olive leaf extracts have different classes of phenolic compounds including simple phenols (hydroxytyrosol and tyrosol), flavonoids (luteolin 7-*O*-glucoside, rutin, apigenin 7-*O*-glucoside, luteolin 4-*O*-glucoside), and secoiridoids such as oleuropein, which is a heterosidic ester of

elenolic acid and hydroxytyrosol and is the most abundant compound in olive leaf extracts[10,11]. These phenolic compounds possess antioxidant, antimicrobial, antiviral, anti-atherogenic, cardioprotective, antihypertensive and anti-inflammatory properties[12-14]. Furthermore, these compounds have hypocholesterolemic and hypoglycemic activities[15] and improve lipid metabolism minimizing obesity problems[16].

The phenolic profile of olive leaf extracts varies based on the extraction method and extraction conditions. Because of that, it is important to evaluate different extraction methods to choose one that keeps the stability of phenolic compounds during the extractions while being efficient and cost-effective at the same time [17]. In this sense, pressurized liquid extraction (PLE), is considered a promising innovative extraction procedure for recovering phenolic compounds from olive leaves and this is a great alternative to the conventional extraction procedures due to its high selectivity, low solvent consumption, short extraction times and non-harmful solvents with lower toxicity causing the minimum environmental impact after their usage[17,18]. Ethanol is flammable, however the Food and Drug Administration (FDA) has labeled ethanol as a Generally Recognized as Safe solvent to use in food products[19]. PLE is an automated extraction technique which runs under constant pressure and varying parameter values such as temperature, extraction time, composition of extraction solvent, etc.[20]. PLE uses elevated temperatures while applying high pressure to keep the solvents in their liquid state, thus improving the extraction efficiency[21,22].

In addition, the technique most suitable to determining phenolic compounds in olive leaves is high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS). This advanced analytical technique affords shorter analysis times, it can easily separate a great variety of chemical mixtures and acquires a high level of versatility not found in other chromatographic systems such as Gas chromatography (GC) and high-performance liquid chromatography with UV detection HPLC-UV[23]. GC has limited application to the separation of phenolic compounds since these have a high polarity and a limited volatility. Therefore, a

derivatization step is necessary, thus increasing the duration of the analysis. Moreover, thermal decomposition may occur at high temperatures of the experiment, impeding the analysis of phenolics with the higher molecular mass[23,24]. The main problem of UV-Vis detection is the need for commercial standards for the identification of compounds, since absorption bands are mostly common to compounds of the same family[25].

The aim of this work was the optimization of PLE procedure with regard to the extraction of phenolic compounds from dried olive leaves and the characterization by HPLC-ESI-TOF-MS. For that purpose, an experimental Box-Behnken design coupled with response-surface methodology was performed to optimize extraction parameters of temperature, percentage solvent (ethanol and water), and extraction time in order to maximize the recovery of simple phenols, secoiridoids, flavonoids, elenolic acids, total compounds and extraction yield.

2. Materials and methods

2.1. Chemicals and reagents

For extractions, ethanol was purchased from Fisher Scientific (Leicestershire, UK), and water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). For HPLC analysis, LC-MS grade acetronitrile was purchased from Fisher (Fisher Scientific UK, Leicesterchire, UK) and ultrapure water was obtained by the aforementioned Milli-Q system. The acetic acid used was purchased from Fluka (Switzerland). Standard compounds used for the quantification were: hydroxytyrosol, oleuropein and apigenin, which were purchased from Sigma-Aldrich (Saint Louis, MO, USA), and oleuropein that was from Extrasynthèse (Lyon, France).

2.2. Samples

Olive leaves were from 'Hojiblanca' cultivar grown in Seville (Spain). Olive leaves were dried under controlled temperature at 22ºC. Subsequently, leaves were ground using an ultra-centrifugal mill ZM 200 (Retsch GmbH, Haan, Germany) and the resulting powder, with an average particle size of 0.2 mm, was stored at -20ºC until the extraction.

2.3. Extraction of phenolic compounds from olive leaves by PLE.

PLE was carried out using an accelerated solvent extractor equipped with a solvent controller (ASE 350, Dionex, Sunnyvale, CA, USA). 4 g of powdered leaves with diatomaceous earth (DE) (1:2 ratio) were placed in a 33 mL extraction cell. PLE was performed in static mode, where the solvent is pressurized in the extractor while the outlet valve is closed[26]. Extraction pressure was constant at 10.34 MPa[20,27]. After extraction, the extracts were collected in 200 mL volumetric flasks, which were immediately cooled in an ice bath to prevent extract degradation[28]. Then, the extracts were centrifuged at 13000 rpm for 15 min at 4ºC in a centrifuge (Sorvall ST 16 R, Thermo Scientific, Leicestershire, UK) and the supernatants were collected and evaporated at 35°C to dryness in a Savan SC250EXP Speed-Vac (Thermo Scientific, Leicestershire, UK). The extracts were stored at -18°C until further use. Dried extracts obtained were reconstituted in a proportion of 1/4 m/v (0.25mg/mL) of MeOH/H_2O (50/50), filtered with single-use syringe filters (0.20 µm pore size) and injected into the HPLC system.

2.4. Experimental design

A Box–Behnken design (BBD) with 3 variables was used to determine the response pattern and then establish a model since it is more simple and efficient than other three-level factorial designs[29,30]. The independent PLE parameters were the extraction temperature (X_1), % ethanol/water (v/v) (X_2) and extraction time (X_3) (**Table 1.**). Temperature range was the same as reported by Herrero et al. 2011[22]

who evaluated PLE temperature conditions (50-200ºC) and extraction solvent (water and ethanol) in the total phenolic content from olive leaves. The percentage of ethanol/water ranged from 0-100%, which was the same as the one previously used in PLE of oleuropein from olive leaves[20]. Furthermore, another study reported a range of static time (5,10 and 15 min), where PLE optimal conditions were 2 cycles, 80°C and 5 min for total polyphenols recovery, whereas 1 cycle with 100 °C and 15 min was optimal PLE conditions for total flavonoid recovery, respectively[27]. Nevertheless, other studies used a constant extraction time of 5 and 20 minutes in PLE for the phenolic recovery from olive leaves[22,31,32].Thus, in the present study the time range was from 5 to 20 min.

The response variables were fitted to a second-order polynomial model equation obtained by the response surface methodology (RSM) (**Eq.1**).

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} {X_{ii}}^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ii} X_i X_j \quad (\mathbf{Eq.\ 1})$$

The response variables (Y) were concentration of simple phenols, secoiridoids, flavonoids elenolic acids, total compounds and extraction yield in olive leaf extracts via HPLC-ESI-TOF-MS. The % of extraction yield was calculated by the following equation % Yield = weight of dried extract x 100/weight of dried sample (w/w)[33]. *Xi* and *Xj* are the independent factors affecting the response, and β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients of the model (intercept, linear, quadratic and interaction term).

Model building, experimental results and designs were processed using Statgraphics Centurion (Stat Point Technologies, Inc., VA, USA). An analysis of variance (ANOVA) with 95% confidence level was carried out for each response. The suitability of the regression model was decided by the regression coefficient (r^2), the *p* value of the model, and the lack of fit. Optimal conditions were chosen after considering the response surfaces for all variables.

Table 1. Concentration of simple phenols, secoiridoids, flavonoids, elenolic acid, total compou
extracts obtained by pressurized liquid extraction by Box Behnken design and quantified by HPLC
expressed as mg g-1 dry matter of olive leaves (mg g^{-1} d.w.) and yield is expressed as percentage
(w/w).

Run	Independent Variables			Dependent variables				
	X_1	X_2	X_3	Simple phenols (mg g^{-1} hydroxytyrosol d.w.)	Secoiridoids (mg g^{-1} oleuropein d.w.)	Flavonoids (mg apigenin g^{-1} d.w.)	Elenolic acids (mg oleuropein g^{-1} d.w.)	Total (mg
1	200	50	20	2.6 ± 0.4	95.4 ± 0.5	2.46 ± 0.02	2.6 ± 0.2	103
2	125	100	5	1.6 ± 0.1	129.93 ± 0.01	3.8 ± 0.2	3.56 ± 0.01	13
3	200	100	12.5	2.03 ± 0.08	75.8 ± 0.1	2.05 ± 0.03	2.47 ± 0.02	82
4	50	0	12.5	1.26 ± 0.05	53.62 ± 0.04	2.440 ± 0.004	2.59 ± 0.05	60.
5	125	50	12.5	1.77 ± 0.03	122.1 ± 0.2	3.65 ± 0.06	3.87 ± 0.06	13
6	125	100	20	2.2 ± 0.1	132.6 ± 0.9	4.15 ± 0.02	4.0 ± 0.1	
7	125	0	20	2.10 ± 0.08	107.3 ± 0.4	3.03 ± 0.04	3.28 ± 0.07	11
8	50	100	12.5	1.68 ± 0.08	114.5 ± 0.3	2.46 ± 0.07	3.57 ± 0.06	12
9	200	50	5	2.6 ± 0.2	102 ± 1	2.416 ± 0.001	3.2 ± 0.2	
10	125	0	5	2.19 ± 0.06	115.4 ± 0.8	3.35 ± 0.01	4.00 ± 0.01	12
11	125	50	12.5	1.78 ± 0.04	102.3 ± 0.1	3.23 ± 0.03	3.7 ± 0.1	11
12	50	50	5	1.66 ± 0.02	104.0 ± 0.6	3.07 ± 0.03	3.837 ± 0.003	11
13	125	50	12.5	2.2 ± 0.1	126.8 ± 0.3	3.5 ± 0.1	3.87 ± 0.07	13
14	50	50	20	1.8 ± 0.1	92.5 ± 0.3	3.28 ± 0.01	3.36 ± 0.04	101
15	200	0	12.5	5.17 ± 0.03	74.2 ± 0.9	2.06 ± 0.06	2.90 ± 0.09	84

X_1: Temperature, X_2: % ethanol/water (v/v). X_3: Time of extraction.

2.5. Analysis of the phenolic composition by HPLC-ESI-TOF-MS

Analyses of compounds of olive leaves were performed in line with a method previously established by Talhaoui et al. (2014)[34], using an Agilent 1200 series Rapid Resolution Liquid Chromatograph (Agilent Technologies, CA, USA), which was comprised of a binary pump, degasser, and auto sampler. Phenolic compounds were separated using a Poroshell 120 EC-C18 (4.6 x 100 mm, 2.7 µm) from Agilent Technologies, at 25ºC and a flow rate of 0.8 mL min^{-1}. The mobile phases were 1% acetic acid as mobile phase A and acetonitrile as mobile phase B. The conditions of the solvent gradient were the following: 0 min, 5% B; 4 min, 9% B; 7 min, 12% B; 8 min, 15% B; 9 min, 16% B; 14 min, 20% B; 15 min, 22% B; 18 min, 28% B; 19 min, 30% B; 20 min, 31% B; 21.50 min, 32% B; 23 min, 34% B; 24 min, 35% B; 25.5 min, 40% B; 27 min, 50% B; 30 min, 100% B; 34 min, 100% B; 36 min, 5%B.

In addition, HPLC system was coupled to a microTOF™ (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer, using an electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA) operating in negative ion mode. The effluent from the HPLC column was split using a T-type phase separator before being introduced into the mass spectrometer (split ratio = 1:3). Analysis parameters were arranged using a negative-ion mode with a scan range from *m/z* 50 to 1000. The optimum values for the ESI−MS parameters were as follows: capillary voltage, +4.0 kV; drying gas temperature, 200°C; drying gas flow, 9.0 L min^{-1}; and nebulizing gas pressure, 2.0 bar. External mass spectrometer calibration was performed with sodium acetate clusters (5 mM sodium hydroxide in water/2-propanol 1/1 (v/v), with 0.2% of acetic) in quadratic high precision calibration (HPC) regression mode.

The data was processed using the software Data Analysis 4.0 (Bruker Daltonik). The identification of compounds was carried out by the generation of the candidate formula with a tolerance of 10 ppm (part per million mass error) using the SmartFormula™ editor and considering the retention time (RT), mass spectrum and the information available in the relative literature. The integration of peak

areas of compounds was carried out using Bruker Compass Target Analysis 1.2 software for compound screening (Bruker Daltonics, Bremen, Germany). Three standards (hydroxytyrosol, oleuropein and apigenin) were used for the quantification of compounds in the olive leaf extracts. The calibration curves were prepared at seven concentration levels from the limit of quantification (LOQ) to 125 mg/L (**Table S2**.).

3. Results and discussion

3.1. Characterization of phenolic and other compounds from olive leaves PLE extracts by HPLC-ESI-TOF-MS

The identification of phenolic compounds from PLE extracts of olive leaves was carried out as previously reported in other works[16,34-36]. In this study, a total of 42 compounds were identified in olive leaf PLE extracts. Compounds were classified in simple phenols, secoiridoids, flavonoids and other compounds (elenolic acids).

Table 2. Compounds identified in PLE olive leaves extracts by HPLC-ESI-TOF-MS.

Peak	*RT*	m/z experimental	m/z calculated	Tolerance (ppm)	Error (ppm)	mSigma	Molecular formula	
1	3.65	389.1093	389.1089	10	-1	14.2	$C_{16}H_{22}O_{11}$	
2	4.3	315.1085	315.1085	10	0.1	3.7	$C_{14}H_{20}O_8$	
3	4.64	389.1104	389.1089	10	-3.7	8.1	$C_{16}H_{22}O_{11}$	
4	5.28	153.0555	153.0557	10	1.1	8.2	$C_8H_{10}O_3$	
5	6.14	341.085	341.0878	10	8.3	32	$C_{15}H_{18}O_9$	C
6	6.17	299.1122	299.1136	10	4.9	26	$C_{14}H_{20}O_7$	T
7	7.75	403.1204	403.1246	15	10.4	7.7	$C_{17}H_{24}O_{11}$	g
8	8.18	403.1213	403.1246	10	8.1	12.1	$C_{17}H_{24}O_{11}$	g
9	8.53	389.1072	389.1089	10	4.5	35.8	$C_{16}H_{22}O_{11}$	
10	10.81	377.1397	377.1453	15	14.8	3.3	$C_{16}H_{26}O_{10}$	O
11	11.44	609.1426	609.1461	10	5.8	16.6	$C_{27}H_{30}O_{16}$	Lu
12	11.94	403.1204	403.1246	15	10.4	7.7	$C_{17}H_{24}O_{11}$	g
13	13.26	525.1588	525.1614	10	4.9	28.8	$C_{24}H_{30}O_{13}$	D
14	13.7	555.1696	555.1719	10	4.1	27.6	$C_{25}H_{32}O_{14}$	H
15	13.78	609.1452	609.1461	10	1.5	16.6	$C_{27}H_{30}O_{16}$	
16	13.9	593.1513	593.1512	10	-0.2	20.5	$C_{27}H_{30}O_{15}$	L
17	14.55	447.0949	447.0933	10	-3.5	43	$C_{21}H_{20}O_{11}$	L
18	15.52	555.1723	555.1719	10	-0.6	19.3	$C_{25}H_{32}O_{14}$	H
19	15.92	623.1996	623.1981	10	-2.3	35.4	$C_{29}H_{36}O_{15}$	
20	15.9	577.155	577.1563	10	2.2	27.2	$C_{27}H_{30}O_{14}$	A

21	16.22	701.2284	701.2298	10	2.1	19.9	$C_{31}H_{42}O_{18}$	Ol
22	16.44	607.1676	607.1668	10	-1.2	19	$C_{28}H_{32}O_{15}$	rha
23	16.47	701.228	701.2298	10	2.7	26	$C_{31}H_{42}O_{18}$	Ol
24	16.54	447.0919	447.0933	10	3.2	22.7	$C_{21}H_{20}O_{11}$	L
25	16.69	701.2277	701.2298	10	3	17.1	$C_{31}H_{42}O_{18}$	Ol
26	17.04	431.097	431.0984	10	3.1	15.1	$C_{21}H_{20}O_{10}$	A
27	17.18	447.0951	447.0933	10	-4	34.4	$C_{21}H_{20}O_{11}$	L
28	17.56	461.1106	461.1089	10	-3.7	11.5	$C_{22}H_{22}O_{11}$	
29	18.03	701.2297	701.2298	10	0.2	23	$C_{31}H_{42}O_{18}$	Ol
30	18.16	541.1927	541.1927	10	-0.1	7.9	$C_{25}H_{34}O_{13}$	
31	18.31	447.0922	447.0933	10	2.4	12.3	$C_{21}H_{20}O_{11}$	L
32	18.6	701.2296	701.2298	10	0.3	25.1	$C_{31}H_{42}O_{18}$	Ol
33	18.87	539.1865	539.177	10	-7.6	51.9	$C_{25}H_{32}O_{13}$	O
34	19.5	539.1779	539.177	10	-1.7	18.7	$C_{25}H_{32}O_{13}$	O
35	19.95	539.1807	539.177	10	-6.8	36.9	$C_{25}H_{32}O_{13}$	O
36	20.22	539.1722	539.177	10	8.9	17.2	$C_{25}H_{32}O_{13}$	O
37	20.54	539.1722	539.177	10	8.1	27.5	$C_{25}H_{32}O_{13}$	O
38	20.82	523.1838	523.1821	10	-3.3	38.4	$C_{25}H_{32}O_{12}$	
39	21.32	285.0401	285.0405	10	1.3	19.8	$C_{15}H_{10}O_6$	
40	21.46	301.0345	301.0354	10	3	26.6	$C_{15}H_{10}O_7$	
41	21.94	553.1874	553.1927	10	9.6	13.8	$C_{26}H_{34}O_{13}$	C
42	23.2	613.1866	613.1927	10	9.9	43.5	$C_{31}H_{34}O_{13}$	

Table S1. Phenolic compounds quantified in PLE olive leaves extracts by HPLC-ESI-TOF-MS e
of olive leaves. Different letters indicate significant differences among the extractions. N.D.: N
quantification. LOQ=0.051mg L^{-1} for flavonoids.

	PLE 1	**PLE 2**	**PLE 3**	**PLE 4**	**PLE 5**	**PL**
			Simple phenols			
Hydroxytyrosol-hexose	1.3 ± 0.1^{c,d}	1.26 ± 0.04^{c,d}	1.04 ± 0.01^{b,c}	0.96 ± 0.05^{b}	1.35 ± 0.04^{d,e,f}	0.4 0.0
Hydroxytyrosol	1.3 ± 0.4^{c}	0.21 ± 0.05^{a}	0.89 ± 0.06^{b}	0.210 ± 0.006^{a}	0.30 ± 0.01^{a}	1.6 ±
Tyrosol glucoside	0.072 ± 0.002 ^{a}	0.11 ± 0.01^{b,c,d}	0.100 ±0.002 ^{b,c}	0.091 ± 0.009 ^{a,b}	0.117 ± 0.001 ^{c,d,e,f}	0.12 0.003
Total simple phenols	2.6 ± 0.5^{f,g}	1.6 ± 0.1^{b,c}	2.03 ± 0.08^{c,d,e,f}	1.26 ± 0.05 ^{a,b}	1.77 ± 0.03 ^{c,d,e,f}	2.2 0.1
			Secoiridoids			
Oleoside	0.081 ± 0.003^{a,b}	0.12 ± 0.01^{a,b,c,d,e}	0.059 ± 0.005^{a}	0.08 ± 0.01^{a,b,c}	0.143 ± 0.001^{b,c,d,e,f}	0.1 0.02
Secologanoside isomer a	2.50 ± 0.04^{b,c}	2.53 ± 0.01^{c,d}	2.05 ± 0.05^{a,b}	2.057 ± 0.003^{a,b}	2.627 ± 0.002^{c,d,e}	3.0 0.3
Secologanoside isomer b	2.7 ± 0.1^{a,b,c}	3.8 ± 0.3^{d,e,f,g}	2.13 ± 0.01^{a}	2.99 ± 0.08^{b,c,d}	3.71 ± 0.07^{d,e,f,g}	3.7 0.3
Oleuropein aglycon	0.4509 ± 0.0003^{b,c}	0.7 ± 0.1^{e,f}	0.29609 ± 0.00002^{a}	0.47 ± 0.04^{c}	0.75 ± 0.03^{e,f,g}	0.7 0.0
Demethyloleuropein	0.08 ± 0.01^{b}	0.174 ± 0.001^{f,g}	0.049 ± 0.005^{a}	0.060 ± 0.005^{a,b}	0.164 ± 0.001^{f}	0.1 0.0
Hydroxyoleuropein/hydroxyoleuroside isomer a	0.43 ± 0.01^{b,c}	1.0 ± 0.1^{e}	0.4271 ± 0.0002^{b,c}	0.74 ± 0.01^{d}	1.21 ± 0.04^{f}	0.7 0.0
Hydroxyoleuropein/hydroxyoleuroside isomer b	1.098 ± 0.001^{e,f}	1.3 ± 0.1^{f}	0.82 ± 0.01^{d}	N.D.	1.125 ± 0.004^{e,f}	2.04 0.0
Oleuropein glucoside isomer a	0.17 ± 0.02^{a,b,c,d}	0.28 ± 0.03^{e}	0.13 ± 0.01^{a}	0.158 ± 0.005^{a,b,c}	0.25 ± 0.02^{d,e}	0.3 0.0
Oleuropein glucoside isomer b	0.245 ±0.001^{c,d}	0.35 ± 0.03^{f}	0.158 ± 0.002^{b}	0.2359 ± 0.0003^{b,c}	0.33 ± 0.02^{d,e,f}	0.4 ±
Oleuropein glucoside isomer c	0.34 ± 0.04^{a}	0.8 ± 0.1^{c}	0.61 ± 0.01^{b}	0.55 ± 0.01^{b}	0.81 ± 0.02^{c}	0.9 0.0

Oleuropein glucoside isomer d	0.14 ± 0.02^{a,b,c}	0.21 ± 0.02^{c,d}	0.140 ± 0.004^{a,b}	0.10 ± 0.01^{a}	0.22 ± 0.03^{d}	0.2 0.0
Hydro-oleuropein/hydro-oleuroside	0.23 ± 0.01^{c}	0.33 ± 0.02^{d,e}	0.162 ± 0.001^{a,b}	0.15 ± 0.01^{a}	0.345 ± 0.004^{e,f}	0.3 0.0
Oleuropein glucoside isomer e	0.06 ± 0.01^{a}	0.11 ± 0.01^{a,b,c}	0.05 ± 0.01^{a}	0.33 ± 0.01^{f,g}	0.17 ± 0.01^{d,e}	0.1 0.0
Oleuropein isomer a	68.6 ± 0.8^{d}	104.1 ± 0.2^{k}	52.52 ± 0.02^{c}	39.1 ± 0.2^{a}	94.6 ± 0.6^{j}	104 0.
Oleuropein isomer b	1.4 ± 0.3^{a,b}	2.36 ± 0.03^{c,d}	1.3 ± 0.1^{a}	1.30 ± 0.03^{a}	2.31 ± 0.04^{c,d}	2.6 0.2
Oleuropein isomer c	13.0 ± 0.3^{i}	8.7 ± 0.1^{f}	10.97 ± 0.01^{h}	3.40 ± 0.06^{a}	9.4 ± 0.1^{g}	9.2 0.0
Oleuropein isomer d	0.9 ± 0.1^{d}	N.D.	1.03 ± 0.07^{d}	N.D.	0.32 ± 0.02^{b}	0.1 0.0
Oleuropein isomer e	0.8 ± 0.1^{b}	N.D.	0.78 ± 0.03^{b}	N.D.	0.21 ± 0.03^{a}	0.2 ±
Ligstroside	1.99 ± 0.02^{b,c}	3.0 ± 0.1^{e,f,g,h}	1.924 ± 0.002^{a,b}	1.9 ± 0.1^{a,b}	3.16 ± 0.04^{g,h}	3.1 ±
Oleuropein/oleuroside methyl ether	0.127 ± 0.003^{b,c}	0.16 ± 0.01^{d,e}	0.108 ± 0.004^{b}	0.05 ± 0.01^{a}	0.172 ± 0.002^{d,e}	0.1 0.0
Total secoiridoids	95.4 ± 0.5^{d}	129.93 ± 0.01^{j}	75.8 ± 0.1^{b}	53.62 ± 0.04^{a}	122.1 ± 0.2^{h}	132 0.
			Flavonoids			
Luteolin-diglucoside a	0.0671 ± 0.0005	0.145 ± 0.002^{e,f}	0.058 ± 0.002^{a,b}	0.100 ± 0.003^{d}	0.13611 ± 0.00001^{e,f}	0.1 0.0
Rutin	0.154 ± 0.004^{d,e,f,g}	0.18 ± 0.01^{g,h,i,j}	0.0918 ± 0.0001^{a,b}	0.126 ± 0.004^{c,d}	0.17 ± 0.01^{g,h,i}	0.2 0.0
Luteolin rutinoside	0.086 ± 0.004^{b,c}	0.16 ± 0.02^{g,h}	0.0809 ± 0.0003^{b,c}	0.118 ± 0.003^{d,e}	0.16 ± 0.01^{g,h}	0.1 0.0
luteolin glucoside isomer a	0.636 ± 0.002^{a}	1.14 ± 0.05^{f,g}	0.65 ± 0.02^{a}	0.88 ± 0.01^{b}	1.13 ± 0.03^{e,f,g}	1.24 0.0
Apigenin rutinoside	0.110 ± 0.001^{a,b,c}	0.19 ± 0.01^{f,g,h}	0.103 ± 0.004^{a}	0.13888 ± 0.00003^{c,d,e}	0.195 ± 0.004^{f,g,h}	0.20 0.0
Diosmetin rhamnoside glucoside (diosmin)	0.004 ± 0.001^{a,b}	0.012 ± 0.001^{b,c,d,e}	0.0011 ± 0.0002^{a}	0.0071 ± 0.0005^{a,b,c}	0.014 ± 0.002^{c,d,e}	0.0 0.0
luteolin glucoside isomer b	0.05 ± 0.01^{c,d}	0.12 ± 0.01^{f}	0.050 ± 0.003^{c,d}	0.032 ± 0.002^{a,b}	0.1181 ± 0.0001^{f}	0.1 0.0
Apigenin glucoside	0.200 ± 0.004^{g}	0.19 ± 0.04^{g}	0.0632 ± 0.0001^{a}	0.090 ± 0.005^{a,b,c}	0.13 ± 0.01^{c,d,e,f}	0.1 0.0

luteolin glucoside isomer c	0.498 ± 0.001^{a,b}	0.79 ± 0.05^{d,e}	0.442 ± 0.003^{a,b}	0.620 ± 0.004^{c}	0.79 ± 0.02^{d,e}	0.9 ±
Chrysoeriol-7-O-glucoside a	0.28 ± 0.02^{b,c,d}	0.51 ± 0.02^{h,i}	0.188 ± 0.003^{a,b}	0.25 ± 0.01^{d}	0.39 ± 0.01^{g,h,i}	0.42 0.0
luteolin glucoside isomer d	0.056 ± 0.003^{a,b,c}	0.12 ± 0.01^{f,g}	0.051 ± 0.001^{a,b}	0.052 ± 0.004^{a,b,c}	0.11 ± 0.01^{e,f,g}	0.1 0.0
Luteolin isomer a	0.24 ± 0.02^{d}	0.018 ± 0.001^{a}	0.167 ± 0.004^{c}	0.025 ± 0.001^{a}	0.025 ± 0.001^{a}	0.02 0.0
Quercetin	0.15 ± 0.01^{d,e}	0.34 ± 0.02^{g}	0.107 ± 0.002^{c}	<LOQ	0.28 ± 0.01^{f}	0.3 0.0
Resinoside	<LOQ	0.003 ± 0.001^{a,b,c}	<LOQ	<LOQ	0.005 ± 0.001^{b,c,d}	0.00 0.00
Total flavonoids	2.46 ± 0.02^{b}	3.8 ± 0.2^{f}	2.05 ± 0.03^{a}	2.440 ± 0.004^{b}	3.65 ± 0.06^{f}	4.1 0.0
Other phenolic compounds						
Caffeoylglucoside	0.006 ± 0.003^{a,b,c}	0.02 ± 0.01^{d,e,f}	0.00221 ± 0.00003^{a}	0.005 ± 0.002^{a,b}	0.016 ± 0.005^{b,c,d}	0.00 0.00
Verbascoside	0.20 ± 0.01^{a}	0.197 ± 0.001^{a}	0.22 ± 0.04^{a,b}	0.291 ± 0.005 ^{a,b,c}	0.496 ± 0.002 ^{d,e}	0.56 ±
Other compounds						
Elenolic acid glucoside isomer a	0.163 ± 0.003^{b,c}	0.305 ± 0.004^{d,e}	0.12 ± 0.01^{a,b}	0.14 ± 0.02^{a,b}	0.41 ± 0.01^{f,g}	0.3 0.0
Elenolic acid glucoside isomer b	1.0 ± 0.1^{b}	1.73 ± 0.01^{d,e}	1.00 ± 0.04^{b}	1.36 ± 0.02^{c}	1.75 ± 0.02^{d,e,f}	1.8 0.0
Elenolic acid glucoside isomer c	1.4 ± 0.1^{b,c}	1.53 ± 0.03^{b,c,d,e}	1.35 ± 0.02^{b}	1.08 ± 0.04^{a}	1.71 ± 0.03^{e,f}	1.7 ±
Total elenolic acids	2.6 ± 0.2^{b,c}	3.56 ± 0.01^{e,f,g}	2.47 ± 0.02^{a,b}	2.59 ± 0.05^{b,c}	3.87 ± 0.06^{g,h}	4.0 ±
Total compounds	103.39 ± 0.06^{c}	139.1 ± 0.1^{j,k}	82.5 ± 0.2^{b}	60.21 ± 0.06^{a}	131.9 ± 0.2^{i}	143

Continue

	PLE9	**PLE10**	**PLE11**	**PLE12**	**PLE13**
Simple phenols					

Hydroxytyrosol-hexose	1.5 ± 0.1e,f,g	1.60 ± 0.06f,g,h	1.36 ± 0.03d,e,f	1.35 ± 0.03d,e,f	1.8 ± 0.1h
Hydroxytyrosol	0.90 ± 0.07b	0.458 ± 0.003a	0.30 ± 0.01a	0.19 ± 0.02a	0.28 ± 0.03a
Tyrosol glucoside	0.124 ± 0.004d,e,f,g	0.136 ± 0.005e,f,g	0.115 ± 0.007c,d,e	0.12 ± 0.01c,d,e,f,g	0.138 ± 0.001f,g,h
Total simple phenols	2.6 ± 0.2g	2.19 ± 0.06d,e,f,g	1.78 ± 0.04b,c,d	1.66 ± 0.02c,d,e	2.2 ± 0.1d,e,f,g
			Secoiridoids		
Oleoside	0.11 ± 0.01 a,b,c,d	0.20 ± 0.01e,f,g	0.16 ± 0.04d,e,f,g	0.164 ± 0.003 d,e,f,g	0.23 ± 0.04g
Secologanoside isomer a	2.71 ± 0.02c,d,e	3.03 ± 0.05e,f,g	2.78 ± 0.07c,d,e	2.9 ± 0.1c,d,e	3.4 ± 0.2g
Secologanoside isomer b	3.2 ± 0.1c,d,e,f	4.3 ± 0.2g	3.8 ± 0.2e,f,g	4.0 ± 0.3f,g	4.5 ± 0.4g
Oleuropein aglycon	0.62 ± 0.01d,e	1.01 ± 0.04i	0.74 ± 0.03e,f,g	0.73 ± 0.06e,f,g	0.9 ± 0.1h,i
Demethyloleuropein	0.10 ± 0.01c	0.155 ± 0.004e,f	0.132 ± 0.005d,e	0.13 ± 0.01d,e	0.17 ± 0.01f
Hydroxyoleuropein/hydroxyoleuroside isomer a	0.78 ± 0.02d	2.78 ± 0.06h	1.1109 ± 0.0001f	0.516 ± 0.003c	1.21 ± 0.02f

Hydroxyoleuropein/hydroxyoleuroside isomer b	1.1 ± 0.1e	0.414 ± 0.004b,c	0.58 ± 0.04c	0.06 ± 0.01a	1.09 ± 0.02e
Oleuropein glucoside isomer a	0.24 ± 0.04b,c,d,e	0.34 ± 0.01e	0.28 ± 0.03e	0.25 ± 0.01c,d,e	0.3 ± 0.1e
Oleuropein glucoside isomer b	0.25 ± 0.01c,d	0.24 ± 0.01b,c	0.198 ± 0.002b,c	0.21 ± 0.01b,c	0.27 ± 0.03c,d,e
Oleuropein glucoside isomer c	1.0 ± 0.1d,e,f	1.11 ± 0.04f,g	0.99 ± 0.01d,e,f	1.01 ± 0.02d,e,f	1.24 ± 0.02g
Oleuropein glucoside isomer d	0.23 ± 0.05d	0.25 ± 0.01d	0.22 ± 0.01d	0.24 ± 0.01d	0.27 ± 0.01d
Hydrooleuropein/hydrooleuroside	0.290 ± 0.002d	0.37 ± 0.01e,f	0.336 ± 0.003e	0.34 ± 0.02e,f	0.43 ± 0.01g
Oleuropein glucoside isomer e	0.09 ± 0.03a,b	0.38 ± 0.01g	0.14 ± 0.02b,c,d,e	0.156 ± 0.002c,d,e	0.194 ± 0.00
Oleuropein isomer a	67.4 ± 0.1d	86.5 ± 0.9h	77.85 ± 0.05e	80.2 ± 0.1f	90.8 ± 0.5i
Oleuropein isomer b	2.0 ± 0.2b,c	2.685 ± 0.002c,d,e	2.43 ± 0.08c,d,e	2.8 ± 0.1d,e	3.0 ± 0.4e
Oleuropein isomer c	16.7 ± 0.1k	8.7 ± 0.2f,g	7.5 ± 0.2d,e	7.13 ± 0.07d	15.17 ± 0.00
Oleuropein isomer d	1.6 ± 0.1d	N.D.	N.D.	N.D.	N.D.
Oleuropein isomer e	1.3 ± 0.2c	N.D.	N.D.	N.D.	N.D.

Ligstroside	2.13 ± 0.03b,c	2.802 ± 0.002d,e	2.84 ± 0.05d,e,f	3.034 ± 0.003e,f,g	3.326 ± 0.004h
Oleuropein/oleuroside methyl ether	0.15 ± 0.01c,d	0.162 ± 0.001d,e	0.159 ± 0.001d,e	0.161 ± 0.004d,e	0.215 ± 0.0
Total secoiridoids	102 ± 1e	115.4 ± 0.8g	102.3 ± 0.1e	104.0 ± 0.6e	126.8 ± 0.3
			Flavonoids		
Luteolin-diglucoside a	0.088 ± 0.001c,d	0.152 ± 0.002f	0.134 ± 0.003e	0.13 ± 0.01e	0.141 ± 0.005e,f
Rutin	0.139 ± 0.003d,e,f	0.162 ± 0.002f,g,h,i	0.16 ± 0.01e,f,g,h	0.177 ± 0.001g,h,i,j	0.188 ± 0.001i,j
Luteolin rutinoside	0.104 ± 0.002c,d	0.16 ± 0.01g,h	0.128 ± 0.003d,e,f	0.138 ± 0.002e,f,g	0.152 ± 0.001f,g,h
luteolin glucoside isomer a	0.73 ± 0.03a	1.02 ± 0.04d,e,f	0.97 ± 0.01b,c,d	0.96 ± 0.02b,c,d	1.01 ± 0.04c,d,e
Apigenin rutinoside	0.13 ± 0.01b,c,d	0.20 ± 0.02g,h	0.16 ± 0.02d,e,f	0.17 ± 0.01e,f,g	0.201 ± 0.001h
Diosmetin rhamnoside glucoside (diosmin)	0.010 ± 0.002c,d	0.020 ± 0.004e,f	0.014 ± 0.004c,d,e,f	0.0178 ± 0.0001d,e,f	0.020 ± 0.001e,f
luteolin glucoside isomer b	0.060 ± 0.002d	0.097 ± 0.002e	0.09 ± 0.01e	0.039 ± 0.001a,b,c	0.098 ± 0.0
Apigenin glucoside	0.08 ± 0.01a,b	0.13 ± 0.01c,d,e,f	0.13 ± 0.01b,c,d,e,f	0.13 ± 0.01c,d,e,f	0.16 ± 0.02

luteolin glucoside isomer c	0.52 ± 0.01b,c	0.732 ± 0.004d	0.73 ± 0.01d	0.81 ± 0.03d,e,f	0.78 ± 0.02d
Chrysoeriol-7-O-glucoside a	0.24 ± 0.01c,d	0.368 ± 0.002f,g,h	0.342 ± 0.001f	0.353 ± 0.002f,g	0.367 ± 0.002f,g,h
luteolin glucoside isomer d	0.0514 ± 0.0004a,b,c	0.09 ± 0.01d,e,f	0.10 ± 0.01d,e,f	0.10 ± 0.01d,e,f	0.11 ± 0.01d,e,f
Luteolin	0.131 ± 0.002b	0.021 ± 0.002a	0.0170 ± 0.0003a	0.014 ± 0.004a	0.014 ± 0.00
Quercetin	0.13 ± 0.01c,d	0.19 ± 0.01e	0.251 ± 0.002f	0.0260 ± 0.0001a,b	0.29 ± 0.01f
Resinoside	<LOQ	0.008 ± 0.001d,e	0.0045 ± 0.0003a,b,c,d	0.005 ± 0.001b,c,d	0.0074 ± 0.0003c,d,e
Total flavonoids	2.416 ± 0.001b	3.35 ± 0.01d,e	3.23 ± 0.03c,d	3.07 ± 0.03c,d	3.5 ± 0.1e,f
			Other phenolic compounds		
Caffeoylglucoside	0.0085 ± 0.0001a,b,c	0.020 ± 0.002d,e,f	0.019 ± 0.004d,e	0.035 ± 0.001g	0.0285 ± 0.0002e,f,g
Verbascoside	0.30 ± 0.07a,b,c	0.324 ± 0.008 a,b,c,d	0.288 ± 0.008a,b,c	0.55 ± 0.04e	0.362 ± 0.002a,b,c,d
			Other compounds		
Elenolic acid glucoside isomer a	0.22 ± 0.04c	0.44 ± 0.02g	0.40 ± 0.01f,g	0.23 ± 0.02c,d	0.41 ± 0.02f

Elenolic acid glucoside isomer b	1.2 ± 0.1b,c	1.75 ± 0.04d,e,f	1.60 ± 0.01d	1.97 ± 0.07f	1.7 ± 0.1d
Elenolic acid glucoside isomer c	1.9 ± 0.1f,g	1.80 ± 0.04f,g	1.68 ± 0.09d,e,f	1.63 ± 0.09c,d,e,f	1.8 ± 0.1f,g
Total elenolic acids	3.2 ± 0.2d,e	4.00 ± 0.01h	3.7 ± 0.1f,g,h	3.837 ± 0.003g,h	3.9 ± 0.1g,h
Total compounds	111 ± 1d	125 ± 1h	111.3 ± 0.3d,e	113.1 ± 0.6e	136.9 ± 0.2j

In **Table 2**, the main parameters are summarized. These allowed the identification of these compounds, which were retention time, *m/z* experimental and calculated error, mSigma that represents the goodness of fit between the measured and theoretical isotopic pattern, and molecular formula.

The following simple phenols previously found in olive leaves were also identified: Peaks 2 at 4.3 min with a molecular ion at *m/z* 315.1085 was identified as hydroxytyrosol-hexose[34,35,37] and peak 4 (RT 5.28 min) at *m/z* 153.0555 as hydroxytyrosol[16,35]. Peak 6 (RT 6.17 min) with a molecular ion at *m/z* 299.1122 correspond to tyrosol-glucoside[34,35,37].

Secoiridoids were also identified in the extracts according to previous studies[3,16,34-38]. Thus, peaks 1, 3 and 9 at 3.65 min, 4.64 min and 8.53 min with a molecular ion at *m/z* 389 correspond to oleoside (peak 1) and secologanoside (peak 3 and 9) (isomer of oleoside). Peak 10 (RT 10.81 min and *m/z* 377.1397) corresponding to oleuropein aglycon and peak 13 (RT 13.26 min and *m/z* 525.1588) was detected as demethyloleuropein. Peak 14 and 18 (RT 13.70 min and 15.521 min and *m/z* 555) were proposed to be isomers of hydroxyoleuroside. Peaks 21, 23, 25, 29 and 32 (RT 16.22 min, 16.47 min, 16.69 min, 18.03 min and 18.60 min) with a molecular ion at *m/z* 701 correspond with oleuropein glucoside and its isomers. Peak 30 at 18.16 min and m/z 541.1927 was proposed to be hydro-oleuropein. Peaks 33-37 (RT 18.87 min, 19.50 min, 19.95 min, 20.22 min, and 20.54 min and *m/z* 539) correspond to isomers of oleuropein. Peak 38 at 20.82 min and *m/z* 523.1838 was proposed to be ligstroside, and finally oleuroside methyl ether corresponds to peak 41 at 21.94 min with a molecular ion at *m/z* 553.1874.

The following flavonoids found in olive leaf extracts have also been identified previously[16,34-36,38,39]: Luteolin-diglucoside (Peak 11 at 11.44 min *m/z* 609.1426), rutin (peak 15 at 13.78 min and *m/z* 609.1452), luteolin-rutinose (peak 16 at 13.89 min and *m/z* 593.1513), luteolin glucoside isomers (peaks 17, 24, 27 and 31 at 14.55, 16.54, 17.18 and 18.31 min at *m/z* 447), apigenin rutinoside (peak 21 at 15.99 min and *m/z* 577.155), diosmetin rhamnoside glucoside (diosmin) (peak 22 at 16.44 min and *m/z* 607.1676), apigenin glucoside (peak 26 at 17.04 min and *m/z* 431.097), chrysoeriol-7-O-glucoside (peaks 28 at 17.56 min and *m/z* 461), luteolin

(peak 39 at 21.32 min and m/z 285.0401), quercetin (peak 40 at 21.46 min and *m/z* 301.0345) and resinoside (peak 42 at 23.20 min and *m/z* 613.1866).

Peaks 7, 8 and 12 at 7.75, 8.18, 11.94 min, presented a molecular ion at *m/z* 403 were identified as elenolic acid glucoside isomers[3,34,38].

Other phenolic compounds were also identified: Peak 5 (RT 6.14 min and *m/z* 341.085) was identified as caffeoylglucoside and peak 19 at 15.92 min and a molecular ion at *m/z* 623.1996 was proposed to be verbascoside[16,34].

Subsequently, the quantification of individual compounds in each experiment was carried out. These were grouped into different families (simple phenols, secoiridoids, flavonoids and elenolic acids) (**Table S1.**). The calibration curve of hydroxytyrosol was used to quantify the simple phenols, elenolic acid derivatives and verbascoside. The curve of oleuropein to quantify secoiridoids and caffeoylglucoside and the calibration curve of apigenin was used to quantify the flavonoids. In addition, all calibration curves showed a good linearity (r^2 > 0.9910). The limit of detection (LOD) and limit of quantification (LOQ) for each standard was calculated as S/N = 3 and S/N = 10, where S/N is the signal-to-noise ratio. For the standards hydroxytyrosol, oleuropein and apigenin, LOD was 0.117, 0.031 and 0.015 mg L^{-1}, whereas LOQ was 0.394, 0.107 and 0.051 mg L^{-1} respectively (**Table S2**). An olive leaf extract was injected (n = 6) on the same day (Intraday precision) and the 3 following days n=18 (Interday precision). The Intraday and Interday repeatability of the peak area among all peaks by the relative standard deviation (RSD) was in the same range as the previously validated method (0.05-1.03% and 0.17-3.6%)[39].

Table S2. Calibration curves of the method proposed. LOD: Limit of detection, LOQ: Limit of quantification

Analyte	LOD (mg/L)	LOQ (mg/L)	Calibration ranges (mg/L)	Calibration curves (mg/L)	r^2
Hydroxytyrosol	0.117	0.394	LOQ-125	y=17616x-39492	0.9963
Oleuropein	0.031	0.107	LOQ-125	y=34393x+95646	0.9945
Apigenin	0.015	0.051	LOQ-125	y=129394x+136218	0.9910

Concerning the simple phenols, a total of 3 compounds were quantified, whose lowest total content of simple phenols was 1.26 ± 0.05 mg g^{-1} d.w. in PLE 4 (50ºC, 0% EtOH and 12.5 min), whereas its major concentration was 5.17 ± 0.03 mg g^{-1} d.w. in PLE 15 at 200ºC, 0% EtOH and 12.5 min. So, the increase in the content of total simple phenols with the increase in temperature can be explained by the breaking of the supramolecular structures generating molecules of simple phenols.

Regarding the secoiridoids, a total of 20 compounds were quantified. The most abundant secoiridoid in olive leaf extracts was oleuropein isomer a, whose value ranged from 39.1 ± 0.2 mg g^{-1} d.w. in PLE 4 (50ºC, 0% EtOH and 12.5 min) to 104.0 ± 0.3 mg g^{-1} d.w. in PLE 6 (125ºC, 100% EtOH and 20 min) and 104.1 ± 0.2 mg g^{-1} d.w. in PLE 2 (125ºC, 100% and 5 min). The lowest content of secoiridoids was 53.62 ± 0.04 mg g^{-1} d.w. in PLE 4 at 50ºC, 0% EtOH and 12.5 min, whereas the highest content of secoiridoids was 132.6 ± 0.9 mg g^{-1} d.w. in PLE 6 (125ºC, 100% EtOH and 20 min) and 129.93 ± 0.01 in PLE 2 (125ºC, 100% EtOH and 5 min). Therefore, the content of secoiridoids increased with the rise in temperature up to 125ºC and with the increase of % EtOH. This result could be attributed to the increased solubility of oleuropein at high temperatures and high percentages of ethanol[40].

With regard to the concentration of flavonoids in olive leaf PLE extracts, 14 compounds were quantified, whose total content of flavonoids ranged from 2.06 ± 0.06 mg g^{-1} d.w. in PLE 15 (200ºC, 0% EtOH and 12.5 min) and 2.05 ± 0.03 d.w. in PLE 3 (200ºC, 100% EtOH and 12.5 min) to 4.15 ± 0.02 mg g^{-1} d.w. in PLE 6 (125ºC, 100% EtOH and 20 min). Temperatures higher than 125ºC lowered the content of flavonoids due to these compounds are degraded due to their thermo-labile nature[41].

Finally, three elenolic acid derivatives were quantified, whose total concentration of elenolic acid ranged from 2.47 ± 0.02 mg g^{-1} d.w. in PLE 3 (200ºC, 100% EtOH and 12.5 min) to 4.0 ± 0.1 mg g^{-1} d.w. in PLE 6 (125ºC, 100% EtOH and 20 min) and 4.00 ± 0.01 mg g^{-1} d.w. in PLE 10 (125ºC, 0% EtOH and 5 min). This result shows that elenolic acids possess the same solubility in water as in ethanol at the

temperature of 125ºC. Also, the highest content of elenolic acid was obtained at 5 min, from which it decreases with the increase of time.

Total compounds ranged from 60.21 ± 0.06 mg g^{-1} d.w. in PLE 4 at 50ºC, 0% EtOH and 12.5 min to 143 ± 1 and 139.1 ± 0.1 mg g^{-1} d.w. in PLE 6 at 125ºC, 100% EtOH and 20 min and in PLE2 (125ºC,100% EtOH and 5 min). According to these results, it is important to highlight the fact that that an increase of more than twice the lowest total content was obtained. Therefore, the highest total content was obtained in the same PLE conditions as those obtained by the greatest content of oleuropein, due to it being the most concentrated phenolic compound in olive leaves.

3.2. Extraction yield

According to the yield obtained in all extractions, its content was from 14 ± 1% in PLE 4 at 50ºC, 0% EtOH and 12.5 min to 44 ± 2% in PLE 9 at 200ºC, 50% EtOH and 5 min) (**Table 1.**). This range was in the same order of magnitude as reported in the previous study (14.7-53.9%)[20]. It has been reported that the increase in yield in PLE is mainly due to the increase in temperature[20,22,32]. High pressure in PLE keeps the solvent in a liquid state at elevated temperatures, increasing the solubility of the analytes and providing an improvement in solvent diffusivity and a decrease of its viscosity, improving its penetration into the matrix. Therefore, these effects provide an increase in the mass transfer improving the extraction yield[42].

3.3. Fitting the model

ANOVA test of the regression model for simple phenols and secoiridoids is provided in **Table S3.**, for flavonoids and elenolic acids in **Table S4.** and for total compounds and yield in **Table S5.** ANOVA table partitioned the variability of the response variables in separate parts for each one of the effects, therefore testing

the statistical significance of each effect comparing its mean squared against an estimated experimental error.

Table S3. ANOVA test for the response variables of simple phenols and secoiridoids

Source	**Simple phenols**					**Secoiridoids**				
	SS	*DF*	*MS*	*F-value*	*p-value*	*SS*	*DF*	*MS*	*F-value*	*p-value*
X₁	4.46	1	4.46	61.06	0.0160[a]	36.77	1	37.06	0.39	0.5589
X₂	1.27	1	1.27	17.38	0.0530[b]	1308.16	1	1308.25	13.93	0.0135[a]
X₃	0.07	1	0.07	1.02	0.4187	69.79	1	69.96	0.74	0.4280
X₁₁	0.51	1	0.51	6.97	0.1186	3358.54	1	3359.91	35.77	0.0019[a]
X₁₂	3.15	1	3.15	43.23	0.0224[a]	880.49	1	880.70	9.38	0.0280[a]
X₁₃	0.003	1	0.003	0.04	0.8561	5.60	1	5.61	0.06	0.8167
X₂₂	0.21	1	0.21	2.82	0.2351	200.71	1	201.25	2.14	0.2036
X₂₃	0.14	1	0.14	1.96	0.2965	29.35	1	29.28	0.31	0.6002
X₃₃	0.06	1	0.06	0.89	0.4449	498.89	1	499.30	5.31	0.0693[b]
Lack of fit	1.48	3	0.58	6.76	0.1316	130.60	3	43.53	0.26	0.8533
Pure error	0.15	2	0.07			338.87.	2	169.44		
Total (corr.)	11.52	14				7016.97	14			
r²	85.88					93.31				
Adj r²	60.46					81.27				

X_1: Temperature
X_2: % ethanol/water (v/v)
X_3: Time,
SS: Sum of squares
DF: Degree of freedom
MS: Mean square
r^2 : Quadratic correlation coefficient
Adj r^2: Quadratic correlation coefficient adjusted
a: Significant ($p < 0.05$)
b: Marginally significant ($p < 0.1$).

Regarding the response of simple phenols, the temperature (X_1) and the interaction between the temperature and % of EtOH (X_{12}) were two significant parameters, due to these having a *p-value* less than 0.05 (*p-value* = 0.0160 and *p-value* = 0.0224), whereas the % of EtOH (X_2) was a marginally significant factor because

the *p*-value was less than 0.1 (*p-value* = 0.0530). For the response variable of secoiridoids, the effect of EtOH (X_2) (*p-value* = 0.0135), quadratic effect of temperature (X_{11}) (*p-value* = 0.00190) and the interaction between temperature with % of EtOH (X_{12}) (*p-value* = 0.0280) were significative variables , whereas the quadratic of time (X_{33}) (*p-value* = 0.0693) was marginally significant. (**Table S3.**).

Table S4. ANOVA test for the response variables of flavonoids and elenolic acids.

Source	**Flavonoids**					**Elenolic acids**				
	SS	DF	MS	F-value	p-value	SS	DF	MS	F-value	p-value
X_1	0.64	1	0.64	13.68	0.0660b	0.58	1	0.58	48	0.0202a
X_2	0.32	1	0.32	6.75	0.1216	0.08	1	0.08	6.88	0.1198
X_3	0.009	1	0.009	0.2	0.6974	0.24	1	0.24	19.91	0.0467a
X_{11}	3.68	1	3.68	78.42	0.0125a	1.74	1	1.74	144.4	0.0069a
X_{12}	0.00002	1	0.00002	0	0.9513	0.5	1	0.5	41.26	0.0234a
X_{13}	0.006	1	0.006	0.13	0.7506	0.005	1	0.005	0.42	0.5839
X_{22}	0.18	1	0.18	3.92	0.1861	0.21	1	0.21	17.58	0.0524b
X_{23}	0.11	1	0.11	2.38	0.2627	0.32	1	0.32	26.63	0.0356a
X_{33}	0.4	1	0.4	8.59	0.0994b	0.07	1	0.07	5.98	0.1343
Lack of fit	0.4	3	0.12	2.6	0.2901	0.13	3	0.04	3.55	0.2275
Pure error	0.09	2	0.05			0.02	2	0.01		
Total (corr.)	5.96	14				3.91	14			
r^2	92.28					96.1				
Adj r^2	78.4					89.07				

X_1: Temperature
X_2: % ethanol/water (v/v)
X_3: Time
DF: Degree of freedom
MS: Mean square
r^2*:* Quadratic correlation coefficient
Adj r^2: Quadratic correlation coefficient adjusted
a: Significant ($p < 0.05$)
b: Marginally significant ($p < 0.1$).

For the variable response of flavonoids, the effect of the quadratic of temperature (X_{11}) was significant (*p-value* = 0.0125), while the effect of temperature (X_1) (*p-value* = 0.0660) and the quadratic of time (X_{33}) (*p-value* = 0.0994) were marginally significant. For the variable response of elenolic acid, the effect of temperature (X_1) (*p-value* = 0.0202), time (X_3) (*p-value* = 0.0467), quadratic of temperature

(X_{11}) (*p-value* = 0.00690), the interaction between temperature and % of EtOH (X_{12}) (*p-value* = 0.0234), the interaction between % of EtOH and time (X_{23}) (*p-value* = 0.0356), were significant, whereas the effect of the quadratic % of EtOH (X_{22}) with a *p*-value of 0.0524 was marginally significant (**Table S4.**).

Table S5. ANOVA test for the response variable of total compounds and percentage of yield.

Source	**Total compounds**					**% Yield (w/w)**				
	SS	*DF*	*MS*	*F-value*	*p-value*	*SS*	*DF*	*MS*	*F-value*	*p-value*
X_1	33.013	1	33.013	0.35	0.5781	196.27	1	196.27	96.13	0.0102[a]
X_2	1295.325	1	1295.325	13.86	0.0137[a]	143.01	1	143.01	70.40	0.0139[a]
X_3	71.61	1	71.61	0.77	0.4214	64.52	1	64.52	31.76	0.0301[a]
X_{11}	3664.77	1	3664.77	39.22	0.0015[a]	20.00	1	20.00	9.84	0.0883[b]
X_{12}	1040..16	1	1040..16	11.13	0.0206[a]	23.30	1	23.30	11.47	0.0772[b]
X_{13}	4.86	1	4.86	0.05	0.8286	19.02	1	19.02	9.36	0.0923[b]
X_{22}	217.31	1	217.31	2.33	0.1878	4.11	1	4.11	2.03	0.2906
X_{23}	47.63	1	47.63	0.51	0.5072	0.08	1	0.08	0.04	0.8592
X_{33}	530.098	1	530.098	5.67	0.0630[b]	40.41	1	40.41	19.89	0.0468[a]
Lack of fit	99.2	3	33.08	0.18	0.9021	64.86	3	21.62	10.64	0.0871
Pure error	367.97	2	183.986			4.06	2	2.03		
Total (corr.)	7542.48	14				584.03	14			
r^2	93.80					88.20				
Adj r^2	82.65					74.96				

X_1: Temperature
X_2: % ethanol/water (v/v)
X_3: Time
SS: Sum of squares
DF: Degree of freedom
MS: Mean square
r^2: Quadratic correlation coefficient
Adj r^2: Quadratic correlation coefficient adjusted
a: Significant ($p < 0.05$)
b: Marginally significant ($p < 0.1$).

For the response of total compounds, the effect of EtOH (X_2) (*p-value* = 0.0137), quadratic effect of temperature (X_{11}) (*p-value* = 0.00150) and the interaction between temperature with % of EtOH (X_{12}) (*p-value* = 0.0206) were significant variables, whereas the quadratic of time (X_{33}) (*p-value* = 0.0630) was marginally

significant. For the response of yield, the effect of temperature X_1 (*p-value* = 0.0102), % of EtOH (X_2) (*p*-value = 0.0139), time (X_3) (*p-value* = 0.0301) and quadratic of time (X_{33}) (*p-value* = 0.0468) were significant, whereas the quadratic of temperature (X_{11}) (*p-value* = 0.0883), the interaction between temperature with % of EtOH (X_{12}) (p-value = 0.0772) and the interaction between temperature with time (X_{13}) (*p-value* = 0.0923) were marginally significant. (**Table S5.**).

The models presented a high correlation between independent factors and response variables with quadratic correlation coefficient (r^2) from 88.2% to 96.1% except for the variable response of simple phenols, whose response possess a good correlation but lower than the other ones (r^2 = 85.9%). The *p*-value of lack-of-fit was used to determinate the adequacy of the model to describe the observed data. The test is performed by comparing the variability of the residuals of the current model with the variability between observations obtained in repeated conditions of the factors. The *p*-value for the lack of adjustment in the ANOVA tables was higher than 0.05, therefore the model seems to be adequate for the data observed at 95.0% confidence level.

3.3.1. Analysis of response surfaces

Three-dimensional response surfaces were plotted where the effectof two PLE factors (temperature, %EtOH and extraction time) appear inthe response variables (simple phenols, secoiridoids,flavonoids, ele-nolic acids, total compounds and yield)fixing the third at its middlevalue (**Figures 1–3**). In these figures**,** it can be seen that the increase in extraction temperature demonstrated a high increase in the content in secoiridoids, flavonoids, elenolic acids and total compounds within the range of 100-150°C, from which they decreased. This could be due to the high effect of the temperature quadratic in these responses. Whereas **Figure 1. and Figure 3** show an increase in simple phenols and yield with the rise in temperature to arrive at maximum value of 200ºC, where the temperature has the greatest effect on the variable responses.

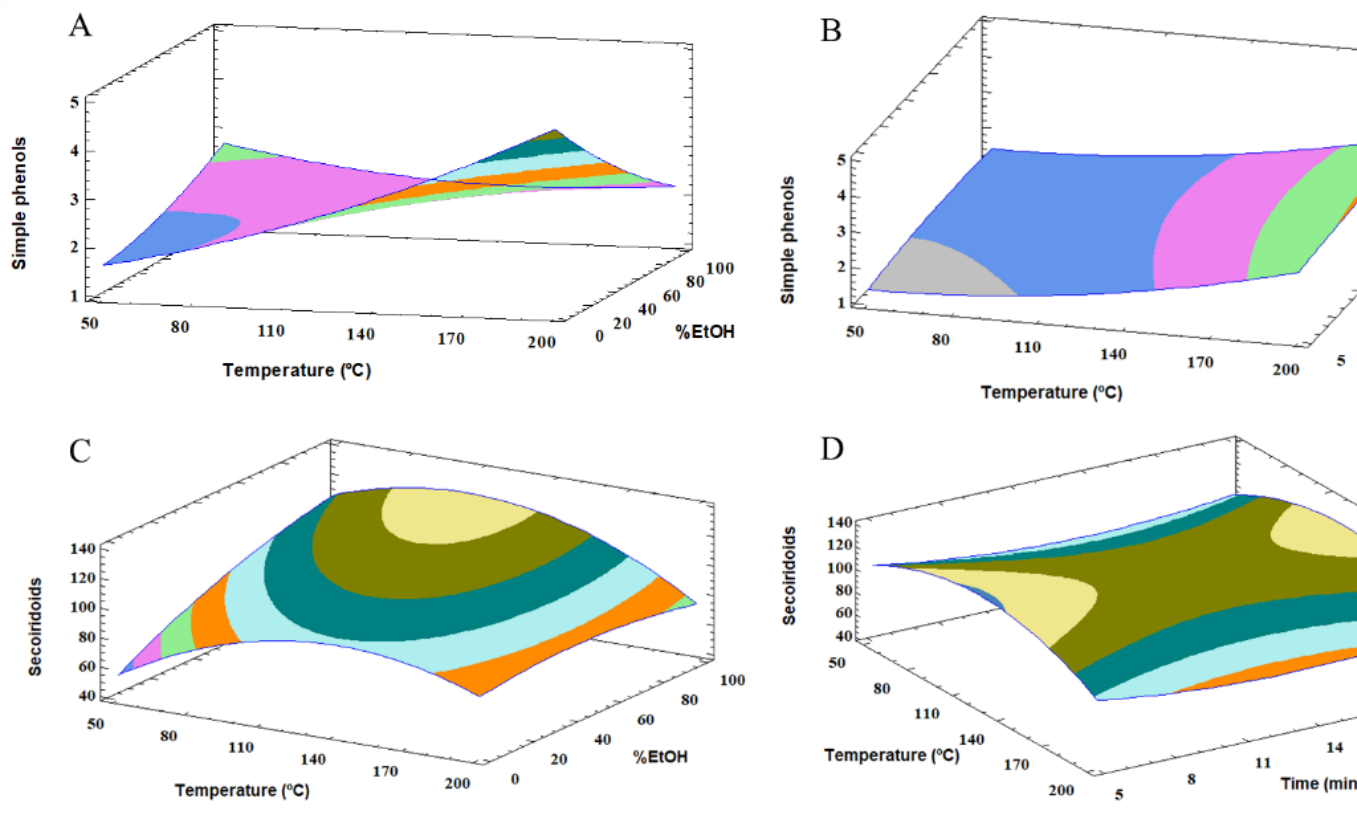

Figure 1. Response surface plots showing the combined effects of the factors and the response secoiridoids): Temperature (ºC) - % EtOH (ethanol/water ratio (% (v/v)) (A, C), Temperature (ºC responses are expressed as mg g^{-1} dry matter of olive leaves (mg g^{-1} d.w.).

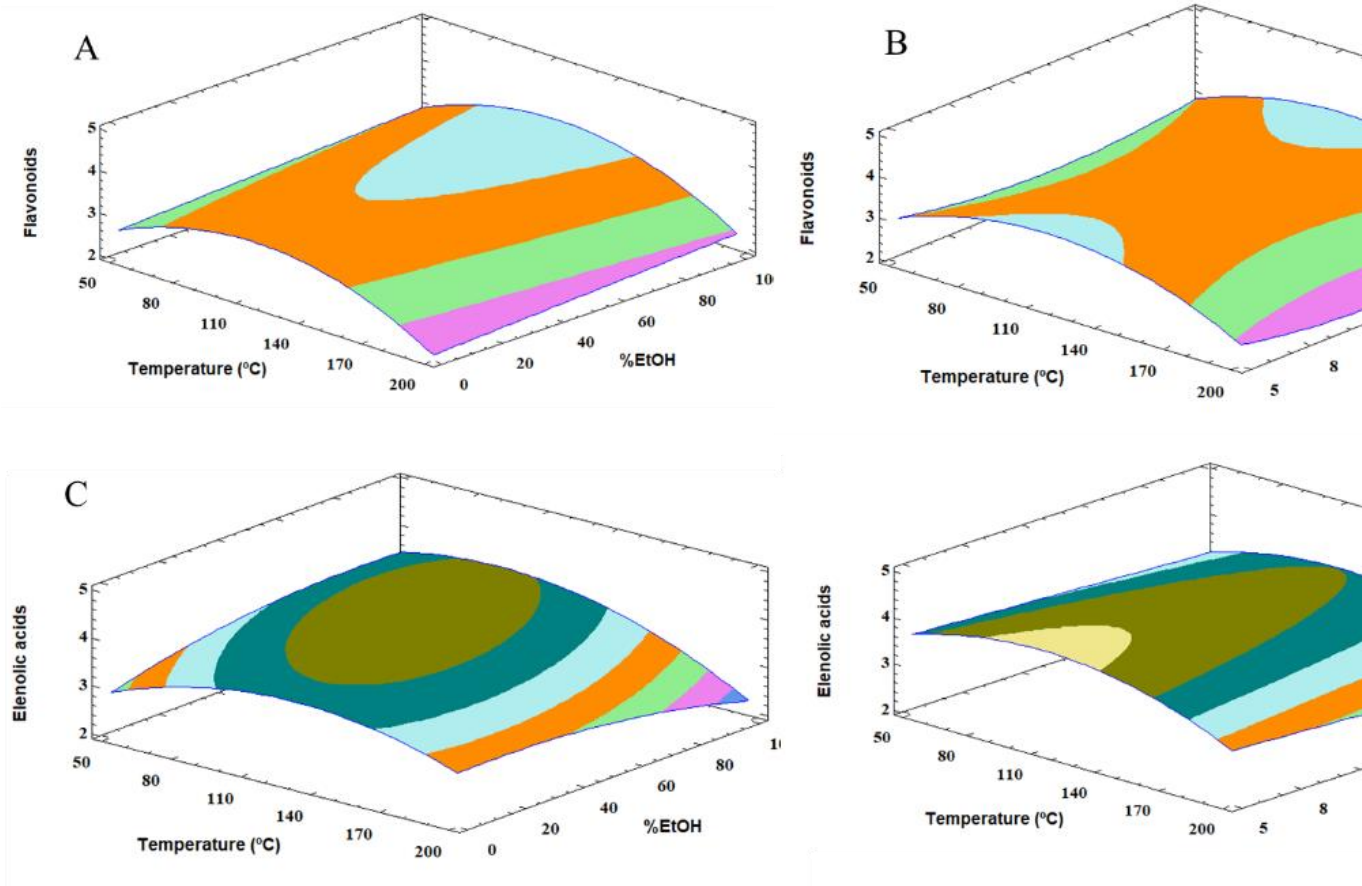

Figure 2. Response surface plots showing the combined effects of the factors and the response vari
acids): Temperature (ºC) - % EtOH (ethanol/water ratio (% (v/v)) (A, C), Temperature (ºC) - time (n
are expressed as mg g^{-1} dry matter of olive leaves (mg g^{-1} d.w.).

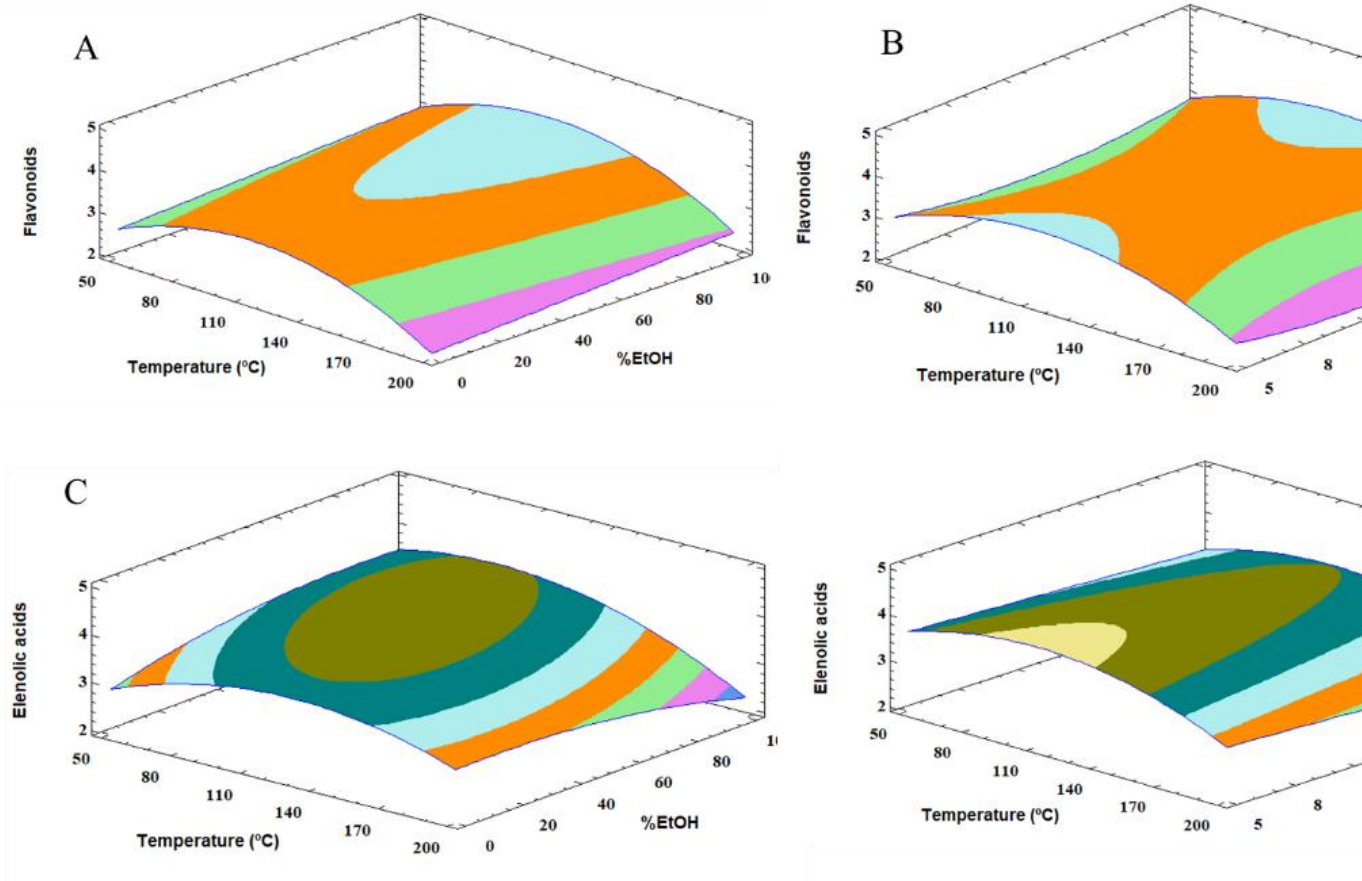

Figure 3. Response surface plots showing the combined effects of the factors and the response varia
and yield): Temperature (ºC) - % EtOH (ethanol/water ratio (% (v/v)) (A, C), Temperature (º
compounds expressed as mg g^{-1} dry matter of olive leaves (mg g^{-1} d.w) and yield expressed in perc

Apart from temperature, in **Figure 1. A** a decrease of the content of simple phenols with the raising of ethanol was also demonstrated, whereas the maximum content of simple phenols was in the time range of 11-16 min (**Figure 1. B**). **Figure 1. C** shows an increase in the concentration of secoiridoids with the increasing of % of EtOH, whereas in **Figure 1.D,** the largest value of secoiridoids appeared at the minimum value of time.

In **Figure 2.A** a slight growth can be seen in the content of flavonoids with the increase in % of EtOH to reach 39-100%, in which the flavonoid content remained constant, while its highest content was obtained at the minimum and maximum of time (**Figure 2.B)**. **Figure 2 C** shows the maximum concentration of elenolic compounds in the range of 20-100% EtOH, while in **Figure 2. D,** a decrease in this response can be seen with the increase in time. In **Figure 3. A,** it can be observed that the maximum value of total compounds appeared in the range of 80-100% EtOH, whereas its maximum content was obtained at 5 min (**Figure 3. B)**. For the yield response, it could be observed in **Figure 3.C** that this response increases with temperature. The maximum yield value appears in the range of 150-200ºC of temperature and 80-100% of EtOH. In **Figure 3.D**, the maximum yield was in the range of 150-200ºC and 5-7 min**.**

Therefore, **Figures 1, 2** and **3** show that the content of compounds in PLE olive leaf extracts increases with the increase in temperature to reach 100-150°C because at high temperatures, the solvent capacity to solubilize the compounds increases, decreasing the viscosity of the liquid solvent and allowing a better penetration in the matrix[22]. At temperatures higher than 150ºC, most phenolic compounds are degraded because they are thermosensitive, especially glucosides of phenolic compounds, because thermal oxidation affect to the bound oxygen atom between the phenolic molecule and the sugar moieties[43,44]. Nevertheless, the major content of simple phenols and extraction yield is obtained at maximum temperature. At high temperature, supramolecular structures are broken generating simple phenols. Concretely, it has been obtained an increase of the content of hydroxytyrosol and a decrease of oleuropein at 200ºC. This is due to the oleuropein degradation rate

and hydroxytyrosol content sharply increased with increasing temperature[45] In addition, at high % EtOH the highest content of secoiridoids, flavonoids, elenolic acids and extraction yield was obtained. Nevertheless, at low percentages of EtOH a major concentration of simple phenols was obtained, this is due to the highest solubility of simple phenols in water[22]. Also, at low values of time was obtained the major content of compounds and the maximum yield.

3.3.2. Optimization of PLE parameters

Pressurized liquid extraction factors were optimized to maximize the content of simple phenols, secoiridoids, flavonoids, elenolic acids, total compounds and yield.

The fitted equation for simple phenols (Y_1) with only the significant parameters appears in **Eq.2** (being X_1 (temperature), X_2 (% EtOH) and X_{12} (combined effect of temperature and % EtOH):

$$Y_1 \text{ (Simple phenols)} = -0.145583 + 0.0218044X_1 + 0.0216496X_2 - 0.00023695X_{12}$$

(**Eq. 2**)

An optimization of PLE parameters to obtain the major content of simple phenols was proposed. Regarding the suggested model, a great value on this response variable could be obtained under the following optimized conditions: 200ºC, 0% EtOH and a total time of 12.5 min to obtain a concentration of simple phenols of 4.21 mg g^{-1} d.w. Temperature and % EtOH were the same that the obtained by a previous work for the highest content of hydroxytyrosol (8.54 mg g^{-1} extract), excluding the time that it was fixed at 20 min[22].

For secoiridoids response (Y_2), model equation with the significant PLE parameters (**Eq.3**) (being X_2 (% EtOH), X_{11} (quadratic value of temperature), X_{12} (cross effect of temperature with % EtOH) and X_{33} (quadratic of time)), and also the model took into account the linear effect of temperature (X_1) and time (X_3) due to these have an effect on the response.

$$Y_2\,(\text{Secoiridoids}) = 26.6666 + 1.51304\,X_1 + 0.750302\,X_2 - 5.41829X_3 - 0.00526089X_{11} - 0.00395642\,X_{12} + 0.216732X_{33} \quad (\textbf{Eq. 3})$$

Regarding the suggested model, a great value on the response variable of concentration of secoiridoids could be obtained under the following optimized conditions: 106ºC, 100% EtOH and 5 minutes to obtain 139 mg g^{-1} d.w of secoiridoids. Temperature was similar to the obtained by Herrero et al. (2011) for the highest content of oleuropein (7.993 ± 0.091 mg g^{-1} dry extract), which was 100ºC, whereas % EtOH and time of extractions (0% EtOH and 20 min) were different to the obtained in this model. These differences could be due to this study fixing the time at 20 min in which the major content of oleuropein was obtained at 0% EtOH, due to the increase of the time of extraction provides an increase on the solubility of oleuropein in water.

The fitted equation of the model with the significant PLE parameters for flavonoids response (Y_3) appear in the **Eq.4** ((being X_1 (temperature), X_{11} (quadratic value of temperature) and X_{33} (quadratic value of time)), and it has also considered the linear effect of time (X_3).

$$Y_3\,(\text{Flavonoids}) = 2.04901 + 0.0398476X_1 - 0.154515X_3 - 0.000174504X_{11} + 0.00618059X_{33}\,(\textbf{Eq. 4})$$

An optimization of PLE parameters to obtain the major content of flavonoids were proposed. Regarding the suggested model, a great value on this response variable could be obtained under the following optimized conditions: 114ºC, 39.7% EtOH and a total time of 20 min to obtain a concentration of flavonoids of 3.70 mg g^{-1} d.w.. Optimum conditions were similar to the obtained by Putnik et al. (2017)[27] to obtain the highest content of flavonoids (26.52 mg g^{-1} d.w. of quercetin equivalents), which was 100ºC, 50% EtOH and 15 minutes. Optimum conditions of temperature and time were similar to those reported by Herrero et al. (2011)[22] (100ºC and 20 min) for the great value of sum of flavonoids (3.36 mg g^{-1} extract), except for the ethanol composition that was 100% EtOH[22].

For elenolic acids (Y_4), the fitted equation with the significant PLE parameters appears in **Eq.5,** being temperature (X_1), time (X_3), the quadratic of temperature (X_{11}), the cross effect between temperature and % EtOH (X_{12}) and the cross effect between % EtOH and time (X_{23}) was significant, whereas the quadratic of % EtOH (X_{22}) was marginally significant (**Eq. 5**). The fitted equation of the model included the effect of time (X_2).

Y_4 (Elenolic acids) = 2.32651 + 0.0321307X_1 - 0.0123311X_2 - 0.0609088X_3 - 0.000124057X_{11} -0.0000940917X_{12} - 0.000100199X_{22} + 0.000756017X_{23} (**Eq.5**).

The highest value of elenolic acids was obtained under the following optimized conditions: 120ºC, 24% EtOH and 5 min to obtain a concentration of elenolic acids of 4.15 mg g^{-1} d.w. Elenolic acids have not been identified previously by other studies in PLE olive leaf extracts, so it was not possible to compare PLE conditions with other studies.

The fitted equation of the model to obtain the maximum value of total compounds (Y_5) includes the effect of temperature (X_1) and time (X_3) and significant PLE parameters of % EtOH (X_2), quadratic of temperature (X_{11}), cross effect between temperature and % EtOH (X_{12}) and quadratic of time (X_{33}) (**Eq.6**):

$$Y_5 \text{ (Total compounds)} = 31.4008 + 1.58889X_1 + 0.792018X_2 - 5.58761X_3 - 0.00549591X_{11} - 0.00430021X_{12} + 0.223504X_{33} \text{ (Eq.6)}$$

Regarding the suggested model, the highest value of total compounds can be obtained under the following optimized conditions: 105ºC, 100% EtOH and 5 min. The predicted value was 149 mg g^{-1} d.w.. To verify the suitability of the quadratic equation for predicting the optimum total content value, the verification experiment was carried out under optimum conditions, whose experimental value was obtained by HPLC-ESI-TOF-MS was 158.7 ± 0.4 mg g^{-1} d.w. An analysis of results revealed an acceptable variance (*CV*= 4.28%) between theoretical and experimental data.

Temperature and time conditions were similar to the optimal PLE conditions reported by Putnik et al. (2017)[27] (2 cycles, 80°C and 5 min) for total polyphenols,

whereas the extracts were obtained with 50% EtOH. According to this previous study, temperature and static extraction time showed higher effects on total polyphenol recovery than the cycle numbers. In addition, the increasing of static extraction time showed a significant decrease in the polyphenol content.

The experimental result of the total compound content obtained was compared to the optimum reported in other studies using different extraction techniques: This was 82.2% higher than the optimum value of the sum of individual compounds obtained by microwave assisted extraction (MAE) in olive leaf extracts (28.5 mg g^{-1} d.w.), which was obtained at a temperature of 80°C, 15.28 min of irradiation time and 49% of water[46]. In addition, this optimum value was compared with the ultrasonic assisted extraction, which was 66.9%, 67.1% and 61.8% higher than the obtained by Talhaoui et al. (2014)[34] in olive leaves of 'Picual', 'Sikitita' and 'Arbequina' cultivars. And this optimum value was 65.5- 91% higher than the mean of all cultivars at different growth stages[3]. UAE conditions in these previous studies were 80% MeOH and 10 min. Thus, compared with MAE and UAE, PLE has shown a higher phenolic content in shorter extraction times. Hence, it has been proven that the PLE extraction technique is effective in the recovery of phenolic and elenolic compounds from olive leaves. Therefore, in PLE optimum conditions, it could be possible to obtain olive leaf extracts enriched in phenolic compounds that could be used as an ingredient to develop functional food.

Below it can be seen that the fitted equation of the model for the yield with the significant PLE parameters of temperature (X_1), % EtOH (X_2), time (X_3), the cross effect of temperature with % of EtOH (X_{12}) and with time (X_{13}) and the quadratic effect of time (X_{33}).(**Eq.4**):

$$Y_6\,(\text{Yield}) = 16.3512 + 0.246335X_1 + 0.165017X_2 - 1.40048X_3 - 0.000399278X_{11} - 0.000643651X_{12} - 0.00387675X_{13} + 0.0602571X_{33}$$ (**Eq.4**)

Optimum parameters to obtain the maximum value of yield were the following:198ºC, 100% of EtOH and 5 min to obtain 43.9% of yield. In order to verify the suitability of the model, these conditions were carried out

experimentally, obtaining a value of 43 ± 3%. Analysis of results revealed an acceptable variance ($CV = 1.52\%$) between theoretical and experimental data obtained in optimum conditions.

According to the results, the optimal temperature and % of EtOH to obtain the highest yield was in concordance with previous studies, which were 100% of EtOH and temperatures of 200ºC and 190ºC[20,22]. Whereas, the optimum time and temperature were in accordance with other studies that reported 190ºC, 60% EtOH and 5 min being required to achieve higher yields[31,32].

Xynos et al. (2014) reported a yield of 46.64 ±6.30 at 190ºC, 100% EtOH and 3 cycles, whereas Lama-muñoz et al. (2020)[32] reported a yield of 36.2-51.5% at 200ºC, 60% EtOH and 5 min in different cultivars of olive leaves. According to these previous works, there is a great difference in the extraction yield due to the different cultivar. Therefore, the optimal yield was compared with a previous study that reported the same cultivar 'Hojiblanca', being 12.7% greater than this one, which was 37.5% of yield at 200ºC, 100% EtOH and 20 minutes[22]. Nevertheless, this difference could be due to many influencing factors such as different teams and agronomic variability of the sample such as harvesting season, etc[3].

The value of temperature for the highest yield was different from that of the highest total compounds. This fact could be down to the degradation of some compounds due to the reactions of hydrolysis and oxidation that may occur at high temperatures[32]. The experimental yield obtained from the highest content of compounds was 36 ± 2%, which was 16.6% lower than that obtained for the maximum yield. Therefore, the highest yield is not related to the highest content of compound in olive leaves by PLE. This result is in accordance with those reported in previous studies, whose yield for the highest phenolic content was 22.7-36.4% lower than the highest yield[20,22]. For that reason, a simultaneous optimization of PLE conditions by multi-response surface for total compounds and yield was carried out using Derringer's desirability function method. This function helps determine the combination of factors that simultaneously optimize various responses[47]

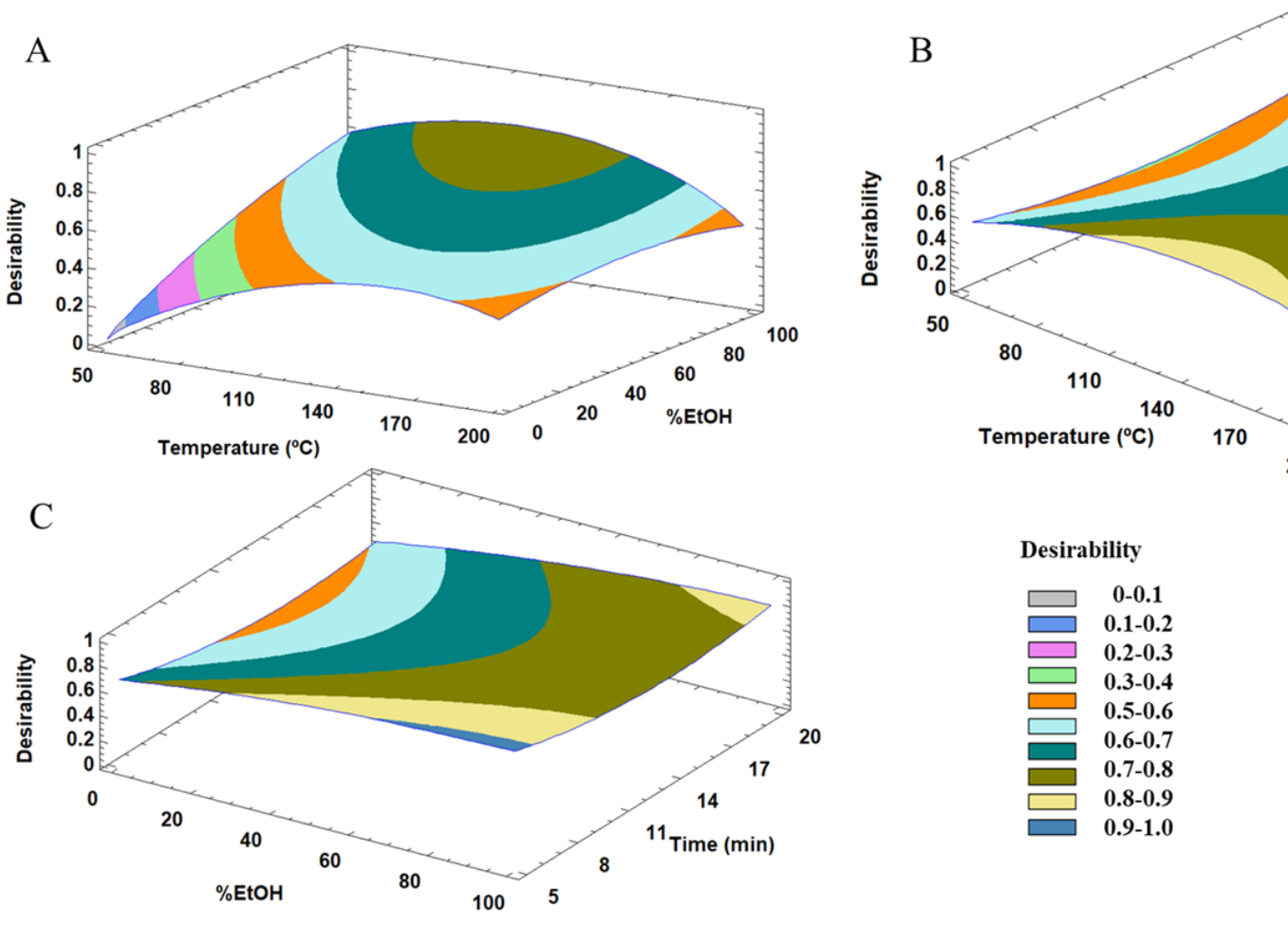

Figure 4. Response surface plots showing the combined effects of the factors: Temperature (ºC) -% EtOH (
temperature (ºC) - time (min) (B) and %EtOH - time (min) (C) in the desirability of total compounds and y

Figure 4 shows the three-dimensional response surfaces for the effect of temperature with %EtOH and with time and %EtOH with time in the desirability**.** In this case, the optimization desirability was 0.96**.** The optimal conditions were 138ºC, 100% EtOH and 5 minutes to obtain 144 mg g^{-1} d.w. of total compounds and 42.2% of yield, respectively. According to these results, optimum conditions of %EtOH and time were the same optimized for individual responses. Nevertheless, temperature was slightly higher than that obtained for the total compounds content. This means that at a temperature of 138ºC, the compounds did not degrade, thus, this temperature is suitable to obtain a high total compound recovery and a high yield.

4. Conclusions

Despite the numerous studies about phenolic composition in olive leaves by PLE, this is the first study that evaluates the effect of PLE parameters of temperature, solvent composition and extraction time in families of compounds of simple phenols, secoiridoids, flavonoids and elenolic acids in olive leaves by HPLC-ESI-TOF-MS. Furthermore, it is important to underline the fact that in PLE conditions of 105ºC, 100% EtOH and 5 min, the total content of compounds in olive leaves was 2.5-3.6 times higher than that obtained in previous studies by UAE and MAE. In addition, the highest yield (43 ± 3%) obtained at 198ºC, 100% EtOH and 5 minutes was not related to the highest total content of compounds.

For that reason, multi-response analysis was evaluated by desirability function, with, 138ºC, 100% EtOH and 5 min being the optimal conditions to obtain the maximum responses of 144 mg g^{-1} d.w. of total compounds and 42.2% of yield. Optimum desirability was 0.96, which indicates the settings seem to achieve favourable results for both responses. Therefore, these PLE optimum conditions could be applied on an industrial scale. In addition, temperatures of 138ºC avoid thermal degradation of compounds and ethanol is safe to provide olive leaf extracts for human consumption, as well as being commonly used in the food industry, and

short extraction times allow lower energy consumption, which could be economically beneficial.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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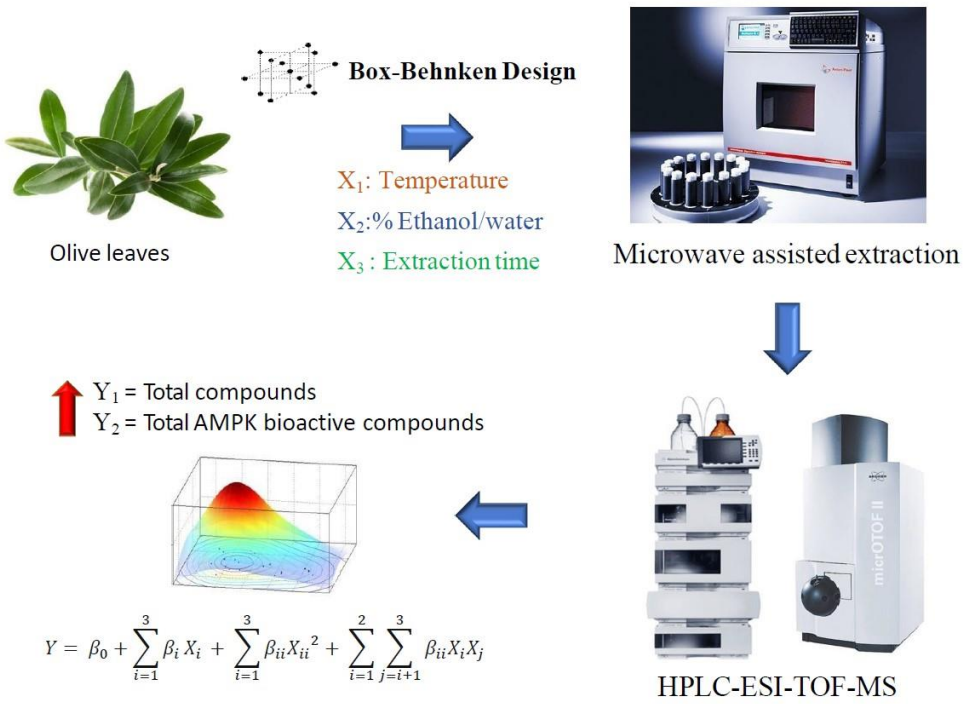
Chapter 2

A Box-Behnken design for optimal green extraction of compounds from olive leaves that potentially activate the AMPK pathway



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A Box-Behnken Design for Optimal Green Extraction of Compounds from Olive Leaves That Potentially Activate the AMPK Pathway

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Abstract

Olive leaves contain bioactive compounds that have been shown to activate AMP-activated protein kinase (AMPK), which decreases intracellular lipid accumulation. Microwave-assisted extraction (MAE) is a green extraction technique that is frequently used in the recovery of phenolic compounds from plants. Thus, in this study, a Box-Behnken design was used to optimize MAE conditions such as temperature, percentage of ethanol and extraction time to obtain the maximum content of total compounds and compounds that activate AMPK. To this end, all extracts were characterized by High-Performance Liquid Chromatography Coupled to Electrospray Ionization Time-of-Flight Mass Spectrometry (HPLC-ESI-TOF-MS). The optimum conditions to obtain the highest content of total compounds were 123 °C, 100% of ethanol/water (v/v) and 23 min, whereas the optimum conditions for the highest amount of compounds that activate AMPK were 111 °C, 42% of ethanol/water (v/v) and 23 min. Thus, a multi-analysis by desirability was carried out to establish MAE optimal conditions for both responses. The optimum conditions were 111 °C, 100% EtOH and 23 min with a desirability of 0.97, which means that the responses are close to their individual optimal values. As a result, the olive leaf extract obtained at

these optimal MAE conditions has great potential to be effective in the treatment of obesity.

Keywords: microwave-assisted extraction; olive leaves; HPLC-ESI-TOF-MS; AMPK; Box-Behnken design

1. Introduction

The large amount of phenolic compounds present in olive leaves has attracted the interest of researchers and many studies have reported that olive leaves provide beneficial effects such as antioxidant capacity, antihypertensive, cholesterol lowering, cardioprotective, anti-inflammatory and as a coadjuvant in the treatment of obesity[1-7].

The extraction process is the main step in the recovery and isolation of bioactive compounds from plant samples[8]. Conventional extraction by maceration has several disadvantages such as low phenolic recovery, longer extraction times, toxic solvents and high energy consumption[9]. Therefore, green extraction techniques such as microwave-assisted extraction (MAE) have been developed to resolve the disadvantages of conventional extraction by reducing the extraction time and solvent consumption. This method is also respectful of the environment and it is economical[10]. Microwaves are electromagnetic fields in the frequency between 0.3 and 300 GHz. Microwaves can penetrate some materials and interact with the polar components to generate heat[11]. Heat improves the diffusivity of phenolic compounds from the matrix to the solvent[12].

Several parameters can affect the extraction efficiency of MAE, including microwave power, extraction time, solvent type and composition, liquid to solid ratio, sample particle size, soaking time, and number of extraction cycles[13]. With regard to the solvent, it is important that it provides high extracting power and strong interplay with the matrix and the bioactive compounds. Thus, ethanol and water are suitable in MAE because they can absorb microwave energy due to their high dielectric constant and dielectric loss, and they are also safe to use in the food industry[14,15]. Other important parameters in MAE are the time of extraction and

the temperature. Short extraction times are used in MAE to avoid the degradation of the chemically active structures of phenolic compounds. On the other hand, high temperatures cause a decrease in viscosity and surface tension, which results in better solvent penetration. However, high temperatures can also break down the molecular structure of bioactive compounds, which results in a decrease in the extraction yield[16]. For these reasons, it is important to determine the optimum value of these MAE parameters in order to improve the extraction recovery of bioactive compounds.

Several studies have reported that secoiridoids present in extra virgin oil and olive leaves have the capacity to activate the AMP-activated protein kinase (AMPK)[17,18]. This protein is the major regulator of energy metabolism at both the cell and the whole body level[19,20]. Acute activation of AMPK in adipose tissue suppresses lipolysis, whereas chronic pharmacological stimulation of this enzyme remodels adipocyte metabolism to promote energy dissipation and prevent re-esterification of fatty acids (FAs). These specific tissue effects indicate that this enzyme is suitable in the treatment of obesity and its related metabolic disorders[21]. In addition, it has been reported that olive leaf extract decreases intracellular lipid accumulation through AMPK dependent mechanisms in hypertrophic adipocytes[22]. It has been suggested that this effect is due to the presence of several fractions in olive leaf extract that contain specific compounds belonging to the secoiridoids, phenylethanoids, phenylpropanoids and flavonoids subclasses[22]. High recovery of these bioactive compounds from olive leaves can be achieved by optimizing the extraction conditions. In this study, an experimental Box-Behnken response surface design was used to optimize the MAE parameters of temperature, solvent composition and extraction time to obtain the highest total compounds content and compounds with the capacity to activate AMPK from olive leaves. To this end, the determination of total compounds in MAE olive leaf extracts was carried out by High-Performance Liquid Chromatography Coupled to Electrospray Ionization Time-of-Flight Mass Spectrometry (HPLC-ESI-TOF-MS).

2. Materials and Methods

2.1. Chemicals and Reagents

Ethanol and methanol were purchased from Fisher Scientific (Leicestershire, UK), and water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). For HPLC analysis, LC-MS grade acetronitrile was purchased from Fisher (Fisher Scientific UK, Leicestershire, UK) and ultrapure water was obtained with the Milli-Q system described above. The acetic acid used was purchased from Fluka (Buchs, Switzerland). The standard compounds used for the quantification were hydroxytyrosol, and apigenin, which were purchased from Sigma-Aldrich (Saint Louis, MO, USA), and oleuropein was from Extrasynthèse (Lyon, France).

2.2. Samples

Olive leaves were purchased from Hojiblanca cultivar grown in Seville (Spain). After collection, the fresh leaves were dried under controlled temperature at 22 °C. These were ground using an ultra-centrifugal mill ZM 200 (Retsch GmbH, Haan, Germany). The resulting powder, with an average particle size of 0.2 mm, was stored to avoid light exposure and kept at room temperature until the extraction.

2.3. Extraction of Phenolic Compounds from Olive Leaves by Microwave-Assisted Extraction (MAE)

MAE extraction was carried out in a microwave extraction reactor (Anton Paar GmbH, Graz, Austria) equipped with two standard magnetrons of 850 W that deliver up to 1500 W microwave power and an autosampler (model MAS 24). Extracts were prepared according to Pimentel-Moral et al. (2018)[23] by adding 3 g of dried olive leaf powder into a closed extraction vessel with 30 mL of ethanol/water solvent mixture[23]. The solvent was selected because ethanol is generally recognized as a safe solvent to use in food products[24]. Extraction time, temperature and percentage of ethanol were varied according to the experimental design. After cooling, samples were centrifuged at 13,000 rpm for 15 min at 4 °C in a centrifuge (Sorvall ST 16 R, Thermo Scientific, Leicestershire, UK) and the

supernatant was collected and evaporated at 35 °C to dryness in a Savan SC250EXP Speed-Vac (Thermo Scientific, Leicestershire, UK). The extract was stored at −18 °C until further use.

2.4. Characterization of MAE Olive Leaf Extract by High-Performance Liquid Chromatography Coupled to Electrospray Ionization Time-of-Flight Mass Spectrometry (HPLC-ESI-TOF-MS)

According to previous studies, dried olive leaf extracts obtained by MAE were reconstituted in the proportion of 1/4 m/v (0.25 mg/mL) of MeOH/H_2O (50/50) and the extracts were filtered prior to analysis with single-use syringe filters (0.20 µm pore size) and then injected into the HPLC system[25,26].

Analyses of the phenolic compounds of olive leaves were carried out following the previously validated method of Talhaoui et al. (2014)[26] using Agilent 1200 Series Rapid Resolution liquid chromatography system (Agilent Technologies, CA, USA), which is comprised of a binary pump, degasser, and auto sampler. Phenolic compounds were separated using a Poroshell 120 EC-C18 (4.6 × 100 mm, 2.7 mm) from Agilent Technologies, at 25 °C and a flow rate of 0.8 mL min^{-1}. The mobile phases were 1% of acetic acid as mobile phase A and acetonitrile as mobile phase B. The conditions of the solvent gradient were as follows: 0 min, 5% B; 4 min, 9% B; 7 min, 12% B; 8 min, 15% B; 9 min, 16% B; 14 min, 20% B; 15 min, 22% B; 18 min, 28% B; 19 min, 30% B; 20 min, 31% B; 21.50 min, 32% B; 23 min, 34% B; 24 min, 35% B; 25.5 min, 40% B; 27 min, 50% B; 30 min, 100% B; 34 min, 100% B; 36 min, 5% B.

In addition, the HPLC system was coupled to a microTOF™ (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer, using an electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The effluent from the HPLC column was split using a T-type phase separator before being introduced into the mass spectrometer (split ratio = 1:3). Analysis parameters were arranged using a negative-ion mode with a scan range from m/z 50 to 1000. The optimum values for the ESI−MS parameters were as

follows: capillary voltage, +4.0 kV; drying gas temperature, 200 °C; drying gas flow, 9.0 L min^{-1}; and nebulizing gas pressure, 2.0 bar.

The data was processed using the software Data Analysis 4.0 (Bruker Daltonik, Bremen, Germany). The identified compounds were carried out by the generation of the candidate formula with a tolerance of 10 ppm (part per million mass error) using the SmartFormula^{TM} editor and considering their retention time (RT), mass spectrum and the information available in the literature. The integration of peak areas of compounds were carried out using Bruker Compass Target Analysis 1.2 software for compound screening (Bruker Daltonics, Bremen, Germany). Three standard calibration graphs were prepared for quantification of the compounds in the olive leaves using three standards (hydroxytyrosol, oleuropein and apigenin).

2.5. Response Surface Methodology for Evaluation of MAE Parameters on Concentration of Compounds in Olive Leaves

The evaluation was carried out using a Box-Behnken design with 3 variables. The MAE independent variables were temperature (X_1) (50–150 °C), percentage of ethanol/water (X_2) (0–100% (v/v)) and extraction time (X_3) (5–40 min). A total of 15 experiments with 3 central points were conducted in a randomized order (**Table 1**). Optimum MAE conditions were estimated considering the maximum responses variables: total compounds and total AMPK bioactive compounds by using Statgraphics Centurion software provided by Statpoint Technologies. The relationship between the independent variables and concentration of compounds was analyzed by a response surface plot, which represents the dependent variable as a function of the two most influential independent variables.

Table 1. Concentration of total compounds and total AMP-activated protein kinase (AMPK) bioactive compounds in olive leaf extracts from microwave-assisted extraction (MAE) obtained by Box Behnken design.

MAE	X_1	X_2	X_3	Total Compounds (mg g^{-1} d.w.)	Total AMPK Bioactive Compounds (mg g^{-1} d.w.)
1	50	100	22.5	35.72 ± 0.17 ^{b}	3.70 ± 0.10 ^{b,c}
2	50	50	40	21.85 ± 0.16 ^{a}	2.81 ± 0.05 ^{a}
3	100	50	22.5	57.37 ± 0.07 ^{f}	8.64 ± 0.08 ^{g,h}
4	150	100	22.5	74.24 ± 0.50 ^{i}	7.93 ± 0.21 ^{g}
5	100	100	5	67.18 ± 0.60 ^{h}	6.99 ± 0.16 ^{f}
6	150	0	22.5	51.36 ± 0.37 ^{d}	6.56 ± 0.21 ^{f}
7	100	100	40	63.55 ± 0.90 ^{g}	7.05 ± 0.24 ^{f}
8	50	0	22.5	21.84 ± 0.37 ^{a}	4.06 ± 0.35 ^{c,d}
9	50	50	5	21.02 ± 0.02 ^{a}	2.87 ± 0.10 ^{a,b}
10	100	0	40	39.19 ± 0.09 ^{c}	6.97 ± 0.13 ^{f}
11	100	50	22.5	58.25 ± 0.37 ^{f}	9.04 ± 0.53 ^{h}
12	100	0	5	35.73 ± 0.07 ^{b}	6.29 ± 0.06 ^{f}
13	150	50	40	38.81 ± 0.57 ^{c}	5.25 ± 0.21 ^{e}
14	100	50	22.5	62.49 ± 0.45 ^{g}	9.39 ± 0.02 ^{h}
15	150	50	5	53.47 ± 0.31 ^{e}	4.80 ± 0.002 ^{d,e}

X_1: Temperature (°C), X_2: % EtOH/H_2O (%(v/v)) and X_3: Time (min). All results are expressed as mg g^{-1} of dry matter of olive leaves. Different letters in the same column indicate significant differences ($p < 0.05$).

3. Results and Discussion

3.1. Determination of Phenolic and Other Compounds in Olive Leaf Samples by HPLC-ESI-TOF-MS

A total of 41 compounds were identified in MAE olive leaf extracts, which were identified by considering their mass spectra as determined via TOF-MS and considering the data reported in the literature[22,26-28].

Figure 1 shows the extracted ion chromatogram obtained by HPLC-ESI-TOF-MS for each compound in the olive leaf extract from MAE 4. The parameters that enabled the identification of these phenolic compounds were retention time, experimental and calculated m/z, error, mSigma and the molecular formula (Supplementary Materials, **Table S1**).

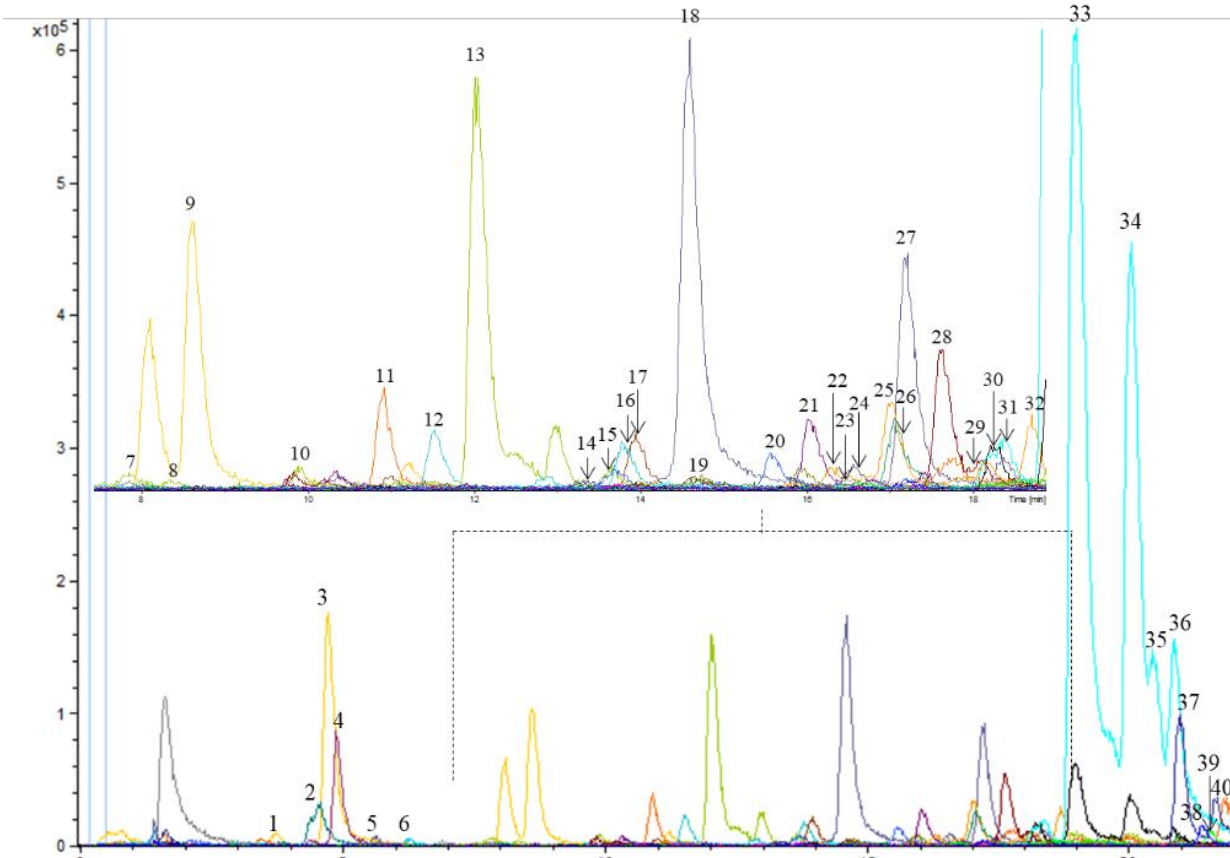

Figure 1. Extracted ion chromatogram (EIC) obtained from HPLC-ESI-TOF-MS analysis of olive le
Peaks have been numbered according to the elution order.

Table S1. Phenolic and other compounds identified in MAE olive leaf extracts by HP

Peak	RT	m/z experimental	m/z calculated	Tolerance (ppm)	Error (ppm)	mSigma	Molecular formula	Compound
1	3.783	389.1092	389.1089	10	-0.7	14.1	$C_{16}H_{22}O_{11}$	Oleoside
2	4.602	315.1064	315.1085	10	6.9	8.2	$C_{14}H_{20}O_{8}$	Hydroxytyroso
3	4.752	389.1092	389.1089	10	-0.7	4.7	$C_{16}H_{22}O_{11}$	Secologanoside
4	4.903	153.0549	153.0557	10	5.1	12.9	$C_{8}H_{10}O_{3}$	Hydroxytyroso
5	6.274	299.1135	299.1136	10	0.5	14.7	$C_{14}H_{20}O_{7}$	Tyrosol glucosi
6	6.926	341.0899	341.0878	10	-6.2	32.7	$C_{15}H_{18}O_{9}$	Caffeoylglucos
7	7.863	403.1254	403.1246	10	-2	34.7	$C_{17}H_{24}O_{11}$	Elenolic acid gl
8	8.331	403.1271	403.1246	10	-6.2	21	$C_{17}H_{24}O_{11}$	Elenolic acid gl
9	8.615	389.1109	389.1089	10	-5.1	4.4	$C_{16}H_{22}O_{11}$	Secologanoside
10	9.885	403.1257	403.1246	10	-2.7	20.9	$C_{17}H_{24}O_{11}$	Elenolic acid gl
11	10.922	377.1458	377.1453	10	-1.4	2.3	$C_{16}H_{26}O_{10}$	Oleuropein agly
12	11.524	609.1446	609.1461	10	2.5	11.9	$C_{27}H_{30}O_{16}$	Luteolin-digluc
13	12.009	403.1254	403.1246	10	-2.1	6.7	$C_{17}H_{24}O_{11}$	Elenolic acid gl
14	13.330	525.1642	525.1614	10	-5.4	35.4	$C_{24}H_{30}O_{13}$	Demethyloleur

15	13.681	555.1727	555.1719	10	-1.4	8.4	$C_{25}H_{32}O_{14}$	Hydroxyoleuro isomer a
16	13.781	609.1455	609.1461	10	1	23.1	$C_{27}H_{30}O_{16}$	Rutin
17	13.932	593.1523	593.1512	10	-1.9	18.8	$C_{27}H_{30}O_{15}$	Luteolin rutino
18	14.584	447.093	447.0933	10	0.6	17.1	$C_{21}H_{20}O_{11}$	luteolin glucosi
19	14.718	623.1969	623.1981	10	2	38	$C_{29}H_{36}O_{15}$	Verbascoside
20	15.554	555.1725	555.1719	10	-1.1	26	$C_{25}H_{32}O_{14}$	Hydroxyoleuro isomer b
21	16.022	577.153	577.1563	10	5.6	36.4	$C_{27}H_{30}O_{14}$	Apigenin rutino
22	16.256	701.2265	701.2298	10	4.8	10.9	$C_{31}H_{42}O_{18}$	Oleuropein glu
23	16.524	607.1637	607.1668	10	5.2	29.7	$C_{28}H_{32}O_{15}$	Diosmetin rhan (diosmin)
24	16.541	447.091	447.0933	10	5.2	34.3	$C_{21}H_{20}O_{11}$	luteolin glucosi
25	16.992	701.2282	701.2298	10	2.4	15.5	$C_{31}H_{42}O_{18}$	Oleuropein glu
26	17.042	431.0978	431.0984	10	1.4	14.8	$C_{21}H_{20}O_{10}$	Apigenin gluco
27	17.159	447.0915	447.0933	10	4	25.6	$C_{21}H_{20}O_{11}$	luteolin glucosi
28	17.241	461.1099	461.1084	10	-2	4.8	$C_{22}H_{22}O_{11}$	Chrysoeriol-7-(
29	18.146	701.2317	701.2298	10	-2.7	35.6	$C_{31}H_{42}O_{18}$	Oleuropein glu

30	18.263	541.1909	541.1927	10	3.3	31.9	$C_{25}H_{34}O_{13}$	Hydro-oleurope
31	18.347	447.0924	447.0933	10	2	7.8	$C_{21}H_{20}O_{11}$	luteolin glucosi
32	18.681	701.2279	701.2298	10	2.8	18.3	$C_{31}H_{42}O_{18}$	Oleuropein glu
33	18.949	539.1784	539.177	10	-2.6	24.4	$C_{25}H_{32}O_{13}$	Oleuropein iso
34	20.019	539.1787	539.177	10	-3.1	22.6	$C_{25}H_{32}O_{13}$	Oleuropein iso
35	20.420	539.1752	539.177	10	3.3	10.5	$C_{25}H_{32}O_{13}$	Oleuropein iso
36	20.822	539.175	539.177	10	3.8	7	$C_{25}H_{32}O_{13}$	Oleuropein iso
37	20.939	523.1804	523.1821	10	3.3	4.7	$C_{25}H_{32}O_{12}$	ligstroside
38	21.34	285.0401	285.0405	10	1.1	15.7	$C_{15}H_{10}O_{6}$	Luteolin
39	21.524	301.0371	301.0354	10	-5.8	19.3	$C_{15}H_{10}O_{7}$	Quercetin
40	21.974	553.1909	553.1927	10	3.3	8.5	$C_{26}H_{34}O_{13}$	Oleuropein/ole
41	23.280	613.1946	613.1927	10	-3.1	39.2	$C_{31}H_{34}O_{13}$	Resinoside

Table S2. Calibration curves of standards.

Analyte	LOD (mg/L)	LOQ (mg/L)	Calibration ranges (mg/L)	Calibration curves (mg/L)
Hydroxytyrosol	0.065	0.215	LOQ-125	y=31916x-75724
Oleuropein	0.036	0.121	LOQ-125	y=29372x+51188
Apigenin	0.016	0.055	LOQ-125	y= 117665x+16542

LOD: Limit of detection, LOQ: Limit of quantification

Table S3. Compounds quantified in MAE olive leaf extracts by HPLC-ESI-TOF-M

Compounds	MAE 1	MAE 2	MAE 3	MAE 4	MAE 5	
Simple phenols						
Hydroxytyrosol-hexose	0.13 ± 0.005^{a,b}	0.11 ± 0.004^{a}	0.30 ± 0.003^{d,e,f}	0.33 ± 0.004^{f}	0.25 ± 0.003^{c,d}	0.5
Hydroxytyrosol	N.D.	N.D.	0.10 ± 0.003^{a,b,c}	0.15 ± 0.01^{b,c,d}	0.08 ± 0.0001^{a,b}	0.7
Tyrosol glucoside	0.05 ± 0.0002^{a}	N.D.	0.07 ± 0.001^{a,b,c,d,e}	0.07 ± 0.002^{d,e}	0.07 ± 0.0002^{c,d,e}	0.(
Total	0.18 ± 0.003^{a}	0.11 ± 0.004^{a}	0.46 ± 0.02^{c,d}	0.54 ± 0.01^{d}	0.40 ± 0.003^{b,c}	1.3
Secoiridoids						
Oleoside	N.D.	N.D.	0.09 ± 0.01^{f}	0.03 ± 0.002^{b}	0.06 ± 0.005^{c,d}	
Secologanoside isomer a	0.47 ± 0.03^{a}	0.42 ± 0.01^{a}	1.39 ± 0.04^{e,f}	1.23 ± 0.01^{d,e}	1.00 ± 0.01^{b}	1.9
Secologanoside isomer b	0.33 ± 0.01^{a}	0.47 ± 0.02^{a,b}	1.77 ± 0.04^{f}	0.71 ± 0.02^{c}	0.75 ± 0.02^{c}	1.(
Oleuropein aglycon	0.01 ± 0.001^{a}	0.03 ± 0.002^{a}	0.23 ± 0.002^{e,f}	0.08 ± 0.007^{b}	0.05 ± 0.004^{a,b}	0.
Demethyloleuropein	0.005 ± 0.00001^{a,b}	0.001 ± 0.00002^{a}	0.03 ± 0.005^{e}	0.03 ± 0.002^{e,f}	0.02 ± 0.001^{d}	(

Hydroxyoleuropein/hydroxyoleuroside isomer a	0.007 ± 0.001[a]	0.03 ± 0.002[a,b,c]	0.40 ± 0.005[e]	0.04 ± 0.002[a,b,c]	0.07 ± 0.01[c]	
Hydroxyoleuropein/hydroxyoleuroside isomer b	0.22 ± 0.0004[c]	N.D.	N.D.	0.53 ± 0.03[e]	0.46 ± 0.02[d]	
Oleuropein glucoside isomer a	< LOQ	0.02± 0.002[b,c]	0.05 ± 0.01[e,f]	0.05 ± 0.002[c,d,e,f]	0.03 ± 0.01[b,c,d]	0
Oleuropein glucoside isomer b	0.01 ±0.001[a,b]	0.05 ± 0.01[b,c]	0.07 ± 0.0004[c,d]	0.06 ± 0.003[c]	0.04 ± 0.0002[a,b,c]	0.
Oleuropein glucoside isomer c	0.08 ± 0.002[b]	N.D.	0.24 ± 0.01[e,f]	0.27 ± 0.00003[e,f]	0.18 ± 0.002[c,d]	
Hydro-oleuropein/hydro-oleuroside	0.04 ± 0.01[a,b]	0.01 ± 0.002[a]	0.12 ± 0.01[e,f,g]	0.13 ± 0.007[f,g]	0.10 ± 0.004[d,e,f]	
Oleuropein glucoside isomer d	0.01 ± 0.003[a,b,c]	0.01 ± 0.003[a,b]	0.05 ± 0.002[c,d]	0.04 ± 0.001[a,b,c,d]	0.04 ± 0.003[a,b,c,d]	0.
Oleuropein isomer a	29.53 ± 0.03[f]	17.28 ± 0.09[c]	42.96 ± 0.04[i]	60.00 ± 0.27[m]	54.58 ± 0.48[l]	
Oleuropein isomer b	0.81 ± 0.09[a,b]	0.59 ± 0.01[a,b]	1.28 ± 0.15[c,d]	1.70 ± 0.05[d,e]	1.81 ± 0.04[e]	5.
Oleuropein isomer c	2.09 ± 0.15[b,c]	1.25 ± 0.04[a]	3.61 ± 0.02[d,e]	4.32 ± 0.25[f]	3.50 ± 0.25[e]	
Oleuropein isomer d	N.D.	N.D.	N.D.	N.D.	N.D.	1.
ligstroside	0.93 ± 0.05[d,e]	0.66 ± 0.01[b]	1.47 ± 0.01[g]	1.70 ± 0.05[h,i]	1.78 ± 0.06[i]	
Oleuropein/oleuroside methyl ether	0.02 ± 0.0005[b,c]	0.009 ± 0.002[a]	0.04 ± 0.001[f,g]	0.06 ± 0.003[h]	0.04 ± 0.003[e,f,g]	
Total	34.57 ± 0.20[c]	20.86 ± 0.14[a]	53.8 ± 0.1[f]	70.98 ± 0.48[j]	64.52 ± 0.58[i]	
			Flavonoids			
Luteolin-diglucoside a	0.007 ± 0.001[a]	0.01 ± 0.001[a,b]	0.05 ± 0.003[g]	0.04 ± 0.001[e,f]	0.02 ± 0.001[c]	
Rutin	0.009 ± 0.001[a]	0.01 ± 0.0003[a]	0.05 ± 0.003[g]	0.04 ± 0.001[e]	0.02 ± 0.0004[b]	
Luteolin rutinoside	0.009 ± 0.001[a]	0.01 ± 0.001[a]	0.04 ± 0.001[e,f]	0.04 ± 0.003[e,f]	0.02 ± 0.001[c]	
Luteolin glucoside isomer a	0.16 ± 0.01[a]	0.16 ± 0.01[a]	0.48 ± 0.01[e,f]	0.47 ± 0.02[e]	0.33 ± 0.001[c,d]	
Apigenin rutinoside	0.02 ± 0.001[a]	0.02 ± 0.0003[a]	0.06 ± 0.004[e,f]	0.06 ± 0.002[e,f,g]	0.04 ± 0.001[b]	

Diosmetin rhamnoside glucoside (diosmin)	<LOQ	0.001 ± 0.0002^{a,b}	0.008 ± 0.001^{e,f}	0.008 ± 0.0001^{e,f}	0.004 ± 0.001^{c,d}	
Luteolin glucoside isomer b	0.004 ± 0.001^{a}	0.004 ± 0.0004^{a}	0.04 ± 0.001^{e,f}	0.03 ± 0.002^{e}	0.02 ± 0.001^{c,d}	
Apigenin glucoside	0.03 ± 0.001^{a}	0.03 ± 0.00003^{a}	0.06 ± 0.0002^{c,d,e}	0.07 ± 0.004^{e,f}	0.06 ± 0.0001^{c,d,e}	
Luteolin glucoside isomer c	0.09 ± 0.001^{a}	0.01 ± 0.007^{a}	0.33 ± 0.01^{g}	0.31 ± 0.003^{f,g}	0.20 ± 0.01^{c,d}	
Chrysoeriol-7-O-glucoside a	0.05 ± 0.004^{a}	0.04 ± 0.001^{a}	0.12 ± 0.003^{g}	0.13 ± 0.005^{g,h}	0.10 ± 0.001^{e}	
Luteolin glucoside isomer d	0.01 ± 0.001^{a}	0.01 ± 0.001^{a}	0.05± 0.004^{d,e}	0.05 ± 0.001^{d}	0.03 ± 0.0006^{c}	
Luteolin	0.001 ± 0.000003^{a}	0.001 ± 0.001^{a}	0.01 ± 0.002^{e,f}	0.02 ± 0.001^{g,h}	0.006 ± 0.001^{c,d}	
Quercetin	0.005 ± 0.0001^{a}	0.002 ± 0.0004^{a}	0.13 ± 0.006^{f}	0.13 ± 0.001^{f}	0.07 ± 0.0004^{c,d}	
Resinoside	0.001 ± 0.0001^{a}	0.0005 ± 0.00001^{a}	0.005 ± 0.001^{c,d}	0.005 ± 0.0009^{c,d}	0.003 ± 0.0004^{b,c}	
Total	0.39 ± 0.01^{a}	0.39 ± 0.02^{a}	1.43 ± 0.01^{g}	1.40 ± 0.02^{g}	0.91 ± 0.005^{d}	1.
			Other phenolic compounds			
Caffeoylglucoside	N.D.	N.D.	0.004 ± 0.0001^{c,d}	0.002 ± 0.0004^{a,b,c}	0.004 ± 0.0004^{c,d}	
Verbascoside	0.07 ± 0.005^{a,b}	0.07 ± 0.003^{a}	0.13 ± 0.007^{e}	0.11 ± 0.002^{d}	0.09 ± 0.001^{c,d}	
			Other compounds			
Elenolic acid glucoside isomer a	< LOQ	N.D.	0.02 ± 0.003^{b}	0.03 ± 0.004^{b,c}	<LOQ	
Elenolic acid glucoside isomer b	0.08 ± 0.01^{b}	0.07 ± 0.01^{b}	0.20 ± 0.01^{d}	0.08 ± 0.0001^{b}	0.19 ± 0.005^{d}	
Elenolic acid glucoside isomer c	0.05 ± 0.01^{a,b}	0.09 ± 0.01^{b}	0.40 ± 0.004^{e}	0.16 ± 0.03^{c}	0.24 ± 0.002^{d}	0.
Elenolic acid glucoside isomer d	0.36 ± 0.02^{a}	0.26 ± 0.001^{a}	0.93 ± 0.01^{d,e,f}	0.93 ± 0.02^{d,e,f}	0.83 ± 0.01^{c,d}	1.
Total	0.49 ± 0.02^{a}	0.42 ± 0.002^{a}	1.56 ± 0.02^{f}	1.20 ± 0.01^{d}	1.26 ± 0.01^{d,e}	1.
Total compounds	35.72 ± 0.17^{b}	21.85 ± 0.16^{a}	57.37 ± 0.07^{f}	74.24 ± 0.50^{i}	67.18 ± 0.60^{h}	

Continue

Compounds	**MAE 9**	**MAE 10**	**MAE 11**	**MAE 12**	**MAE 13**
			Simple phenols		
Hydroxytyrosol-hexose isomer a	0.12 ± 0.01^{a}	0.29 ± 0.03^{d,e,f}	0.33 ± 0.003^{f}	0.26 ± 0.01^{d,e}	0.28 ± 0.04^{d,e}
Hydroxytyrosol	N.D.	0.19 ± 0.01^{c,d}	0.09 ± 0.00003^{a,b,c}	1.06 ± 0.006^{f}	0.24 ± 0.01^{d}
Tyrosol glucoside	0.06 ± 0.003^{a,b}	0.07 ± 0.004^{a,b,c,d,e}	0.07 ± 0.0008^{b,c,d,e}	0.06 ± 0.0007^{a,b,c,d}	0.07 ± 0.002^{a}
Total	0.18 ± 0.01^{a}	0.55 ± 0.04^{d}	0.49 ± 0.004^{c,d}	1.38 ± 0.02^{e}	0.59 ± 0.05^{d}
			Secoiridoids		
Oleoside	0.02 ± 0.001^{b}	0.08 ± 0.01^{e,f}	0.14 ± 0.006^{h}	0.07 ± 0.0005^{d,e}	0.01 ± 0.003^{a}
Secologanoside isomer a	0.47 ± 0.02^{a}	1.35 ± 0.02^{s,e}	1.62 ± 0.001^{g}	1.18 ± 0.04^{c,d}	1.24 ± 0.10^{d,e}
Secologanoside isomer b	0.59 ± 0.01^{b,c}	1.58 ± 0.12^{c}	1.97 ± 0.02^{g}	1.51 ± 0.05^{c}	0.63 ± 0.03^{b,c}
Oleuropein aglycon	0.05 ± 0.001^{a,b}	0.24 ± 0.01^{f,g}	0.27 ± 0.01^{g,h}	0.20 ± 0.02^{d,e}	0.18 ± 0.0004
Demethyloleuropein	0.007 ± 0.002^{a,b,c}	0.01 ± 0.001^{c,d}	0.04 ± 0.004^{f}	0.02 ± 0.0003^{d}	<LOQ
Hydroxyoleuropein/hydroxyoleuroside isomer a	0.04 ± 0.002^{a,b,c}	0.74 ± 0.01^{g}	0.42 ± 0.01^{e,f}	0.82 ± 0.04^{h}	0.02 ± 0.001^{a}
Hydroxyoleuropein/hydroxyoleuroside isomer b	N.D.	N.D.	0.11 ± 0.01^{b}	N.D.	0.14 ± 0.01^{b}
Oleuropein glucoside isomer a	<LOQ	0.06 ± 0.01^{f}	0.05 ± 0.01^{f}	0.03 ± 0.005^{b,c,d}	0.02 ± 0.003^{a}
Oleuropein glucoside isomer b	0.004 ± 0.0002^{a}	0.06 ± 0.008^{c}	0.08 ± 0.01^{c,d}	0.05 ± 0.001^{b,c}	0.01 ± 0.001^{a}
Oleuropein glucoside isomer c	0.06 ± 0.009^{b}	0.24 ± 0.01^{e,f}	0.29 ± 0.04^{f}	0.18 ± 0.01^{d}	0.24 ± 0.005^{e}

Hydro-oleuropein/hydro-oleuroside	0.03 ± 0.005^{a,b}	0.08 ± 0.004^{c,d,e}	0.15 ± 0.005^{g}	0.05 ± 0.004^{a,b,c}	0.07 ± 0.01^{b,c}
Oleuropein glucoside isomer d	0.007 ± 0.001^{a}	0.20 ± 0.005^{f}	0.05 ± 0.01^{d}	0.17 ± 0.006^{e,f}	0.06 ± 0.01^{d}
Oleuropein isomer a	16.18 ± 0.07^{b}	26.92 ± 0.29^{e}	42.27 ± 0.29^{i}	23.97 ± 0.05^{d}	23.77 ± 0.05^{d}
Oleuropein isomer b	0.53 ± 0.05^{a}	0.98 ± 0.11^{b,c}	1.71 ± 0.02^{d,e}	0.86 ± 0.04^{a,b,c}	5.65 ± 0.20^{g}
Oleuropein isomer c	1.25 ± 0.03^{a}	2.58 ± 0.04^{c,d}	3.45 ± 0.52^{e}	2.09 ± 0.07^{b,c}	1.57 ± 0.11^{a,b}
Oleuropein isomer d	N.D.	N.D.	N.D.	N.D.	1.67 ± 0.02^{b}
Ligstroside	0.60 ± 0.06^{a,b}	0.84 ± 0.05^{d}	1.62 ± 0.01^{h}	0.83 ± 0.01^{c,d}	0.70 ± 0.01^{b,c}
Oleuropein/oleuroside methyl ether	0.01 ± 0.003^{a,b}	0.03 ± 0.01^{d,e}	0.05 ± 0.002^{g,h}	0.02 ± 0.0002^{c,d}	0.05 ± 0.003^{g}
Total	19.84 ± 0.01^{a}	36.01 ± 0.06^{c}	54.34 ± 0.32^{f}	32.08 ± 0.07^{b}	36.03 ± 0.47^{c}
			Flavonoids		
Luteolin-diglucoside a	0.01 ± 0.002^{a,b,c}	0.04 ± 0.0001^{g}	0.05 ± 0.003^{h}	0.04 ± 0.001^{e,f}	0.03 ± 0.003^{e}
Rutin	0.01 ± 0.001^{a}	0.04 ± 0.0003^{d}	0.06 ± 0.001^{h}	0.03 ± 0.001^{d}	0.04 ± 0.001^{d}
Luteolin rutinoside	0.01 ± 0.001^{a,b}	0.04 ± 0.003^{e,f}	0.05 ± 0.003^{f,g}	0.03 ± 0.001^{d}	0.04 ± 0.004^{d}
luteolin glucoside isomer a	0.18 ± 0.002^{a,b}	0.37 ± 0.005^{d}	0.55 ± 0.003^{g}	0.29 ± 0.02^{c}	0.34 ± 0.01^{d}
Apigenin rutinoside	0.02 ± 0.00004^{a}	0.05 ± 0.003^{c,d}	0.07 ± 0.004^{f,g}	0.04 ± 0.001^{b,c}	0.05 ± 0.0000
Diosmetin rhamnoside glucoside (diosmin)	0.0008 ± 0.0001^{a}	0.07 ± 0.001^{d,e,f}	0.01 ± 0.001^{f}	0.006 ± 0.001^{c,d,e}	0.006 ± 0.000
Luteolin glucoside isomer b	0.007 ± 0.0001^{a,b}	0.02 ± 0.003^{c,d}	0.04 ± 0.002^{e,f}	0.01 ± 0.0004^{b,c}	0.02 ± 0.001^{d}
Apigenin glucoside	0.03 ± 0.002^{a}	0.05 ± 0.004^{b}	0.07 ± 0.005^{d,e}	0.04 ± 0.0002^{a}	0.05 ± 0.0002
Luteolin glucoside isomer c	0.12 ± 0.009^{b}	0.21 ± 0.005^{d}	0.39 ± 0.02^{h}	0.18 ± 0.01^{c}	0.23 ± 0.002^{d}
Chrysoeriol-7-O-glucoside a	0.05 ± 0.003^{a}	0.10 ± 0.003^{e}	0.13 ± 0.01^{h}	0.08 ± 0.003^{c}	0.10 ± 0.002^{e}

Luteolin glucoside isomer d	0.02 ± 0.001^{a,b}	0.02 ± 0.002^{b,c}	0.06 ± 0.005^{e}	0.02 ± 0.002^{b,c}	0.03 ± 0.0001
Luteolin	0.003 ± 0.001^{a,b}	0.01 ± 0.0001^{f,g}	0.01 ± 0.0001^{f,g}	0.006 ± 0.0003^{b,c}	0.06 ± 0.0006
Quercetin	0.004 ± 0.0002^{a}	0.02 ± 0.001^{b}	0.13 ± 0.001^{f}	0.004 ± 0.001^{a}	0.06 ± 0.003^{c}
Resinoside	<LOQ	0.0008 ± 0.00002^{a}	0.006 ± 0.001^{d}	N.D.	N.D.
Total	0.47 ± 0.002^{a}	0.98 ± 0.01^{d,e}	1.61 ± 0.03^{h}	0.78 ± 0.03^{c}	1.05 ± 0.01^{e}
			Other phenolic compounds		
Caffeoylglucoside	N.D.	0.003 ± 0.0002^{b,c}	0.002 ± 0.0008^{b,c}	<LOQ	0.003 ± 0.000
Verbascoside	0.07 ± 0.0003^{a,b}	0.10 ± 0.007^{c,d}	0.13 ± 0.003^{e}	0.09 ± 0.001^{c}	0.10 ± 0.002^{c}
			Other compounds		
Elenolic acid glucoside isomer a	N.D.	0.04 ± 0.001^{d}	0.007 ± 0.004^{a}	0.02 ± 0.002^{a,b}	N.D.
Elenolic acid glucoside isomer b	0.07 ± 0.002^{b}	0.10 ± 0.003^{b}	0.25 ± 0.003^{e}	0.15 ± 0.008^{c}	N.D.
Elenolic acid glucoside isomer c	0.08 ± 0.009^{a,b}	0.52 ± 0.01^{g}	0.44 ± 0.01^{e,f}	0.48 ± 0.02^{f,g}	<LOQ
Elenolic acid glucoside isomer d	0.30 ± 0.007^{a}	0.89 ± 0.01^{d,e,f}	0.99 ± 0.02^{e,f,g}	0.75 ± 0.03^{c}	1.03 ± 0.03^{f,g}
Total	0.46 ± 0.0004^{a}	1.54 ± 0.02^{f}	1.68 ± 0.1^{f}	1.39 ± 0.05^{e}	1.03 ± 0.03^{c}
Total compounds	21.02 ± 0.02^{a}	39.19 ± 0.09^{c}	58.25 ± 0.37^{f}	35.73 ± 0.07^{b}	38.81 ± 0.57^{c}

Different letters in the same line indicate significant differences among the concentration of phenc
different extractions in the design. N.D.: Not detected, LOQ: Limit of quantification.

Consequently, individual compounds in olive leaf extracts were quantified by calibration curves of the standards: hydroxytyrosol, oleuropein and apigenin. All calibration curves revealed good linearity among different concentrations, and the determination coefficients were higher than 0.9958 in all cases (Supplementary Materials, **Table S2**). Quantification of individual compounds were classified by families of simple phenols, secoiridoids, flavonoids, other phenolic compounds and elenolic acids (Supplementary Materials, **Table S3**).

With regard to the simple phenols and derivatives, a total of three compounds were quantified. According to these results, the extraction of simple phenols improved with the use of water as their maximum content was obtained in MAE 12 and MAE 6 with 0% EtOH. This is due to the polar nature of simple phenols that make them water soluble, and water is more efficient in solvent extraction than ethanol[29]. In addition, the highest content of simple phenols was obtained in the extraction time range of 5–22.5 min and a temperature of 100–150 °C.

With regard to secoiridoids, a total of 18 phenolic compounds were quantified. Of these, oleuropein isomer a was the most abundant, with a high content of 60.00 ± 0.27 mg g^{-1} d.w. in MAE 4 (150 °C, 100% EtOH and 22.5 min). In addition, MAE 4 conditions resulted in the best total secoiridoids content. Therefore, this result showed that an increase in temperature provides an increase in the concentration of secoiridoids. This can be attributed to the increase in the solubility of oleuropein at high temperatures and 100% of ethanol[30].

A total of 14 of flavonoids were found and the highest content was obtained at 100 °C, 50% EtOH and 22.5 min. The most concentrated flavonoid was luteolin glucoside isomer a (0.16 mg g^{-1} d.w.–0.55 mg g^{-1} d.w.). These results suggest that the content of flavonoids increases with the temperature up to 100 °C. Thus, temperatures higher than 100 °C decreased the content of flavonoids because these compounds are thermosensitive, especially flavonoids glycosides. This decrease was attributed to the thermal oxidation of the hydroxyl groups of glycoside[31]. Regarding elenolic acids, four compounds were quantified and 100 °C, 50% EtOH and 22.5 min were the best conditions for maximizing elenolic content. Hence, an increase in the temperature up to 100 °C increases the content of these compounds,

whereas an increase in the extraction time from 22.5 min to 40 min decreases elenolic acid content. This can be due to overexposure to microwave radiation, which leads to overheating and results in thermal degradation and oxidation of elenolic compounds[32].

Finally, it is important to note that the lowest total content of compounds was 21.02 ± 0.02 mg g^{-1} d.w., which was obtained in MAE 9 (50 °C, 50% EtOH and 5 min) and 21.84 ± 0.37 mg g^{-1} d.w. in MAE 8 (50 °C, 0% EtOH and 22.5 min), whereas the highest total content was 74.24 ± 0.50 mg g^{-1} d.w. which was obtained in MAE 4 at 150 °C, 100% ethanol and 22.5 min. Therefore, the highest content of total compounds was obtained at the maximum temperature and percentage of ethanol. This is because oleuropein, which is the most abundant secoiridoid in olive leaves, was extracted in the highest quantity at 100% of ethanol. Ethanol has the capacity to absorb the microwave radiation and heats up faster, thus, the increase in temperature improves the solubility of phenolic and elenolic compounds in ethanol and improves matrix penetration due to a decrease in surface tension and solvent viscosity[33]. In addition, the highest content of total compounds was obtained at 22.5 min. However, the total content decreased in extended extraction times of 40 min because overheating the solvent can lead to the degradation of most compounds[34].

3.2. Optimization of MAE Extraction Conditions

3.2.1. Optimization of Extraction Conditions for the Content of Total Compounds

The effect of optimization of temperature, time and % of ethanol amount on the content of the total compounds was analyzed by response surface methodology (RSM). **Table 2** shows the results of the ANOVA test of the regression model for the response variable (total compounds). The data revealed that the value of the determination coefficient (R^2) was 97.03%. This result explained a considerable part of the variance within the data. The proof of the lack of fit is a function of

determining the model. The test is performed by comparing the variability of the residuals in the current model with the variability in the observations under repeated conditions of the factors. Lack of fit for this model has a *p*-value greater than 0.05 (p = 0.1716). Therefore, the model seems to be adequate for the data observed at the 95.0% confidence level. ANOVA partitions the variability of total compounds into separate pieces for each of the effects. Then it tests the statistical significance of each effect by comparing its average square against an estimate of the experimental error. In this case, temperature (X_1) (p = 0.0043), % EtOH (X_2) (p = 0.0069), the quadratic effect of the temperature (X_{11}) (p = 0.0082) and quadratic of time (X_{33}) (p = 0.0198) had significant effects on the response variable (*p*-value less than 0.05, indicating that they were significantly different from zero with a confidence level of 95.0%).

Table 2. ANOVA test for the response variable of total compounds.

Total Compounds					
Source	**SS**	**DF**	**MS**	**F-Value**	***p*-Value**
X_1	1723.74	1	1723.74	230.05	0.0043 *
X_2	1071.32	1	1071.32	142.98	0.0069 *
X_3	24.51	1	24.51	3.27	0.2122
X_{11}	898.99	1	898.99	119.98	0.0082 *
X_{12}	20.26	1	20.26	2.70	0.2418
X_{13}	59.98	1	59.98	8.01	0.1055
X_{22}	15.05	1	15.05	2.01	0.2921
X_{23}	12.60	1	12.60	1.68	0.3242
X_{33}	367.86	1	367.86	49.09	0.0198 *
Lack of fit	112.07	3	37.36	4.99	0.1716
Pure error	14.99	2	7.49		
Total (corr.)	4280.1	14			
R^2	97.03				
Adj R^2	91.69				

X_1: temperature, X_2: % EtOH, X_3: Time, SS: Sum of squares, DF: Degree of freedom, MS: Mean square, R^2: Quadratic correlation coefficient; Adj R^2: Quadratic correlation coefficient adjusted, * Significant ($p < 0.05$).

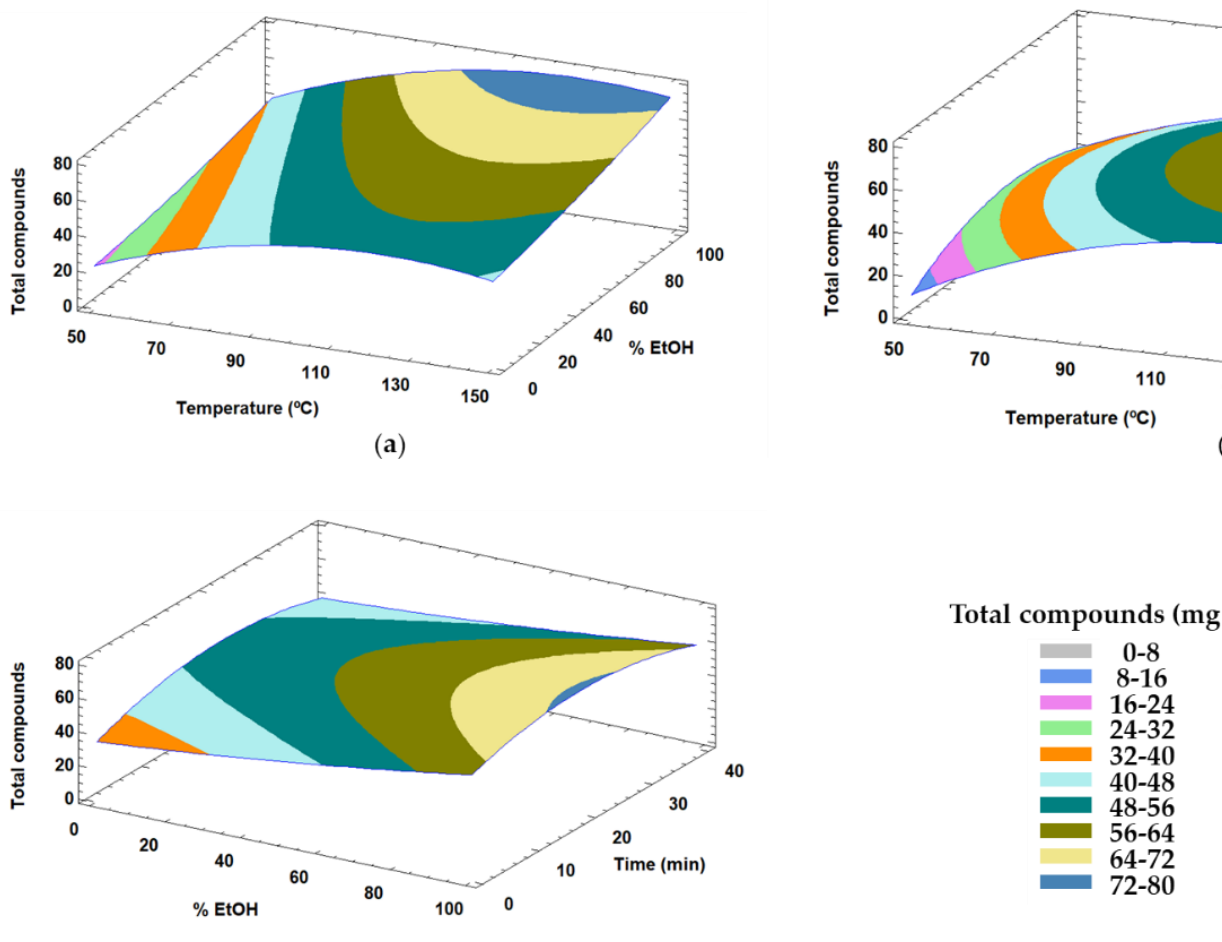

Figure 2. Response surface plots showing combined effects of process variables for total comp
% EtOH (**a**), temperature (°C)–time (min) (**b**) and % EtOH–time (min) (**c**)

Response surface plots show the effect of two MAE parameters on the total content of compound while keeping the third parameter constant, and these are displayed in **Figure 2**. **Figure 2a** shows the positive effect of temperature and percentage ethanol on the total compounds, with the highest content appearing at 100–150 °C and 80–100% EtOH, while **Figure 2b** shows the maximum value of total compounds at 100–150 °C and 10–30 min. Finally, **Figure 2c** shows the effect of EtOH with time, with the highest content appearing in the range of 15–30 min and 95–100% EtOH.

The fitted equation of the model for total compounds with the significant MAE parameters of X_1 (temperature), X_2 (% EtOH), X_{11} (quadratic of temperature) and X_{33} (quadratic of time) can be seen below. The fitted equation of the model includes the effect of time (X_3) (Equation (1)):

$$\text{Total compounds} = -57.8562 + 1.55431X_1 + 0.231443X_2 + 1.38944X_3 - 0.00630364X_{11} - 0.0330993X_{33} \quad (1)$$

Considering the equation to explain the model for total compounds behavior and understanding the influence of each independent variable, an optimization of the conditions to obtain the highest content of total compounds was proposed under the following optimized conditions: 123 °C, 100% EtOH and an extraction time of 23 min to obtain a predictable value of 75.60 mg g^{-1} d.w. of total compounds.

To verify the suitability of the model for total compounds, the predictable value of total compounds was compared with experimental values obtained at optimal conditions. The theoretical and experimental values were 75.60 mg g^{-1} d.w. and 86.7 ± 0.4 mg g^{-1} d.w., respectively. Analysis of the results revealed an acceptable variance (CV = 9.72%) between the theoretical and experimental data, therefore, the model was considered suitable.

Optimal conditions for the MAE of olive leaves have been reported previously in other research. Rafiee et al. (2011)[35] reported the highest phenolic content, expressed as mg TAE/g power (dw), in 'Koroneiki', 'Roghani' and 'Mission' cultivar at 50% EtOH and 15 min. This study used a constant composition of

different solvents (water, 80% methanol, 50% ethanol and acetone). Japón-Luján et al. (2006)[36] found that the optimum conditions of 200 W, 8 min and 80% ethanol obtained the maximum value of oleuropein and flavonoids: 2.32% of oleuropein, verbascoside 631 mg Kg^{-1} d.w., apigenin-7-glucoside 1076 mg Kg^{-1} d.w., luteolin-7-glucoside 1016 mg Kg^{-1} d.w. in olive leaves. The optimum conditions found in these previous studies are different to ours because these studies did not evaluate temperature as the third MAE parameter and nor is it indicated even though it significantly affects phenolic recovery from olive leaves in MAE. At high temperatures, the solvent power increases because of a decrease in viscosity and surface tension, which helps the solvent to solubilize solutes and improves matrix wetting and penetration. In addition, in a closed vessel microwave extraction, the temperature exceeds the boiling point of solvent, leading to improved extraction efficiency[37]. Moreover, the pressure of the system increases the boiling temperature of the solvent[38].

There are few studies that have been carried out on the optimization of three MAE parameters, including the extraction temperature. Taamalli et al. (2012)[28] reported the optimal MAE parameters of 80 °C, 80% methanol and 6 min to obtain a yield of 16.70% of fresh leafweight. Alañón et al. (2020)[39] optimized the MAE conditions for the highest content of total compounds in olive leaves by using deep eutectic solvents with optimum conditions of 79.98 °C, 15.28 min and 48.63% of water, to obtain 28.52 mg g^{-1} d.w. of total compounds from olive leaves by HPLC-ESI-TOF-MS. Thus, there is a great difference between these MAE optimum conditions compared to those obtained in the present study. This could be because these previous studies used methanol and water as an extraction solvent. Nevertheless, ethanol has been shown to be a good solvent for phenolic extraction and also it is safe for human consumption[40]. Therefore, this study is the first time that the optimization of these MAE parameters has been carried out using a mixture of ethanol/water as an extraction solvent in order to obtain the maximum total compounds from olive leaves samples by HPLC-ESI-TOF-MS.

Furthermore, the experimental value of the content of total compounds obtained under optimum MAE conditions in this study was 65.9% higher than the value of

the sum of individual compounds obtained by Alañón et al.(2020)[39] at optimum MAE conditions. Besides, the experimental result of this study was 40.5%, 39.9% and 31.3% higher than that obtained in 'Sikitita', 'Picual' and 'Arbequina' olive leaves, respectively, by ultrasonic assisted extraction (UAE)[26]. Also this was 38.7%, 81.3%, 59.8% and 37.6% higher than the means for all 'Arbequina'; 'Arbosana', 'Changlot Real', 'Picual', 'Koroneiki' and 'Sikitita' cultivars, respectively, at four sampling times (June, August, October and December) obtained by UAE[25]. Therefore, a high concentration of compounds in olive leaf extract was obtained by using optimum MAE conditions compared with that obtained in previous studies using MAE and UAE. However, this difference may be due to agronomic variability in the sample such as harvesting season, cultivar, etc.[25].

3.2.2. Optimization of Extraction Conditions for the Content of Compounds from Olive Leaves with Capacity to Modulate AMPK

According to Jimenez-Sanchez et al. (2017)[22], there are compounds in different fractions of olive leaf extract that decrease intracellular lipid accumulation through AMPK-dependent mechanisms in hypertrophic adipocyte[22].
However, these bioactive compounds were not quantified in that previous study. For that reason, it has been proposed that these compounds extracted by MAE should be quantified in order to establish the best conditions to obtain their highest recovery. Among a total of 41 compounds determined in olive leaf MAE extracts, 18 of them possess the capacity to activate the AMPK pathway (**Table 3**)[22]. It is important to note that according to Jimenez-Sanchez et al. (2017)[22], demethyoleouropein, verbascoside, hydroxyoleouropein, rutin, luteolin rutinoside and luteolin glucoside are the compounds that provide the highest activation of AMPK and these were quantified in the previous study.

Table 3. Compounds identified in olive leaf extracts with the potential to modulate AMPK.

Peak	AMPK Bioactive Compounds
9	Secologanoside isomer b
10	Elenolic acid glucoside isomer c
14	Demethyloleuropein
15	Hydroxyoleuropein/hydroxyoleuroside isomer a
16	Rutin
17	Luteolin rutinoside
18	luteolin glucoside isomer a
19	Verbascoside
22	Oleuropein glucoside isomer a
23	Diosmetin rhamnoside glucoside (diosmin)
24	luteolin glucoside isomer b
35	Oleuropein isomer c
36	Oleuropein isomer d
37	ligstroside
38	Luteolin
39	Quercetin
40	Oleuropein/oleuroside methyl ether
41	Resinoside

Table 1 shows the total content of these 18 compounds obtained in each extraction. Total AMPK bioactive compounds ranged from 2.81 ± 0.05 mg g^{-1} d.w. in MAE 2 (50 °C, 50% EtOH and 40 min) to 9.39 ± 0.02 mg g^{-1} d.w. in MAE 14 (100 °C, 50% EtOH and 22.5 min). Therefore, high temperatures, an intermediate percentage of ethanol and extraction time of 22.5 min generated the highest content of total compounds with the capacity to modulate the AMPK activation route. The increasing temperature speeds up the mass transfer of analytes from the sample to the extraction solvent due to the increase in the diffusion speed and solubility, and the decrease in viscosity, surface tension and the strength of the links between compounds and the matrix[41]. Therefore, high temperatures of 100 °C provided an increase in the recovery of total compounds, whereas an extraction time of 40 min resulted in a decrease in the content of these compounds. This could be because prolonging the exposure to the extraction microwave could lead to degradation of the compounds with overheating of the solvent[42].

A Box-Behnken design was used to maximize the content of these compounds with the capacity to activate the AMPK pathway. **Table 4** includes the results of the ANOVA test of the regression model for the response variable (total AMPK

bioactive compounds). The results indicated that the value of the regression coefficient (R^2) was 98.47%. This value justified a considerable part of the variance within data. Lack of fit for this model has a *p*-value greater than 0.05 ($p = 0.3957$). Therefore, the model seems to be adequate for the data observed at the 95.0% confidence level. The obtained parameters of ANOVA confirmed that this model provides a suitable approach to the experimental conditions. Temperature (X_1) ($p = 0.0091$), quadratic of temperature (X_{11}) ($p = 0.0038$) and the quadratic of time (X_{33}) ($p = 0.0104$) were the significant variables ($p \leq 0.05$).

Table 4. ANOVA test for the response variable of total AMPK bioactive compounds.

Total AMPk Bioactive Compounds					
Source	**SS**	**DF**	**MS**	**F-Value**	***p*-Value**
X_1	15.41	1	15.41	108.22	0.0091 *
X_2	0.40	1	0.40	2.79	0.2366
X_3	0.16	1	0.16	1.11	0.4033
X_{11}	37.2	1	37.2	261.32	0.0038 *
X_{12}	0.75	1	0.75	5.25	0.1490
X_{13}	0.07	1	0.07	0.48	0.5612
X_{22}	0.29	1	0.29	2.06	0.2874
X_{23}	0.10	1	0.10	0.70	0.4922
X_{33}	13.50	1	13.50	94.82	0.0104 *
Lack of fit	0.71	3	0.24	1.67	0.3957
Pure error	0.28	2	0.14		
Total (corr.)	65.46	14			
R^2	98.47				
Adj R^2	95.73				

X_1: temperature, X_2: % EtOH, X_3: Time, SS: Sum of squares, DF: Degree of freedom, MS: Mean square, R^2: Quadratic correlation coefficient; Adj R^2: Quadratic correlation coefficient adjusted, * Significant ($p < 0.05$).

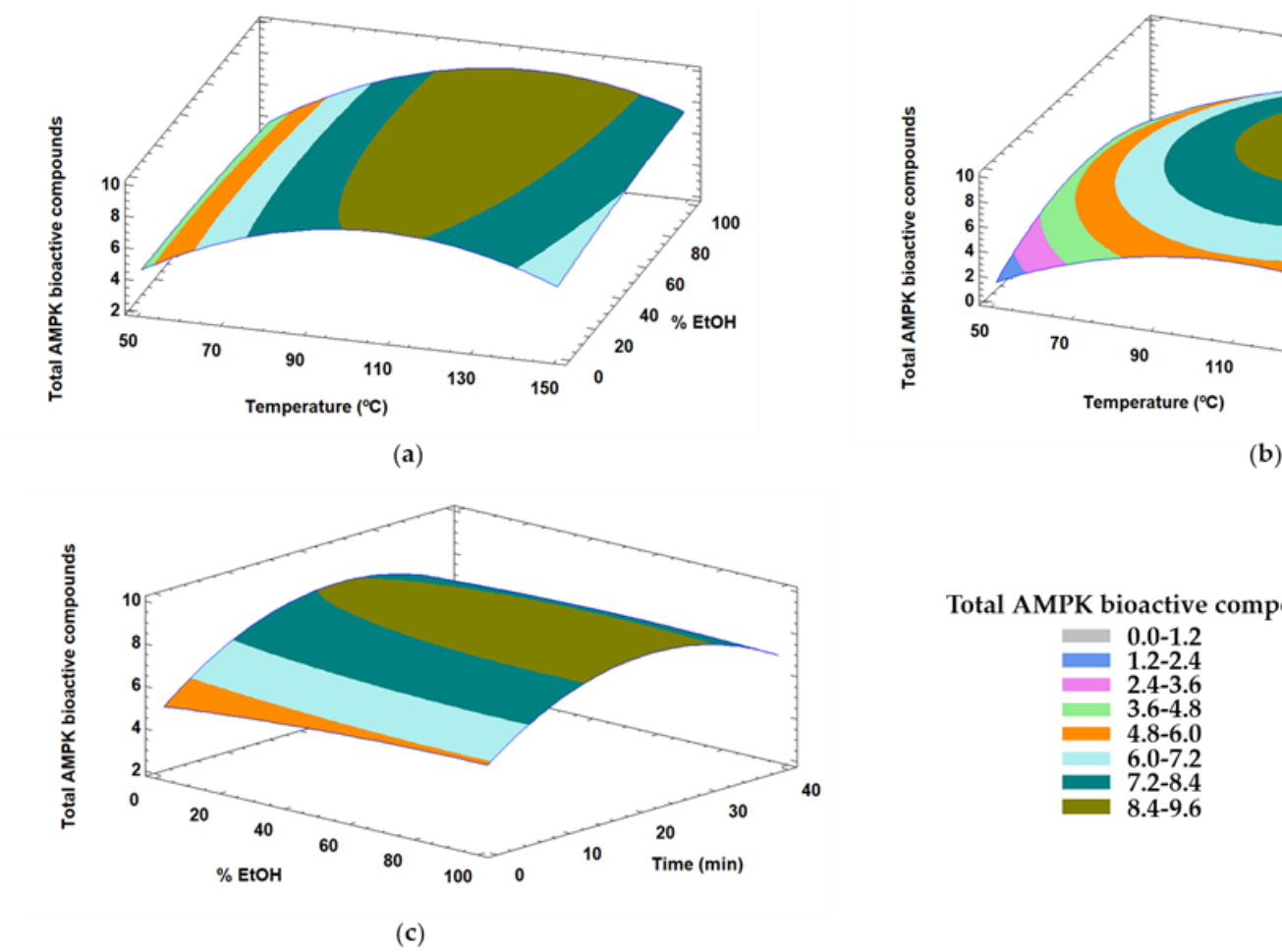

Figure 3. Response surface plots showing combined effects of process variables for total AM
Temperature (°C)–% EtOH (**a**), temperature (°C)–time (min) (**b**) and % EtOH–time (min) (**c**)

In **Figure 3**, the surface response plots show the effect of temperature with % EtOH (a), temperature with time (b), and % EtOH with time (c) on total AMPK bioactive compounds. The significant effect of temperature on the content of total AMPK bioactive compounds can be observed, with the maximum value appearing at 90–130 °C, after which this response decreases (**Figure 3a**). **Figure 3b** shows that the maximum content of total AMPK bioactive compounds is in the range of 100–130 °C and 20–30 min. **Figure 3c** shows that the maximum value of this response was obtained in the range of 15–30 min, after which it decreases, whereas the % EtOH did not has a significant effect on this response. This is because high temperatures of 100–130 °C increase the solubility of these compounds so they have a similar solubility in different ethanol compositions, while an extraction time higher than 20–30 min results in a decrease in content. This is probably because these compounds were degraded due to thermal degradation after a prolonged period of microwave radiation[43].

The fitted equation of the model for total AMPK bioactive compounds (Equation (2) includes the significant effects of temperature (X_1), quadratic of temperature (X_{11}) and quadratic of time (X_{33}). The model also considers the linear effect of time (X_3).

$$\text{Total AMPK bioactive compounds} = -9.84797 + 0.280015X_1 + 0.285851X_3 - 0.00126127X_{11} - 0.00617407X_{33} \quad (2)$$

Considering the equation to explain the model for total AMPK bioactive compounds behavior and understanding the influence of each independent variable, an optimization of conditions to obtain the highest content of these compounds was proposed. A high value for this response variable can be obtained under the following optimized conditions: 111 °C, 42% EtOH and a total extraction time of 23 min to obtain a predicted value of 9.00 mg g^{-1} d.w. of total AMPK bioactive compounds.

This theoretical optimum was experimentally verified. Analysis of the results revealed an acceptable variance (CV = 3.81%) between the theoretical (9.00 mg g^{-1} d.w.) and experimental data (9.5 ± 0.1 mg g^{-1} d.w.). This means that the experimental validation was consistent with the mathematical description of the

model, which indicates the suitability of RSM in optimizing the total compounds that have the capacity to activate AMPK from olive leaf extracts by MAE. However, these MAE optimal conditions were different to those for total compounds. For example, ethanol composition was not a significant variable in total AMPK bioactive compounds. Therefore, a simultaneous optimization of MAE conditions by multi-response surface for total compounds and total AMPK bioactive compounds was carried out using Derringer's desirability function method.

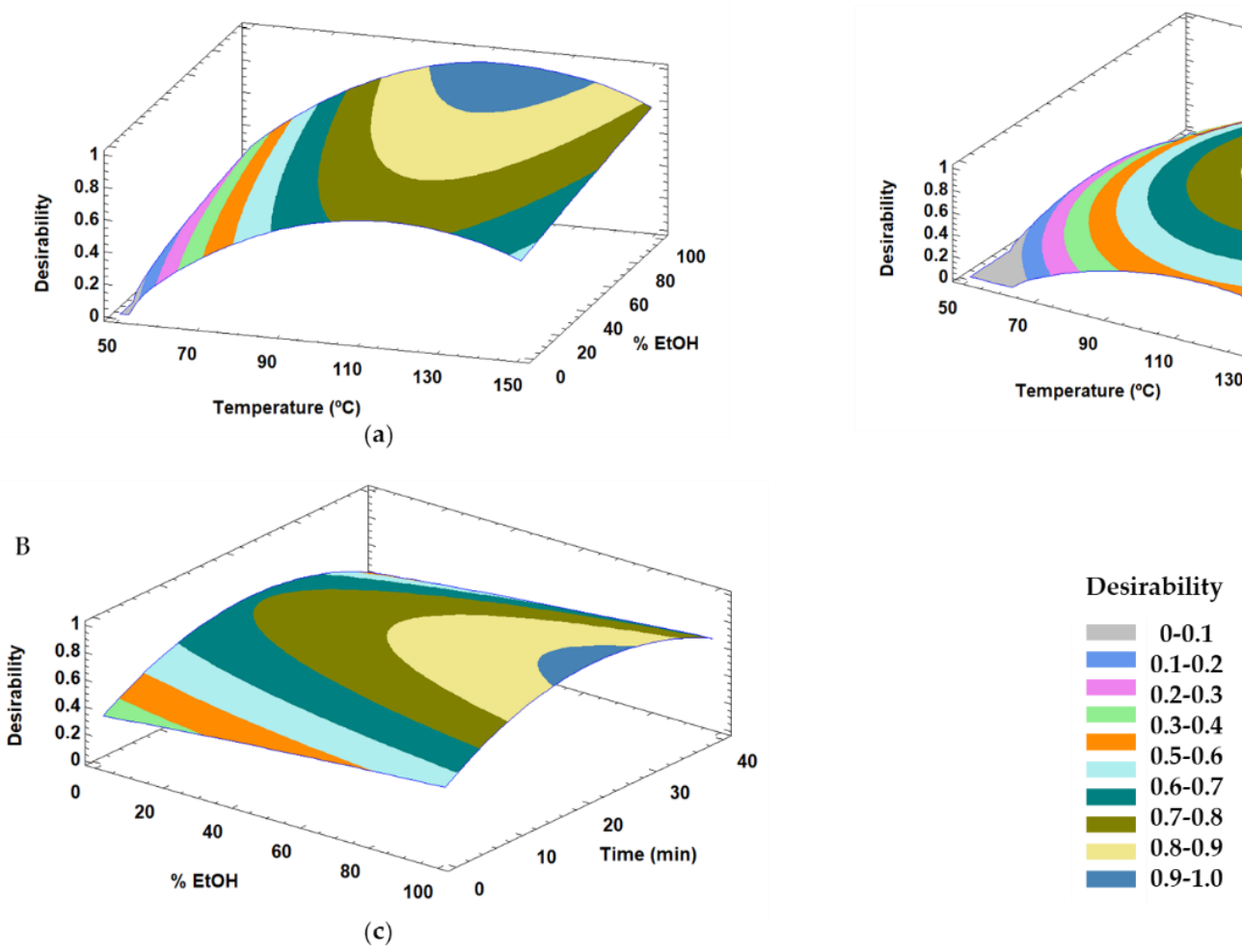

Figure 4. Response surface plots showing combined effects of process variables for desirabil
total AMPK bioactive compounds: Temperature (°C)–% EtOH (**a**), Temperature (°C)–time (r
(min) (**c**)

Surface response plots are shown in **Figure 4** for the effect of temperature with % of EtOH (a), with time (b) and % EtOH with time (c) by desirability. The optimal conditions were 111 °C, 100% EtOH and 23 min to obtain 74.39 mg g^{-1} d.w. of total compounds and 9.00 mg g^{-1} d.w. of total AMPK bioactive compounds, respectively. The optimized desirability was 0.97, that is close to 1, which means that the responses are close to their individual optimum values[44]. Therefore, the total content of compounds that possess the capacity to activate AMPK is related to the total compounds. In addition, the optimum 100% EtOH was the same as the optimum obtained for total compounds and this is because the % of ethanol did not have a significant effect in the total AMPK bioactive compounds. Moreover, the temperature of 111 °C was slightly lower than the optimum for total compounds (123 °C), whereas this was the same as the AMPK bioactive compounds. Nevertheless, the optimum content of total compounds predicted at 111 °C was similar to its individual optimum value at 123 °C. In addition, the bioactivity of the extracts is decreased due to the degradation of the compounds at high temperatures[32]. For this reason, establishing an optimum temperature in which the AMPK bioactive compounds content was the maximum was considered. Besides, according to our results, the highest content of compounds with a demonstrated effect on the bioactivity of AMPK was obtained at 111 °C. Therefore, these conditions are suitable for obtaining the maximum content for both responses.

4. Conclusions

In this study, for the first time, we carried out an optimization of the MAE factors of temperature, % EtOH and time for the total compounds (phenolic compounds and elenolic acids) obtained by HPLC-MS. The optimum MAE conditions for the highest recovery of total compounds from olive leaves were 123 °C, 100% EtOH and 23 min. An optimization of MAE conditions was also carried out to obtain the maximum content of compounds from olive leaves that have shown the capacity to activate AMPK, whereby the optimum MAE conditions were 111 °C, 42% ethanol/water (v/v) and extraction time of 23 min. Thus, these optimum conditions

for temperature and % EtOH were different compared to those for total compounds. For that reason, a multi-response analysis by desirability was carried out to obtain optimum MAE conditions for both responses. The optimum conditions were 111 °C, 100% EtOH and 23 min to obtain 74.39 mg g^{-1} d.w. of total compounds and 9.00 mg g^{-1} d.w. of total AMPK bioactive compounds. Optimum desirability was 0.97, thus, these responses are close to their individual optimum values. Therefore, these optimum MAE conditions can be applied to obtain an olive leaf extract enriched in total compounds and in compounds with the capacity to activate AMPK; thus, this could be used as a treatment in diseases such as obesity.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Phenolic and other compounds identified in MAE olive leaf extracts by HPLC-ESI-TOF-MS., Table S2: Calibration curves of standards., Table S3: Compounds quantified in MAE olive leaf extracts by HPLC-ESI-TOF-MS (mg g^{-1} d.w.).

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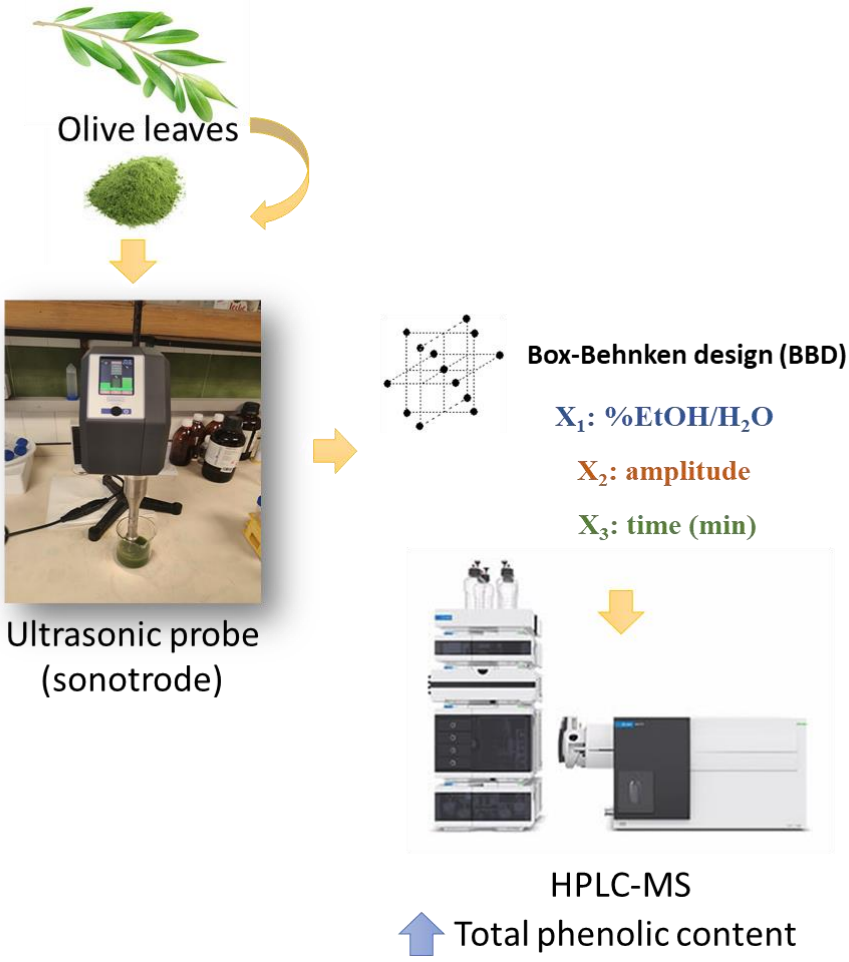
CHAPTER 3

Set-up of sonotrode based extraction to recover phenolic compounds from olive leaves



Abstract

Olive leaves are a waste by-product obtained during the olive oil production and pruning. They contain phenolic compounds that possess antioxidative, antimicrobial, anti-atherogenic and anti-inflammatory properties, among others. For that reason, a procedure based on ultrasound-assisted extraction via sonotrode was developed to evaluate the recovery of these phenolic compounds from olive leaves. To establish the sonotrode extraction, a Box-Behnken design based on response surface methodology (RSM) was used to optimize the effects of factors such as solvent composition (30-100% EtOH), extraction time (1-10 min) and amplitude (20-100%). Qualitative and quantitative analyses of phenolic compounds were performed using HPLC coupled to DAD and mass spectrometer detectors. The highest content of phenolic compounds was 40.9 ± 0.2 mg/g d.w. obtained using 55:45 ethanol/water (v/v), 8 minutes and 100% of the amplitude. The optimal conditions selected for the sonotrode were compared with the result obtained by a conventional ultrasonic bath achieving similar concentrations. Therefore, sonotrode could be considered as an efficient extraction technique that allows a good recovery of phenolic substances from olive leaf that could be easily scale-up at industrial level.

Keywords: Olive leaves, phenolic compounds, sonotrode, Box-Behnken, HPLC-MS

1. Introduction

Olive leaves represents around 10% of the weight of olives collected for the oil production (25 kg per olive tree) during tree pruning[1]. Part of this by-product is used in the animal food or energetic biomass, whereas a great quantity of olive leaves are discarded generating a great cost and a high environment impact[2]. Nevertheless, olive leaves are a potential source of phenolic compounds that

possess numerous beneficial properties attributed to their antioxidant activity[3]. Therefore, its reutilization can be profitable for the Food Industry in order to obtain nutraceuticals or functional foods. The phenolic composition of olive leaves varies according to many factors such as the date of collection[2,4], cultivation zone [5] and cultivar[2,6,7]. Phenolic compounds in olive leaves can be classified in phenolic acids, phenolic alcohols, flavonoids and secoiridoids[7]. The main phenolic compounds in olive leaves are hydroxytyrosol, rutin, verbascoside, luteolin-7-glucoside, luteolin-4-o-glucoside, oleuropein, oleuropein aglycone, and ligstroside aglycone[3,7,8].

The extraction process is the most important step in the phenolic recovery. Conventional techniques such as maceration have been used for a long time. However, they require high volume of solvents, long extraction times and possess a low selectivity, low reproducibility and low efficiency[9,10]. Nowadays, in order to reduce extraction times, new techniques such as microwave extraction, supercritical fluid extraction and pressurized liquid extraction have been applied in the phenolic recovery from olive leaves[2,11-13]. Nevertheless, most of these techniques generate high energy costs because they operate at high pressures. For that reason, ultrasound assisted extraction (UAE) can be the best choice due to it is an effective and low-cost extraction technique[14]. Ultrasound assisted extraction can be carried out by using two types of devices, ultrasonic bath or ultrasonic probe (sonotrode) (US) equipment[15]. The ultrasonic bath is the most used for the phenolic extraction because they are cheap, available and allow the extraction of various samples simultaneously. However, by comparison with probe systems, they possess a low reproducibility and low power of ultrasound delivered directly to the sample[15]. Nevertheless, the sonotrode system is more powerful because of an ultrasonic intensity delivered through a smaller surface (the tip of the probe), in comparison with the ultrasonic bath[16]. In addition, quantification of phenolic compounds in olive leaves is carried out by high-performance liquid chromatography (HPLC). This technique is coupled to a diode array detector (DAD) and mass spectrometer detector (MS)[2,3,7,12].

In view of the above, the purpose of this work was to evaluate the recovery of phenolic compounds from olive leaves by optimization of a sonotrode ultrasonic-assisted extraction method. For that purpose, response surface methodology (RSM) was performed to evaluate extraction parameters % EtOH/H_2O (v/v), amplitude and extraction time with an experimental Box–Behnken design. In addition, it was carried out a conventional ultrasonic bath extraction in order to compare with the optimized by sonotrode technique.

2. Materials and Methods

2.1. Chemicals and Reagents

Ethanol and methanol were purchased from Fisher Scientific (Leicestershire, UK), and water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). For HPLC analysis, LC-MS grade acetronitrile was purchased from Fisher (Fisher Scientific UK, Leicestershire, UK) and ultrapure water was obtained with the Milli-Q system described above. The acetic acid used was purchased from Fluka (Buchs, Switzerland). The standard compounds used for the quantification were hydroxytyrosol, and apigenin, which were purchased from Sigma-Aldrich (Saint Louis, MO, USA), and oleuropein was from Extrasynthèse (Lyon, France).

2.2. Samples

Olive leaves ‘Koroneiki’ were collected from at “IFAPA, Centro Alameda del Obispo” in Córdoba, Spain (37°51′36.5″ N4°47′53.7″ W). Samples were harvested at mid-December (fruit-ripening) in 2020. Olive leaves were air dried under controlled temperature. Subsequently, leaves were ground using IKA A 10 Basic Mill (Retsch GmbH, Haan, Germany) and the resulting powder was stored at -20ºC until the extraction.

2.3. Extraction of phenolic compounds from olive leaves by US sonotrode and ultrasonic bath extraction.

The extraction was achieved with an US sonotrode UP400St (Hielscher Ultrasonics GmbH, Teltow, Germany). 0.25 g of powdered olive leaves were extracted using 100mL of EtOH/H_2O. The percentage of ethanol/water, extraction time and the US amplitude were varied according to the experimental design.

The ultrasonic bath extraction of phenolic compounds was performed as described previously by Talhaoui et al. 2015 with certain modifications[2]. Briefly, powdered leaves (0.1 g) were extracted using 10 mL of EtOH/H2O (80:20, v/v) by using an ultrasonic bath (Bandelin, Sonorex, RK52, Berlin, Germany) operating at a frequency of 35 kHz during 20 minutes. Two replicates of each sample were processed.

After the extraction, the olive leaf extracts were centrifugated at 1000g for 10 min, the supernatant was collected, evaporated, and reconstituted in 5 mL of methanol/water (1:1, v/v). The final extracts were filtered through 0.2 μm polytetrafluoroethylene (PTFE) syringe filters and stored at -18 °C until the analyses.

2.4. Experimental design

A Box-Behnken design with 3 variables was carried out to optimize the extraction parameters to obtain the highest phenolic content from olive leaves. In this study, three independent variables were %EtOH/H_2O (X_1), Amplitude (X_2) and time (X_3), with 3 levels for each variable and the response variable (Y) was the sum of the phenolic compounds and elenolic acids (total compounds). The parameters range established were percentage of ethanol/water (30, 65 and 100 %), amplitude (20, 60, and 100%) and extractions times (1, 5.5 and 10 min), which were similar to a previous study that reported UAE factors of 20-80% EtOH, 20-70% of amplitude and 5-15 min in olive mill leaves[17]. Amplitude percentage refers to the percentage of maximum power used. The extraction time was limited to 10 min due to during the extraction the temperature increased. In addition, the range of extraction time was chosen from 1 minute according to a previous study, which employed short

sonication times from 1 to 5 min in olive leaves[18]. The design consisted of 15 combinations including 3 center points (**Table 1**).

Table 19. Box-Behnken design with sonotrode parameters and values of dependent variable obtained (total compounds) quantified by HPLC-MS 'koroneiki' in olive leaves.

Runs	X1	X2 (Power)	X3	Total compounds (mg/g d.w.)
1	30	20 (38W)	5.5	26.5 ± 0.6b
2	30	100 (149W)	5.5	33.7 ± 0.9g,h
3	100	20 (29W)	5.5	27.5 ± 0.5b,c
4	100	100 (126W)	5.5	28.31 ± 0.06c,d
5	65	20 (36W)	1	31.20 ± 0.05e
6	65	100 (136W)	1	32.5 ± 0.5e,f,g
7	65	20 (37W)	10	33.0 ± 0.3f,g,h
8	65	100 (140W)	10	33.0 ± 0.2f,g,h
9	30	60 (89W)	1	28.9 ± 0.5c,d
10	100	60 (88W)	1	24.92 ± 0.06a
11	30	60 (87W)	10	31.82 ± 0.08e,f
12	100	60 (85w)	10	29.2 ± 0.5d
13	65	60 (86 W)	5.5	34.1531 ± 0.0008h
14	65	60 (87W)	5.5	33.29 ± 0.4f,g,h
15	65	60 (85W)	5.5	34.0 ± 0.7g,h

X_1: %EtOH/H_2O (v/v), X_2: amplitude X_3: time (min)

The response variables were fitted to a second-order polynomial model equation obtained by the response surface methodology (RSM) (Eq.1).

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} {X_{ii}}^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ii} X_i X_j \quad (\mathbf{Eq.\,1})$$

Were Y is the response variable, which was the total compounds in olive leaves obtained by HPLC-MS. *Xi* and *Xj* are the independent factors, whereas β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients of the model for the mean, linear, quadratic and interaction term calculated from the experimental results by the least of squares method. Model building, experimental results and designs were processed using STATISTICA 7.0 (2002, StatSoft, Tulsa,OK).

2.5. Analysis of the phenolic compunds by High-Performance Liquid Chromatography Coupled to Mass Spectrometry (HPLC- MS)

Analyses of the phenolic compounds of olive leaves were carried out following the previously validated method of Talhaoui et al. (2014)[7] using Agilent 1200 Series Rapid Resolution liquid chromatography system (Agilent Technologies, CA, USA), which is comprised of a binary pump, degasser, and auto sampler. Phenolic compounds were separated using a Poroshell 120 EC-C18 (4.6 × 100 mm, 2.7 mm) from Agilent Technologies, at 25 °C and a flow rate of 0.8 mL min^{-1}. The mobile phases were 1% of acetic acid as mobile phase A and acetonitrile as mobile phase B. The conditions of the solvent gradient were as follows: 0 min, 5% B; 4 min, 9% B; 7 min, 12% B; 8 min, 15% B; 9 min, 16% B; 14 min, 20% B; 15 min, 22% B; 18 min, 28% B; 19 min, 30% B; 20 min, 31% B; 21.50 min, 32% B; 23 min, 34% B; 24 min, 35% B; 25.5 min, 40% B; 27 min, 50% B; 30 min, 100% B; 34 min, 100% B; 36 min, 5% B.

Hydroxytyrosol, tyrosol, oleuropein, rutin, luteolin-7-glucoside, apigenin-7-glucoside and luteolin were the standard used for the quantification of compounds in the olive leaf extracts. The calibration curves were prepared at seven concentration levels from the limit of quantification (LOQ) to 100 mg/L.

3. Results and discussion

3.1. Characterization of phenolic and other compounds from olive leaves US sonorode extracts by HPLC- MS.

Phenolic compounds were identified by rendering their mass spectra using the data reported in in previous studies[7,19-21]. A total of 36 compounds were identified and quantified in olive leaf obtained by US sonotrode. The quantification of individual compounds in each experiment was carried out by the calibration curve of standards. In addition, all calibration curves showed a good linearity ($r^2 > 0.9910$) (**Table S1**).

Table S1. Phenolic compounds quantified in olive leaves extracts by HPLC -MS expressed as mg

Different letters indicate significant differences among the extractions. LOQ: Limit

Peak	Compound	SON-1	SON-2	SON-3	SON-4	SON
1	Hydroxytyrosol-hexose isomer a[1]	0.0027 ± 0.0007	0.0028 ± 0.0003	0.00026 ± 0.0008	0.0016 ± 0.0003	0.0028 ±
2	Oleoside[2]	0.45 ± 0.01	0.40 ± 0.01	0.2988 ± 0.0007	0.32 ± 0.01	0.362 ±
3	Hydroxytyrosol-hexose isomer b[1]	0.62302 ± 0.00009	0.70 ± 0.02	0.642 ± 0.003	0.66 ± 0.03	0.656 ±
4	Hydroxytyroso[l]	0.071 ± 0.007	0.0843 ± 0.0007	0.067 ± 0.003	0.069 ± 0.001	0.077 ±
5	Secologanoside isomer a[2]	2.5 ± 0.1	2.2 ± 0.1	1.893 ± 0.006	1.929 ± 0.001	2.10 ±
6	Tyrosol glucoside[3]	0.181 ± 0.009	0.191 ± 0.003	0.167 ± 0.009	0.166 ± 0.008	0.1783 ±
7	Caffeoyl glucoside[2]	0.035 ± 0.001	0.042 ± 0.004	0.0234 ± 0.0003	0.024 ± 0.002	0.029 ±

8	Tyrosol[3]	0.0052 ± 0.0002	0.00587 ± 0.0008	<LOQ	0.00330 ± 0.00005	0.0020 ±
9	Elenolic acid glucoside isomer a[2]	0.10 ± 0.02	0.088 ± 0.004	0.046 ± 0.009	0.0486 ± 0.0007	0.065 ±
10	Secologanoside isomer b[2]	1.26 ± 0.03	1.17 ± 0.03	0.68 ± 0.01	0.80 ± 0.01	1.104 ±
11	Elenolic acid glucoside isomer b[2]	0.7318 ± 0.0006	0.71 ± 0.09	0.37 ± 0.01	0.421 ± 0.009	0.559 ±
12	Oleuropein aglycon[2]	2.29 ± 0.08	2.19 ± 0.07	1.070 ± 0.001	1.24 ± 0.02	1.98 ±
13	Elenolic acid glucoside isomer c[2]	0.40 ± 0.01	0.363 ± 0.005	0.303 ± 0.002	0.31 ± 0.02	0.33 ±
14	Luteolin diglucoside[4]	0.029 ± 0.003	0.027 ± 0.002	0.0143 ± 0.0004	0.018 ± 0.002	0.0215 ±
15	Elenolic acid glucoside isomer d[2]	0.1410 ± 0.009	0.12 ± 0.01	0.08690601	0.086 ± 0.001	0.112 ±
16	Demethyloleuropein[2]	0.24 ± 0.02	0.32 ± 0.02	0.256 ± 0.002	0.295 ± 0.004	0.31 ±
17	Hydroxyoleuropein isomer a[2]	0.48 ± 0.03	0.59 ± 0.02	0.034 ± 0.002	0.032 ± 0.005	0.12 ±

18	Rutin[5]	0.43 ± 0.03	0.45 ± 0.02	0.260 ± 0.008	0.286 ± 0.003	0.375 ±
19	Luteolin rutinoside[4]	0.067 ± 0.003	0.061 ± 0.006	0.0365 ± 0.0005	0.043 ± 0.002	0.053 ±
20	Luteolin glucoside isomer a[4]	1.23 ± 0.03	1.25 ± 0.05	0.92 ± 0.01	0.98 ± 0.03	1.109 ±
21	Verbascoside[1]	0.00180 ± 0.00006	0.007208 ± 0.000006	0.00537 ± 0.00006	0.0050 ± 0.0003	0.0057 ±
22	Hydroxyoleuropein isomer b[2]	0.0030 ± 0.0005	0.0028 ± 0.0002	0.0120 ± 0.0007	0.0132 ± 0.0002	<LC
23	Apigenin rutinoside[6]	0.0268 ± 0.0002	0.0248 ± 0.0004	0.0154 ± 0.0003	0.018 ± 0.001	0.022 ±
24	Oleuropein diglucoside isomer a[2]	0.023 ± 0.003	0.0270 ± 0.0009	0.0151 ± 0.0003	0.0192 ± 0.0001	0.023 ±
25	Apigenin-7-glucoside[6]	0.059 ± 0.002	0.0541 ± 0.0001	0.051 ± 0.004	0.049 ± 0.002	0.058 ±
26	Oleuropein diglucoside isomer b[2]	0.051 ± 0.002	0.069 ± 0.006	0.054 ± 0.002	0.0466 ± 0.0009	0.053 ±
27	Luteolin glucoside isomer b[4]	0.73 ± 0.03	0.713 ± 0.006	0.47 ± 0.01	0.54 ± 0.02	0.62 ±

28	Oleuropein diglucoside isomer c[2]	0.102 ± 0.003	0.090 ± 0.003	0.094 ± 0.007	0.083 ± 0.002	0.103 ±
29	Chrysoeriol-7-O-glucoside[4]	0.253 ± 0.008	0.262 ± 0.002	0.1988 ± 0.0006	0.206 ± 0.001	0.236 ±
30	Luteolin glucoside isomer c[4]	0.101 ± 0.005	0.137 ± 0.002	0.10 ± 0.01	0.119 ± 0.005	0.106 0.00
31	Oleuropein isomer 1[2]	12.24 ± 0.01	19.3 ± 0.4	17.1 ± 0.4	17.35 ± 0.03	18.37 ±
32	Oleuropein isomer 2[2]	0.29 ± 0.04	0.43 ± 0.01	0.47 ± 0.01	0.47 ± 0.02	0.436 ±
33	Oleuropein/Oleuroside [2]	1.08 ± 0.07	1.263 ± 0.005	1.40 ± 0.04	1.30 ± 0.03	1.30 ±
34	Ligstroside aglycone[2]	0.012 ± 0.004	0.024 ± 0.002	N.D.	0.008 ± 0.003	0.014 ±
35	Ligstroside [2]	0.28 ± 0.01	0.30 ± 0.03	0.34 ± 0.03	0.32 ± 0.01	0.289 ±
36	Luteolin[7]	0.0240 ± 0.0009	0.014 ± 0.001	0.00273 ± 0.00007	0.0042 ± 0.0003	0.0059 ±
	Total	26.5 ± 0.6	33.7 ± 0.8	27.5 ± 0.5	28.31 ± 0.06	31.20 ±

[1] mg/g hydroxytyrosol. [2] mg/g oleuropein. [3] mg/g tyrosol. [4] mg/g luteolin-7-glucoside. [5] mg/g rutin, [6]mg/g apige

Continued

Peak	Compound	SON-8	SON-9	SON-10	SON-11	SON-12	SO
1	Hydroxytyrosol-hexose isomer a[1]	0.0023 ± 0.0002	0.0028 ± 0.0002	0.00094 ± 0.00004	0.0022 ± 0.0004	0.0013 ± 0.0001	0. 0
2	Oleoside[2]	0.350 ± 0.005	0.33 ± 0.01	0.240 ± 0.009	0.351 ± 0.005	0.297 ± 0.002	0 0
3	Hydroxytyrosol-hexose isomer b[1]	0.664 ± 0.004	0.66 ± 0.03	0.58 ± 0.01	0.67 ± 0.05	0.64 ± 0.02	0.7
4	Hydroxytyroso[l]	0.079 ± 0.005	0.0756 ± 0.0004	0.0627 ± 0.0007	0.07632 ± 0.00009	0.076 ± 0.001	0 0
5	Secologanoside isomer a[2]	1.92 ± 0.01	2.02 ± 0.04	1.72180602	1.911 ± 0.002	1.81 ± 0.07	1.9
6	Tyrosol glucoside[3]	0.182 ± 0.001	0.1870 ± 0.0002	0.16 ± 0.01	0.1831 ± 0.0003	0.175 ± 0.006	0.1
7	Caffeoyl glucoside[2]	0.02844 ± 0.00004	0.0279 ± 0.0005	0.018 ± 0.001	0.0286 ± 0.0008	0.021 ± 0.002	0. 0
8	Tyrosol[3]	0.004 ± 0.001	0.004 ± 0.001	<LOQ	0.0023 ± 0.0004	0.0019 ± 0.0003	0. 0
9	Elenolic acid glucoside isomer a[2]	0.072 ± 0.009	0.070 ± 0.007	0.029 ± 0.004	0.076 ± 0.001	0.047 ± 0.002	0 0

10	Secologanoside isomer b[2]	1.13 ± 0.02	1.12 ± 0.05	0.60 ± 0.02	1.12 ± 0.01	0.75 ± 0.06	1.1
11	Elenolic acid glucoside isomer b[2]	0.65 ± 0.02	0.56 ± 0.03	0.34 ± 0.01	0.70 ± 0.01	0.41 ± 0.03	0
12	Oleuropein aglycon[2]	2.030 ± 0.001	1.98 ± 0.06	0.920 ± 0.004	1.983 ± 0.003	1.25 ± 0.02	1.9
13	Elenolic acid glucoside isomer c[2]	0.029 ± 0.001	0.029 ± 0.006	0.289 ± 0.009	0.34 ± 0.02	0.30 ± 0.02	0
14	Luteolin diglucoside[4]	0.168 ± 0.004	0.146 ± 0.001	0.013 ± 0.001	0.025 ± 0.002	0.0163 ± 0.0008	0.
15	Elenolic acid glucoside isomer d[2]	0.1073 ± 0.0006	0.108 ± 0.001	0.077 ± 0.005	0.108 ± 0.009	0.082 ± 0.003	0
16	Demethyloleuropein[2]	0.33 ± 0.01	0.31 ± 0.01	0.237 ± 0.008	0.303642 ± 0.000004	0.282 ± 0.002	0
17	Hydroxyoleuropein isomer a[2]	0.312 ± 0.002	0.267 ± 0.004	0.018 ± 0.006	0.493 ± 0.007	0.041 ± 0.003	0.
18	Rutin[5]	0.418 ± 0.007	0.395 ± 0.007	0.22 ± 0.01	0.42 ± 0.01	0.289 ± 0.005	0
19	Luteolin rutinoside[4]	0.059 ± 0.002	0.0534 ± 0.0008	0.033 ± 0.003	0.0596 ± 0.0001	0.0425 ± 0.0005	0
20	Luteolin glucoside isomer a[4]	1.10 ± 0.01	1.06 ± 0.02	0.78 ± 0.02	1.12 ± 0.01	0.93 ± 0.03	1.1

21	Verbascoside[1]	0.00639 ± 0.00009	0.0051 ± 0.0003	0.0040 ± 0.0001	0.00591 ± 0.00003	0.0052 ± 0.0001	0.0
22	Hydroxyoleuropein isomer b[2]	0.00027 ± 0.00002	<LOQ	0.006 ± 0.002	<LOQ	0.0123 ± 0.0001	<
23	Apigenin rutinoside[6]	0.0244 ± 0.0002	0.0222 ± 0.0003	0.01309 ± 0.00004	0.021 ± 0.001	0.0189 ± 0.0005	0.
24	Oleuropein diglucoside isomer a[2]	0.023 ± 0.001	0.022 ± 0.006	0.008 ± 0.001	0.021 ± 0.004	0.019 ± 0.001	0.
25	Apigenin-7-glucoside[6]	0.059 ± 0.002	0.0513 ± 0.0009	0.044263	0.057 ± 0.001	0.053 ± 0.003	0.
26	Oleuropein diglucoside isomer b[2]	0.047 ± 0.004	0.044 ± 0.006	0.0412731	0.046 ± 0.002	0.045 ± 0.003	0.
27	Luteolin glucoside isomer b[4]	0.65 ± 0.02	0.574 ± 0.009	0.382 ± 0.005	0.63 ± 0.01	0.54 ± 0.03	0.6
28	Oleuropein diglucoside isomer c[2]	0.105 ± 0.003	0.093 ± 0.007	0.0773 ± 0.0004	0.09 ± 0.01	0.09 ± 0.02	0.
29	Chrysoeriol-7-O-glucoside[4]	0.240 ± 0.002	0.21 ± 0.01	0.164 ± 0.007	0.231 ± 0.007	0.21 ± 0.01	0.
30	Luteolin glucoside isomer c[4]	0.137 ± 0.003	0.119 ± 0.005	0.084 ± 0.009	0.123 ± 0.004	0.107 ± 0.006	0.
31	Oleuropein isomer 1[2]	20.1 ± 0.2	16.6 ± 0.4	15.8 ± 0.3	18.8 ± 0.1	18.6 ± 0.4	21.0

32	Oleuropein isomer 2[2]	0.402 ± 0.009	0.343 ± 0.008	0.39 ± 0.02	0.386 ± 0.004	0.43 ± 0.01	0.4
33	Oleuropein/Oleuroside[2]	1.20 ± 0.03	1.14 ± 0.03	1.24 ± 0.07	1.14 ± 0.06	1.29 ± 0.04	1.2
34	Ligstroside aglycone[2]	0.009 ± 0.002	0.015 ± 0.001	<LOQ	0.0116 ± 0.0008	<LOQ	0
35	Ligstroside [2]	0.28 ± 0.02	0.259 ± 0.006	0.283 ± 0.004	0.267 ± 0.009	0.298 ± 0.009	0
36	Luteolin[7]	0.0050 ± 0.0003	0.0191 ± 0.0002	0.00170 ± 0.00005	0.0124 ± 0.0003	0.00373 ± 0.00007	0.
	Total	33.0 ± 0.3	28.9 ± 0.5	24.92 ± 0.06	31.82 ± 0.08	29.2 ± 0.5	34

[1] mg/g hydroxytyrosol. [2] mg/g oleuropein. [3] mg/g tyrosol. [4] mg/g luteolin-7-glucoside. [5] mg/g rutin, [6] m
luteolin

Regarding the results, the minimum content of total compounds was 24.92 ± 0.06 in SON 10 (100% EtOH, 60% of amplitude and 10 min), whereas the maximum total content was 33.0-34.15 in SON 2 (30% EtOH, 100% amplitude and 5.5 min), SON 7 (65% EtOH, 20% amplitude and 10 min), SON 8 (65% EtOH, 100% amplitude and 10 min) and in the central points in SON 13, 14 and 15 at 60% EtOH, 60% amplitude and 5.5 minutes. Therefore, it was obtained an increase of 37 % in the total phenolic content.

3.2. Fitting the model

The experimental design employed the data from **Table 1** to find the combined effect of ethanol/water ratio, ampplitude and extraction time on the response variable during the sonotrode UAE. The regression coefficients of the model and the results of the analysis of variance (ANOVA) shows in the **Table 2 and Table 3.**

The evaluation of the model was carried out according to the significance of the regression coefficients. According with other works, the level of significance was $\alpha<0.1$ in order to increase the number of significant variables. The significant variables on the response variable of sum of phenolic compounds were the intercept (X_0), the linear effect of amplitude (X_1), the linear effect of EtOH/H2O (X_2) and its quadratic effect (X_{22}), the linear effect of time (X_3) and its quadratic effect (X_{33}) and the cross effect between the amplitude and %EtOH/H2O (X_{12}).

An analysis of variance (ANOVA) with 95% confidence level was generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. ANOVA revealed that the models presented a high correlation between independent factors and the variable response with a coefficient of determination (r^2) of 0.92365. In addittion, the p-value of lack-of-fit was used to verify the adequacy of the model, which was non-significant ($p > 0.05$), so this means that the model fits well (**Table 3**).

Table 20. Regression coefficients of the model

Regression coefficients	**Coefficients**	**p value**
β_0	11.80061	0.012806
Linear		
β1	0.15374	0.020174
β_2	0.46774	0.003687
β_3	0.68413	0.062779
Cross product		
β_{12}	-0.00114	0.019798
β_{13}	-0.00190	0.272439
β_{23}	0.00225	0.258975
Quadratic		
β_{11}	-0.00034	0.149246
β_{22}	-0.00347	0.003091
β33	-0.04123	0.071892

Table 21. ANOVA test for the response variable of total compounds SS: Sum of squares, DF: Degree of freedom, MS: Mean square, r^2 : Quadratic correlation coefficient.

	SS	**DF**	**MS**	**F value**	**p value**
Lack of fit	11.2031	6	1.86718	9.0156	0.103196
Pure error	0.4142	2	0.20711		
Total	125.2392	14			

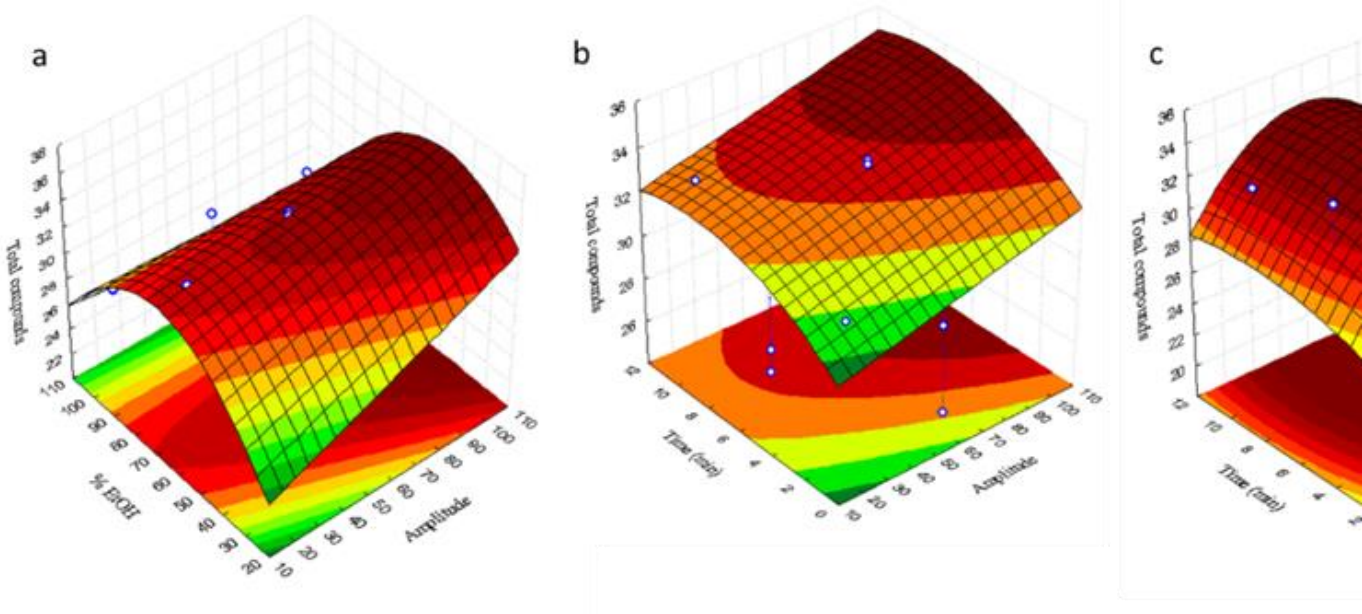


Figure 1. Response surface plots showing combined effects of process variables for total compounds amplitude–time (min) (b) and % EtOH–time (min) (c).

3.3. Analysis of response surfaces

In order to determine the optimal levels of independent variables for the extraction of the total content of phenolic compounds from olive leaves, responses surfaces were plotted. Each pair of variables was depicted in three-dimensional surface plots, while the other one variable was kept constant at central level. Figures 1 (a, b and c) are the three-dimensional plots showing the effects of amplitude (X_1) with % EtOH (X_2) (a) , amplitude (X_1) with time (X_2) (b) and %EtOH (X_2) with time (X_3) (c) on the total content of compounds.

In the **Figure 1** (**a**) can be observed that at maximum value of ampltide of 100%, an on the content of total compounds with the increasing of % etahnol is obtained to arrive at its maximum value of 40-60 % of ethanol, from which the response starts to decrease. In the **Figure 1** (**b**) the maximum concentration of total compounds shows at 8-10 min and 100 % of amplitude. In the **Figure 1** (**c**) can be observed one maximum value on the response at 50-70% EtOH and 4-10 min.

3.3.1. Optimization of sonotrode UAE parameters

Determination the optimal conditions through the 3-D plots, the final step of the RSM after selecting the optimal conditions was to predict the accuracy of the mathematical model. Results of the optimal conditions to obtain the highest content of total compounds from olive leaves show in the Table 5. The highest phenolic content was obtained at optimum conditions: 100% Amplitude, 55% EtOH and water to obtain a predictable value of total compounds of 35.54 mg g-1 d.w.

Table 22. Optimal conditions for UAE sonotrode. CV: coefficient of variation

Optimal conditions	
Amplitude (%) (Power)	100 (151W)
EtOH/ water (% (v/v))	55
Time (min)	8
Predicted (total compounds (mg g^{-1} d.w.))	36 ± 2
Obtained ((total compounds (mg g^{-1} d.w.))	40.9 ± 0.2
CV(%)	9.05

To verify the suitability of the model for total compounds, the predictable value of total compounds was compared with experimental values obtained at optimal conditions. Analysis of the results revealed an acceptable variance (CV = 9.05%) between the theoretical and experimental data, therefore, the model was considered suitable (**Table 4**). The total phenolic content by UAE sonotrode and with bath was in the same order of magnitude than the obtained in a previous study by UAE bath in the same cultivar 'Koroneiki´ (39.55 mg/g d.w.) collected in december[2]. Another study reported a quantity of oleuropein of 13.386 mg/g d.w. obtained by optimum conditions of UAE: 10 cycles, ratio of 15 mL/g and sonication time of 4 min, which value was 27.6-36.3% lower than the obtained in the present study[18].

Optimum conditions for UAE sonotrode were compared with those obtained in a previous study in olive mill leaves, which reported the highest phenolic content of 42 mg gallic acid eq/ g olive tree pruning biomass at 51.9%EtOH, 70% Amplitude and 15 min[17]. Therefore, in the present study a shorter extraction time was obtained in comparison with this previous study[17]. In addition, **Table 5** shows a similar total phenolic content obtained by UAE sonotrode and UAE bath. Therefore, the probe system is more powerful due to an ultrasonic intensity delivered through a smaller

surface (only the tip of the probe), when comparing to the ultrasonic bath providing a high phenolic recovery in shorter extraction times[16].

Table 23. Comparison of phenolic content (mg/g d.w.) with UAE sonotrode obtained at optimum conditions and with bath. LOQ= Limit of quantification.

Phenolic compounds	**UAE sontrode**	**UAE bath**
Hydroxytyrosol-hexose isomer a	0.0048 ± 0.0005	<LOQ
Oleoside	0.48 ± 0.01	0.53 ± 0.04
Hydroxytyrosol-hexose isomer b	0.90 ± 0.02	0.83 ± 0.04
Hydroxytyrosol	0.1031 ± 0.0004	0.131 ± 0.001
Secologanoside isomer a	7.3 ± 0.1	3.8 ± 0.12
Tyrosol glucoside	0.196 ± 0.007	0.29 ± 0.02
Caffeoyl glucoside	0.039 ± 0.007	0.030 ± 0.001
Tyrosol	0.007 ± 0.001	<LOQ
Elenolic acid glucoside isomer a	0.21 ± 0.01	0.13 ± 0.01
Secologanoside isomer b	2.149 ± 0.004	1.73 ± 0.12
Elenolic acid glucoside isomer b	0.94 ± 0.06	0.87 ± 0.08
Oleuropein aglycon	2.7147 ± 0.0001	2.63 ± 0.02
Elenolic acid glucoside isomer c	0.53 ± 0.02	0.04 ± 0.01
Luteolin diglucoside	0.013 ± 0.002	0.281 ± 0.008
Elenolic acid glucoside isomer d	0.202 ± 0.008	0.143 ± 0.005
Demethyloleuropein	0.37 ± 0.01	0.47 ± 0.01
Hydroxyoleuropein isomer a	0.40 ± 0.02	0.17 ± 0.03
Rutin	0.46 ± 0.05	0.61 ± 0.04
Luteolin rutinoside	0.038 ± 0.001	0.09 ± 0.01
Luteolin glucoside isomer a	1.74 ± 0.03	1.89 ± 0.07

Verbascoside	0.0054 ± 0.0004	0.0092 ± 0.0006
Hydroxyoleuropein isomer b	0.0166 ± 0.0009	<LOQ
Apigenin rutinoside	0.0157 ± 0.0004	0.038 ± 0.001
Oleuropein diglucoside isomer a	0.0135 ± 0.0004	0.055 ± 0.001
Apigenin-7-glucoside	0.064 ± 0.002	0.107 ± 0.003
Oleuropein diglucoside isomer b	0.021 ± 0.002	0.09 ± 0.01
Luteolin glucoside isomer b	0.68 ± 0.01	0.84 ± 0.06
Oleuropein diglucoside isomer c	0.0391 ± 0.0003	0.147 ± 0.002
Chrysoeriol-7-O-glucoside	0.029 ± 0.002	0.35 ± 0.03
Luteolin glucoside isomer c	0.19 ± 0.01	0.232 ± 0.002
Oleuropein isomer 1	18.5 ± 0.4	20.7 ± 0.3
Oleuropein isomer 2	0.56 ± 0.1	0.63958158
Oleuropein/Oleuroside	1.5 ± 0.1	2.2 ± 0.2
Ligstroside aglycone	0.074 ± 0.004	<LOQ
Ligstroside	0.35 ± 0.02	0.49 ± 0.02
Luteolin	0.0061 ± 0.003	0.0053 ± 0.0007
Total compounds	40.9 ± 0.2	41 ± 1

4. Conclusions

A Box-Behnken experimental design was used in order to optimize the sonotrode ultrasound-assisted extraction parameters to obtain the maximum phenolic content from olive leaves. The highest value of total phenolic content was obtained at 55% ethanol/water (v/v), 100% amplitude and 8 minutes. Finally, it has been proved that sonotrode ultrasonic extraction is a more effective technique than conventional extraction method with bath, providing a similar recovery of phenolic compounds from olives leaves in a shorter time.

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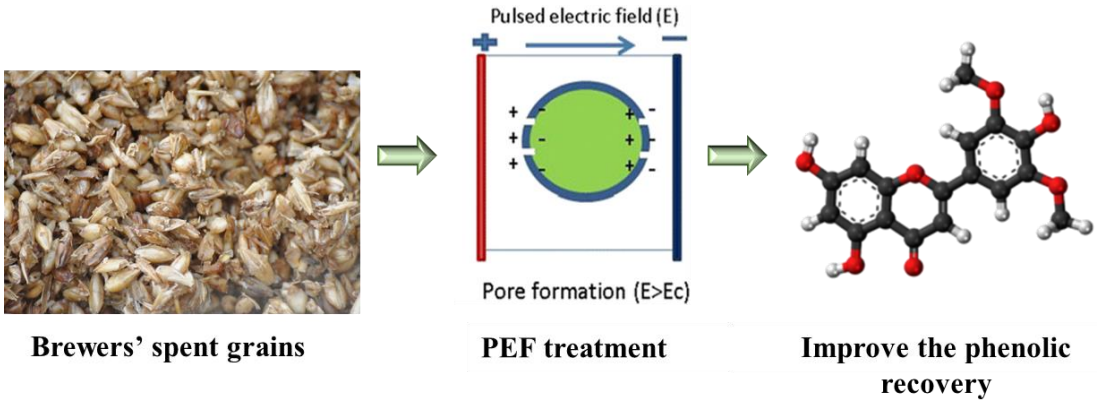
SECTION II

Use of fractionation technologies applied to obtain bioactive enriched cereal and pseudocereal flours fractions and the optimization of different treatments and extraction techniques in cereal by-products.

Chapter 4

Pulsed electric field (PEF) as pre-treatment to improve the phenolic compounds recovery from brewers' spent grains



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Pulsed electric field (PEF) as pre-treatment to improve the phenolic compounds recovery from brewers' spent grains

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Abstract

Brewers' spent grain (BSG) is the most abundant by-product obtained from beer production. However, it contains some bioactive compounds such as phenolic compounds, therefore, the valorization of BSG is important to recovery these compounds and reused them as functional ingredients in food industry. Therefore, in this work, pulsed electric field (PEF) has been used as extraction pre-treatment. PEF parameters such as electric field strength E (0.5, 1.5, 2.5 kV/cm), frequency (50, 100, 150 Hz) and total time of treatment (5, 10, 15 s) were optimized in order to maximize the content of flavan-3-ols, flavonoids, phenolic acid derivates and total free phenolic compounds. Optimal conditions to the maximum value of total free phenolic compounds were the following: 2.5 kV/cm, 50 Hz and 14.5 s. Concentrations of total free and bound phenolic compounds from BSG under these PEF optimum conditions were 2.7 and 1.7 times, respectively, higher than in case of the extraction without PEF pre-treatment, indicating an improvement in the phenolic recovery with the use of PEF as a pre-treatment in brewers spent grain samples.

Keywords: Pulsed Electric Field, free and bound phenolic compounds, brewers' spent grain, Box-Behnken design

1. Introduction

Brewers' spent grain (BSG) is the most abundant brewing by-product, corresponding to approximately 85% of total by-products generated. BSG may consist of the residues from malted barley, or those from malted barley and adjuncts (non-malt sources of fermentable sugars), such as wheat, rice, or maize added during mashing[1].

Chemical composition of BSG varies according to barley variety, harvest time, malting and mashing conditions, and the quality and type of adjuncts added in the brewing process[1]; but in general, BSG is considered as a lignocellulosic rich in fiber and proteins and also contains appreciable amounts of lipids, carbohydrates, polyphenols and minerals[2-4]. These compounds, when incorporated into human diets, may provide a number of benefits by lowering the risk of certain diseases including cancer, gastrointestinal disorders, diabetes, obesity and coronary heart disease[2]. Therefore, the valorization of BSG is important in order to recovery these high-value compounds that can be extracted, purified and reused as functional ingredients in food industry and in others industries[5].

BSG consists predominantly of the husk, pericarp, and seed coat and is largely made up of cell walls[3]. Barley provides a broad range of phenolic compounds that includes derivatives of benzoic and cinnamic acids, flavonoids, proanthocyanidins, tannins, and amino phenolic compounds, which are located mainly in the husk and hydroxycinnamic acids accumulate in the cell wall. Therefore, BSG is a potentially valuable source of these compounds[6], which are an important source of antioxidants in cereals, and they are found in free and in the bound form. The majority of free phenolics in barley are flavanols, whereas the bound phenolics are mainly phenolic acids, which are ester-linked to cell wall polysaccharides[6,7].

The re-emergence of nutraceuticals from agricultural by-products is achieved due to the existence of some conventional and emerging technologies, which allow both their recovery and also their reutilization inside foods[8]. Five distinct recovery stages of high-added value components from food waste are usually applied: macroscopic pre-treatment, macro- and micro-molecules separation, extraction,

purification and nutraceuticals formation. Although, some steps are sometimes deleted or over-subscribe each other. Processing often advances from the macroscopic pre-treatment to the macro and micro molecular separation, after that, to the extraction of specific micro-molecules before the purification and finally to the encapsulation of the target ones. The objective of the macroscopic pre-treatment is the setting of the food waste matrix according to the water content, enzymatic activity and permeability of the bioresource tissues[9]. Extraction technique represents the most important step in the recovery and isolation of phenolic compounds from brewers' spent grain. Many factors such as solvent composition, extraction temperature and solvent-to-solid ratio, may significantly influence the extraction efficiency, antioxidant activity and phenolic content. Therefore, it is necessary to optimize the extraction conditions to improve phenolic recovery[6,10]. Solid–liquid extractions (SLE) are the most commonly used procedures prior to analysis of phenolics in BSG samples, due to their ease of use, efficiency, and wide applicability[11,12]. Some studies have reported different extractions techniques for the recovery of phenolic compounds from brewers' spent grain such as ultrasound-assisted extraction (UAE)[10] and microwave assisted extraction (MAE)[12,14]. UAE and MAE have been considered as an alternative to SLE for the extraction of phenolic compounds from plants for various reasons: reduced extraction time, reduced solvent usage, and improved extraction yield[6]. Recently, pulsed electric field (PEF) has been used for the extraction in plants. The principle of PEF is to disintegrate the cell membrane structure for increasing extraction. When an electric field is applied to a living cell, an electric potential pass through the membrane of that cell. Based on the dipole nature of membrane molecules, electric potential separates molecules according to their charge in the cell membrane. After exceeding a critical value of approximately 1 V of transmembrane potential, repulsion occurs between the charge carrying molecules that form pores in weak areas of the membrane and causes a drastic increase of permeability. The effectiveness of PEF treatment strictly depends on the process parameters including electric field strength, pulse shape, pulse width, number of pulses, pulse specific energy, and frequency[15,16]. PEF can increase mass transfer

during extraction by drilling of the membrane structure of plant materials for enhancing extraction and decreasing extraction time. PEF has been applied to improve the release of intracellular compounds from plant tissue with the help of increasing cell membrane permeability[17]. PEF could be also applicable on plant materials as a pre-treatment process prior to conventional extraction to lower extraction time[18,19]. Moreover, previous studies reported an increase in the phenolic content when PEF treatment was applied as the pre-treatment step in food samples such as grapes or grape pomace, onion, orange peel, sorghum flour and apple pomace[20-23]. In general PEF intensities ranging from 0.5 to 2 kV/cm are used for fresh materials whereas high dry matter containing materials require higher intensity e.g. 20 kV/cm[20,24]). A recent study, which applied PEF in Panax ginseng at electrical field strengths varying from 0.5 to 2.5 kV/cm, the pulse number of 500, the pulse frequency of 50 Hz, and the pulse width of 25 μs, showed a higher phenolic content in samples treated at 1.5 to 2.5 kV/cm than in the control one or the one treated at 0.5 kV/cm[24]. For that, PEF could be applied to BSG samples as a pre-treatment process to conventional extraction to lower extraction effort.

One study has reported the effect of PEF treatment with an electric field strength of 2.8 kV/cm, frequency of 10 Hz and a total of 3000 pulses with a pulse width of 20 μs on the contents of bioactive constituents in dark and light BSG extracts as well as on their antioxidant, antimicrobial and immunomodulatory properties. Light BSG extracts pre-treated with PEF showed higher antimicrobial activity compared to the untreated extracts. Nevertheless, this study did not show significant differences on the total phenolic content, antioxidant activity and on the immunomodulatory activity in PEF treated extracts compared to untreated extracts for both the BSG samples[25].

Therefore, this work was focused on the extraction by PEF treatment and identification and quantification, by HPLC-MS, of phenolic compounds from brewers spent grain. An experimental design response-surface Box-Behnken has been performed to optimize the extraction parameters of PEF: electric field strength, frequency and total time. In addition, in order to show the improvement on the efficiency of extraction by PEF as pre-treatment, a comparison on the

content of free and bound phenolic compounds in PEF brewers spent grain extracts with those obtained without PEF treatment was carried out.

2. Material and methods

2.1. Chemicals

HPLC-grade acetonitrile, water, methanol, acetone, acetic acid, ethanol were purchased from Merck KGaA (Darmstadt, Germany). Ferulic acid, catechin and quercetin were from Sigma-Aldrich (St. Louis, MO).

2.2. Samples

Brewers' spent grain samples were obtained in a microbrewing plant after special beer production (Mastrobirraio, Cesena, Italy, 44°08′00″N 12°14′00″E).

2.3. Experimental design for Pulsed electric field extraction (PEF) in brewers' spent grain

The protocol of PEF pre-treatment was the following: 60 g of brewers spent grain with water ratio 1:1, was placed into a rectangular treatment chamber (5 x 5 x 5 cm) equipped with two stainless steel electrodes (5 x 5 cm) with a gap between them of 22 mm. The conductivity of the mix was of 463 mS/cm (measured by EC-Meter basic 30+, Crison). PEF treatments were applied by using pulse generator S-P7500 60A 8kV (Alintel srl., Bologna). The pulse width was fixed to 10 μs.
Box-Behnken design (BBD) was chosen for the optimization of PEF parameters since it is simpler and more efficient than other three-level factorial designs[26]. The experimental design consisted of 15 experimental runs, with three levels (− 1, 0, 1) for each factor, and three center points. PEF parameters and values of the response variables in each experiment appear in **Table 1**. Independent variables of PEF were the electric field strength - E (0.5, 1.5, 2.5 kV/cm), frequency (50,100,

150 Hz) and total time of treatment (5,10,15 s). Total time refers to the treatment time that is the number of pulses applied multiplied by the pulse width (or pulse duration)[27]. Also, the total energy input of each experiment was calculated according to Raso et al. (2016)[27] and it is reported in **Table 1**.

The response variables were fitted to a second-order polynomial model equation obtained by the response surface methodology (RSM) (**Eq.1**).

Where Y correspond with the response variables, which were the concentration of free phenolic compounds (Y_1), flavan-3-ols (Y_2), flavonoids (Y_3), phenolic acids derivates (Y_4) obtained from brewers' spent grain extracts by HPLC-MS, X_i and X_j are the independent factors affecting the response, and β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients of the model (intercept, linear, quadratic and interaction term).

Table 1. Box-Behnken design with PEF parameters, values of total energy input in each experiment and dependent variables obtained (free phenolic compounds, flavan-3-ols, flavonoids and phenolic acid derivates) quantified by HPLC-MS in brewers' spent grain expressed by μg g^{-1} d.w.

	Independent parameters				**Dependent parameters**			
Exp	X_1	X_2	X_3	Total energy input (kJ/kg)	Total free phenolic compounds	Flavan-3-ols	Flavonoids	Phenolic acid derivatives
1	0.5	50	10 (500)	0.25	68.664	6.432	34.368	34.296
2	2.5	50	10 (500)	6.25	95.187	8.809	54.933	40.254
3	0.5	150	10 (1500)	0.75	96.842	6.020	39.925	56.916
4	2.5	150	10 (1500)	18.75	82.510	7.824	47.411	35.099
5	0.5	100	5 (500)	0.25	86.541	7.729	45.112	41.429
6	2.5	100	5 (500)	6.25	73.503	5.106	33.853	39.650
7	0.5	100	15 (1500)	0.75	82.891	7.181	41.860	41.031
8	2.5	100	15 (1500)	18.75	89.971	8.406	45.073	44.897
9	1.5	50	5 (250)	1.13	89.747	7.859	46.883	42.864
10	1.5	150	5 (750)	3.38	73.940	5.229	34.915	39.025
11	1.5	50	15 (750)	3.38	87.609	7.434	46.546	41.063
12	1.5	150	15 (2250)	10.13	83.371	6.068	43.863	39.508
13	1.5	100	10 (1000)	4.50	84.593	7.041	41.942	42.651
14	1.5	100	10 (1000)	4.50	84.906	6.583	42.289	42.617
15	1.5	100	10 (1000)	4.50	84.253	6.663	41.717	42.537

X_1: E (kV/cm), X_2: Frequency (Hz), X_3: Total time (s) (pulses per second)

$$Y = \beta_{0+} \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} {X_{ii}}^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ii} X_i X_j \quad (\mathbf{1})$$

The range of electric field strength was the same that the established by Liu, Zeng, and Ngadi (2018)[20] for the extraction of phenolic compounds from onion. Moreover, the range of electric field strength and the minimum value of frequency chosen in this work was the same reported by Kim et al (2019)[24] on raw gingesng samples and the pulse width was the same that the used in orange peel[21].

The model building, experimental results, and designs were carried out using STATISTICA 7.0 (2002, StatSoft, Tulsa, OK). The results of quantification reported in this work are the averages of three repetitions (n=3).

2.4. Extraction of phenolic fractions of brewers' spent grain

After PEF treatment, extraction of free fraction was carried out according the protocol established by Hung and Morita (2008)[28], with certain differences: 15 g from brewers' spent grain (2 g of dry matter), previously submitted to PEF treatment was extracted by shaking twice with 30 mL of ethanol/water (4:1 v/v). The supernatants were collected and evaporated at 35 °C in a rotary evaporator, and finally the dried extract was reconstituted with 2 mL of methanol/ water (1:1 v/v). The extracts were stored at -18 ºC before the analysis.

In order to compare the effect of PEF treatment on the extraction of phenolic compounds, extraction of brewers spent grain without PEF treatment (Control) was carried out.

After establishing the PEF conditions the samples were extracted according to the previous methodology and the residue after phenolic extraction was submitted to alkaline hydrolysis as reported by Verardo et al. (2011)[29] in order to recover the bound phenolic compounds.

2.5. Determination of phenolic compounds by HPLC- MS

Determination of free and bound phenolic compounds was carried out by using a liquid chromatography apparatus HP 1100 Series (Agilent Technologies, Palo Alto, CA, USA) equipped with a degasser, a binary pump delivery system and an automatic liquid sampler and coupled to single quadrupole mass spectrometer detector was used. Separation these phenolic compounds from brewers spent grains was carried out by using a C-18 column (Poroshell 120, SB-C18, 3.0×100 mm, 2.7 μm from Agilent Technologies, Palo Alto, CA, USA). The gradient elution was the same that the previously established by Gómez-Caravaca, Verardo, Berardinelli, Marconi, and Caboni 2014[7] using as a mobile phase A acidified water (1% acetic acid) and as mobile phase B acetonitrile. MS analysis was carried out using an electrospray ionization (ESI) interface in negative ionization mode at the following conditions: drying gas flow (N2), 9.0 L/min; nebulizer pressure, 50 psi; gas drying temperature, 350°C; capillary voltage, 4000 V. The fragmentor and m/z range used for HPLC-ESI/MS analyses were 80 V and m/z 50-1000, respectively. Data were processed by the software MassHunter Workstation Qualitative Analysis Version B.07.00 (Agilent Technologies, Santa Clara, CA, USA).

3. Results and discussion

3.1. Characterization of phenolic compounds from brewers spent grain extracts by HPLC-MS

3.1.1. Analytical parameters of the method

Analytical validation of the method was performed considering linearity and sensitivity. In order to quantify phenolic compounds, four calibration curves were elaborated with the standards ferulic acid, catechin, quercetin and gallic acid. **Table S-1** lists the analytical parameters of the standards used containing linear range, calibration curve, determination coefficients, limit of determination (LOD) and limit of quantification (LOQ).

Table S-1. Analytical parameters of the method proposed, LOD: Limit of detection, LOQ: Limit of quantification.

Analytes	calibration ranges (mg/L)	calibration curves (mg/L)	R^2	LOD (mg/L)	LOQ (mg/L)
Ferulic acid	LOD-100	y=119572x+16157	0.9985	0.0136	0.0452
Catechin	LOD-100	y=170925x+8609.5	0.9994	0.0095	0.0316
Quercetin	LOD-100	y=402162x + 44862	0.9996	0.0040	0.0134
Gallic acid	LOD-100	y=123892x-4971.6	0.9984	0.0131	0.0437

Calibration curves were carried out by using the peak areas analyte standard against the concentration of the analyte for the analysis by HPLC. The external calibration of the standards was elaborated at different concentration levels from LOQ to 100 mg L^{-1}. All calibration curves revealed good linearity among different concentrations, and the determination coefficients were higher than 0.9984 in all

cases. The method used for analysis showed LOD within the range 0.0040-0.0136 mg L^{-1} and the LOQ within 0.0134-0.0452 mg L^{-1}.

3.1.2. Identification of phenolic compounds

Free phenolic compounds in BSG extracts were analyzed by HPLC with MS detection. Free phenolic compounds were identified by rendering their mass spectra using the data reported in the literature and, when available, by co-elution with commercial standards.

Table 2. Table of identification of free phenolic compounds from brewers' spent grain extracts by HPLC-MS

Peak	RT (min)	m/z experimental	Free phenolic compound
1	2.039	451.1	Catechin-3-glucose
2	3.665	577	Procyanidin B3
3	4.149	289	Catechin
4	4.737	167	Vanilllic acid
5	4.483	771	Quercetin-3-hexosylrutinoside
6	5.221	121	p-Hydroxybenzaldehyde
7	6.267	151	Vannilin
8	6.416	593	Prodelphinidin B3
9	6.838	163	p-coumaric acid
10	7.506	371	Hydroferuloyl glucose
11	7.638	193	Ferulic acid
12	9.792	385	Sinapoyl hexose
13	17	329	Tricin

A total of 13 free phenolic compounds were identified in beer by-products and they were previously identified in barley, millet, hop and brewers' spent grain extracts[7,30,31] (**Table 2**). The peak 1 at 2.0 min with a m/z 451 corresponded with catechin-3-glucose and the peak 2 at 3.7 min presented the molecular ion at m/z 577 was identified as procyanidin B3, which was present in barley extracts[7]. The peak 3 at 4.1 min with a molecular ion at m/z 289 was identified as catechin and

was present in barley extracts and brewers' spent grain[7,32]. The peak 4 at 4.7 min at m/z 167 correspond with vanillic acid, which was identified in millet and brewers' spent grain extracts[30,32]. The peak 5 at 4.5 min with a molecular ion at m/z 771 correspond with quercetin-3-hexosylrutinoside and was identified previously in hop extracts[31]. The peak 6 at 5.2 min at m/z 121 was identified as p-hydroxybenzaldehyde and the peak 7 at 6.3 min at m/z 151 was identified as vanillin, both peaks were detected previously in millet extracts[30]. The peak 8 at 6.4 min at m/z 593 was identified as prodelphinidin B3, which was detected in barley extracts[7]. The peak 9 at 6.8 min with a molecular ion at m/z 163 corresponded with p-coumaric acid, which was identified in brewers' spent grain and in barley [14,32,33]. The peak 10 at 7.5 min at m/z 371 was identified as hydroferuloyl-glucose according to the identification of this compound in barley samples[7], the peak 11 at 7.6 min with a molecular ion at m/z 193 was identified as ferulic acid and it was identified previously in brewers' spent grain[14,32,33]. The peak 12 at 10.0 min with a molecular ion at m/z 385 was identified as sinapoyl hexose and was detected in barley samples[7]. The peak 13 at 15.0 min with a molecular ion at m/z 329 was identified as tricin, which was previously identified in millet and rice extracts[30].

3.1.3. Quantification of phenolic compounds

Free phenolic compounds were quantified through calibration curves of standards. Therefore, the calibration curve of ferulic acid was used to quantify vanillic acid, vanillin, p-coumaric acid, hydroferuloyl glucose and ferulic acid, the calibration curve of catechin was used to quantify catechin 3-glucose, procyanidin B3, catechin, prodelphinidin B3 and sinapoyl hexose, the calibration curve of gallic acid was used to quantify p-hydroxybenzaldehyde and the calibration curve of quercetin was used to quantify tricin. A total of 12 free phenolic compounds were quantified in brewers spent grain (**Table 3**). Quercetin-3-hexosylrutinoside was not quantified due to its value of concentration was less than the limit of quantification.

Tricin was the most concentrated flavonoid in brewers spent grain, which value

varied from 27.936 µg g^{-1} d.w. in PEF-1 to 46.125 µg g^{-1} in PEF 2, whereas the most concentrated phenolic acid derivates was sinapoyl hexose, which ranged from 21.080 µg g^{-1} d.w. in PEF-1 to 36.108 µg g^{-1} d.w. in PEF-3.

Stefanello et al. (2018)[32] quantified some phenolic compounds in brewers spent grain, in their study concentration of catechin was 68.4 µg g^{-1} d.w., which was higher than the obtained in this work, whereas concentration of p-coumaric acid (8.4 µg g^{-1} d.w.) and ferulic acid (5.6 µg g^{-1} d.w.) were in the same order of magnitude than the obtained in this work. These differences in the concentration of phenolic compounds in brewers spent grain could be mainly due to barley variety, harvest time, malting and mashing conditions, and the quality and type of adjuncts added in the brewing process[1]. With respect to the study performed by Gómez-Caravaca, Verardo, Berardinelli, Marconi, and Caboni (2014)[7] about the content of free phenolic compounds in barley samples, which is the main component in brewing by-products, the highest content of sinapoyl hexose obtained in the present work was 88-51-99.35 %, which was higher than the obtained in barley extracts (0.3-5.3 µg g^{-1} d.w.) and ferulic acid was in the same order than in the obtained barley extracts (1.4-7.3 µg g-1 d.w.), whereas the concentration of catechin-3-glucose (9.2-45 µg g^{-1} d.w.), procyanidin B3 (276.2-514.8 µg g^{-1}), catechin (68.8-350.6 µg g^{-1} d.w.) and prodelphinidin B3 (232-482.7 µg g^{-1} d.w.) in barley samples were higher than the obtained in the present work.

Table 3. Free phenolic compounds quantified in Brewers' spent grain (µg g-1 d.w.) in each PE

LOQ: Limit of quantification

	PEF-1	**PEF-2**	**PEF-3**	**PEF-4**	**PEF-5**	**PEF-6**	**PEF-7**	**PEF-8**	**PEF-9**	**PEF-10**	**PE 11**
Catechin-3-glucose	0.389	0.574	0.198	0.440	0.453	0.234	0.250	0.386	0.416	0.098	0.4
Procyanidin B3	1.183	2.786	1.302	2.393	1.763	1.282	1.593	1.893	1.776	1.346	1.3
Catechin	2.739	3.369	2.598	3.183	3.081	2.236	2.996	3.612	3.056	2.209	3.2
Quercetin-3-hexosylrutinoside	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<L
Vanillic acid	<LOQ	0.244	0.021	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<L
p-Hydroxybenzaldehyde	3.058	3.747	3.221	2.921	3.375	2.534	3.248	3.452	3.548	2.886	3.8
Vanillin	0.725	1.483	0.704	0.759	1.213	0.590	1.282	1.326	1.162	0.726	1.2
Prodelphinidin B3	2.120	2.080	1.922	1.808	2.432	1.354	2.343	2.515	2.611	1.576	2.3
p-coumaric acid	0.000	3.035	3.093	2.570	3.937	1.936	3.506	3.643	3.097	2.100	3.2
Hydroferuloyl glucose	8.656	8.453	13.200	6.266	7.362	7.978	8.370	7.981	10.139	8.766	8.9
Ferulic acid	0.777	0.592	0.570	0.629	0.921	0.655	0.891	1.074	1.087	0.432	1.2
Sinapoyl hexose	21.080	22.700	36.108	21.955	24.623	25.957	23.734	27.423	23.774	24.115	22. 9
Tricin	27.936	46.125	33.906	39.587	37.383	28.747	34.679	36.667	39.024	29.685	39. 2
Total	68.664	95.187	96.842	82.510	86.541	73.503	82.891	89.971	89.747	73.940	87. 9
Flavan-3-ols	6.432	8.809	6.020	7.824	7.729	5.106	7.181	8.406	7.859	5.229	7.4
Flavonoids	34.368	54.933	39.925	47.411	45.112	33.853	41.860	45.073	46.883	34.915	46. 6
Phenolic acid derivatives	34.296	40.254	56.916	35.099	41.429	39.650	41.031	44.897	42.864	39.025	41. 3

Content of total free phenolic compounds ranged from 68.664 µg g-1 d.w. in PEF-1 (0.5 kV/cm, 50 Hz and 10 seconds) to 96.842 µg g-1 d.w. in PEF-3 (0.5 kV/cm, 150 Hz and 10 seconds), content of flavan-3-ols ranged from 5.106 µg g-1 d.w. in PEF-6 (2.5 kV/cm, 100 Hz and 5 seconds) to 8.809 µg g-1 d.w. in PEF-2 (2.5 kV/cm, 50 Hz and 10 seconds). Concentration of flavonoids ranged from 33.853 µg g-1 d.w. in PEF-6 (2.5 kV/cm, 100 Hz and 5 seconds) to 54.933 µg g-1 d.w. in PEF-2 (2.5 kV/cm, 50 Hz and 10 seconds) and the content of phenolic acid derivates ranged from 34.296 µg g-1 d.w. in PEF-1 (0.5 kV/cm, 50 Hz and 10 seconds) to 56.916 µg g-1 d.w. in PEF-3 (0.5 kV/cm, 150 Hz and 10 seconds) (**Table 1**).

Comparing the content of free phenolic compounds obtained in PEF extracts with the obtained in control samples it was possible to observe that concentration of flavan-3-ols was 55.8 % higher, whereas, flavonoids content was 64.34 % higher than the obtained in control samples, content of phenolic acid derivates was 68.39 % higher in PEF treated sample and, finally, the total free phenolic content in PEF extract was 61.20 % higher than the obtained in control one. Therefore, these results have shown that the application of PEF treatment improves the phenolic extraction efficiency in brewers spent grains.

3.2. Fitting the model

The response surface methodology (RSM) was applied for the optimization of three PEF parameters to obtain the highest content of free phenolic compounds in brewers spent extracts. For that purpose, an experimental Box-Behnken design (BBD) was applied to evaluate the effects of electric field strength (0.5, 1.5 and 2.5 kV/cm) (X_1), frequency (50, 100 and 150 Hz) (X_2), and total time (5, 10 and 15 s) (X_3) on the response variable of free phenolic compounds, flavan-3-ols, flavonoids and phenolic acid derivates via HPLC- MS from brewers spent grain.

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Table 4. Regression coefficients of the model

Regresion coefficients	**Free phenolic compounds Coefficients**		**Flavan-3-ols**		**Flavonoids**	
	Coefficients	p value	Coefficients	p value	Coefficients	p value
β_0	12.9834*	0.000001	-0.49365*	0.000102	-4.39453*	0.000004
Linear						
β_1	87.472**	0.006226	9.54454*	0.023825	50.55570*	0.000720
β_2	0.9686*	0.017308	0.16624*	0.025020	1.00745*	0.004786
β_3	-0.2828*	0.001961	-0.01935*	0.032560	-1.00923*	0.002751
Cross product						
β_{12}	-1.1005*	0.000256	-0.15341	0.362347	-0.97762*	0.001940
β_{13}	0.1770*	0.001052	-0.15821*	0.015798	0.81990*	0.001586
β_{23}	0.0116*	0.003173	0.00126	0.122936	0.00928*	0.003839
Quadratic						
β_{11}	-20.1473**	0.062937	-1.98423**	0.062937	-5.72219	0.183873
β_{22}	-0.0024*	0.039608	-0.00083	0.859846	-0.00466*	0.006337
β_{33}	-0.0698*	0.009345	-0.00560	0.386344	-0.03232*	0.032837

*Significant at $\alpha \leq 0.05$,**Significant at $\alpha \leq 0.1$

The data of the response variable were used to fit the model to a second order-polynomial equation by means of least squares method (LSM). Relied on Fisher test, the evaluation of the model was carried out according to the significance used in other works ($\alpha = 0.1$)[34,35]. Regression coefficients that describe free phenolic compounds, flavan-3-ols, flavonoids and phenolic acid derivates responses appear in the **Table 4**. Most of the single factors, interactions between them and their cross-products reported a significant effect ($p < 0.1$) on the response variables, being the linear effect of electric field strength (X_1) the most influent, followed by the quadratic effect of electric field strenght (X_{11}).

Table 5. Analysis of variance (ANOVA) of the model

	Free phenolic compounds	**Flavan-3-ols**	**Flavonoids**	**Phenolic acid derivates**
R^2	0.9991	0.9590	0.9987	0.9999
p (Lack of fit)	0.1497	0.3220	0.3026	0.1010
Pure error	0.1067	0.0599	0.0832	0.0034

The model was recalculated only with significant effects and the results of the analysis of variance (ANOVA) appears in the **Table 5**. Models presented a strong correlation between independent variables and response variables with coefficients of determination (R^2) between 0.9590 to 0.9999. In addition, the validity of the model was also verified by the p-value of the lack of fit as non-significant in all models ($p > 0.05$) and pure errors were also low. Therefore, models were accepted.

Figure 1. Response surface plots showing combined effects of process variables for free phenolic
flavan-3-ols (d, e and f). a and d: Frequency with electric field strength, b and e: total time with el
f: total time with frequency

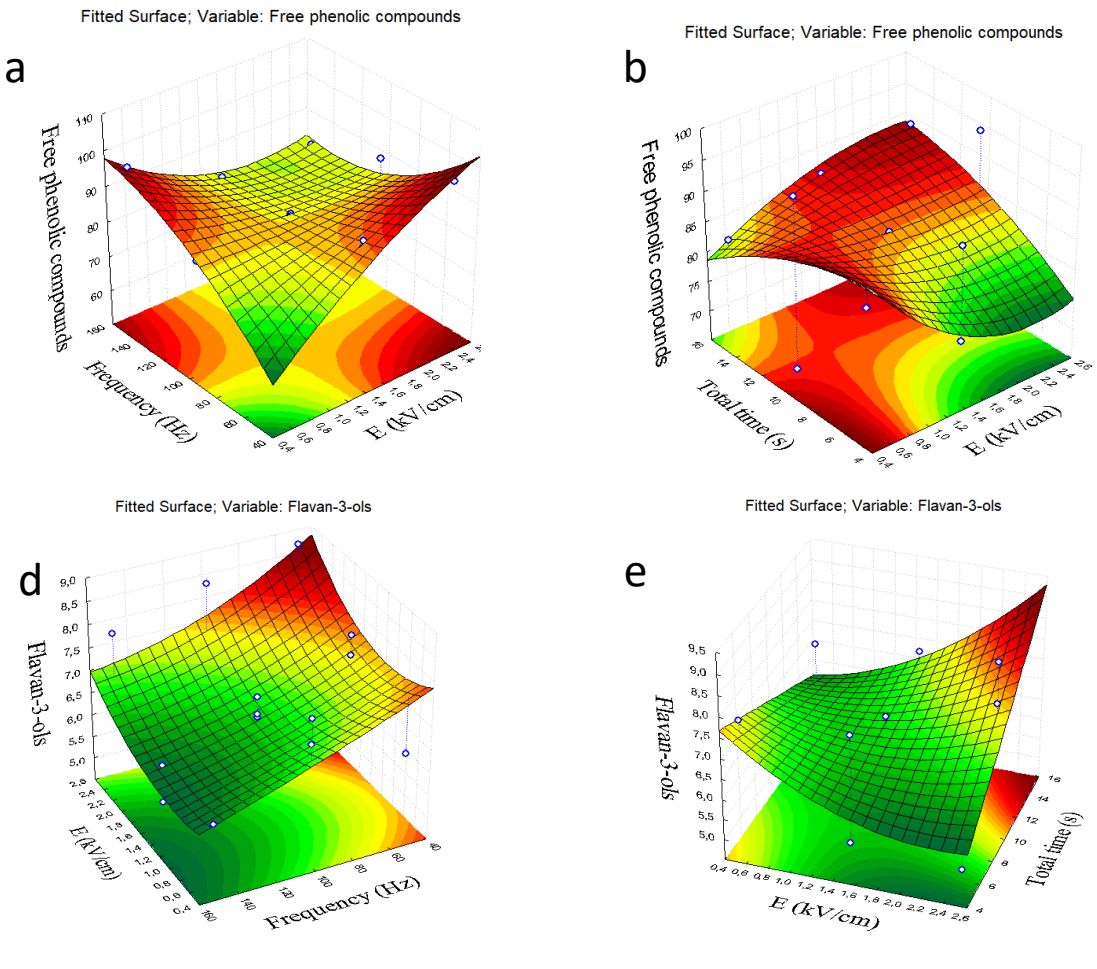
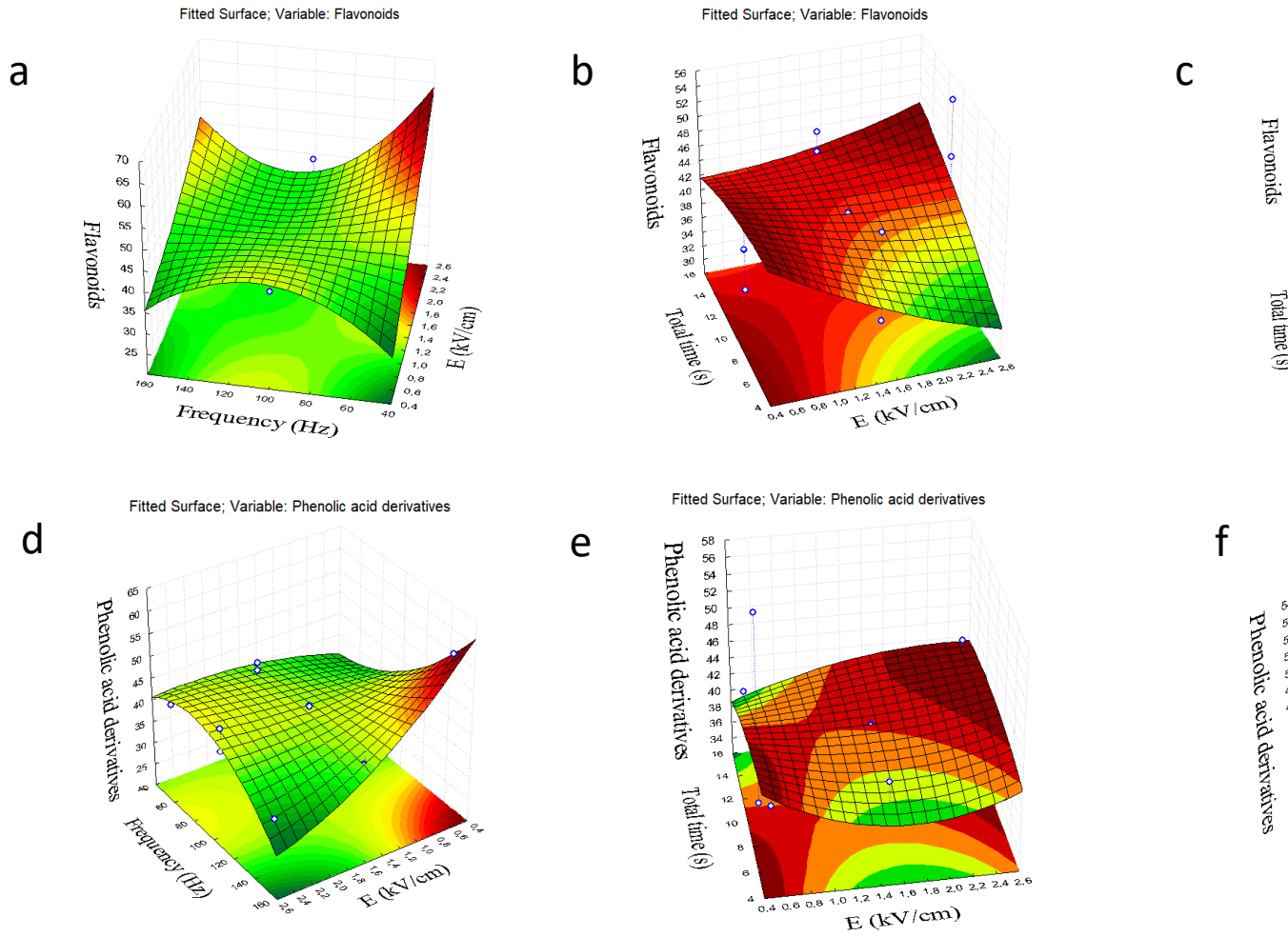


Figure 2. Response surface plots showing combined effects of process variables for flavonoids (a, derivates (d, e and f). a and d: Frequency with electric field strength, b and e: total time with elect total time with frequency



Three-dimensional response surface plots for the variables of free phenolic compounds, flavan-3-ols are presented in **Figure. 1**, while those for flavonoids and phenolic acid derivates are presented in **Figure. 2.** Electric field strength (X_1) has demonstrated the highest effect on the response variables.

In the **Figure 1.a** it can be observed the positive effect of electric field strength (X_1) and the positive effect of frequency (X_2), which had a higher effect than the negative effect of the quadratic term of frequency (X_{22}) in the response of free phenolic compounds, whereas the quadratic effect of electric field did not have a significant effect. In addition, the negative effect between electric field strength and frequency appears in the **Figure 1.a.** In the **Figure 1.b** it can be observed the positive effect between electric field strength and total time (X_{13}), which was higher than the quadratic effect of total time (X_{33}) and lower than the positive effect of electric field strength (X_1) and the negative effect of time (X_3). **Figure 1.c** shows the positive effect between total time and frequency, which had a lower effect than the positive effect of frequency (X_2), negative effect of total time (X_3) and the quadratic negative effect of time (X_{33}). Nevertheless, it had a higher effect than the quadratic effect of frequency (X_{22}). In the **Figure 1.d it** can be observed the positive effect of electric field strenght(X_1) in the response of flavan-3-ols, which was higher than the negative effect of the quadratic electric field strength (X_{11}) and the negative effect of frequency (X_2). The **Figure 1.e** shows the negative effect of cross product between electric field strength and total time (X_{13}), which had less influence than the linear (X_1) and quadratic effect (X_{11}) of electric field strength (X_1). The most influence on the response was attributed to the positive linear effect of electric field (X_1). In the **figure 1.f** appears the influence of total time and frequency on the content of flavan-3-ols, linear effect of frequency (X_2) has the most influence on the response following by the effect of the linear effect of total time (X_3).

In the **Figure 2.a** appears the positive effect of electric field strength (X_1), which has the most influence in the response of flavonoids. Negative cross effect between electric field strength and frequency (X_{12}) is lower than the positive linear effect of frequency (X_2) and higher than the negative quadratic effect of frequency (X_{22}).

In the **Figure 2.b** it is possible to observe the positive effect of electric field strength and total time (X_{13}) that was lower than the positive linear effect of electric field strength (X_1) and negative linear effect of total time (X_3) and higher than the negative quadratic effect of total time (X_{33}). In the **Figure 2.c** the positive effect between frequency and total time (X_{23}) in the response can be observed, which had a lower effect than the linear positive effect of frequency (X_2) and negative linear (X_3) and quadratic effect of total time (X_{33}). In the **Figure 2.d** it can be observed the negative influence between electric field strength and frequency (X_{12}). The **Figure 2.e** shows the positive effect of electric field strength (X_1) and total time (X_3) that had a higher influence on the response than the negative effect between the electric field strength and total time (X_{13}), which was higher than the quadratic negative effect of total time (X_{33}) and in the **Figure 2.f** it can be observed the positive effect between frequency and total time (X_{23}).

3.2.1. Optimization of PEF parameters

PEF factors were optimized in order to maximize the content for each family of free phenolic compounds and their total: flavan-3-ols, flavonoids, phenolic acid derivates and total free phenolic compounds. (**Table 6**) Optimization of these factors was carried out by response surface plots of the combined effects of the factors.

Table 6. Optimal conditions for PEF, N.S.: Not significant differences.

Optimal conditions	**Free phenolic compounds**	**Flavan-3-ols**	**Flavonoids**	**Phenolic acid derivates**
E (KV/cm)	2.5	2.5	2.5	0.5
Frequency (Hz)	50	50	50	150
Total time (s)	14.5	15	15	5
Predicted (µg g^{-1} d.w.)	99 ± 2	10 ± 1	59 ± 1	56.1 ± 0.3
Obtained value (µg g^{-1} d.w.)	101 ± 2	10.1 ± 0.8	60 ± 2	55 ± 2

Significant differences	N.S.	N.S.	N.S.	N.S.

Regarding the suggested model, a great value on free phenolic compounds could be obtained under the following optimized conditions: 2.5 kV/cm, 50 Hz and 14.5 seconds (energy input of 9.06 kJ/kg) to obtain a maximum value of 99 ± 2 µg g^{-1} d.w. This optimum extraction conditions are in concordance with a study by Kim et al. (2019)[24], where the highest phenolic content was observed in ginseng samples following the application of PEF at frequency of 50 Hz and electric field strength higher than 0.5 kV/cm (1.5 and 2.5 kV/cm), showing that by increasing of electric field strength there is an increase of phenolic compounds extraction yield[24]. Also other authors observed the similar effect of electric field strength on the polyphenols extraction yield, in particular, TPC of date palm fruit extract was of 64.20, 65.90 and 67.35 mg GAE/100 g for samples treated at 1, 2 and 3 kV/cm, respectively[36]. Also the frequency, which indicates the number of pulses applied by unit of time, is an important parameter to consider during the PEF application since it determines the amount of electrical energy delivered per unit of time on the treated product[27]. In our study the lowest frequency and long time of the treatment (14.5 s) was more beneficial than short time and high frequency (e.g. treatment 4 with 2.5 kV/cm, 150 Hz and 10 s, and 18.75 kJ/kg) in the extraction of free phenolic compounds, indicating that, probably, energy input of 9.06 kJ/kg was sufficient for the electroporation of the majority of cells.

Optimal conditions to obtain the maximum value of families of phenolic compounds were the following: 2.5 kV/cm, 50 Hz and 15 second to obtain 10 ± 1 µg g^{-1} d.w. of flavan-3-ols, 2.5 kV/cm, 50 Hz and 15 seconds to obtain 59 ± 1 µg g^{-1} d.w. of flavonoids and 0.5 kV/cm, 150 Hz and 5 seconds to obtain 56.1 ± 0.3 µg g^{-1} d.w. of phenolic acid derivatives. These optimal conditions have been applied to obtain the experimental values of each responses and as reported in **Table 5** any statistical difference was noticed between the predicted and obtained values.

Table 7. Table of identification of bound phenolic compounds from brewers' spent grain extracts by HPLC-MS

Peak	RT	m/z experimental	Bound phenolic compounds
1	4.175	289	Epicatechin
2	4.996	179	Caffeic acid
3	6.873	163	trans-p-coumaric acid
4	7.146	163	cis-p-coumaric acid
5	7.656	193	trans ferulic acid
6	7.972	193	cis ferulic acid
7	9.247	385	Sinapoyl-hexose a
8	9.809	385	Sinapoyl-hexose b
9	11.224	385	Sinapoyl-hexose c
10	11.637	385	Sinapoyl-hexose d
11	12.675	341	Caffeoyl-hexose
12	12.525	385	Sinapoyl-hexose e
13	12.683	385	Sinapoyl-hexose f

The established PEF conditions that allowed the highest value of total free phenolic compounds were applied to obtain enriched phenolic extracts from BSGs and the phenolic content was compared with those obtained without the PEF pre-treatment. Moreover, the determination of bound phenolic compounds has been carried out at the optimum conditions established for free phenolic compound (2.5 KV/cm, 50 Hz and 14.5 s). These compounds were identified by HPLC-MS by rendering their mass spectra using the data reported in the literature and, when available, by co-elution with commercial standards (**Table 7**). The peak 1 at 4.2 min with a molecular ion at m/z 283 was identified as epicatechin, which was identified previously in buckwheat samples and in BSG[30,32]. The peak 2 at 5.0 min with a molecular ion at m/z 179 was identified as caffeic acid, which was identified in barley, beer and BSG samples[7,32,37]. The peaks 3 and 4 at 6.9 min and 7.146 min respectively, at m/z 163 were identified as trans-p-coumaric acid and cis-p-coumaric acid, according to the identification of these compounds in barley samples[7]. The peaks 5 and 6 at 7.656 min and 7.972 min respectively, at m/z 193 were identified as tras ferulic acid and cis ferulic acid[7]. The peaks 7, 8, 9, 10, 12 and 13 at 9.2, 9.8, 11.2, 11.6, 12.5, 12.7 min respectively, with a molecular ion at m/z 385 were identified as sinapoyl-hexose isomers, which were identified

previously in barley samples[7]. The peak 11 with a molecular ion at m/z 341 was identified as caffeoyl-hexose, which was identified in barley samples[7].

Table 8 reports the content of free and bound phenolic compounds obtained from BSGs with and without PEF pre-treatment at optimal conditions.

The total content of free phenolic compounds, flavan-3-ols, sum of flavonoids and phenolic acid derivatives were 62.8 %, 61.5 %, 67.3 % and 67.3 % higher than the obtained in the control samples. Furthermore, the total bound content and flavan-3-ols were 39.6 % and 39.8 % higher than the obtained in the control samples (**Table 8.**). In addition, the total phenolic content (sum of free and bound) obtained in brewers' spent grains after the PEF treatment at optimum conditions (2.5 kV/cm, 50 Hz and 14.5 s) (640.46 µg g^{-1} d.w.) was 43.23 % higher than the obtained in the control sample (363.58 µg g^{-1} d.w.). This increase in the recovery of phenolic content with the application of PEF was in concordance with the study conducted by Barba et al. 2015.[38]. They applied the PEF (13.3 kV/cm), and ultrasounds (USN) (400W; 24 kHz) treatments in blackberries in order to evaluate the effects of processing on protein, total phenolics and anthocyanins, showing that the phenolic content obtained following the PEF application (108.0 mg/100 g) was 57.2 % higher than the one obtained by ultrasounds (46.2 mg/100 g). Also, Kim et al. (2019)[24] reported the highest phenolic content at 1.5 and 2.5 kV/cm (893.83 and 877.40 mg tannic acid equivalent/ 100 g), which were 8-10 % higher than the obtained in the control samples without PEF treatment (807.02 mg tannic acid equivalent/ 100g). Other study reported an increase on the phenolic content of 23 % in blueberries after PEF treatment (electric field strength 2.0 kV/cm, 100 pulses per s for 4 minutes, and pulse width 1 µs)[39]. This substantial increase in the phenolic recovery after PEF treatment is due to the disintegration of the structure of cell cytomembrane and change its selective permeability properties, which caused an increased mass transfer through the cells[40]. In fact, Kim et al. (2019)[24] reported that the conductivity of ginseng samples increased with the application of PEF at 1.5 and 2.5 kV/cm, while no effect was observed when 0.5 kV/cm was applied in comparison to untreated samples. This increase in electrical conductivity values shows that PEF treatment at 1.5 and 2.5 kV/cm led to

biological cell membrane disruption[24]. Therefore, PEF can be used as a pre-treatment to increase the recovery of phenolic compounds in brewers spent grain. The results of the total free and bound phenolic compounds content obtained in our study were lower than those obtained previously in barley samples[7], this probably because part of the content of these compounds are extracted from brewers spent grain during the beer production, and some of them could have been degraded since high temperatures are used during the brewing processing[1]. Nevertheless, bound phenolic compounds are in the same order than the obtained in barley samples[7], because these phenolic compounds are ester linked to the cell wall, for that reasons most of them are kept during the beer production[41]. Comparing the content of bound phenolic compounds of caffeic acid, p-coumaric acid and ferulic acid obtained in the present work, these were lower than the obtained previously in BSG[42,43], whereas, the content of free phenolic compounds of catechin and ferulic acid were higher than the obtained previously in BSG[43]. These differences could be because phenolic content of BSG varies according to barley variety, harvest time, malting and mashing conditions, and the quality and type of adjuncts added in the brewing process[1].

Table 8. Comparison of phenolic content (µg/g d.w.) (free and bound) with and with

Free phenolic compounds	Control	PEF treated	Bound phenolic compounds	Control
Catechin-3-glucose	0.18 ±0.02	0.73 ±0.10	Epicatechin	3.34 ±0.
Procyanidin B3	0.87 ±0.03	3.02 ±0.21	Caffeic acid	6.89 ±0.
Catechin	1.34 ±0.01	3.96 ±0.34	trans-p-coumaric acid	76.74 ±
Quercetin-3-hexosylrutinoside	n.d.	<LOQ	cis-p-coumaric acid	27.73 ±
Vanillic acid	n.d.	0.30 ±0.17	trans ferulic acid	85.28 ±
p-Hydroxybenzaldehyde	3.38 ±0.13	3.80 ±0.05	cis ferulic acid	21.42 ±
Vanillin	0.58 ±0.02	1.50 ±0.22	Sinapoyl-hexose a	15.12 ±
Prodelphinidin B3	1.49 ±0.06	2.40 ±0.02	Sinapoyl-hexose b	13.30 ±
p-coumaric acid	1.21 ±0.10	3.30 ±0.05	Sinapoyl-hexose c	6.46 ±0.
Hydroferuloyl glucose	3.15 ±0.08	8.60 ±0.12	Sinapoyl-hexose d	26.61 ±
Ferulic acid	0.25 ±0.01	0.90 ±0.03	Caffeoyl-hexose	11.77 ±
Sinapoyl hexose	9.43 ±0.21	23.00 ±0.73	Sinapoyl-hexose e	19.48 ±
Tricin	15.70 ±0.19	49.76 ±1.20	Sinapoyl-hexose f	11.86 ±
Sum	37.58 ±2.06	101 ± 2	Sum	326 ±3.0
Sum flavan-3-ols	3.89 ±0.19	10.1 ± 0.8	Sum flavan-3-ols	3.34 ±0.
Sum flavonoids	19.59 ±0.47	60 ± 2	Sum flavonoids	3.34 ±0.
Sum phenolic acid derivatives	17.99 ±0.90	55 ± 2	Sum phenolic acid derivatives	322.66±

According to the high content of phenolic compounds obtained from BSG with PEF treatment, these phenolic extracts could be beneficial as ingredients in food Industry because of the low cost and high nutritional value of BSG. For example, these extracts could be used to enriche bakery products such as bread, biscuits, cookies, muffins, cakes, waffles, pancakes, tortillas, snacks, doughnuts, brownies and pasta[44-46].

4. Conclusions

The valorization of BSG is an important goal in order to recover the phenolic compounds that can be extracted, purified and reused as functional ingredients in food and cosmeceutical industry. Solid–liquid extractions are the most commonly used procedures to extract the phenolic compounds in BSG samples, due to their ease of use, efficiency, and wide applicability. However, to improve the phenolic recovery, pulsed electric field (PEF) have been used as extraction pre-treatment. PEF parameters were optimized and this pre-treatment at electric field strength of 2.5 kV/cm, frequency of 50 Hz for 14.5 s was able to improve the total free and bound phenolics recovery of 2.7 and 1.7 times, respectively, compared to the control samples without PEF treatment, probably due to the increase of the permeability of the cell membrane, which facilitates the extraction of bioactive compounds. These promising results encourage further studies in order to check the extraction efficiency of PEF coupled to ultrasounds or microwave extraction technology and the possibility of the scale-up of the process.

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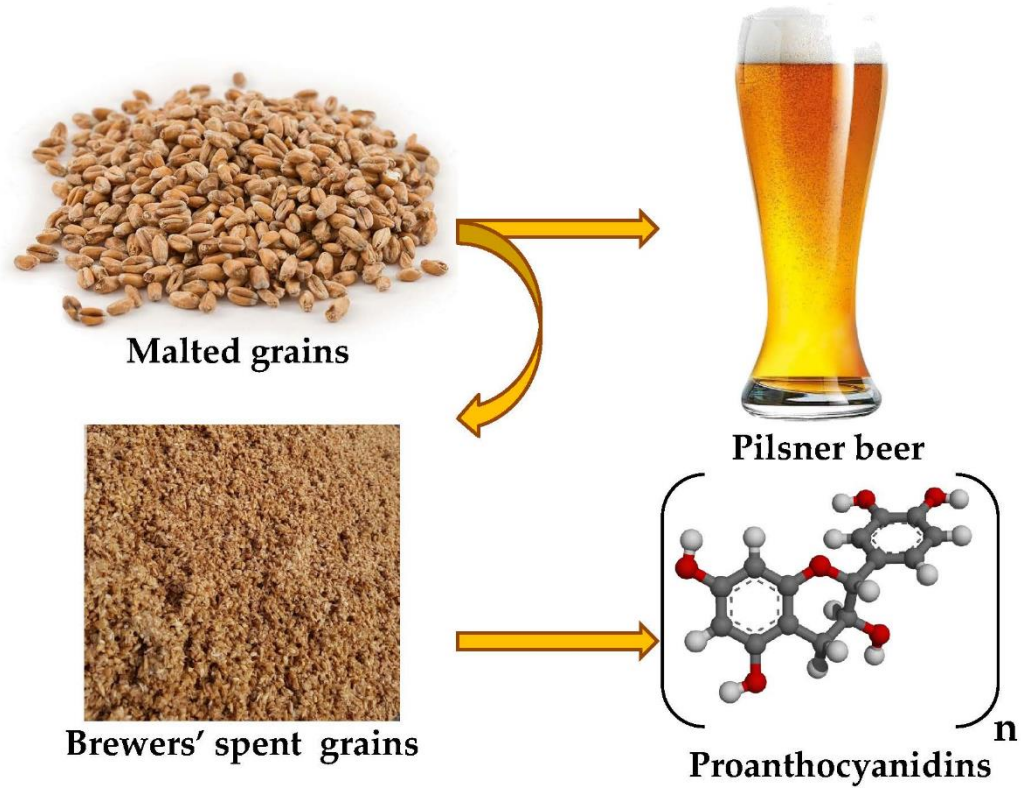
Chapter 5

Optimization of sonotrode ultrasonic-assisted extraction of proanthocyanidins from brewers' spent grains



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Optimization of Sonotrode Ultrasonic-Assisted Extraction of Proanthocyanidins from Brewers' Spent Grains

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Abstract

Brewing spent grains (BSGs) are the main by-product from breweries and they are rich of proanthocyanidins, among other phenolic compounds. However, literature on these compounds in BSGs is scarce. Thus, this research focuses on the establishment of ultrasound-assisted extraction of proanthocyanidin compounds in brewing spent grains using a sonotrode. To set the sonotrode extraction up, response surface methodology (RSM) was used to study the effects of three factors, namely, solvent composition, time of extraction, and ultrasound power. Qualitative and quantitative analyses of proanthocyanidin compounds were performed using HPLC coupled to fluorometric and mass spectrometer detectors. The highest content of proanthocyanidins was obtained using 80/20 acetone/water (v/v), 55 min, and 400 W. The established method allows the extraction of 1.01 mg/g dry weight (d.w.) of pronthocyanidins from BSGs; this value is more than two times higher than conventional extraction.

Keywords: Box–Behnken design; proanthocyanidins; Brewers' spent grains; sonotrode ultrasonic-assisted extraction; HPLC-fluorometric detector (FLD)–MS.

1. Introduction

Barley is the basic raw material for brewing. Phenolic compounds identified in barley include flavonoids, phenolic acids, and proanthocyanidins (PCs)[1,2]. There are more than 50 PCs in barley, comprising flavan-3-ol oligomers and their polymers[3]. The oligomers include dimers (prodelphinidin B3 and procyanidin B3), trimers, tetramers, and pentamers, while polymers are formed by oxidation and polymerization of simple flavan-3-ols[4]. Barley PCs ranged from 25 to 250 mg/100 g of grain[5-8]. Among them, proanthocyanidin trimers, such as catechin–gallocatechin–catechin (C–GC–C), prodelphinidin B3 and procyanidin B2[9] are the most representative in barley. In addition, hops also contribute to the proanthocyanidin content in brewing spent grains (BSGs); in fact, according to several authors, this ingredient contains high amounts of catechin and procyanidins[10,11].

Furthermore, PCs showed anti-bacterial[12], anti-viral[13], anti-carcinogenic[14], anti-inflammatory[15], and cardioprotective effects[16]. Some studies demonstrated the potential of PCs for prevention or treatment of oxidative stress-associated diseases due to their antioxidant capacity[17]. In addition, PCs are easily extracted, affordable, and demonstrated low toxicity[17].

During the process of brewing, many BSGs are generated from barley grains after separation of the wort, and they consist of the residues from malted barley which could contain adjuncts (non-malt sources of fermentable sugars) such as wheat, rice, or maize and hop added during mashing[1]. Consequently, this by-product is rich in protein, fibers, arabinoxylans, and β-glucan, and also contains PCs in low concentration; thus, its reutilization could be useful for the food industry, and offers an opportunity for cereal-based baked and extruded products with acceptable sensory and nutritional characteristics[1].

In this sense, the challenge is to increase the efficient collection of PC-rich extracts with high bioactivity by the optimization of the extraction process. Thus far, conventional solid/liquid extraction was often used, employing as an extraction solvent a mixture of acetone and water in proportions from 50/50 to 80/20[4,8,18,19]. due to the large number of OH groups in PCs. In addition, bath-ultrasound-assisted extraction is the most used extraction technique. Some authors carried out pressurized solvent extraction, which is a static solid/liquid extraction with high pressure and eventually high temperature in stainless-steel extraction cells. Nevertheless, conventional extractions using ultrasonic-assisted extraction seem to be the best choice, since it is an economical technique, can be performed at atmospheric pressure and ambient temperature, and it could be developed on an ultrasound (US) bath or even with an US probe (or sonotrode)[20,21].

To carry out the determination of PCs in cereal, high-performance liquid chromatography (HPLC) is the analytical technique usually applied to this aim. In many instances, this technique was coupled to a diode array detector (DAD), fluorometric detector (FLD), and mass spectrometer detector (MSD)[8,22,23], or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis[24].

In view of the above, the objective of this work was to evaluate the recovery of proanthocyanidins from BSGs by establishing a sonotrode ultrasonic-assisted extraction method. For that purpose, response surface methodology (RSM) was performed to evaluate extraction parameters with an experimental Box–Behnken design.

2. Materials and Methods

2.1. Samples

Brewers' spent grain (BSG) samples were obtained in a micro-brewing plant after pilsner beer production (Mastrobirraio, Cesena, Italy, 44°08′00″ north (N), 12°14′00″ east €).

2.2. Chemicals

HPLC-grade water and solvents were purchased from Merck KGaA (Darmstadt, Germany). Catechin was purchased from Sigma-Aldrich (St. Louis, MO).

2.3. Experimental Design

Response surface methodology (RSM) is the most popular tool for modeling. In RSM, statistical models and polynomial equations are always combined to provide an approximate relationship between the dependent and independent variables[25]. In the present work, a Box–Behnken design (BBD) with three factors was carried out in order to optimize the extraction parameters of proanthocyanidins in BSGs. The parameters of ultrasound-assisted extraction (US) can be divided into US parameters (ultrasound frequency, duration, acoustic power/intensity, and treatment mode) and non-US parameters (solvent type, solvent/sample ratio, particle size, temperature)[25]. In this work, the factors investigated were acetone/water (X_1), time (X_2), and potency (X_3), with three levels for each factor, and the response variable (Y) was the sum of the total content of proanthocyanidins (PCs). The range for the percentage of acetone/water was chosen based on the conditions previously established in other works (50, 75, and 100%)[4,8]; the extraction time (5, 30, and 55 min) and the US power (80, 240, and 400 W) were the same as those previously used in a study where a sonotrode US was employed to optimize these parameters for the extraction of phenolic compounds from *Psidium guajava* L. leaves[26]. The design consisted of 15 combinations including three center points (**Table 1**), and the experiments were randomized to maximize the effects of unexplained variability in the observed response, due to extraneous factors.

Table 1. Box-Behnken design (BBD) with the values of the sonotrode ultrasound (US) parameters with the experimental values for the dependent response of proanthocyanins (PCs) quantified by HPLC-fluorometric detector (FLD) in brewers' spent grain (BSG) extracts; d.w.- dry weight.

Experiment	Independent factors			Dependent factor
	X_1	X_2	X_3	Total (µg g-1 d.w.)
1	50	5	240	540.04
2	100	5	240	548.25
3	50	55	240	690.90
4	100	55	240	802.25
5	50	30	80	547.91
6	100	30	80	849.32
7	50	30	400	601.43
8	100	30	400	792.07
9	75	5	80	796.40
10	75	55	80	977.69
11	75	5	400	993.15
12	75	55	400	1002.31
13	75	30	240	832.04
14	75	30	240	857.04
15	75	30	240	752.68

X_1: acetone/water, X_2: time, and X_3: US power.

The determination of optimal US sonotrode parameters was carried out using STATISTICA 7.0 (2002, StatSoft, Tulsa, OK).

2.4. Extraction of Proanthocyanidins from Brewers' Spent Grains by Sonotrode Ultrasonic Extraction

The extraction was achieved with a US sonotrode UP400St (Hielscher Ultrasonics GmbH, Teltow, Germany) and, during the extraction, an ice bath was used to avoid rises in temperature. The temperature ranged between 23 and 25 °C in all extractions, and it was measured with a thermometer at the end of each extraction. The percentage of acetone/water, the extraction time, and the US power were varied according to the experimental design. After the extraction, samples were

centrifuged at 1000× g for 10 min; supernatants were collected, evaporated, and reconstituted in 1 mL of methanol/water (1/1, v/v). The final extracts were filtered through 0.2-μm polytetrafluoroethylene (PTFE) syringe filters and stored at −18 °C until the analyses.

2.5. Conventional Extraction of Proanthocyanidins

The results obtained by the US sonotrode at the optimal conditions were compared with a PC extract from BSGs obtained via conventional solid/liquid extraction. The extraction methodology was carried out according to Carciochi et al. (2018)[27]. Briefly, BSGs were subjected to mechanical agitation with a w/v ratio of 1/30, temperature of 80 °C, 72/28 ethanol/water (v/v), and an extraction time of 60 min.

2.6. Determination of Proanthocyanidins in Brewing Spent Grain Extracts by HPLC-FLD-MS Analysis

The separation of proanthocyanidins was performed on an Agilent 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a binary pump delivery system, a degasser, an autosampler, and FLD and MS detectors (MSD, model G1946A, Santa Clara, CA, USA). A Develosil Diol 100 Å column (250 × 4.6 mm, 5 μm particle size) purchased from Phenomenex (Torrance, CA, USA) was used for the analyses.

All solvents were HPLC-grade and were filtered in a filter disc of 0.45 μm. According to Robbins et al. (2009)[28], the elution binary gradient consisted of CH3CN/HOAc, 98/2 (v/v) as solvent A, and CH_3OH/H_2O/HOAc 95/3/2 v/v/v as solvent B. The analyses started with 7% of phase B from 0 to 3 min. Thus, solvent B was increased to 37.6% (from 3.1 to 57 min) and then to 100% B over the next 3 min for 7 min. After that, the initial condition was established, and they were maintained for 16 min. The injection volume was 5 μL and all the analyses were run at 35°C. Additionally, fluorescence detection was conducted with an excitation wavelength of 230 nm and an emission wavelength of 321 nm.

Moreover, identification of proanthocyanidins was carried out by HPLC-MS according to Verardo et al. (2015)[8]. Furthermore, quantification of PCs was done employing a calibration curve of (+)-catechin done from the limit of quantitation (LOQ) to 250 µg/mL (LOQ = 0.193 µg/mL). In addition, the quantification of dimers, trimers, tetramers, pentamers, and the polymers was done using the correction factors suggested by Robbins et al. (2009)[28].

3. Results and Discussion

3.1. Determination of Proanthocyanidin Compounds in Brewers' Spent Grains

Table 1 shows the sum of the total content of proanthocyanidins according to the experimental design (**Table 1**).

Peak	Rt (min)	Compound	[M-H]-
1	6.7	Catechin/epicatechin	289
2	17.6	Procyanidin dimer	577
3	19.0	Prodelphinidin dimer	593
4	21.2	Prodelphinidin dimer II	593
5	24.4	Procyanidin trimer	865
6	26.8	Prodelphinidin trimer I (monogalloylated)	881
7	29.5	Prodelphinidin trimer II (digalloylated)	897
8	32.8	Procyanidin tetramer	1153
9	33.9	Prodelphinidin tetramer (digalloylated)	1457
10	36	Procyanidin pentamer	1441
11	51.7	Polymer	

Table 2. Table of identification of proanthocyanidins from brewers' spent grain extracts by HPLC-MS; Rt—retention time.

A total of 11 PCs were identified in BSGs according to their degree of polymerization and their mass spectra. As shown in **Table 2** (and in **Figure S1**), the elution order depended on the number of flavan-3-ol units. Therefore, monomers eluted first and then the different oligomers eluted. In addition, for the

same degree of polymerization, a higher degree of galloylation meant a higher retention time[8].

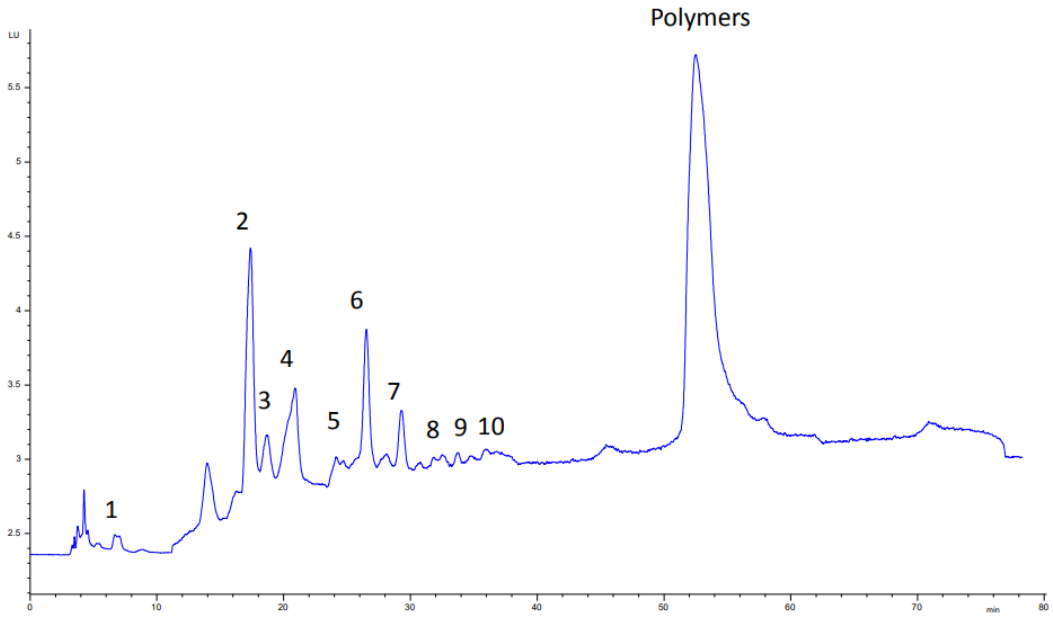

Figure S1. Separation of BSG proantocyanidins by HPLC-FLD.

Moreover, quantification of PCs in brewing by-products was carried out using HPLC-FLD. The calibration curve of catechin was used to quantify the PCs. The correction factors were applied according to Robbins et al. (2009)[28]. The concentration values of PCs obtained in each experiment in the BBD are presented in **Table 3**. Briefly, the total content of PCs varied from 540.04 $\mu g \cdot g^{-1}$ dry weight (d.w.) to 1002.31 $\mu g \cdot g^{-1}$ d.w. Comparing the quantification of each compound, experiment 11, whose parameters of extraction were 75% acetone, 5 min, and 400 W of US power, recovered higher amounts of catechin/epicatechin, dimers, trimers, and tetramers than the rest of the experiments. Finally, the major concentrations of procyanidin pentamer, the polymer, and the total content of PCs were obtained in experiment 12 with 75% acetone, 55 min, and 400 W of US power.

Table 3. Table of quantification of proanthocyanidins from brewers' spent grain extracts by

µg·g^{-1} d.w. UAE—ultrasound-assisted extraction; LOQ—limit of quantit

	UAE 1	UAE 2	UAE 3	UAE 4	UAE 5	UAE 6	UAE 7	UAE 8	UAE 9	UAE 10
catechin/epicatechin	8.34	9.17	10.16	9.71	8.05	10.03	10.07	10.37	9.59	10.33
procyanidin dimer	50.08	70.49	52.50	85.90	40.06	73.45	44.02	82.34	57.47	76.36
prodelphinidin dimer	22.68	33.01	26.09	25.96	30.44	38.86	31.93	43.95	49.16	57.03
prodelphinidin dimer II	25.69	35.62	51.16	66.60	38.16	78.03	37.09	79.55	59.02	72.00
procyanidin trimer	73.11	28.69	61.50	54.93	54.45	67.35	37.20	64.29	88.65	92.85
prodelphinidin trimer I (monogalloylated)	35.58	73.86	56.78	97.85	49.08	101.98	45.60	95.27	92.53	122.39
prodelphinidin trimer II (digalloylated)	<LOQ	48.58	<LOQ	82.52	<LOQ	80.67	<LOQ	71.26	79.53	92.77
procyanidin tetramer	<LOQ	29.46	<LOQ	46.57	<LOQ	51.15	<LOQ	44.52	45.68	56.49
prodelphinidin tetramer (digalloylated)	<LOQ	32.70	<LOQ	52.06	<LOQ	58.55	<LOQ	51.20	50.76	64.87
procyanidin pentamer	<LOQ	17.64	<LOQ	26.50	<LOQ	28.01	<LOQ	19.34	24.84	35.28
Polymers	324.57	169.04	432.71	253.66	327.67	261.23	395.52	229.98	239.17	297.31
Total	540.04	548.25	690.90	802.25	547.91	849.32	601.43	792.07	796.40	977.69

Proanthocyanidins were grouped as monomer, dimers, trimers, tetramers, pentamers, and polymers.

3.2. Fitting the Model

The regression model for the BBD was fitted employing the data from **Table 1** in order to find the combined effect of extraction time, acetone/water ratio, and sonotrode US power on the response variable during the sonotrode US. For that, an analysis of variance (ANOVA) with 95% confidence level was employed to analyze the regression model and to evaluate the effect of the coefficients for each factor (linear and quadratic terms) and the interaction between them (cross-product term). In fact, the evaluation of the model was carried out according to the significance of the regression coefficients which are displayed in **Table 4**. According to other works, the level of significance could be fixed at $\alpha < 0.1$ in order to increase the number of significant terms[26]. In the present work, the model was analyzed at $\alpha < 0.05$ and $\alpha < 0.1$ The significant variables for the total content of PCs were the intercept (X_0) ($p = 0.000426$), the linear effect of acetone/water (X_1) ($p = 0.058033$) and its quadratic effect (X_{11}) ($p = 0.018319$), the linear effect of time (X_2) ($p = 0.060966$), and the quadratic effect of the power (X_{33}) ($p = 0.085914$). Furthermore, ANOVA revealed that the model presented a high correlation between the factors and the response variables with a coefficient of determination (R^2) of 0.8999 (**Table 4**). In addition, the *p*-value of the regression model and the *p*-value of the lack-of-fit (LOF) were also used to verify the adequacy of the model. In fact, a high correlation term, a significant regression model ($p < 0.05$), and a non-significant LOF ($p > 0.05$) demonstrated the validity of the model (**Table 4**).

Table 4. Regression coefficients and ANOVA table.

Regression coefficients	**Total proanthocyanidins**
β_0	-1256.27*
Linear	
β_1	53.07**
β_2	-1.19**
β_3	-0.68
Cross product	
β_{12}	0.04
β_{13}	-0.01
β_{23}	-0.01
Quadratic	
β_{11}	-0.33*
β_{22}	0.06
β_{33}	0.00**
R^2	0.8999
p (Regression model)	0.0074
p (Lack of fit)	0.3420

3.2.1. Analysis of Response Surfaces

In order to determine the optimal value of each factor for the extraction of PCs from BSGs, response surfaces were plotted. Each pair of variables was depicted in three-dimensional surface plots, while the other factor was kept constant at a central level. **Figure 1** shows the three-dimensional plots for the effects of acetone/water (% (*v/v*)) (X_1) with time (X_2), acetone/water (% (*v/v*)) (X_1) with US power (X_3), and time (X_2) with US power (X_3) on the concentration of the total content of PCs.

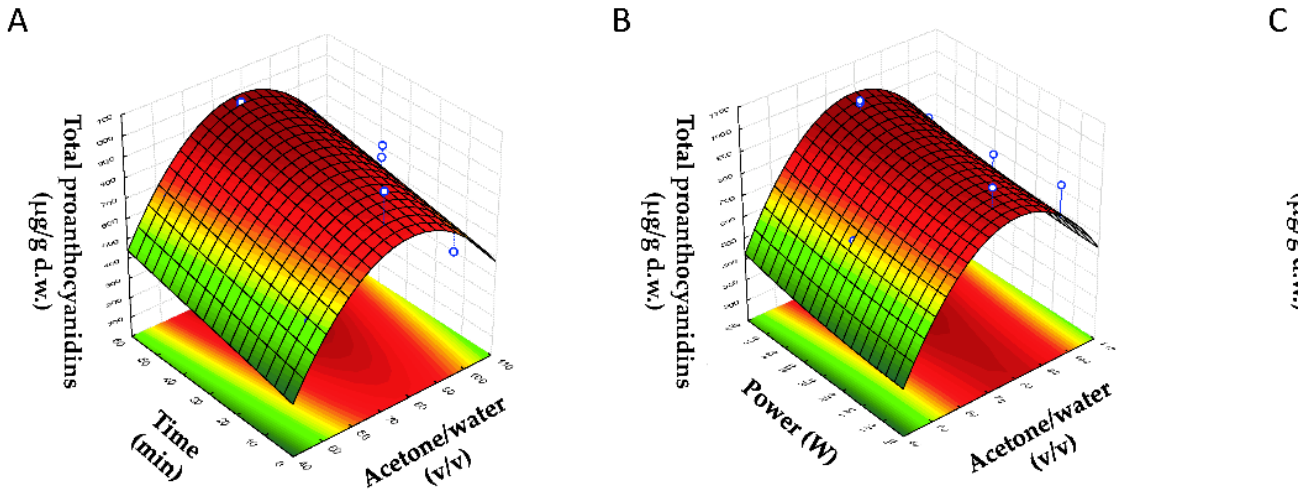

Figure 1. Response surface plots showing the combined effects of process variables for total proanth
(*v/v*)) vs. time (min); (**B**) acetone/water (% (*v/v*)) vs. ultrasound (US) power (W); (**C**) time (mi

In **Figure 1A**,**B**, it can be observed that the response of the total content of PCs increased when the concentration of acetone increased at first. After that, a decrease in response was observed when the maximum response was achieved. This shape was a consequence of the quadratic effect of acetone, which had a negative value, showing that an increase in this parameter more than a certain value tended to decrease the response. For example, **Figure 1A** shows an increase in total concentration of PCs if the content of acetone rose until the maximum value (75–85%), for which the increase time caused a slight increase in the total concentration of PCs. Additionally, in **Figure 1B**, an increase in the content of total PCs up to 70–85% acetone was observed where it started to reduce, whereas the response increased slightly at 70–85% if the power increased. At last, **Figure 1C** shows the positive linear effect of time and power on the response; there was an increase in response with time and power.

3.2.2. Optimization of Sonotrode US Parameters

The optimal conditions were selected through the three-dimensional (3D) plots to obtain the highest content of PCs from BSGs, as shown in **Table 5**.

Table 5. Optimal conditions for sonotrode UAE.

Optimal conditions	**Sum of proanthocyanidins (µg g^{-1} d.w.)**
Acetone/ water ratio (% (v/v))	80
Time (min)	55
US power	400
Predicted (µg g^{-1} d.w.)	1012.7 ± 15.1
Obtained value (µg g^{-1} d.w.)	1023.0 ± 8.9
Significant differences between predicted and obtained value	N.S.

N.S.: non-significant difference

Briefly, optimal extraction conditions were 80% acetone/water (*v*/*v*), 55 min, and 400 W for US power. The final step of the RSM after selecting the optimal conditions was to verify the accuracy of the mathematical model. For that, an extraction at optimal conditions was done with the same methodology; the obtained value did not report significant differences with the predicted value.

According to the results, the maximum content of PCs was obtained at 80% acetone/water, because PCs with a high degree of polymerization were the most concentrated, and they were better extracted at a high percentage of acetone, since they were less polar than the other PCs, increasing their solubility in this solvent. Also, acetone was not an efficient solvent when used pure, showing good results when it was combined with water. This occurred due to increased solvation provided by the presence of water. Additionally, at a high time of extraction and maximum power, cell walls were disrupted, releasing proanthocyanidins from the cell constituents. The predicted values of the model were in accordance with the experimental data under the same conditions. In fact, no significant differences were noted between the two data.

3.3. Comparison between Conventional and Established Sonotrode Extraction

Table 6 displays the comparison between the extraction of flavan-3-ols using sonotrode US at the optimal conditions established by our model and that using conventional extraction carried out according to Carciochi et al. (2018)[27].

Table 6. Comparison of proanthocyanidin content using sonotrode and conventional extractions (µg/g d.w.).

Proanthocyanidin compounds	Sonotrode extraction	Conventional extraction
Catechin/epicatechin	8.96 ± 0.23	3.89 ± 0.36
Procyanidin dimer	66.21 ± 1.10	21.34 ± 1.04
Prodelphinidin dimer	26.08 ± 0.29	10.25 ± 0.92
Prodelphinidin dimer II	80.43 ± 1.62	39.41 ± 1.37
Procyanidin trimer	53.19 ± 1.06	18.69 ± 2.06
Prodelphinidin trimer I (monogalloylated)	83.70 ± 2.12	42.16 ± 1.89
Prodelphinidin trimer II (digalloylated)	76.14 ± 0.98	35.47 ± 1.25
Procyanidin tetramer	47.09 ± 0.63	19.36 ± 0.47
Prodelphinidin tetramer (digalloylated)	65.22 ± 1.52	20.93 ± 1.12
Procyanidin pentamer	46.81 ± 1.70	18.71 ± 0.43
Polymers	469.21 ± 6.69	200.36 ± 2.89
Total	1023.04 ± 8.9	430.57 ± 3.62

According to the results obtained, the proposed methodology recovered 57.9% more total content of PCs than conventional extraction. Therefore, sonotrode ultrasound-assisted extraction is a more effective technique than conventional extraction for the recovery of PCs from BSGs. These data are in agreement with the data presented by Carciochi et al. (2018)[27].

Moreover, comparison with the literature is difficult because the information about the proanthocyanidin composition of BSGs is scarce. Comparing the values of proanthocyanidins obtained in this work with that obtained in barley samples, the contents of catechin, procyanidins, and prodelphynidins obtained in this work were on the same order of magnitude as those obtained in barley samples[4,8]. According to Moreira and co-workers[29], the present data also confirmed that light malt types as used for pilsner beer production contain high amounts of phenolic compounds. In spite of proanthocyanidins being degraded at high temperatures during malting, where barley is milled, mixed with water in the mash tun, and the temperature of mash slowly increased from 37 to 78 °C to promote enzymatic hydrolysis of malt constituents[1], and during beer production, it was confirmed that a part of barley and hop proanthocyanidins still remain in the beer spent grains after beer

production. Concentrations of catechin obtained at optimum sonotrode US conditions and in conventional extraction (8.96 ± 0.23 and 3.89 ± 0.36 mg·g^{-1} d.w., respectively) were higher than that reported by Ikram et al. (2017)[30] in brewers spent grain samples (1.08 ± 0.04 µg·100 g^{-1} d.w.). These differences could be because the catechin content of BSG varies according to barley variety, harvest time, malting and mashing conditions, and the quality and type of adjuncts added in the brewing process[1], but could also be due to the extraction method adopted for the proanthocyanidin extraction.

4. Conclusions

HPLC-FLD-MS was used for the determination of proanthocyanidins in brewers spent grains for the first time. A Box–Behnken experimental design was used in order to optimize the sonotrode ultrasound-assisted extraction parameters to obtain the maximum proanthocyanidin content from BSG. According to the model, the most important effect on the response came from the quadratic term of acetone/water ratio, followed by the linear term of acetone/water, the linear term of the time of extraction, and the quadratic term of US power. The highest value of proanthocyanidins was obtained at 80% acetone/water (*v/v*), 55 min, and 400 W. Finally, it was proven that sonotrode ultrasonic extraction is a more effective technique than conventional extraction method, providing a higher recovery of proanthocyanidins from BSG.

To conclude, BSGs represent a good raw material that could be used for the extraction of bioactive compounds or could be reused for the production of functional flours. In this way, further work will be done in order to validate this hypothesis.

Supplementary Materials

The following are available online at **https://www.mdpi.com/2076-3921/8/8/282/s1**, Figure S1: Separation of BSG proantocyanidins by HPLC-FLD.

Author Contributions

Conceptualization, V.V. and A.M.G.; data curation, B.M.G. and E.D.C.; investigation, B.M.G., F.P., and U.T.; supervision, V.V., A.M.G., and M.F.C.; writing—original draft, B.M.G.; writing—review and editing, F.P., V.V., E.D., U.T., A.M.G., and M.F.C.

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Conflicts of Interest

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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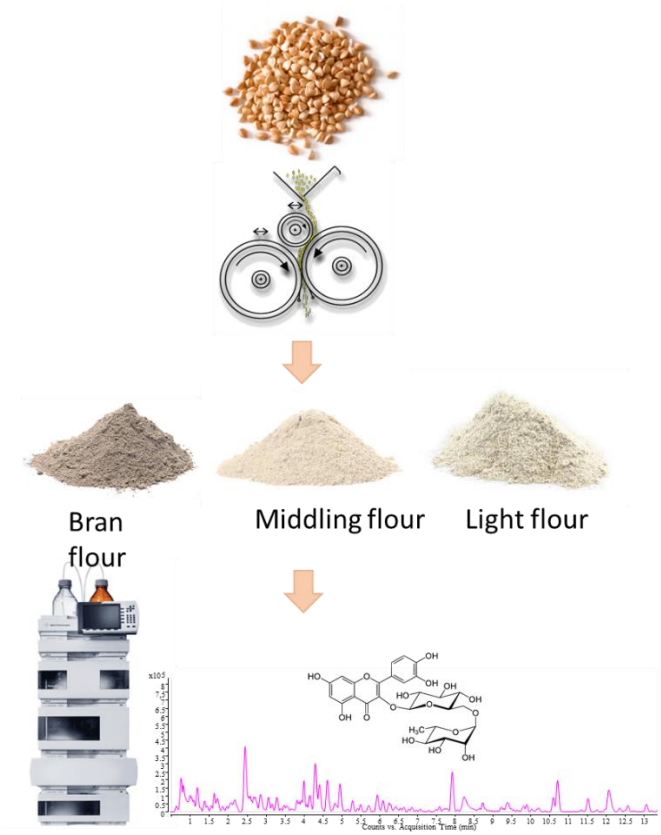
Chapter 6

Distribution of free and bound phenolic compounds in buckwheat milling fractions



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Distribution of Free and Bound Phenolic Compounds in Buckwheat Milling Fractions

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Abstract

Buckwheat is a rich source of phenolic compounds that have shown to possess beneficial effect to reduce some diseases due to their antioxidant power. Phenolic compounds are present in the free and in the bound form to the cell wall that are concentrated mainly in the outer layer (hull and bran). Hull is removed before the milling of buckwheat to obtain flours. In order to evaluate the phenolic composition in dehulled buckwheat milling fractions, it was carried out a determination of free and bound phenolic compounds in dehulled whole buckwheat flour, light flour, bran flour, and middling flour by high-performance liquid chromatography-mass spectrometry (HPLC–MS). The most abundant free phenolic compounds were rutin and epiafzelchin–epicatechin-*O*-dimethylgallate, whereas the most abundant bound phenolic compounds were catechin and epicatechin in all buckwheat flours. Besides, the highest content of free phenolic compounds was obtained in bran flour (1249.49 mg/kg d.w.), whereas the greatest

bound phenolic content was in middling (704.47 mg/kg d.w.) and bran flours (689.81 mg/kg d.w.). Thus, middling and bran flours are naturally enriched flours in phenolic compounds that could be used to develop functional foods.

Keywords: free and bound phenolic compounds; buckwheat flours; HPLC–MS; milling fractions

1. Introduction

Buckwheat (*Fagopyrum esculentum* Moench) as a traditional pseudocereal crop which belongs to the Polygonaceae is extensively utilized as food and as a medicinal plant[1]. Buckwheat is a rich source of starch, protein, and vitamins[2]. In addition, buckwheat is well known for containing phenolic compounds, including phenolic acids such as protocatechuic, syringic acid, and caffeic acid and flavonoids such as rutin (quercetin 3-rutinoside), quercetin, hyperoside (quercetin 3-*O*-b-D-galactoside), quercitrin (quercetin 3-*O*-a-L-rhamnoside), epicatechin, orientin, vitexin, isovitexin, and isoorientin[3-5]. Rutin is the most concentrated phenolic compound in Tartary and some common buckwheats, which have a content higher than most other plants[2]. Phenolic compounds in buckwheat have shown to possess antioxidant activity which has been associated with a lower incidence of cardiovascular disease, cancers, and age-related degenerative process[6-10].

Phenolic compounds in buckwheat are present in the free and in the bound form to cell wall[11], however, the majority of phenolic compounds are present in the free form, which has a distribution and concentration that is different in each part of the grain: pericarp (hull, husk), coat, endosperm, embryo with axis, and two cotyledons[12]; phenolic compounds are concentrated in the outer layers (hull and bran) of buckwheat grain[2]. Nevertheless, during buckwheat seeds processing into flour, the hull (17–20% of buckwheat grain) is removed by stone dehuller. The resulting product, called groat (intact achene), is milled into bran flour (10–24%),

which is a by-product that it is not commonly used in foods, and light flour (55–70%), which consists principally of endosperm and is used in human nutrition[13]. In addition, middling is a by-product from buckwheat milling that is not a flour that comprises different fractions and it includes 12% of the original grain, consisting of fractions of endosperm, bran, and germ[14]. Milling techniques used in the food industry employ mechanical force to break the grains into smaller fragments or fine particles[15]. Previous studies reported the use of roller milling process in dehulled whole buckwheat to obtain a flour and the separation of this flour into various fractions from outer to inner parts[2,16]. These studies have shown that outer layers are richer in protein, lipid, dietary fiber, and ash content than the inner layers. Also, the antioxidant capacity in flour fractions in the outer layers is higher than that in the inner layers by the increase of phenolic compounds from bran[2,16]. In addition, it has reported that milling fractions that contain outer layers possess a higher concentration of phenolic compounds than whole grain and groat flour fractions[6]. Therefore, the aim of this work was the determination of free and bound phenolic content in different buckwheat meals/flours: whole grain flour, light flour, bran meals, and middling flour in order to evaluate the phenolic concentration in each buckwheat meal fraction. These analyses will furnish new information about the total content of phenolic compounds in each fraction, taking into account the free or extractable fraction and bound or nonextractable phenolic fraction (NEPP). For that purpose, phenolic compounds were extracted and then were analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS).

2. Materials and Methods

2.1. Sample

Buckwheat meals/flours were obtained from whole buckwheat grain (cv. Darja) harvested in Matrice (Italy) (41°37′00″N 14°43′00″E), situated in a hilly location at 750 m above sea level. The field presented high tenacity of the soil due to the presence of clay. Harvesting took place on September 2018. The grain was

dehulled by stone dehuller (GRANO 200 SCHNITZER Stein-Getreidemuhle, Offenburg, Germany), and the groat (dehulled grain) was roller-milled by using an experimental mill (Labormill 4RB Bona, Monza, Italy). This mill is able to produce three milling fractions with different particle sizes that constituted the basis for differentiation between bran meal, middling flour, and light flour (**Figure 1**). In the bran meal, the majority of particles were >505 μm, while in middling flour, between 219–363 μm, and in light flour, <183 μm. Granulometry analysis was performed using an automatic sieve (Buhler ML1-300, Uzwil, Switzerland).

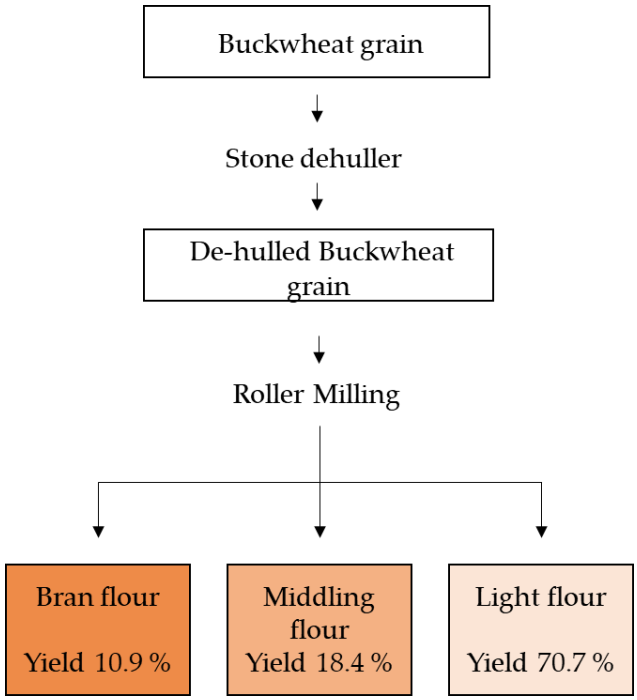

Figure 1. Flowchart of the milling process used for the production of buckwheat flours.

2.2. Reagents and Chemicals

HPLC-grade acetonitrile, water, methanol, acetone, acetic acid, ethanol, hexane, ethyl acetate, diethyl ether, and hydrochloric acid were purchased from Merck KGaA (Darmstadt, Germany). Hydroxide sodium was from Fluka (Buchs, Switzerland). Ferulic acid, catechin, quercetin, and rutin (Sigma-Aldrich (St. Louis, MO, USA)) were used for the calibration curves.

2.3. Extraction Method

Extraction of free phenolic compounds from buckwheat flour fractions has been carried out according with the method established by Hung & Morita (2008)[2] with certain modifications. One gram of buckwheat flour was extracted thrice in an ultrasonic bath with a solution of ethanol/water (4:1 *v*/*v*). The supernatants were collected, centrifugated at 2500 rpm for 10 minutes, evaporated, and reconstituted with 1 mL of methanol/water (1:1 *v*/*v*). The extracts were stored at −18 °C until use.

Extraction of bound phenolic compounds was carried out according to the method established by Verardo et al. (2011)[5]: residues of free phenolic extraction were digested with 25 mL of 1M NaOH at room temperature for 18 h by shaking under nitrogen gas. The mixture was acidified (pH 2.2–2.5) with hydrochloric acid in a cooling ice bath and extracted with 250 mL of hexane to remove the lipids. The aqueous solution was extracted five times with 50 mL of 1:1 diethyl ether/ethyl acetate (*v*/*v*). The organic fractions were collected and evaporated at 40 °C in a rotary evaporator. The dry extract was reconstituted in 1 mL of methanol/water (1:1 *v*/*v*) and stored at −18 °C until use.

2.4. Determination of Free and Bound Phenolic Compounds by HPLC–MS

A liquid chromatography apparatus HP 1100 Series (Agilent Technologies, Palo Alto, CA, USA) equipped with a degasser, a binary pump delivery system, and an automatic liquid sampler and coupled to a single quadrupole mass spectrometer detector was used. Separation of free and bound phenolic compounds from buckwheat flour fractions was carried out using a C-18 column (Poroshell 120, SB-C18, 3.0 × 100 mm, 2.7 μm from Agilent Technologies, Palo Alto, CA, USA). The gradient elution was the same as that previously established by Gómez-

Caravaca et al. (2014)[17] using as a mobile phase A acidified water (1% acetic acid) and as mobile phase B acetonitrile. MS analysis was carried out using an electrospray ionization (ESI) interface in negative ionization mode at the following conditions: drying gas flow (N_2), 9.0 L/min; nebulizer pressure, 50 psi; gas drying temperature, 350 °C; capillary voltage, 4000 V. The fragmentor and m/z range used for HPLC–ESI/MS analyses were 80 V and m/z 50–1000, respectively. Data were processed by the software MassHunter Workstation Qualitative Analysis Version B.07.00 (Agilent Technologies, Santa Clara, CA, USA).

2.5. Statistical Analysis

The results of quantification reported in this work are the averages of three repetitions (n = 3). Tukey's honest significant difference multiple comparison (one-way ANOVA) at the $p < 0.05$ level was evaluated by using the Statistica 7.0 software (StatSoft, Tulsa, OK, USA)

3. Results and Discussion

3.1. Analytical Parameters of the Method

An analytical validation of the method was performed considering linearity and sensitivity. In order to quantify phenolic compounds in buckwheat fractions, five calibrations curves were elaborated with the standards ferulic acid, catechin, quercetin, gallic acid, and rutin. **Table 1** includes the analytical parameters of the standards used, containing calibration ranges, calibration curves, determination coefficients, limit of detection (LOD), and limit of quantification (LOQ).

Table 1. Analytical parameters of the method proposed.

Standards	Calibration Ranges (mg/L)	Calibration curves (mg/g)	r2	LOD (mg/L)	LOQ (mg/L)

Ferulic acid	LOQ-100	y = 119572x + 16157	0.9995	0.0136	0.0452
Catechin	LOQ-100	y = 170925x + 8609.5	0.9994	0.0095	0.0316
Quercetin	LOQ-100	y = 402162x + 44862	0.9996	0.0040	0.0134
Gallic acid	LOQ-100	y = 123892x − 4971.6	0.9984	0.0131	0.0437
Rutin	LOQ-100	y = 199694x − 2067.2	0.9999	0.0081	0.0271

LOD: Limit of detection, LOQ: Limit of quantification.

Calibration curves were carried out by using the peak areas analyte standard against the concentration of the analyte for the analysis by HPLC. The external calibration of the standards was elaborated at different concentration levels from LOQ to 100 mg L^{-1}. All calibration curves revealed good linearity among different concentrations, and the determination coefficients were higher than 0.9994 in all cases. The method used for analysis showed LOD within the range 0.0040–0.0136 mg L^{-1}, the LOQ were within 0.0134–0.0452 mg L^{-1}.

3.2. Identification of Phenolic Compounds from Buckwheat Extracts by HPLC–MS

Free and bound phenolic compounds in buckwheat flour fractions extracts were analyzed by HPLC with MS detection, and the identification of these compounds was carried out by comparison of molecular weight in bibliography and when available, by co-elution with commercial standards.

A total of 25 free phenolic compounds were identified in buckwheat flours, among them five were phenolic acids and 20 were flavonoids, and they were previously identified in other works[4,18]. (**Table 2**).

Twenty-four bound phenolic compounds were identified in buckwheat flours: seven were phenolic acid derivatives and 17 were flavonoids, which were identified in previous works (**Table 3**)[5,18].

Table 2. Identification table of free phenolic compounds in buckwheat flours.

Peak	Retention Time	[M-H]	Molecular Formula	Compound	In Source Fragments
1	2.1	315	C13H15O9	2-hydroxy-3- O-β-d-glucopyranosylbenzoic acid	153
2	2.6	315	C13H15O9	Protocatechuic-4-O-glucoside acid	153
3	3.3	451	C21H23O11	Catechin-glucoside	289
4	4.1	341	C15H17O9	Caffeic acid hexose	179
5	4.2	289	C15H13O6	Catechin	
6	4.4	487	C21 H27 O13	Swertiamacroside	179
7	5.0	179	C9H7O4	Caffeic acid	
8	5.5	289	C15H13O6	Epicatechin	
9	6.2	561	C30H25O11	(Epi)afzelchin-(epi) catechin isomer A	543,289,271,435
10	6.8	447	C21H19O11	Orientin	
11	7.0	447	C21H19O12	Isorientin	
12	7.8	431	C21H19O10	Vitexin	
13	7.9	609	C27H29O16	Rutin	
14	7.9	441	C22H17O10	Epicatechin-gallate	289
15	8.0	833	C45H37O16	Epiafzelchin–epiafzelchin–epicatechin	
16	8.2	487	C21H27O13	Swertiamacroside	
17	8.3	463	C21H19O12	Hyperin	
18	8.7	727	C38H31O15	Epiafzelchin-epicatechin-O-methylgallate	455,289,271
19	9.4	455	C23H19O10	(−)-Epicatechin-3-(3”-O-methyl) gallate	289
20	9.5	561	C30H25O11	(Epi)afzelchin-(epi)catechin isomer B	543,425,289,271
21	9.9	757	C39H33O16	Procyanidin B2-dimethylgallate	
22	10.7	741	C39H33O15	Epiafzelchin–epicatechin-O-dimethylgallate	
23	11.5	469	C24H21O10	Epicatechin-O-3,4-dimethylgallate	
24	12.3	463	C21H19O12	Isoquercitrin	
25	12.6	301	C15H10O7	Quercetin	

Table 3. Identification of bound phenolic compounds in buckwheat flours.

Peak	Retention Time	[M-H]	Molecular Formula	Compound
1	2.1	315	C13H15O9	2-hydroxy-3-O-β-d-glucopyranosylbenzoic acid
2	2.6	315	C13H15O9	Protocatechuic-4-O-glucoside acid
3	3.2	341	C15H17O9	Caffeic acid hexose isomer a
4	4.1	341	C15H17O9	Caffeic acid hexose isomer b
5	4.2	289	C15H13O6	Catechin
6	4.4	487	C21H27O13	Swertiamacroside isomer a
7	5.0	179	C9H7O4	Caffeic acid
8	5.5	289	C15H13O6	Epicatechin
9	6.3	197	C9H9O5	Syringic acid
10	6.8	447	C21H19O11	Orientin
11	6.9	163	C9H7O3	p-coumaric acid derivative
12	7.0	575	C30H23O12	Procyanidin A
13	7.5	317	C15H9O8	Myricetin
14	7.8	431	C21H19O10	Vitexin
15	7.9	609	C27H29O16	Rutin
16	7.9	441	C22H17O10	Epicatechin gallate
17	8.2	451	C21H23O11	Catechin-glucoside
18	8.2	487	C21H27O13	Swertiamacroside isomer b
19	8.7	727	C38H31O15	Epiafzelchin–epicatechin-O-methylgallate
20	9.3	163	C9H7O3	p-coumaric acid
21	9.4	455	C23H19O10	(−)-epicatechin-3-(3’’-O-methyl) gallate
22	11.5	469	C24H21O10	Epicatechin-O-3,4-dimethylgallate
23	12.3	463	C21H19O12	Isoquercitrin
24	12.6	301	C15H10O7	Quercetin

3.3. Quantification of Free and Bound Phenolic Compounds in Buckwheat Fractions

Free phenolic compounds were quantified through of calibration curves of standards. A total of 25 free phenolic compounds were quantified in buckwheat meals/flours: de-hulled grain meal, bran meal, middling flour, and light flour (Table 4).

Table 4. Free phenolic compounds quantified in buckwheat meals/flours (mg/kg d.w.) determined by HPLC-MS.

Free Phenolic Compounds	**Bran Meal**	**Middling Flour**	**Light Flour**	**De-hulled Grain Meal**
2-hydroxy-3-O-β-Dglucopyranosylbenzoic acid	42.17 b	78.22 a	2.67 d	32.71 c
Protocatechuic-4-O-glucoside acid	79.69 b	120.59 a	2.93 d	65.56 c
Catechin-glucoside	23.87 b	34.97 a	1.88 d	13.53 c
Caffeic acid hexose	41.02 a	37.39 b	1.06 d	30.95 c
Catechin	20.40 a	17.25 b	1.36 d	7.33 c
Swertiamacroside	33.14 a	22.81 b	0.85 d	9.84 c
Caffeic acid	36.82 a	22.35 b	0.15d	0.96 c
Epicatechin	69.56 a	26.48 b	2.60 d	14.01 c
(Epi)afzelchin-(epi) catechin isomer A	58.11 a	35.49 b	1.71 d	20.06 c
Orientin	5.18 a	3.79 b	0.02 d	1.58 c
Isorientin	4.61 a	2.84 b	<LOQ	0.82 c
Vitexin	9.14 a	6.26 b	0.06 d	2.02 c
Rutin	214.99 a	148.63 b	7.03 d	87.33 c
Epicatechin-gallate	18.56 a	7.82 b	0.28 d	5.22 c
Epiafzelchin–epiafzelchin–epicatechin	20.37 a	12.69 b	0.84 d	8.01 c

Swertiamacroside	27.41 a	20.92 b	4.23 d	9.47 c
Hyperin	2.84 a	1.59 b	<LOQ	0.13 c
Epiafzelchin–epicatechin-O-methyl gallate	76.84 a	39.84 b	1.00 d	28.73 c
(−)-Epicatechin-3-(3”-O-methyl) gallate	31.61 a	17.77 b	0.51 d	15.18 c
(Epi)afzelchin-(epi) catechin isomer B	25.04 a	15.03 b	0.47 d	9.95 c
Procyanidin B2-dimethylgallate	51.46 a	29.22 b	0.67 d	21.06 c
Epiafzelchin–epicatechin-O-dimethylgallate	216.94 a	176.67 b	13.11 d	93.83 c
Epicatechin-O-3,4-dimethylgallate	98.07 a	8.05 c	2.31 d	39.10 b
Isoquercitrin	1.41 a,b	2.05 a	0.54 d	1.09 c
Quercetin	33.21 a	12.39 b	0.06 d	2.27 c
Flavonoids	982.23 a	598.23 b	34.47 d	371.25 c
Phenolic acids	260.26 b	302.28 a	11.89 d	149.49 c
Sum	1242.49 a	901.10 b	46.36 d	520.74 c

Different letters in the same line show significant differences ($p < 0.05$), LOQ: Limit of quantification.

The most concentrated free phenolic compound in all buckwheat flours was epiafzelchin–epicatechin-*O*-dimethylgallate, whose content was 13.11 mg/kg d.w. in light flour, 93.83 mg/kg d.w. in de-hulled grain meal, 176.67 mg/kg d.w. in middling flour, and 216.94 mg/kg d.w. in bran meal. The second most concentrated in buckwheat flours was rutin, whose content was from 7.03 mg/kg d.w in light flour, 87.33 mg/kg d.w. in de-hulled grain meal, 148.63 mg/kg d.w. in middling flour, to 214.99 mg/kg d.w in bran meal. Thus, the most abundant free flavonoids are present in buckwheat bran meal, followed by middling flour, de-hulled buckwheat meal, and light flour. Besides, 2-hydroxy-3-*O*-β-D-

glucopyranosylbenzoic and protocatechuic-4-*O*-glucoside acid appear in buckwheat fractions in significant quantities, whose values were 2.67–2.93 mg/kg d.w. in light flour, 32.71–65.56 mg/kg in de-hulled grain meal, 42.17–79.69 mg/kg d.w. in bran meal, and 78.22–120.56 mg/kg d.w. in middling flour. Therefore, the highest content of phenolic acids appears in middling flour, followed by bran meal, de-hulled grain meal, and light flour. The third most abundant phenolic compound in middling and de-hulled grain meal was protocatechuic-4-*O*-glucoside acid (120.59 and 65.56 mg/kg d.w.), whereas in light flour was swertiamacroside (4.23 mg/kg d.w.), and in bran meal was epicatechin-*O*-3,4-dimethylgallate (98.07 mg/kg d.w.).

The total free phenolic content in buckwheat flours was decreasing in the following order: bran meal > middling flour > de-hulled buckwheat meal > light flour (1242.49, 901.10, 520.74, and 46.36 mg/kg d.w.). These results are due to the most abundant free phenolic compounds being flavonoids, which corresponded to 66–79% of total free phenolic compounds, and these are found in higher concentration in outer layers than in inner layers of buckwheat grain[2]. For that reason, bran meal contains the highest content of free phenolic compounds, followed by middling flour, as it contains seed coat.

The concentration of free phenolic compounds obtained in buckwheat was compared with that obtained previously in other works. Verardo et al. (2011)[5] quantified the individual free phenolic compounds in de-hulled buckwheat grain, where rutin was the most concentrated, whose value was 35.12% higher than that obtained in the present work and total content of free phenolic compounds was 48.39% higher than in the present work. Nevertheless, the most concentrated free phenolic compound in our work was epiafzelchin–epicatechin-*O*-dimethylgallate, whose value was 50% higher than that obtained by Verardo et al. (2011)[5]. These differences of concentration could be due to the different buckwheat cultivar. Besides, Inglett et al. (2011)[18] quantified the free flavonoid content in different buckwheat flours (fancy, farinetta, supreme, and whole), fancy corresponded with light flour, supreme flour is similar to bran meal, farinetta consists of a fine granulated mixture of aleurone layer of hulled achene and achene embryo, a

composition similar to middling flour[19,20]. The value of free flavonoids obtained in our study in light flour, de-hulled grain meal, bran meal, and middling flour (34.47 mg/kg d.w., 371.25 mg/kg d.w., 982.23 mg/kg d.w., and 598.83 mg/kg d.w) were in the same order of magnitude than that obtained in fancy (71.40 mg/kg d.w.), whole buckwheat flour (417.03 mg/kg d.w.), supreme (525.27 mg/kg d.w.), and farinetta (671.50 mg/kg d.w.) by Inglett et al. (2011)[18].

Hung et al. (2008)[2] reported the content of rutin in the free form obtained in different buckwheat flour fractions, and its concentration was 2.5–3 mg/kg d.w. in the innermost layers, whereas in the outer layers, it was 274-337.8 mg/kg. These results were similar to those obtained in the present work in the light flour (7.03 mg/kg dw.) and bran meal (214.99 mg/kg d.w.). Kalinová et al. (2019)[21] reported the free phenolic compounds in the seed coat (553.18 mg/kg d.w.), in the endosperm (2.59 mg/kg d.w.), and in the groat (139.66 mg/kg d.w.). These values were lower than those obtained in bran meal, light flour, and de-hulled grain meal, and also, the content of rutin in seed coat (54.23 mg/kg d.w.) represents a quart of the phenolic bran meal (214.99 mg/kg d.w.) obtained in our study. This could be due to the different cultivar and/or the different methodology of determination of phenolic compounds (by MS detection a higher number of compounds are determined).In addition, Liu et al. (2019)[22] reported the concentration of rutin in common buckwheat (62.19 mg/kg d.w.) that was in the same order as that obtained in de-hulled grain meal (87.33 mg/kg d.w.). According to the results obtained in these previous works, it has shown that rutin in the free form is concentrated in the outer layers, which is in concordance with our results.

The Table 5 reports the content of bound phenolic compounds in buckwheat flours. Bound phenolic compounds composition in buckwheat flours was similar than that obtained in free phenolic fraction; nevertheless, flavonoids such as isorientin, epiafzelchin–epiafzelchin–epicatechin, Procyanidin B2-dimethylgallate, hyperin, and (epi)afzelchin-(epi)catechin were not detected in bound fraction, whereas some phenolic acids such as syringic and p-coumaric acid, procyanidin A, and myricetin were determined only in bound fraction.

Table 5. Bound phenolic compounds quantified in buckwheat meals/flours (mg/kg d.w.) determined by HPLC–MS.

Bound Phenolic Compounds	Bran Meal	Middling Flour	Light Flour	De-Hulled Grain Meal
2-hydroxy-3-O-β-d-glucopyranosylbenzoic acid	23.02 b	34.56 a	6.19 c,d	7.88 d
Protocatechuic-4-O-glucoside acid	18.44 b	25.50 a	5.51 c	5.95 c
Caffeic acid hexose isomer a	5.52 b	11.34 a	0.67 c	0.43 c,d
Caffeic acid hexose isomer b	40.42 b	56.73 a	13.28 d	26.35 c
Catechin	207.74 a	200.17 a	54.67 c	95.45 b
Swertiamacroside	23.25 c,d	31.84 a,b	25.40 d	33.66 a
Caffeic acid	<LOQ	<LOQ	<LOQ	<LOQ
Epicatechin	59.08 b	97.50 a	34.67 d	41.55 c
Syringic acid	85.86 a	43.57 b	7.74 d	35.62 c
Orientin	0.46 a	0.56 a	0.19 c	0.22 b
p-coumaric acid derivative	9.65 a	3.53 b	1.39 d	3.24 c
Procyanidin A	8.82 a	9.03 a	0.95 c	4.95 b
Myricetin	4.12 a	3.80 a	2.06 b,c	2.92 b
Vitexin	4.22 a	3.86 a	0.67 c	2.30 b
Rutin	51.64 a	45.19 b	6.82 d	33.71 c
Epicatechin gallate	16.24 a	15.57 a	4.21 c	10.75 b
Catechin-glucoside	16.48 a	17.51 a	1.04 c	13.26 b
Swertiamacroside	39.40 a	32.37 b	23.52 d	30.43 c
Epiafzelchin–epicatechin-O-methylgallate	28.04 a	27.81 a	3.57 c	9.72 b
p-coumaric acid	3.96 b	6.91 a	0.67 d	2.74 c
(−)-epicatechin-3-(3”-O-methyl) gallate	6.09 a	6.05 a	2.06 c	4.17 b

Epicatechin-O-3,4-dimethylgallate	4.65 a	4.11 a	0.50 c	1.78 b
Isoquercitrin	6.06 a	5.89 a	1.03 c	3.64 b
Quercitrin	26.64 a	21.05 b	10.94 d	18.78 c
Flavonoids	440.29 b	458.11 a	123.37 d	243.20 c
Phenolic acids	249.52 a	246.35 b	84.37 d	146.31 c
Total	689.81 b	704.47 a	207.74 d	389.51 c

Different letters in the same line show significant differences ($p < 0.05$), LOQ: Limit of quantification.

Catechin was the most concentrated bound phenolic compound in all buckwheat flours, representing 25–30% of total bound phenolic compounds, and its concentration was 54.67 mg/kg d.w. in light flour, 95.45 mg/kg d.w. in de-hulled grain meal, 200.17 mg/kg d.w. in middling flour, and 207.74 mg/kg d.w. in bran meal, respectively. The second component most abundant was epicatechin, whose content was 34.67 mg/kg d.w. in light flour, 41.55 mg/kg d.w. in de-hulled grain meal, 59.08 mg/kg d.w. in bran flour, and 97.50 mg/kg d.w. in middling flour. The third most abundant phenolic compound in de-hulled grain meal and bran meal was syringic acid (35.62 mg/kg d.w. and 85.86 mg/kg d.w.), whereas in middling flour it was caffeic acid hexose (56.73 mg/kg d.w.), and in light flour it was swertiamacroside.

The total bound phenolic content in buckwheat flours was increasing in the following order: light flour < de-hulled grain meal < bran meal < middling flour (207.74, 389.51, 689.81, and 704.47 mg/kg d.w.). Therefore, the highest concentration of bound phenolic compounds is in middling and bran meal due to these compounds being linked to the cell wall of buckwheat layers. Flavonoids represented 59–65% of the bound phenolic fraction. Whereas, phenolic acids represented 35–41% of bound phenolic fraction.

Concentrations of catechin, epicatechin, syringic, and total bound phenolic compounds in de-hulled whole buckwheat flour obtained by Verardo et al. (2011)[5]

were 23.88%, 48.54%, and 53.18% higher than those obtained in the present work. Inglett et al. (2011)[18] reported the content of total bound flavonoid in buckwheat flour fractions obtained was 59.25 mg/kg d.w. in fancy, 389.68 mg/kg in farinetta, 530.21 mg/kg in supreme, and 613.77 mg/kg d.w. in whole flour, which are in the same order of magnitude as that obtained in our work. Nevertheless, in this study, the highest bound phenolic content was obtained in whole buckwheat flour, whereas in our work, the maximum value of phenolic content corresponded with the middling flour. This could be due to the different cultivar or because Inglett et al. (2011)[18] could include the hull in the buckwheat grain flour.

Table 6. Total content of flavonoids, phenolic acids, and phenolic compounds in buckwheat flours. Results are expressed as mg/kg d.w.

	Flavonoids	**Phenolic Acids**	**Total**
Bran meal	1422.52 a	509.78 b	1932.30 a
Middling flour	1056.94 b	548.63 a	1605.57 b
Light flour	157.84 d	96.261 d	254.10 d
De-hulled grain meal	614.46 c	295.80 c	910.25 c

Different letters in the same column show significant differences ($p < 0.05$).

The total content of flavonoids was from 157.84 mg/kg d.w. in light flour to 1422.52 mg/kg d.w. in bran meal, whereas the content of phenolic acids was from 96.261 mg/kg d.w. in light flour to 548.63 mg/kg d.w. in middling flour. Total phenolic content was from 254.10 mg/kg d.w. in light flour to 1932.30 mg/kg d.w. in bran meal (**Table 6**). According to the results, the total phenolic content was increasing in the following order: light flour < de-hulled grain meal < middling flour < bran meal Therefore, middling flour and bran meal possess the highest phenolic content due to bran and the aleurone layer being richer in many phenolic compounds than the others buckwheat flours[23]. Total flavonoid obtained in de-hulled grain meal, bran meal, and middling flour was 49.22%, 71.21%, and 27.83% higher than that obtained in whole grain meal, supreme, and farinetta by liquid chromatography-electrospray ionization- mass spectrometry (LC–ESI-

MS)[18]. According to Guo and co-workers, free rutin was determined in a range of 51–81%[24].

4. Conclusions

An HPLC–MS has been used for the determination of free and bound phenolic compounds in buckwheat flours: middling flour, bran meal, light flour, and whole meal. The results of this study have shown that the total free phenolic compounds are found in the highest concentration in bran meal, whereas the bound content of phenolic compounds are concentrated in middling flour and bran meal. In buckwheat flours, the main flavonoids were rutin and epiafzelchin–epicatechin-O-dimethylgallate, which had the greatest content in bran meal. By contrast, catechin and epicatechin were the main bound flavonoids in buckwheat meal/flours that existed in the greatest quantities in middling and bran fours.

To conclude, the bran meal and middling flour could be considered as flours enriched in phenolic compounds that could be used to elaborate food with health benefits. Moreover, it has been proved, as the distribution of some phenolic compound varied from bran to middling fraction.

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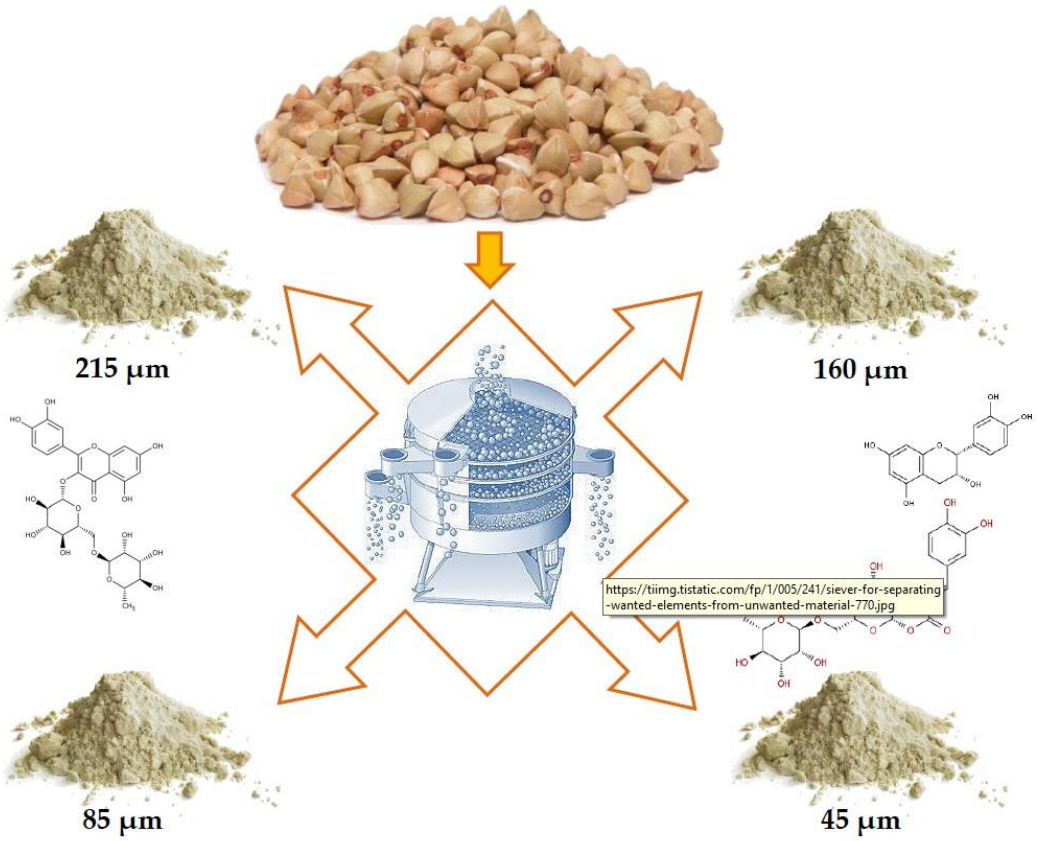
Chapter 7

Use of sieving as a valuable technology to produce enriched buckwheat flours: A Preliminary study



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Use of Sieving as a Valuable Technology to Produce Enriched Buckwheat Flours: A Preliminary Study

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Abstract:

Fractionation processes based on physical separation are a good strategy to produce enriched cereal flours. Therefore, the aim of this work is to evaluate the suitability of sieving of buckwheat flours to produce protein and phenolic (especially rutin) enriched fractions. Because of that, dehulled whole buckwheat flour (GSTQ) was sieved obtaining fractions with a particle size of 215 µm, 160 µm, 85 µm, and 45 µm (GS215, GS160, GS85, and GS45). For that purpose, the determination of protein, ash, and total starch content and free and bound phenolic compounds was carried out. The highest content of total phenolic compounds was obtained in GS215 (3118.84 mg Kg^{-1} d.w.), followed by GS160 (2499.11 mg Kg^{-1} d.w.), GS85 (989.46 mg Kg^{-1} d.w.), GSTQ (983.15 mg Kg^{-1} d.w.), and GS45 (481.31 mg Kg^{-1} d.w.). Therefore, the phenolic content decreased with the particle size decrease from 215 µm to 45 µm. Besides, there were no significant differences between the total phenolic content in GS85 and GSTQ. The fraction with 215 µm reported the highest

protein and mineral salt content and presented rutin amounts four times higher than GSTQ.

Keywords: common buckwheat; free and bound phenolic compounds; HPLC-MS; sieving; proteins; starch

1. Introduction

Buckwheat (*Fagopyrum esculentum Moench*) is a rich source of protein, vitamins, starch, dietary fiber, and essential minerals[1-3]. Buckwheat also contains a high quantity of phenolic compounds, including rutin, orientin, vitexin, quercetin, isovitexin, kaempferol-3-rutinoside, isoorientin, and catechins[4]. Buckwheat contains more rutin than most of the other plants, which exhibits anti-inflammatory, antimutagenic, anticarcinogenic, antihemorrhagic, antioxidative, hypotensive, antihemorrhagic, and blood vessel protecting properties[5-8]. Phenolic compounds are presented in both free and bound forms. Whole buckwheat contains 2–5 times more phenolic compounds than oats or barley, while buckwheat bran and hulls have 2–7 times higher antioxidant activity than barley, triticale, and oats[9]. Most studies have reported that phenolic compounds are mostly bound to cell wall components in the bran and hull of most cereal grains[10]. Nevertheless, in buckwheat most phenolic compounds are found in the free form distributed throughout the entire grain (hull, seed coat, endosperm embryo axis, and cotyledons)[5,11]. The greatest concentration of these phenolic compounds is presented in the outer layers (seed coat and hull) of the grain[5]. During buckwheat flour processing, hull is removed from buckwheat seeds by impact milling and the resulting groat (or the intact achene) is roller-milled and the product is sieved to remove the fragmented hull to obtain bran flour that contains seed coat and light flour that is composed mainly of the endosperm[1,12]. One study has shown that seed coat is the part with the highest total content of phenolics from all parts of the groat[13]. Inglett et al. (2011)[9] evaluated the phenolic content in fancy (endosperm), farinetta (seed coat), supreme (whole groat), and whole buckwheat flour (whole grain), being the farinetta (seed coat) flour the most concentrated in phenolic compounds.

Therefore, consumption of buckwheat flours that contents seed coat is considered to have significant nutritional or medicinal benefits[4].

The trend toward fractionation/enrichment and recombination techniques has captured the attention of the food industry in order to identify and develop green new processes respectful of the nutritional and hygienic quality of the matrix and increasing the quality of foods. In this way, separation and/or enrichment with dry fractionation technologies such as pearling/grinding, sieving, and air classification could be useful to obtain grain fractions with added value. Moreover, the products obtained with these technologies have considerable high quality in the safety point of view, compared with those obtained with other traditional methods that use the solvent extraction or chemical fractionation as enrichment process[14-16].

One study reported the distribution of phenolic compounds in buckwheat graded fractions, where the hull was removed from whole buckwheat grains by dehulling apparatus with disks, and the remained groats with endosperm and bran were milled to buckwheat flours and separated by weight from outer to inner parts in 16 fractions, with the fraction that contained the outermost part of the grain (bran) being the most concentrated in phenolic content[5]. It has been reported that whole grain rice flours, whole grain wheat flours, and wheat bran fours sieved with different particle size have shown different phenolic concentrations because of the different parts obtained from the buckwheat after the sieving[17-19]. Nevertheless, there is no study about buckwheat flour fractions from whole grain with different particle size, which would allow a gradual reduction milling system and this could be advantageous in order to obtain enriched flour fractions for the obtention of desired end-use products of high functionality[11].

For that reason, in this work the sieving of whole buckwheat flours at different particle size was carried out in order to evaluate the fractions enriched in phenolic compounds with particular attention to rutin and protein. The determination of ashes, proteins, total starch, and free and bound phenolic compounds in buckwheat flours was carried out.

2. Materials and Methods

2.1. Sample

Dehulled buckwheat grain meal (GSTQ) was obtained from buckwheat (cv. Darja) harvested in Matrice (Italy) (41°37′00″ N 14°43′00″ E), situated in a hilly location 750 m above sea level. The field presented high tenacity of the soil because of the presence of clay. Harvesting took place on September 2018. Dehulled buckwheat achenes were milled by hammer mill (model 8/B, Beccaria srl, Scarnafigi (CN), Italy); GSTQ meal was sieved to obtain four fractions with different particle size: 215 µm (GS215), 160 µm (GS160), 85 µm (GS85), and 45 µm (GS45).

2.2. Reagents and Chemicals

HPLC-grade acetonitrile, water, methanol, acetone, acetic acid, ethanol, hexane, ethyl acetate, diethyl ether, hydrochloric acid, sulphuric acid, ammonium sulphate, and boric acid were purchased from Merck KGaA (Darmstadt, Germany). Hydroxide sodium was from Fluka (Buchs, Switzerland). Ferulic acid, catechin, quercetin, and rutin (Sigma-Aldrich, St. Louis, MO, USA) were used for the calibration curves. Glucosidase, amyloglucosidase, peroxidase, and α-amylase were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Protein, Ashes, and Total Starch Determination in Buckwheat Samples

2.3.1. Determination of Protein

Determination of protein in buckwheat samples was carried out according to ICC method 105/2 (1995)[20]. Briefly, 1 g of sample was subjected to mineralization of organic matter with 10 mL of sulphuric acid in the presence of copper sulphate. Hence, nitrogen was changed in ammonium sulphate and treated with NaOH. The ammonia

released was gathered in a solution of 4% boric acid and titrated with 0.1 N sulphuric acid.

2.3.2. Determination of Ashes Content

Determination of ashes content was carried out according to ICC method 104/1 (1995)[21]. A total of 1 g of buckwheat flour was collected in a porcelain crucible in muffle furnace at 525 °C for 1 h and then cooled. After that, the sample was charred with ethanol and put in muffle at 525 °C. Ashing was completed when the cooled residue was white or nearly white. Finally, porcelain crucibles were weighed, and ashes content was calculated.

2.3.3. Determination of Total Starch

The total starch in buckwheat samples was determined according to an enzymatic colorimetric method, AOAC International method 996.11 (AOAC, 2007)[22], with an assay kit from Megazyme International Ltd. (Wicklow, Ireland). Samples were ground through a 0.5-mm screen and 100.0 mg of sample was incorporated to a test tube. After that, 0.2 mL of ethanol solution (80%, *v/v*) was added into the tube and mixed to wet the sample. Then, 3 mL of thermostable α-amylase was added, and the tubes were boiled for 6 min and were shaken at intervals of 2 min. Tubes were placed in a 50 °C bath to rest for 5 min. Next, 0.1 mL of amyloglucosidase was added into each tube. Tubes were then shaken and incubated over 30 min and then filled to a volume of 10 mL with distilled water followed by centrifugation at 1800 rpm for 10 min. Then, 1.0 mL of aliquots from the supernatant was diluted in a proportion of 1/10. Next, 0.1 mL of this solution was placed into a test tube. Total of 3 mL of glucose oxidase/peroxidase reagent was added to each tube and incubated at 50 °C for 20 min. A total of 0.1 mL of water was used for blanks rather than 0.1 mL of diluted solution, and the other added reagents were all the same. Samples were read at 510 nm.

2.4. Extraction Methods

Extraction of free phenolic compounds from buckwheat flour fractions was carried out according to the method established by Hung and Morita (2008)[5] with certain modifications in the extraction technique and the solvent used to reconstitute the dry extract. One gram of buckwheat flour was extracted thrice in an ultrasonic bath Starsonic 90 Liarre (Bologna, Italy) equipment with frequency 34 kHz, output power (W) 190RMS, dimensions (H × W × D) 345 × 315 × 246 cm with a solution of ethanol/water (4:1 *v/v*) for 10 min. The supernatants were collected, centrifuged at 2500 rpm for 10 min, evaporated and reconstituted with 1 mL of methanol/water (1:1 *v/v*). The extracts were stored at −18 °C until analysis.

Extraction of bound phenolic compounds was carried out according to the method established by Verardo et al. (2011)[23]: Residues of free phenolic extraction were digested with 25 mL of 1M NaOH at room temperature for 18 h by shaking under nitrogen gas. The mixture was acidified (pH = 2.2–2.5) with hydrochloric acid in a cooling ice bath and extracted with 250 mL of hexane to remove the lipids. The aqueous solution was extracted five times with 50 mL of 1:1 diethyl ether/ethyl acetate (*v/v*). The organic fractions were collected and evaporated at 40 °C in a rotary evaporator. The dry extract was reconstituted in 1 mL of methanol/water (1:1 *v/v*) and stored at −18 °C until analysis.

2.5. Determination of Free and Bound Phenolic Compounds by HPLC- MS

A liquid chromatography apparatus HP 1100 Series (Agilent Technologies, Palo Alto, CA, USA) equipped with a degasser, a binary pump delivery system, and an automatic liquid sampler, and coupled to single quadrupole mass spectrometer detector was used. Separation of free and bound phenolic compounds from buckwheat flour fractions was carried out using a C-18 column (Poroshell 120, SB-C18, 3.0 × 100 mm, 2.7 μm from Agilent Technologies, Palo Alto, CA, USA). The gradient elution was the same as previously established by Gómez-Caravaca et al. (2014)[24] using a mobile phase A acidified water (1% acetic acid) and mobile phase B acetonitrile. MS analysis were carried out using an electrospray ionization (ESI) interface in negative ionization mode at the following conditions: drying gas flow (N_2), 9.0 L/min; nebulizer pressure,

50 psi; gas drying temperature, 350 °C; capillary voltage, 4000 V. The fragmentor and m/z range used for HPLC-ESI/MS analyses were 80 V and *m/z* 50–1000, respectively.

Calibration curves were arranged from LOQ-500 mg/L at six concentration levels, plotting peak area vs. analyte concentration.

2.6. Statistical Analysis

The results of quantification reported in this work are the averages of three repetitions ($n = 3$). Tukey's honest significant difference multiple comparison (one-way ANOVA) at the $p < 0.05$ level were evaluated by using the Statistica 7.0 software (StatSoft, Tulsa, OK, USA)

3. Results and Discussion

3.1. Yield, and Protein, Starch, and Ashes Composition in Buckwheat Samples

One of the main trends in food technologies is the use of the technological model known as fractionation/enrichment and food recombination. It consists of a preliminary extraction of constituents or enrichment of fractions (proteins, lipids, carbohydrates, fibers, flavors, dyes, etc.,), which are subsequently recombined in order to obtain improved products in terms of nutritional value and dietary value. **Table 1** shows the values of yield and some chemical components (protein ashes and total starch) in dehulled buckwheat flour (GSTQ) and its sieved fractions with 215 µm, 160 µm, 85 µm, and 45 µm in order to evaluate the most nutritionally adequate fraction.

Table 1. Evaluation of some chemical components (g/100 g d.w.) of dehulled buckwheat and fractions results from sieving.

	GSTQ	**GS215**	**GS160**	**GS85**	**GS45**
Yield	100	13.5	8.7	32.0	43.3
Protein (N × 6.25)	16.4 ± 0.04	35.2 ± 0.03	29.8 ± 0.04	11.3 ± 0.02	8.1 ± 0.06
Ashes	2.36 ± 0.003	6.05 ± 0.002	5.56 ± 0.001	1.51 ± 0.003	0.31 ± 0.002
Total Starch	72.6 ± 1.49	34.4 ± 1.10	43.3 ± 1.43	76.7 ± 2.00	81.2 ± 1.13

GSTQ : Dehulled buckwheat flour): ; GS215: ; GS160: ; GS85: ; GS45: Sieved fractions with 215, 160, 85 and 45 µm

As expected, the yield of GS215 and GS160 fraction is enormously lower than GS85 and GS45 fraction that correspond to the inner layers of buckwheat achene.

Protein content increased two-fold in GS215 and GS160 fractions; in contrary, it halves in GS85 and GS45 fractions. According to Schutyser et al.(2011)[25] these results confirmed that dry fractionation technologies such as sieving are a valuable tool to produce enriched protein fractions, moreover, the same authors declared that this type of technology is extremely energy efficient and is able to produce enriched fractions with retained (native) functionality compared to other green technologies such as wet fractionation.

GS 215 and GS 160 also triple the ashes content that could be related to the mineral amount; otherwise, the fractions with highest particle size showed middle content of total starch compared with GS 85 and GS45 samples.

3.2. Analytical Parameters of the Method Proposed

An analytical validation of the method was performed considering linearity and sensitivity. In order to quantify phenolic compounds in buckwheat fractions, five calibration curves were elaborated with the standards ferulic acid, catechin, quercetin, gallic acid, and rutin. **Table 2** includes the analytical parameters of the standards used containing calibration ranges, calibration curves, determination coefficients, limit of detection (LOD), and limit of quantification (LOQ).

Table 2. Analytical parameters of the method proposed.

Standards	Calibration Ranges (mg/L)	Calibration Curves (mg/g)	R^2	LOD (mg/L)	LOQ (mg/L)
Ferulic acid	LOQ-500	y = 119572x + 16157	0.9985	0.0136	0.0452
Catechin	LOQ-500	y = 170925x + 8609.5	0.9994	0.0095	0.0316
Quercetin	LOQ-500	y = 402162x + 44862	0.9996	0.0040	0.0134
Gallic acid	LOQ-500	y = 123892x − 4971.6	0.9984	0.0131	0.0437
Rutin	LOQ-500	y = 199694x − 2067.2	1	0.0081	0.0271

LOD: limit of detection, and LOQ: limit of quantification.

Calibration curves were carried out by using the peak areas of analyte standard against the concentration of the analyte for the analysis by HPLC. All calibration curves revealed good linearity among different concentrations, and the determination coefficients were higher than 0.9984 in all cases. The method used for analysis showed LOD within the range 0.0040–0.0136 mg L^{-1}, the LOQ were within 0.0134–0.0452 mg L^{-1}.

3.3. Identification of Phenolic Compounds in Buckwheat Fractions

Free and bound phenolic compounds in buckwheat flour fractions extracts were analyzed by HPLC with MS detection and were identified by rendering their mass spectra using the data reported in the literature and, when available, by co-elution with commercial standards (**Table 3**). A total of 32 phenolic compounds were identified in whole buckwheat flours fractions, which have been identified in previous works[9,23,26]. Among the 32 total phenolic compounds, 25 were free phenolic compounds and 26 were bound phenolic compounds (**Figure S1**), identifying some of them both in the free and in the bound form.

Table 3. Table of identification of free and bound phenolic compounds from whole buckwheat flour and its fractions.

Peak	Retention Time	[M–H]−	Molecular Formula	Compound	Free	Bound	Ion Source Fragments
1	2.07	315	C13H15O9	2-Hydroxy-3-O-βD-glucopyranosyl benzoic acid	+	+	
2	2.58	315	C13H15O9	Protocatechuic-4-O-glucoside acid	+	+	
3	3.22	341	C15H17O9	Caffeic acid hexose	N.D.	+	251
4	3.30	451	C21H23O11	Catechin-glucoside isomer A	+	+	289
5	4.08	341	C15H17O9	Caffeic acid hexose	+	+	179
6	4.17	289	C15H13O6	Catechin	+	+	
7	4.40	487	C21H27O13	Swertiamacroside isomer A	+	+	451
8	4.96	179	C9H7O4	Caffeic acid	+	+	
9	5.49	289	C15H13O6	Epicatechin	+	+	
10	6.25	561	C30H25O11	(Epi)Afzelchin-(Epi) catechin Isomer A	+	+	543, 425, 289
11	6.26	197	C9H9O5	Syringic acid	N.D.	+	
12	6.77	447	C21H19O11	Orientin	+	+	357
13	6.96	447	C21H19O11	Isoorientin	+	N.D.	
14	6.86	163	C9H7O3	p-Coumaric acid	N.D.	+	
15	7	575	C30H23O12	Procyanidin A	N.D.	+	289,285
16	7.46	317	C15H9O8	Myricetin	N.D.	+	
17	7.76	431	C21H19O10	Vitexin	+	+	
18	7.92	609	C27H29O16	Rutin	+	+	
19	7.94	441	C22H17O10	Epicatechin gallate	+	+	289, 169
20	7.96	833	C45H37O16	Epiafzelchin–epiafzelchin–epicatechin	+	N.D.	
21	8.21	451	C21H23O11	Catechin-glucoside isomer B	N.D.	+	289
22	8.23	487	C21H27O13	Swertiamacroside isomer B	+	+	451
23	8.28	463	C21H19O12	Hyperin	+	N.D.	
24	8.73	727	C38H31O15	Epiafzelchin–epicatechin-O-methylgallate	+	+	461, 289
25	9.31	163	C9H7O3	p-Coumaric acid	N.D.	+	
26	9.43	455	C23H19O10	(−)-Epicatechin-3-(3′’-O-methyl) gallate	+	+	289, 183
27	9.47	561	C30H25O11	(Epi)afzelchin-(Epi) catechin Isomer B	+	N.D.	543, 425, 289
28	9.9	757	C39H33O16	Procyanidin B2-dimethylgallate	+	N.D.	289
29	10.71	741	C39H33O15	Epiafzelchin–epicatechin-O-Dimethylgallate	+	N.D.	469, 319, 271

30	11.50	469	C24H21O10	Epicatechin-O-3,4-Dimethylgallate	+	+	319, 271
31	12.35	463	C21H19O12	Isoquercitrin	+	+	
32	12.56	301	C15H10O7	Quercetin	+	+	

+: detected, N.D.: not detected.

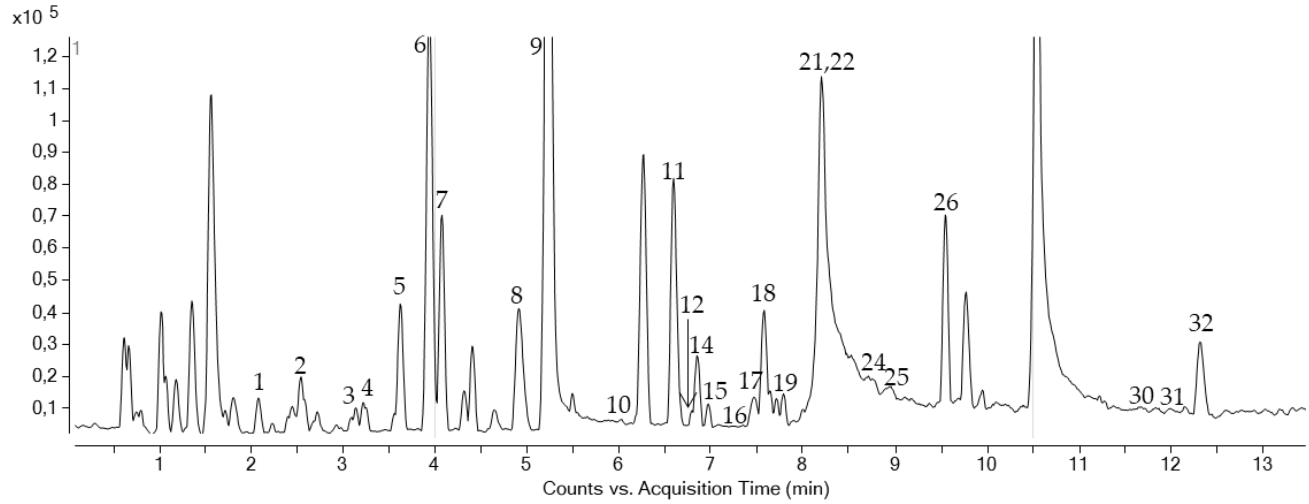

Figure S1. Base peak chromatogram (BPC) of bound phenolic compounds in buckwheat flour fraction GST215, obtained by HPLC-MS. See Table 3 for identification numbers

3.4. Quantification of Phenolic Compounds in Buckwheat Fractions

A total of 25 free phenolic compounds were quantified in whole grain flour (GSTQ) and its fractions (GS215, GS160, GS85, and GS45) (**Table 4**). Flavonoids are the most abundant free phenolic compounds in buckwheat, which represented 73%, 66.2%, 65.6%, 75.8, and 75.8% of total phenolic content in whole grain flour and fractions (GSTQ, GS215, GS85, and GS45). The most concentrated flavonoid was epiafzelchin–epicatechin-*O*-dimethylgallate, which corresponded around 14–16% of total free phenolic compounds in whole grain flour and its fractions. The highest content of epiafzelchin–epicatechin-*O*-dimethylgallate was obtained in GS215 (225.36 mg Kg^{-1} d.w.), in which the value was 58.4%, 17.3%, 63.4%, 79.5% higher than in GSTQ, GS160, GS85, and GS45. Besides that, the most concentrated phenolic acid derivative was protocatechuic-4-*O*-glucoside acid, which represented 11.1%,

15.7%, 15.4%, 9.4%, and 9.4% of the total free phenolic content, in which the highest value was obtained for GS215 followed by GS160, GSTQ, GS85, and GS45. Rutin was the second most abundant phenolic compound in whole grain flour and its sieved fractions with 45 µm and 85 µm, whereas this compound was the third most abundant in sieved fractions with 215 µm and 160 µm. Concentration of rutin in buckwheat flours decreased in the following order: GS215 > GS160 > GSTQ > GS85 > GS45 (195.47, 175.70, 87.33, 77.84, and 43.59 mg kg^{-1} d.w.).

Total free phenolic concentration decreased in the following order: GS215 > GS160 > GSTQ > GS85 > GS45. Therefore, the greatest content of free phenolic compounds was obtained in GS215 (1153.52 mg Kg^{-1} d.w.), in which the value was 14.7%, 66.9%, 61.9%, and 81.5% higher than that obtained in GS160, GS85, GSTQ, and GS45.

Comparing our results of phenolic content in whole buckwheat flour (GSTQ) with previous works, Verardo et al. (2011)[23] obtained a total free phenolic content in whole buckwheat flour of 1008.91 mg Kg^{-1} d.w., which was 41.43% higher than that obtained in our work. But these differences could be due to the different cultivar. Verardo et al. (2011)[23] reported that the highest free flavonoid was the rutin, whereas in our work, the most concentrated flavonoid was epiafzelchin–epicatechin-*O*-dimethylgallate followed by rutin. Kalinová et al. (2019)[13] reported the phenolic content in different parts of common buckwheat, in which the content of catechin, epicatechin, and rutin (20.87, 56.51 and 52.48 mg Kg^{-1} d.w.) in groat was in the same order of magnitude as that obtained for whole buckwheat flour in our work . Liu et al. (2019)[27] reported the phenolic profiles and antioxidant capacities of common buckwheat and Tartary buckwheat, in which the content of rutin in common buckwheat was 62.19 mg Kg^{-1} d.w. and this value was similar to that obtained in whole buckwheat in the present study (87.33 mg Kg^{-1} d.w.)[27]. Hence, our results are in accordance with the previous studies.

Table 4. Table of quantification of free phenolic compounds from whole buckwheat flour (GSTQ GS160, GS85, and GS45) analyzed by HPLC-MS expressed as mg Kg^{-1} d.w

Phenolic Compound	GSTQ-Free	GS215-Free	GS160-Free	GS8
2-Hydroxy-3-O-β D-glucopyranosyl benzoic acid	42.71 ± 1.07c	144.52 ± 1.88a	128.46 ± 2.46b	33.4
Protocatechuic-4-O-glucoside acid	65.56 ± 2.07c	242.95 ± 2.41a	203.55 ± 1.93b	48.5
Catechin-glucoside	23.53 ± 0.33c	45.91 ± 0.70a	40.24 ± 0.58b	22.8
Caffeic acid hexose	30.95 ± 0.74c	107.51 ± 1.55a	100.22 ± 0.92b	23.2
Catechin	27.33 ± 0.12c	72.30 ± 2.04a	64.31 ± 1.36b	21.9
Swertiamacroside	9.84 ± 0.16c	15.79 ± 1.44a	10.96 ± 1.02b	8.25
Caffeic Acid	0.01 ± 0.001c	0.06 ± 0.003a	0.024 ± 0.001b	<LO
Epicatechin	44.01 ± 1.48c	118.75 ± 3.02a	96.29 ± 2.64b	43.5
(Epi)Afzelchin-(epi) catechin isomer A	20.06 ± 1.11c	39.05 ± 0.81a	31.44 ± 0.69b	20.3
Orientin	1.58 ± 0.20c	5.64 ± 0.39a	3.12 ± 0.37b	1.00
Isorientin	0.82 ± 0.14c	3.17 ± 0.21a	1.84 ± 0.11b	0.65
Vitexin	2.02 ± 0.10c	6.00 ± 0.26a	4.11 ± 0.13b	1.49
Rutin	87.33 ± 1.11c	195.47 ± 3.62a	175.70 ± 1.87b	77.8
Epicatechin-gallate	7.22 ± 0.06c	19.44 ± 0.82a	14.81 ± 0.17b	7.65
Epiafzelchin–epiafzelchin–epicatechin	8.01 ± 0.35c	15.69 ± 0.29a	11.64 ± 0.40b	8.31
Swertiamacroside	10.17 ± 0.02c	14.59 ± 0.09a	12.76 ± 0.04b	10.7
Hyperin	1.13 ± 0.01c	3.72 ± 0.22a	1.85 ± 0.08b	0.72
Epiafzelchin-epicatechin-O-methyl gallate	28.73 ± 1.37c	75.39 ± 2.60a	62.88 ± 3.08b	24.3
(−)-Epicatechin-3-(3”-O-methyl) gallate	15.18 ± 0.10c	35.97 ± 3.58a	28.43 ± 2.19b	12.9

(Epi)afzelchin-(epi) catechin isomer B	9.95 ± 0.16c	23.25 ± 1.66a	19.29 ± 2.07b	8.62
Procyanidin B2-dimethylgallate	21.06 ± 0.08c	58.03 ± 2.01a	50.67 ± 1.59b	18.1
Epiafzelchin–epicatechin-O-dimethylgallate	93.83 ± 1.83c	225.36 ± 4.12a	186.37 ± 3.36b	82.3
Epicatechin-O-3,4-dimethylgallate	39.10 ± 0.07c	82.65 ± 1.31a	74.51 ± 2.24b	36.4
Isoquercitrin	0.46 ± 0.01d	0.72 ± 0.02a	0.63 ± 0.01b	0.58
Quercetin	0.32 ± 0.01c	1.68 ± 0.003a	1.09 ± 0.01b	0.18
Total	590.92 ± 13.25c	1553.62 ± 32.16a	1325.19 ± 18.14b	514.
Flavonoids	431.68 ± 20.86c	1028.19 ± 19.88a	869.22 ± 22.09b	389.
Phenolic acid derivatives	159.24 ± 6.48c	525.42 ± 11.79a	455.97 ± 13.93b	124.

Different letters (a–e) in the same line indicate significant differences ($p < 0.05$

Second, a total of 26 bound phenolic compounds were quantified in whole buckwheat flours (GSTQ) and its fractions (GS215, GS160, GS85, and GS45) (**Table 5**). Among them, flavonoids represented 63–68% of total bound phenolic content. The most concentrated flavonoid was catechin, in which the highest value was obtained in GS215 (320.22 mg Kg^{-1} d.w.), followed by GS160 (241.04 mg Kg^{-1} d.w.), GS85 (80.05 mg Kg^{-1} d.w.), GSTQ (77.79 mg Kg^{-1} d.w.), and GS45 (36.05 mg Kg^{-1} d.w.). The second most abundant flavonoid was epicatechin, in which the greatest value appeared in fraction with 215 µm (202.64 mg Kg^{-1}), this value was 32.4, 75.3, 76.3, and 89.1% higher than that obtained in GS160, GS85, GSTQ, and GS45. Rutin was an abundant flavonoid in all fractions that represented 10–14% of total phenolic compounds, in which the highest value was obtained in GS215 (173.97 mg Kg^{-1} d.w.), follow by GS160 (127.24 mg Kg^{-1} d.w.), GS85 (59.09 mg Kg^{-1} d.w.), GSTQ (40.09 mg Kg^{-1} d.w.), and GS45 (27.09 mg Kg^{-1} d.w.). The most abundant phenolic acid derivative was syringic acid, in which the greatest concentration was obtained in GS215 (100.73 mg Kg^{-1} d.w.), this concentration was 21.5%, 68.9%, 69%, and 89.4% higher than that obtained in GS160, GSTQ, GS85, and GS45. There were no significant differences between the concentration of syringic acid obtained in GSTQ and GS85.

Total bound phenolic content was higher in GS215 (1565.22 mg Kg^{-1} d.w.), in which the value was 25%, 69.6%, 74.9%, and 87.6% higher than that obtained in GS160, GSTQ, GS85, and GS45. Therefore, bound phenolic content decreases as the particle size falls.

By comparison of bounds phenolic compounds analyzed in whole buckwheat flours, Verardo et al. (2011)[23] reported that the total bound phenolic compounds in buckwheat was 612.33 mg Kg^{-1} d.w. and this value was in the same order of magnitude as that obtained in our work. Catechin, epicatechin, and syringic acid were the most concentrated bound phenolic compounds; these results coincided with ours.

Table 5. Table of quantification of bound phenolic compounds from whole buckwheat flour (GST
GS160, GS85, and GS45) analyzed by HPLC-MS expressed as mg Kg^{-1} d.w. flour. Different let
indicate significant differences ($p < 0.05$).

Phenolic Compound	**GSTQ**	**GS215**	**GS160**	**GS-85**
2-hydroxy-3-O-β-D-glucopyranosyl benzoic acid	6.34 ± 0.08d	30.53 ± 1.22a	26.42 ± 1.43b	11.65 ±
Protocatechuic-4-O-glucoside acid	4.26 ± 0.13d	18.50 ± 1.05a	15.77 ± 0.49b	8.24 ±
Caffeic acid hexose	0.51± 0.04e	3.27 ± 0.05a	2.89 ± 0.18b	1.07 ±
Catechin-glucoside isomer a	0.48 ± 0.01c	2.03 ± 0.04a	1.12 ± 0.01b	0.50 ±
Caffeic acid hexose	20.33 ± 0.12d	82.34 ± 2.10a	56.26 ± 0.86b	30.25 ±
Catechin	77.79 ± 2.61c	320.22 ± 3.09a	241.04 ± 1.82b	80.05 ±
Swertiamacroside	38.30 ± 3.28c	130.85 ± 1.28a	88.47 ± 1.56b	40.03 ±
Caffeic acid	0.13 ± 0.001c	1.02 ± 0.04a	0.64 ± 0.02b	0.13 ±
Epicatechin	47.93 ± 0.09d	202.64 ± 3.01a	136.89 ± 2.74b	50.05 ±
(Epi)afzelchin-(epi) catechin	0.48 ± 0.03d	3.52 ± 0.11a	2.91 ± 0.004b	1.05 ±
Syringic acid	31.28 ± 0.90c	100.73 ± 1.99a	79.03 ± 0.69b	31.26 ±
Orientin	0.48 ± 0.02d	3.15 ± 0.09a	2.31 ± 0.003b	0.96 ±
p-Coumaric acid	2.11 ± 0.10d	9.47 ± 0.11a	6.42 ± 0.30b	3.11 ±
Procyanidin A	4.06 ± 0.08c	11.88 ± 0.32a	9.60 ± 0.24b	4.04 ±
Myricetin	0.05 ± 0.001c	0.12 ± 0.01a	0.09 ± 0.001b	0.05 ±
Vitexin	3.10 ± 0.10d	14.29 ± 0.46a	11.08 ± 0.29b	5.01 ±
Rutin	40.09 ± 2.24d	173.97 ± 2.08a	127.24 ± 1.75b	59.09 ±
Epicatechin gallate	13.24 ± 0.69c	50.94 ± 1.30a	39.92 ± 0.84b	12.07 ±

Catechin-glucoside isomer b	18.04 ± 0.25d	78.06 ± 0.92a	70.34 ± 1.37b	30.04 ±
Swertiamacroside	30.04 ± 0.38d	105.31 ± 1.56a	89.39 ± 2.61b	35.07 ±
Epiafzelchin–epicatechin-O-methylgallate	8.05 ± 0.11d	35.64 ± 0.86a	26.18 ± 1.27b	18.05 ±
p-Coumaric acid	5.44 ± 0.44d	22.47 ± 0.19a	16.77 ± 1.06b	13.55 ±
(−)-Epicatechin-3-(3’’-O-methyl) gallate	14.22 ± 0.16c	49.21 ± 0.88a	28.58 ± 1.63b	11.83 ±
Epicatechin-O-3,4-dimethylgallate	1.31 ± 0.07d	5.36 ± 0.20a	3.89 ± 0.14b	2.10 ±
Isoquercitrin	4.10 ± 0.04d	17.61 ± 0.17a	13.62 ± 1.31b	6.11 ±
Quercitrin	20.10 ± 1.13c	92.09 ± 3.44a	77.05 ± 1.83b	20.01 ±
Total	392.23 ± 12.63d	1565.22 ± 14.88a	1173.92 ± 20.47b	475.37
Flavonoids	253.51 ± 4.80d	1060.73 ± 13.57a	791.86 ± 11.08b	301.01
Phenolic acids	138.72 ± 8.15d	504.49 ± 5.76a	382.06 ± 6.94b	174.36

Figure 1. shows the sum of free and bound content of phenolic acid derivatives, flavonoids, and phenolic compounds in whole grain flour and its sieved fractions.

From total phenolic content in GSTQ and its fractions GS215, GS160, GS85, and GS45, the total phenolic acid derivatives corresponded to 27.6–33.5% of its total, in which the highest content was obtained in GS215 (1029.92 mg Kg^{-1} d.w.), in which the value was 18.6%, 71%, 71.1%, and 87.1% higher than in GS160 (838.03 mg Kg^{-1} d.w.), GS85 (298.61 mg Kg^{-1} d.w.), GSTQ (297.96 mg Kg^{-1} d.w.), and GS45 (133.07 mg Kg^{-1} d.w.) (**Figure 1a**).

Whereas flavonoids are the most abundant phenolic compounds in buckwheat, which represented 66.5–72.4% of total compounds in all fractions. The greatest flavonoid content in GS215 (2088.92 mg Kg^{-1} d.w.) was 20.5%, 66.9%, 67.2%, 83.3% higher than that obtained in GS215, GS160, GS85, GSTQ, and GS45 (2088.92, 1661.08, 690.86, 685.19, and 348.23 mg Kg^{-1} d.w.) (**Figure 1b**). There were no significant differences between the total content of flavonoids and phenolic acids obtained in GSTQ and GS85.

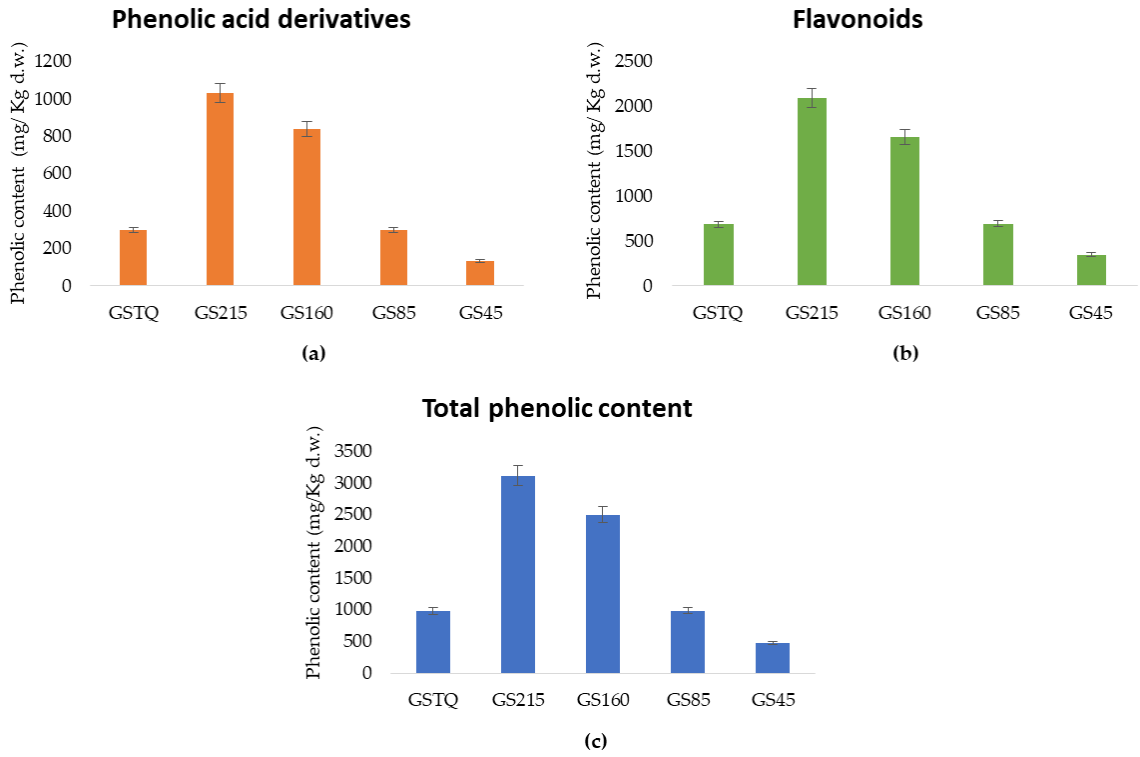

Figure 1. Total content of phenolic acid derivatives (**a**), total content of flavonoids (**b**), and total phenolic content (**c**) in whole grain (GSTQ) flour and its fractions (GS215, GS160, GS85, and GS45).

Total phenolic content was obtained in GS215 (3118.84 mg Kg^{-1} d.w.), which was 19.8%, 68.5%, 68.3%, 84.5% higher than that obtained in GS160 (2499.11 mg Kg^{-1} d.w.), GS85 (989.46 mg Kg^{-1} d.w.), GSTQ (983.15 mg Kg^{-1} d.w.), and GS45 (481.31 mg Kg^{-1} d.w.). Hence, according to these results, as the particle size decreases from 215 µm there is a decrease in the phenolic content (**Figure 1**), this trend was similar to that obtained in previous works. Bressiani et al. (2017)[19] evaluated the total phenolic concentration in sieved whole grain wheat flours, which was higher in the fraction with the particle size of 194.9 µm (3.06 mg gallic acid/100 g flour), followed by 608.44 µm (2.23 mg gallic acid/100 g flour), 830 µm (2.11 mg gallic acid/100 g flour), and finally at 82.67 µm (1.69 mg gallic acid/100 g flour); therefore, as the particle size decreases from 194.9 µm, the phenolic content decreases. Bolea and Vizireanu (2017)[17] evaluated the phenolic content in different black rice flours that were sieved at 630, 550, 315, 180, 125, and 90 µm, the fraction with 180 µm had the highest phenolic content (483 ± 5.32 mg gallic acid/g flour), closely followed by the fraction with 315 µm (432.13 ± 7.32 mg gallic acid/g flour); whereas fractions with 125 µm and 90 µm had almost the same content (402.26 ± 8.01 and 405.32 ± 6.32 mg gallic acid/g flour, respectively). Therefore, it has been reported in the previous works that the highest phenolic content was obtained in flours sieved with a particle size of 180–194.9 µm, whose particle size was similar to our enriched fraction (215 µm), concluding that as the particle size of the fractions decreases a decrease in the concentration of phenolic compounds is obtained. This could be due to the fact that the most enriched fraction contains bran in a high proportion which possess a higher phenolic content than endosperm, and bran could be lost with the sieving at lower particle size, obtaining thereby a fine fraction which is composed mainly of endosperm that contain lower phenolic content than bran.

4. Conclusions

In this study, sieving was tested as a dry green technology in order to produce functional buckwheat flours. An HPLC-MS has been used for the determination of free and bound phenolic compounds in whole grain flour and its fractions sieved with 215 µm, 160 µm, 85 µm, and 45 µm of particle size. According to the results, the highest free and the

bound phenolic content was obtained in buckwheat fraction with 215 µm (GS215), in which the value decreases as the particle size decreases.

Therefore, the process of milling and sieving could be used with success to increase/enrich meaningfully the content of phenolic compounds in sieved fractions from buckwheat. In fact, the concentration of rutin was 40 mg Kg^{-1} d.w. in GSTQ, whereas it increased in GS215 (174 mg Kg^{-1} d.w.). At the same time, the GS215 fraction reported protein and ashes amounts two times higher than the GSTQ flours.

To our knowledge, this is the first report on the use of sieving to enrich buckwheat flour with phenolic compounds (rutin among them) and protein. These preliminary results showed that this technology could be used to produce buckwheat flours, naturally enriched in proteins and phenolic compounds (rutin among others); while other fractions could be concentrated of starch. Briefly, sieved GS215 flour could be considered as naturally rich in phenolic compounds and protein buckwheat flour that could be used as an ingredient/raw material to develop functional food.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Base peak chromatogram (BPC) of bound phenolic compounds in buckwheat flour fraction GST215, obtained by HPLC-MS. See Table 3 for identification numbers.

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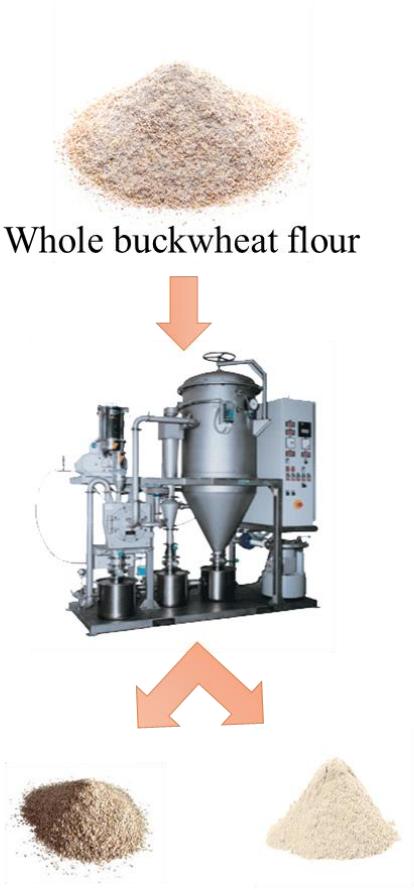
Chapter 8

Air classification as a useful technology to obtain phenolics-enriched buckwheat flour fractions



Submitted to a journal

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Abstract:

Air classification is a valuable process to obtain enriched flours in phenolic compounds. In this study, the use of this technology has been carried in dehulled buckwheat grain to obtain 70 % and 80 % of fine fractions and 20 % and 30 % of coarse fractions. As reported, the total content of phenolic compounds in coarse fractions increased 1.7-2.1 times compared to fine fractions. In addition, coarse fractions have shown a total phenolic content of 1.4-1.7 times more than dehulled buckwheat flour. Moreover, it has been evaluated the antioxidant activity by FRAP and DPPH assays in buckwheat fractions. Coarse fractions have shown a FRAP of 1.2-1.3 times higher than fine fractions, whereas the DPPH show a slight increase in coarse fractions in comparison with WF and fine fractions. In addition, FRAP and DPPH have exhibited a significative positive correlation with the flavonoids, phenolic acid derivatives and total phenolic content. These results have shown that air classification is an effective technique in order to obtain coarse flour fractions enriched in phenolic compounds such as swertimacroside, (epi)afzelchin-(epi)catechin-O-dimethyl gallate, rutin and caffeic acid hexose, among others.

Keywords: Buckwheat; fine and coarse fractions; air classification; phenolic compounds; rutin

1. Introduction

Buckwheat contains a great quantity of phenolic compounds, including rutin, that have shown beneficial health effects such as antioxidant, antitumor, antihypertensive, and anti-inflammatory activities[1]. Phenolic compounds in buckwheat are found in higher concentrations in the outer layers (bran and hull) than in the inner layers[2,3]. Nevertheless, buckwheat seeds are dehulled to obtain groat (intact achene), which is also milled into various grades of flour fractions, being the most common the light flour and bran flour[4-6].

Dehulled buckwheat milling fractions concentrate certain components based on the different proportion of tissues present. Light flour is mainly endosperm that is rich in starch, whereas bran flour is composed by the pericarp with seed coat, nuclear remnants, aleurone, sub aleurone layers and embryo fragments that has low quantities of starch and high content of proteins, lipids, dietary fibers, ashes and phenolic compounds such as rutin[4,7-9]. In mature buckwheat seeds, the outer of the two cotyledons adheres to the seed coat and during milling tears off and divides with bran[9]. However, bran flour is not commonly used for human consumption because buckwheat bran could have negative effects on technological and sensorial properties of the final product[10].

For that reason, it would be interesting to be able to obtain useful milling fractions of bran with specific functional and technological properties to produce enriched flours and bakery products has led to the development of several fractionation processes (dry or wet fractionation)[11]. Dry fractionation is a sustainable and economical process based on progressive grinding of whole cereal grains to decrease the particle size of whole flour, and then separate the fractions which contain most of the peripheral tissues, rich in dietary fiber and phenolic compounds. In many cases, pretreatment practices such as tempering, cryogenic pre-treatment, degerming, dehulling, peeling, pearling etc. are applied previously the use of grinders and millers (roller, impact, hammer, ball, abrasive, etc.). Finally, other physical separation techniques (sieving, air-classification, electrostatic separation etc) can be applied[12-15].

A previous study has reported the use of sieving as a valuable technology to produce enriched buckwheat flours in total phenolic compounds with 215 µm of particle size that is composed mainly by the bran[8]. Other study has also reported the phenolic content in different buckwheat milling fractions: dehulled whole grain flour, light flour, bran meals and middling flour, being the bran meals the one with the highest phenolic content[16]. Also, there are studies about enriched milling fractions obtained from buckwheat flours where they have separated from the innermost part to the outermost part of grain using a gradual milling system and the result was that the highest phenolic content was in the flour from the outer layers[3,17]. Therefore, these technologies have proven to be effective to obtain enriched buckwheat flour fractions in phenolic compounds that are composed mainly by the outer layers.

Air fractionation is a technological process to obtain enriched cereal flours composed by bran in order to use them as valuable ingredients or additives in food[18-20]. The air-classification consists of air currents with centrifugal force that can separate flour particles into different fractions according to size and density. Variation of certain air-classification parameters, such as air flow rate may make possible the collection of fractions with higher contents of compounds of interest[12]. This technique has been applied in barley andwheat obtaining coarse flour fractions with a high phenolic content[12,18,21]. Moreover, processing may positively or negatively affect the content of phenolic compounds which possibly impacts their bioactive properties and health benefits[22-24].

Thus, this is the first time that has been proposed the evaluation of phenolic content in buckwheat flour fractions (fine fraction and coarse fraction) obtained by the air classification technology.

2. Materials and Methods

2.1. Samples

Common buckwheat meals/flours were obtained from whole buckwheat (cv.). Harvesting took place on 2019. The grain was dehulled by stone dehuller (GRANO

200 SCHNITZER Stein-Getreidemuhle, Offenburg, Germany). The groat (dehulled grain) was milled by a hammer mill model 8/B (Beccaria S.r. L. Italy) and successively pin-milled by a model TMX 500 (Separ Micro System, Brescia, Italy). The pin-milled buckwheat flour was fractionated by an air classifier, model SX/LAB (Separ Micro System, Brescia, Italy). Different yields of coarse fraction (20 % and 30 % CF) and fine fraction (80 % and 70 % FF) were obtained by regulating the air flow inlet valve of the air-classifier. The particle size of these fractions is reported in **Table 1**.

Table 1. Particle size of the coarse (CF) and fine fracions (FF).

Dimension of the flour particles	CF 20	FF 80	CF 30	FF 70
>477	57	0	62	0
215-477	28.6	2.2	26	2.0
160-215	4.0	4.8	3.4	4.1
85-160	1.6	34.4	1.0	26.5
45-85	3.2	56.4	2	62
<45	1.8	0	2	4
sum	96.2	97.8	96.4	98.6

2.2. Reagents and Chemicals

HPLC grade acetonitrile, diethyl ether, ethyl acetate were purchased from Fisher Scientific (Leicestershire, UK), methanol, ethanol, sodium hydroxide, hydrochloric acid and hexane were purchased from Labscan (Dublin, Ireland). Acetic acid analytical grade (assay >99.5%) was purchased from Fluka (Buchs, Switzerland). Water was purified by using a Milli-Q system (Millipore, Bedford, MA). The following standards were purchased from Sigma-Aldrich (St. Louis, MO): Syringic acid, (+)-catechin, rutin, and myricetin. Potassium persulfate, TPTZ (2, 6-tripyridyl-s-triazine), ferric chloride and ferrous sulfate were acquired

from VWR (Chemicals Prolabo, Fontenay-sous-Bois, France). 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was also from Sigma-Aldrich (Steinheim, Germany).

2.3. Extraction of free and bound phenolic compounds from buckwheat flour fractions

Determination of free phenolic compounds was carried out according to the protocol established by Van Hung and Morita et al. (2008)[3] with certain modifications. Briefly, 1 g of buckwheat flour was extracted twice in an ultrasonic bath at 40 °C with 10 mL of ethanol/water (4:1 v/v) for 10 min. The supernatants were collected, centrifugated at 2500 rpm for 10 minutes and evaporated at 40 °C in a rotary evaporator, and finally reconstituted with 1 mL of methanol/ water (1:1 v/v).

Extraction of the bound phenolic compounds was carried out with the residues of the free phenolic extraction which were digested with 25 mL of 1 M NaOH at room temperature for 18 h by shaking under nitrogen gas as reported by Verardo et al. (2011)[25]. The mixture was acidified (pH = 2-3) with hydrochloric acid in a cooling ice bath and extracted with 250 mL of hexane to remove the lipids. The final solution was extracted five times with 50 mL of 1:1 diethyl ether/ethyl acetate (v/v). The organic fractions were pooled and evaporated to dryness at 40 °C in a rotary evaporator. The dry extract was finally reconstituted in 1 mL of methanol/water (1:1 v/v). The extracts were stored at -18 °C until use.

2.4. RP-HPLC- ESI-TOF-MS analysis

RP-HPLC analyses were performed by an Agilent 1200 series rapid resolution LC system (Agilent Technologies, Palo Alto, CA) consisting of a vacuum degasser, an autosampler, and a binary pump equipped with a reversed-phase C18 analytical column (4.6 x 250 mm, 1.8 μm particle size, Agilent ZORBAX Eclipse plus). The mobile phase and gradient program were used as previously described by Verardo et al. (2010)[26]. The RP- HPLC system ACQUITY UPLC M-Class System from

Waters (Waters Corp., Milford, MA, USA), including a degasser, a binary pump delivery system and an automatic liquid sampler, was coupled to a microTOF (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated time-of-flight mass spectrometer (oaTOF-MS) with an ESI interface. MS analysis was carried out using negative ionization mode at the following conditions: drying gas flow (N2), 7.0 L/min; nebulizer pressure, 21.7 psi; gas drying temperature, 190 °C; capillary voltage, 4500 V. The m/z range used for HPLC–ESI/MS analyses was m/z 50–1300. Data were processed by the software Data Analysis 4.0 (Bruker Daltonics). Identification of buckwheat free and bound phenolic compounds has been performed as previously described by Verardo et al. (2010)[26].

2.5. Antioxidant assays

The antioxidant activity of whole buckwheat flour, coarse and fine flours extracts was measured by two different methods: DPPH and FRAP. The free radical scavenging activity of the extracts was determined by measuring the reduction power against 2,2-diphenyl-1-picrylhydrazyl (DPPH) at 515 nm following the protocol proposed by Bran- Williams et al. (1995)[27]. The samples were analyzed in triplicate and the results were expressed as μg extract/mL. The ferric reducing antioxidant power (FRAP) assay that measures the capacity of antioxidant compounds to reduce the ferric ions by a single electron-transfer mechanism, was performed according to Al-Duais et al. (2009)[28]. The samples were analyzed in triplicate and results expressed as μmol FeSO4 equivalents/g dry sample.

2.6. Statistical Analysis

Tukey's honest significant difference multiple comparison (one-way ANOVA) at the $p < 0.05$ level was evaluated by using the Statistica 7.0 software (StatSoft, Tulsa, OK, USA)

3. Results and discussion

3.1. Quantification of the free and the bound phenolic compounds in buckwheat extracts

HPLC-ESI-TOF-MS has been used to determine 23 free phenolic compounds: 4 phenolic acid derivatives, 16 flavan-3-ols, 1 flavonol, 1 isoflavan and vitexin, which have been identified previously in other studies[2,26]. **Table 2** shows the quantification results of these free phenolic compounds in dehulled whole buckwheat flour (WF) and in their coarse fractions (CF30 and CF20) and fine fractions (FF70 and FF80). Flavonoid content represented the 72.72, 80.94, 85.14, 66.31 and 65.79% of the total free phenolic content in whole fraction, fine fractions (FF70 and FF80) and coarse fractions (CF30 and CF20), respectively. The most concentrated free phenolic compound was swertiamacroside in whole buckwheat flour and in its fine and coarse fractions; it was in the range 21.99-24.28% of the total free phenolic compounds. The highest content of swertiamacroside was obtained in the coarse fraction (CF20) (221.40 mg/Kg d.w), which value was 17.03, 47.87, 53.97 and 55.75% higher than the obtained in the coarse fraction CF30 (183.69 mg/Kg d.w.), de-hulled whole fraction (115.42 mg/Kg d.w.) and fine fractions FF80 and FF70 (101.90 and 97.96 mg/Kg d.w.). The second most concentrated phenolic compound in the coarse fractions and the fourth most concentrated in the dehulled whole buckwheat flour and fine fractions was rutin, which content decreased in the following order: CF20> CF30>WF>FF80>FF70 (129.05, 94.17, 54.89, 49.05 and 45.28 mg/Kg d.w.). The content of rutin obtained in CF20 fraction was 27.03, 57.47, 62 and 64.21% higher than the obtained in CF30, in the dehulled whole buckwheat flour and fine in fractions FF80 and FF70 respectively. The fact the highest rutin content was obtained in CF20 than CF30 can be due to the different composition of bran layers[29].

The third most concentrated free phenolic compound was the flavonoid (epi)afzelchin-(epi)catechin-O-dimethylgallate in the coarse fractions which highest content was obtained in CF20 (90.55 mg/Kg d.w.). This value was 14.15,

18.43, 41.93 and 43.84% higher than the obtained in the coarse fraction CF30, dehulled whole buckwheat flour and in their fine fractions FF70 and FF80, respectively. Furthermore, the second most concentrated phenolic compounds in fine fractions FF70 and FF80 was (-)-epicatechin that represented 13.02 % and 13.29 % of its total phenolic content, being the third most concentrated phenolic compound in the dehulled whole buckwheat.

Table 2. Free phenolic compounds determined by HPLC–ESI-TOF-MS in dehulled whole buckwheat flour (WF) and in their air classified fractions: 30% and 20% of coarse fractions (CF30 and CF20) and 70 % and 80% of fine fractions (FF70 and FF80). Different letters in the same line indicate significantly different values ($p < 0.05$).

Peak	Compounds	WF	FF70	CF30	FF80	CF20
1	2-hydroxy-3-O-β-D glucopyranosil-benzoic acid1	8.89c	8.38d	9.30b	9.52b	14.37a
2	Caffeic acid hexose1	7.82c	7.79c	8.57b	8.35b	12.87a
3	Protochatecuic-4-O-glucoside acid1	11.06c	8.27d	13.74b	8.97d	19.93a
4	Catechin—glucoside2	4.69c	3.58d	7.10b	3.82d	11.95a
5	Catechin—glucoside2	37.17c	30.01d	63.38b	32.28d	84.83a
6	(+)-Catechin2	17.49c	16.15d	28.24b	16.38d	37.03a
7	Catechin—glucoside2	15.37c	12.44d	26.14b	13.88d	33.74a
8	Swertiamacroside1	115.42c	97.96d	183.69b	101.90d	221.40a
9	Procyanidin B2 2	5.40c	4.75d	7.10b	4.96d	11.95a
10	(-)-Epicatechin2	62.58b	56.61c	63.38b	60.07b	84.83a
11	(Epi)afzelchin-(epi)catechin isomer a2	18.35c	17.94c	28.24b	18.87c	37.03a
12	Procyanidin B2-3-O-gallate2	5.78c	3.90d	26.14b	4.40d	33.74a
13	Rutin3	54.89c	45.28d	94.17b	49.05d	129.05a

14	Vitexin3	2.47c	2.11c	3.26b	2.29c	4.58ca
15	(Epi)afzelchin-(epi)afzelchin-(epi)catechin2	12.18b	10.59c	13.27a	9.88c	13.83a
16	(Epi)catechin-gallate2	5.32c	3.33e	9.47b	4.10d	14.27a
17	(Epi)afzelchin-(epi)catechin isomer b2	0.89d	0.78d	24.48b	1.22c	29.08a
18	(Epi)afzelchin-(epi)catechin-O-methyl gallate2	9.65b	7.02c	9.57b	7.60c	10.19a
19	(-)-epicatechin-3-(3''-O-methyl) gallate2	5.15b	4.61c	4.42c	5.29b	5.83a
20	ProcyanidinB2 dimethyl gallate2	8.76b	4.59c	9.07a	4.43c	9.54a
21	(Epi)afzelchin-(epi)catechin-O-di methyl gallate2	73.86b	52.58c	77.74b	50.85c	90.55a
22	(Epi)catechin-O-3,4-dimethylgallate2	34.14b	31.25c	35.92b	28.37c	38.61a
23	Dihydroxy-trimethoxyisoflavan2	7.57c	4.81d	10.05b	5.34d	15.99a
	Sum	524.89c	434.72d	756.44b	451.81d	965.18a
	Sum phenolic acid derivatives	143.19c	123.33d	214.37b	128.73d	268.57a
	Sum flavan-3-ols	316.76c	299.68d	394.10b	327.98c	485.42a
	Sum flavonols	54.89c	45.28d	94.17b	49.05c	129.05a
	Sum isoflavans	7.57c	4.81d	10.05b	5.34d	15.99a
	Sum flavones	2.47c	2.11d	3.26b	2.29c,d	4.58a
	Sum flavonoids	381.70c	351.88d	501.58b	384.66c	635.03a

[1] mg syringic acid/kg; [2] mg (+)-catechin/kg; [3] mg rutin/kg; n.d. = not detected

It has been obtained an increase of all free phenolic compounds in coarse fractions (CF20 and CF30) with respect to dehulled whole buckwheat flour (WF) (Table 2.): Among them, the highest increase was obtained in the compound 17 ((epi)afzelchin-(epi)catechin) in CF30 and CF20, which concentration was 96.36% and 96.94% higher than in WF. In addition, a high increase of 59.15% and 91.82% was obtained in the swertiamacroside content in coarse fractions CF30 and

CF20 compared with WF. Nevertheless, the fine fraction FF70 has shown a decrease of all phenolic compounds with regard to whole buckwheat flour. Furthermore, in the fine fraction FF80 was obtained a decrease of most phenolic compounds excepting 2-hydroxy-3-O-β-D glucopyranosil-benzoic acid, caffeic acid hexose, (epi)afzelchin-(epi)catechin and (-)-epicatechin-3-(3''-O-methyl) gallate, which were increased slightly with regard WF.

By comparison of the results of the most concentrated free phenolic compounds with the obtained in the literature, Verardo et al. (2011)[25] reported rutin as the most abundant in whole buckwheat grain, with a concentration of 331.38 mg/Kg d.w. followed by swertiamacroside (235.93 mg/Kg d.w.), these concentrations were 83.4 % and 51.1 % higher than the obtained for our dehulled whole buckwheat flour. Nevertheless, in the present study, the content of (epi)afzelchin-(epi)catechin-O-dimethylgallate and epicatechin in dehulled buckwheat flour were 36.5 % and 50.2 % higher than the obtained by Verardo et al. (2011)[25] (46.90 and 31.17 mg/kg d.w.). In addition, Kalinová et al. (2019)[29] determined the content of rutin in the groat (55.78 mg/Kg d.w.), which content was in the same order of magnitude than the obtained in the present study in dehulled whole buckwheat (54.9 mg/Kg d.w.), whereas the content obtained in coarse fractions was 42.4% and 58.0% higher than the obtained by Kalinová et al. (2019)[29] in the seed coat (54.2 mg/Kg d.w.) This fact could be due to that coarse fractions are composed by outer layers, which are enriched in rutin[2,30]. In addition, Kalinová et al. (2019)[29] obtained epicatechin as the most concentrated phenolic compounds in the seed coat (254.4 mg/Kg d.w.), whereas in our case, epicatechin was the fourth most concentrated in buckwheat coarse fractions. In addition, Liu et al. (2019)[31] also reported rutin as the most concentrated flavonoid in raw common buckwheat (Lehuoshi) (62.19 mg/kg d.w.) and it was in the same order of magnitude as that obtained in dehulled whole grain flour in our study (54.89 mg/kg d.w.).

Previous studies about buckwheat reported (epi)afzelchin-(epi)catechin-o-dimethylgallate as the most concentrated compound followed by rutin, but both compounds were in similar concentrations to those found in the present work in dehulled buckwheat meal[8,16]. Concerning the concentration of these compounds in

sieved buckwheat fractions at 85 µm (82.38 and 77.84 mg/Kg d.w.) and 45 µm (46.14 and 43.59 mg/Kg d.w.) were similar to the obtained in the fine fractions[8]. This fact is due to fine fractions contains a high flour proportion with the particle size of 45-85 µm. Another study reported the content of (epi)afzelchin-(epi)catechin-o-di methyl gallate and rutin obtained in bran meal (216.94 mg/Kg d.w and 214.99 mg/Kg d.w., respectively) and middling flour (176.67 mg/Kg d.w. and 148.63 mg/Kg d.w., respectively), which were higher than the obtained in fine and coarse fractions in the present study[16]. Nevertheless, these studies obtained a lower concentration of swertiamacroside than in our buckwheat fractions. These differences found with previous studies could be due to the different cultivars of buckwheat used that could provide variations in phenolic profiles. In addition, also the environment plays a significant role in determining variations in phenolics amount[32].

Besides, the total flavonoid content obtained in our study were in the same order of magnitude than the obtained by Inglett et al. (2011)[2] in different buckwheat flours: whole buckwheat flour (417.03 mg/kg d.w.) and supreme (525.27 mg/kg d.w., composed mainly by bran of buckwheat), and farinetta (671.50 mg/kg d.w.) and fancy that is constituted by the endosperm and was 71.4 mg/Kg d.w. Considering our results, coarse fractions could contain the same composition of supreme and fine fractions could contain part of the composition of farinetta[30].

The total free phenolic content in buckwheat flours decreased in the following order: CF20>CF30>WF>FF80 >FF70 (965.18, 756.44, 524.89, 451.81 and 434.72 mg/kg d.w., respectively). These differences could be observed in Table 2. Free phenolic content in CF30 was 42.53 % higher than the obtained in FF70, whereas free phenolic compounds in CF20 was 53.19% higher than the obtained in FF80. In addition, the free phenolic content increased 1.44 and 1.84 times in coarse fractions CF30 and CF20 compared to whole meal. These results showed that coarse fractions are enriched in phenolic compounds due to free phenols are concentrated in the external layers (aleurone, bran) of the kernel, which are crushed during the milling resulting in bigger particles, which generates the coarse fraction[18].

Table 3. Bound phenolic compounds determined by HPLC–ESI-TOF-MS in dehulled whole buckwheat flour (WF) and in their air classified fractions: 30% and 20% of coarse fractions (CF30 and CF20) and 70 % and 80% of fine fractions (FF70 and FF80). Different letters in the same line indicate significantly different values ($p < 0.05$).

Peak	Compounds	WF	FF70	CF30	FF80	CF20
1	2-hydroxy-3-O-β-D-glucopyranosil-benzoic acid1	22.70c	14.43d	33.67b	13.44d	43.02a
2	Protochatecuic-4-O-glucoside acid1	15.92b	13.19c	24.78a	10.19d	23.46a
3	Caffeic acid hexose isomer a1	88.78c	66.20d	95.98b	62.87d	123.57a
4	Caffeic acid hexose isomer b1	16.54b	9.14c	21.88a	5.40d	23.14a
5	Swertiamacroside1	28.52c	25.51d	36.05b	25.40d	47.21a
6	(Epi)afzelchin-(epi)catechin2	6.17b	5.59b	10.07a	4.89b	10.25a
7	Myricetin3	3.14c	2.03d	5.15b	2.22d	6.36a
8	Syringic acid1	51.78d	50.27d	82.03b	58.91c	88.23a
9	Procyanidin A2	1.60b	0.62c	3.35a	0.50c	3.93a
10	Rutin4	18.83c	7.18d	23.63b	4.87e	30.56a
11	Vitexin4	2.54c	1.76c	5.05b	2.23c	8.36a
12	Epicatechin-gallate2	5.74c	3.35d	12.53a	3.86d	8.90b
13	Epiafzelchin-epicatechin-O-methyl gallate 2	0.74b	0.42c	1.55a	0.28d	1.46a
14	(-)-epicatechin-3-(3’’-O-methyl) gallate2	32.28b	27.36c	39.04a	28.35c	42.85a
	Sum	295.28c	227.04d	394.75b	223.40d	461.29a
	Sum phenolic acid derivates	224.24c	178.73d	294.39b	176.21d	348.63a

Sum flavan-3-ols	46.53b	37.34c	66.54a	37.86c	67.39a
Sum flavonols	21.97c	9.21d	28.78b	7.09e	36.92a
Sum flavones	3.14c	2.03d	5.15b	2.22d	6.36a
Flavonoids	71.64c	48.58d	100.46b	47.17d	110.67a

[1] mg syringic acid/kg; [2] mg (+)-catechin/kg; [3]mg myricetin/kg, [4] mg rutin/kg; LOQ= limit of quantification.

HPLC-ESI-TOF-MS has also been used to determine 14 bound phenolic compounds (**Table 3**): 7 phenolic acid derivatives, 5 flavan-3-ols, 2 flavonols and vitexin, which were previously identified by Verardo et al. (2011)[25]. Phenolic acid derivatives represent 75.94%, 78.72%, 74.58%, 78.88% and 75.58% of total bound phenolic compounds in dehulled whole buckwheat, FF70, CF30, FF80 and CF20, respectively. The most abundant phenolic acid derivative was caffeic acid hexose isomer a that represented 24.31-30.07 % of the total bound phenolic compounds in all fractions, which concentration decreased in the following order: CF20>CF30>WF>FF70=FF80 (123.57, 95.98, 88.78, 66.20, and 62.87 mg/Kg d.w.). The second most concentrated bound phenolic acid derivative was syringic acid, its greatest concentration was found in CF20 (88.23 mg/kg d.w.) followed by CF30, FF80, WF and FF70. Besides that, the most abundant bound flavonoid was (-)-epicatechin-3-(3''-O-methyl) gallate and the highest content was obtained in CF20, which content represented 24.67 %, 8.89%, 33.84% and 36.15% more than the concentration obtained in WF, CF30, FF80 and FF70 ,respectively. The second most abundant flavonoid was rutin, CF20 also showed the highest content followed by CF30, WF, FF70 and FF80 .The content of rutin obtained in CF20 and CF30 was 84.06 and 69.61% higher than the obtained in their fine fractions.

According to the results in the **Table 3**. It could be observed that most bound phenolic compounds present FF70 and FF80 decreased compared with WF, as exception of compound 8 (syringic acid), which increased about 12.08% in FF80. Nevertheless, all bound phenolic compounds increased in coarse fractions in comparison with WF. Whereas CF20 sample provided the highest amounts of bound phenolic compounds. In addition, a high increase of 145.63% and 229.13%

was obtained in the procyanidin and vitexin content in coarse fractions CF20 compared with whole buckwheat flour.

Based on the results of sum of bound phenolic compounds in all samples. The highest bound phenolic content was obtained CF20 (461.29 mg/Kg d.w.), followed by CF30 (394.75 mg/Kg d.w.), WF (295.28mg/Kg d.w.) and FF70 (227.04mg/Kg d.w.) and FF80 (223.40 mg/Kg d.w.). Thus, the value of the bound phenolic content in CF20 was 51.57% higher than the obtained in FF80, whereas in CF30 was 42.49 % higher than the obtained in FF80. In addition, the content of bound phenolic compounds in coarse fractions CF20 and CF30 increased 56.22% and 33.69% with regard the whole buckwheat flour. This result is due to coarse fraction contain the aleurone layer, which is richer in phenolic compounds than the whole buckwheat flour[33].

By comparing the bound phenolic content obtained with literature, the content of caffeic acid hexose found in sieved buckwheat at 215 µm and 160 µm (82.34 and 56.26 mg/Kg d.w., respectively)[8] and in bran meal and middling flour (40.42 and 56.73 mg/Kg d.w., respectively)[16] was similar to those obtained in our fine and coarse fractions. Furthermore, total bound phenolic content in de-hulled buckwheat (389.51-392.93 mg/Kg d.w.) matched with the present results[8,16]. However, in these previous studies, the most concentrated bound phenolic compound was catechin and the content of bound phenolic compounds was higher than the obtained in our fine and coarse fractions. Besides, total bound phenolic concentration obtained by Verardo et al. (2011)[25] in de-hulled buckwheat was 51.78% higher than the obtained in our work. These differences could be related to the study of different cultivars grown under different conditions. In addition, also the environment plays a significant role in determining variations in phenolics amount[32].

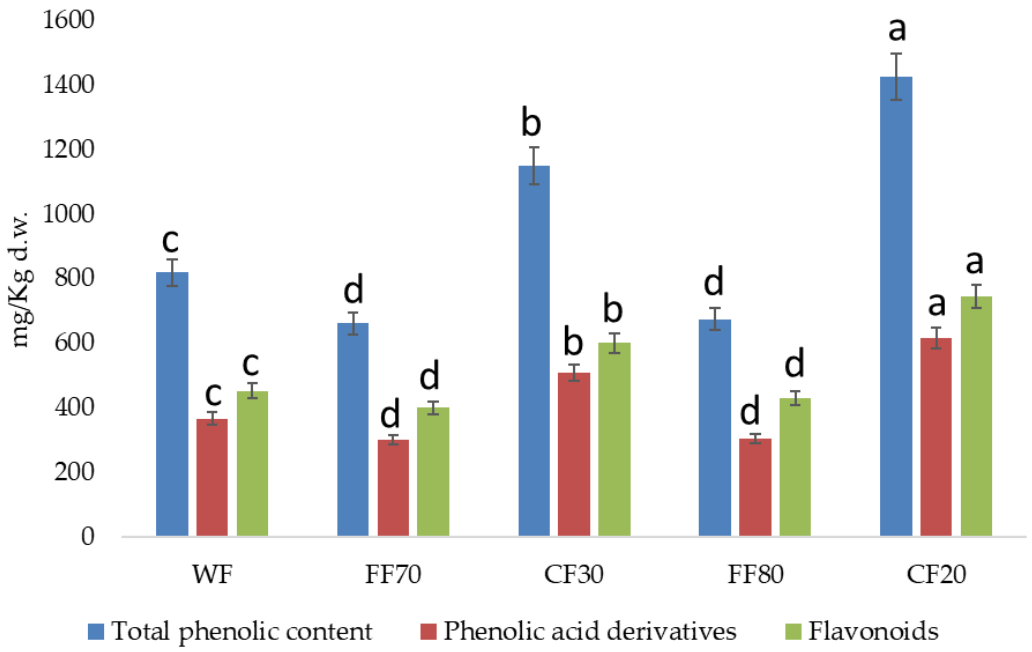

Figure 1. Content of total phenolics, phenolic acid derivatives found in dehulled whole buckwheat flour (WF), 30 % and 20 % of coarse fractions (CF30 and CF20) and 70 % and 80 % of fine fractions (FF70 and FF80). Different letters indicate significantly different values ($p < 0.05$)

In **Figure 1** shows plotted the results of total (sum of free and bound) phenolic acid derivatives, flavonoids and total phenolic content in dehulled buckwheat, fine and coarse fractions. Total flavonoids content represented 51.8-56.6 % of the total phenolic content. The highest flavonoid content was obtained in CF20 (745.70 mg/Kg d.w.) followed by CF30 (602.05 mg/Kg d.w.) and WF (453.33 mg/Kg d.w.) and fine fractions FF80 and FF70 (431.83 and 400.46 mg/Kg d.w.). The highest content of phenolic acid derivatives was also obtained in CF20 with a content of 617.20 mg/Kg, followed by CF30 (508.76 mg/Kg d.w.), WF (367.43 mg/Kg d.w.) and fine fractions FF80 and FF70 (304.94 and 302.06 mg/Kg d.w.). The total phenolic content in the coarse fractions CF20 and CF30 (1426.48 and 1151.19 mg/Kg d.w.) was 52.67% and 42.51 % higher than the obtained in the fine fractions FF80 and FF70 (675.21 and 661.76 mg/Kg d.w.). In addition, total phenolic content in coarse fractions CF20 and CF30 was 42.5% and 28.75% greater than the whole flours (820.17 mg/Kg d.w.). These results are in concordance with the

obtained previously by Verardo et al. (2011)[18] that reported an increase in the phenolic content in the barley coarse fraction in comparison with fine fractions and whole meal . Furthermore, Inglett et al. (2012)[2] reported the lowest phenolic content in fancy (5.75 mg gallic eq/g) that is composed by endosperm, followed by whole buckwheat (5.85 mg gallic eq/g), supreme (11.97 mg gallic eq/g) that corresponded with bran fraction and farinetta (18.9 mg gallic eq/g) that contains a considerable amount of aleurone layer along with embryo. Therefore, these results confirm that fractions which contain bran and aleurone layer (coarse fractions) possess the highest phenolic content with regard to the whole grain and fine fractions[2]. In addition, the total phenolic content obtained in dehulled buckwheat flour was in the same order of magnitude than the obtained in previous studies[8,16,25]. These results suggest that the use air classification in buckwheat is suitable to obtain coarse flour fractions composed by bran that content a high content in phenolic compounds

3.2. Antioxidant activity of buckwheat fractions

Table 4. Antioxidant activity of de-hulled whole buckwheat flour (WF) and their air classified fractions: 30% and 20% of coarse fractions (CF30 and CF20) and 70 % and 80% of fine fractions (FF70 and FF80). Different letters in the same line indicate significantly different values ($p < 0.05$).

Compounds	WF	FF70	CF30	FF80	CF20
Free phenolic compounds					
DPPH	332.76^{c}	334.17^{c}	341.42^{b}	335.29^{c}	363.95^{a}
FRAP	1267.13^{d}	1315.24^{d}	1563.41^{b}	1371.41^{c}	1659.53^{a}
Bound phenolic compounds					
DPPH	170.71^{c}	143.36^{e}	195.81^{b}	165.81^{d}	216.89^{a}
FRAP	386.42^{c}	316.24^{e}	397.14^{b}	327.14^{d}	413.60^{a}

DDPH was expressed as µg extract/mL, FRAP as µmol Fe^{2+}/ g extract; DPPH= 2,2-diphenyl-1-picrylhydrazyl; FRAP= The ferric reducing antioxidant power.

Results of antioxidant activities in dehulled buckwheat flour and its fine and coarse fractions are presented in **Table 4**. DPPH and FRAP assays were carried out. DPPH assay measures a change in the stable radical DPPH by the electron donating ability of the sample and FRAP value measures the reduction of the ferric ion (Fe3+) to the ferrous ion (Fe2+) by donor electrons in the sample. The antioxidant activity of free phenolic extracts was significantly higher than the obtained for bound phenolic extracts. DPPH measured in free phenolic extracts of dehulled buckwheat flour, CF30, CF20, FF70 and FF80 were 48.69%, 42.9%, 42.65%, 50.55% and 40.41% higher than the obtained in bound phenolic extracts for the same fractions. Similarly, FRAP of the free phenolic extracts of WF, CF30, CF20, FF70 and FF80 were 69.5%, 74.6%, 75.03%, 75.96% and 76.15% respectively,

higher than the obtained in the bound phenolic extracts for the same fractions. These results are in concordance with Guo et al. (2012)[34] that reported the antioxidant activity in Tartary buckwheat fractions and obtained a higher antioxidant power in the free phenolic extracts than in the bound phenolic extracts. The results confirm that antioxidant activities are related with the concentration phenolic compounds due to the concentration of free phenolic c was higher than the obtained for the bound phenolic compounds.

DPPH in the free phenolic extracts show a slight increase in CF20 and CF30 in comparison with WF and fine fractions. Furthermore, the highest value of FRAP was found in the free phenolic extracts of CF20 and CF30, followed by FF80, FF70 and WF. In addition, FRAP in coarse fractions were 17.4% and 15.87% higher than the obtained in their fine fractions. Regarding the bound phenolic extracts, the highest DPPH and FRAP value was obtained in CF20 followed by CF30, WF, FF80 and FF70. DPPH and FRAP in the bound phenolic extracts show an increase of 14.7-27% and 25.6-26.4% in CF20 and CF30 in comparison with their fine fractions. These results suggested that coarse fractions possess the highest antioxidant activity in comparison with WF and their fine fractions due to their higher content in phenolic compounds.

Table 5. Correlation analysis of phenolic content and antioxidant activities of free phenolic extracts in de-hulled buckwheat flour and their air classified fractions

Free phenolic compounds	FRAP	DPPH
2-Hydroxy-3-O-β-D glucopyranosil-benzoic acid	0.80	0.97*
Caffeic acid hexose	0.83	0.99*
Protochatecuic-4-O-glucoside acid	0.88*	0.95*
Catechin—glucoside isomer a	0.90*	0.97*
Catechin—glucoside isomer b	0.95*	0.93*
(+)-Catechin	0.96*	0.94*

Catechin—glucoside	0.95*	0.92*
Swertiamacroside	0.95*	0.90*
Procyanidin B2	0.88*	0.99*
(-)-Epicatechin	0.80	0.97*
(Epi)afzelchin-(epi)catechin isomer a	0.97*	0.96*
Procyanidin B2-3-O-gallate	0.97*	0.88*
Rutin	0.95*	0.94*
Vitexin	0.92*	0.97*
(Epi)afzelchin-(epi)afzelchin-(epi)catechin	0.74	0.72
(Epi)catechin-gallate	0.93*	0.94*
(Epi)afzelchin-(epi)catechin isomer b	0.97*	0.85
(Epi)afzelchin-(epi)catechin-O-methyl gallate	0.59	0.61
(-)-epicatechin-3-(3’’-O-methyl) gallate	0.30	0.63
ProcyanidinB2 dimethyl gallate	0.58	0.57
(Epi)afzelchin-(epi)catechin-O-dimethyl gallate	0.72	0.76
(Epi)catechin-O-3,4-dimethylgallate	0.72	0.76
Dihydroxy-trimethoxyisoflavan	0.87	0.94*
Sum	0.93*	0.92*
Sum phenolic acid derivatives	0.94*	0.92*
Sum flavan-3-ols	0.95*	0.97*
Sum flavonols	0.95*	0.94*
Sum isoflavans	0.87	0.94*
Sum flavones	0.92*	0.97*
Sum flavonoids	0.95*	0.96*

Results are expressed as Pearson correlation coefficients with indicated level of significance. TPC, total phenolic content. * $p<0.05$; FRAP= The ferric reducing antioxidant power; DPPH= 2,2-diphenyl-1-picrylhydrazyl.

The relationship between free phenolic compounds and antioxidant activities appears in **Table 5**. FRAP exhibit a significant positive correlation with protochatecuic-4-O-glucoside acid (r= 0.88, p= 0.049), catechin glucoside isomer a (r= 0.90, p= 0.035) and b (r=0.95, p=0.014), (+)-catechin (r= 0.96, p= 0.011), catechin-glucoside (r= 0.95, p= 0.012), swertiamacroside (r=0.95, p=0.013), procyanidin B2 (r= 0.88, p=0.046), (epi)afzelchin-(epi)catechin (r= 0.97, p= 0.008), procyanidin B2-3-O-gallate (r= 0.97, p= 0.007), rutin (r=0.95, p= 0.013), vitexin (r=0.92, p=0.027), (epi)catechin-gallate (r= 0.93, p= 0.022), and (epi)afzelchin-(epi)catechin (r=0.97, p=0.005). Furthermore, DPPH scavenging activities exhibited a high significant positive correlation with the following free phenolic compounds: 2-hydroxy-3-O-β-Dglucopyranosil-benzoic acid (r= 0.97, p= 0.005), caffeic acid hexose (r= 0.99, p= 0.001), protochatecuic-4-O-glucoside acid (r= 0.95, p= 0.014), catechin glucoside isomer a (r=0.97, p= 0.005) and b (r=0.93, p= 0.022), catechin (p= 0.94, r= 0.017), catechin—glucoside (r= 0.92, p= 0.026), swertiamacroside (r=0.90, p= 0.038), procyanidin B2 (r=0.99, p= 0.001), (-)-epicatechin (r= 0.97, p= 0.007), (epi)afzelchin-(epi)catechin isomer a (r= 0.96, p=0.010), procyanidin B2-3-O-gallate (r= 0.88, p= 0.046), rutin (p= 0.94, p= 0.017), vitexin (r= 0.97, p= 0.007), (epi)catechin-gallate (r= 0.94, p= 0.017) and dihydroxy-trimethoxyisoflavan (r= 0.94. p= 0.015). According to these results, the most abundant free phenolic acids swertimacroside and rutin possess antioxidant activities, excepting (epi)afzelchin-(epi)catechin-O-di methyl gallate that did not showed a correlation with FRAP and DPPH. Antioxidant activities by FRAP and DPPH have showed a high correlation with sum of free phenolic compounds, sum phenolic acid derivatives and sum of flavonoids. Nevertheless, FRAP did not exhibit a correlation with sum of isoflavans.

Table 6. Correlation analysis of phenolic content and antioxidant activities of bound phenolic extracts in de-hulled buckwheat flour and their air classified fractions

Bound phenolic compounds	FRAP	DPPH
2-hydroxy-3-O-β-D-glucopyranosil-benzoic acid	0.92*	0.95*
Protochatecuic-4-O-glucoside acid	0.88	0.84
Caffeic acid hexose isomer a	0.93*	0.92*
Caffeic acid hexose isomer b	0.95*	0.84
Swertiamacroside	0.84	0.94*
(Epi)afzelchin-(Epi)catechin	0.85*	0.89*
Myricetin	0.90*	0.97*
Syringic acid	0.75	0.94*
Procyanidin A	0.92*	0.94*
Rutin	0.97*	0.90*
Vitexin	0.81	0.95*
Epicatechin-gallate	0.81	0.79
Epiafzelchin-epicatechin-O-methyl gallate isomer b	0.89*	0.88*
(-)-epicatechin-3-(3''-O-methyl)gallate	0.93*	0.97*
Sum	0.92*	0.95*
Sum phenolic acid derivates	0.91*	0.96*
Sum flavan-3-ols	0.91*	0.93*
Sum flavonols	0.97*	0.92*
Sum flavones	0.90*	0.97*
Sum flavonoids	0.95*	0.94*

Results are expressed as Pearson correlation coefficients with indicated level of significance. TPC, total phenolic content. * $p<0.05$; FRAP= The ferric reducing antioxidant power; DPPH= 2,2-diphenyl-1-picrylhydrazyl.

Finally, **Table 6** describes the correlation of DPPH and FRAP with bound phenolic compounds. FRAP and DPPH showed a positive significative correlation with most of bound phenolic acids and with sum of bound phenolic compounds, sum of phenolic acid derivatives and flavonoids. Nevertheless, FRAP did not show a correlation with protochatecuic-4-O-glucoside acid, swertiamacroside, syringic acid, vitexin and epicatechin gallate. DPPH did not show a correlation with protochatecuic-4-O-glucoside acid, caffeic acid hexose isomer b and epicatechin-gallate. Therefore, according to the results, the most concentrated bound phenolic compounds such as caffeic acid hexose isomer a, (-)-epicatechin-3-(3''-O-methyl) gallate and rutin have shown a high correlation in FRAP and DPPH. Excepting syringic acid, which has shown a high correlation only with DPPH.

4. Conclusions

Air classification has been used for the first time in the dehulled buckwheat grain to obtain fine and coarse fractions in order to evaluate their phenolic content. The

total phenolic content in coarse fractions CF20 and CF30 was 1.74 and 1.40 times greater than the whole flours. Moreover, an increase of most free and bound phenolic compounds has been found in CF20 and CF30 with regard to whole buckwheat flour. In addition, the total phenolic content in the coarse fractions CF20 and CF30 was twice that their fine fractions. Moreover, FRAP and DDPH were higher in the coarse flour fractions than in the fine flour fractions and dehulled buckwheat flours.

To conclude, the use of an air classification could be a valuable technology in order to obtain coarse fractions (CF20 and CF30) enriched in phenolic compounds such as rutin, among others. Therefore, these enriched flour fractions could be used as ingredients to develop functional food.

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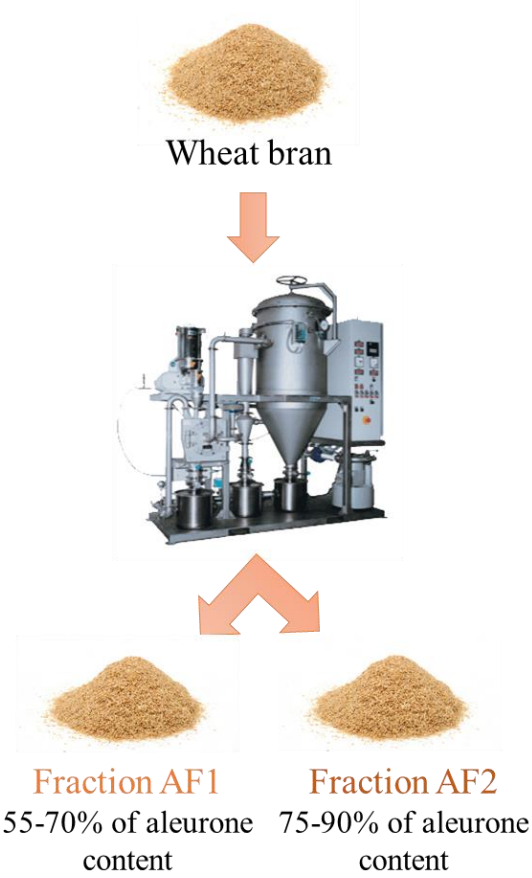
CHAPTER 9

Distribution of free and bound phenolic compounds, and alkylresorcinols in wheat aleurone enriched fractions



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Distribution of free and bound phenolic compounds, and alkylresorcinols in wheat aleurone enriched fractions

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Abstract

Several companies have focused their attention on the development of technologies able to enrich/isolate the wheat aleuronic layer because it is a source of bioactive compounds. In this work two different wheat bran fractions enriched in aleurone (AF1, 55-70% aleurone and AF2, 75-90% aleurone) were obtained by a dry fractionation based on air classification. Free and bound phenolic compounds, and alkylresorcinols were determined in the two fractions by HPLC-DAD-ESI-TOF-MS and GC-MS, respectively. To our knowledge, feruloyl di-hexoside was described for the first time in wheat aleurone and flavonoids were quantified for the first time in this fraction. The results have shown that the most concentrated free phenolic compounds were flavonoids, and AF1 was the fraction that presented the highest flavonoid content; whereas *trans* ferulic acid was the most abundant bound phenolic acid, which highest content was obtained in AF2. Besides, total content of ferulic acid monomers in AF2 was 33.63% higher than in

AF1, whereas total content of ferulic acid dimers/trimers in AF1 was 33.9% higher than in AF2. The highest content of alkylresorcinols was obtained in AF1 and it was 10.30% higher than the obtained in AF2. Therefore, it can be stated that this green technology could be used to produce enriched aleurone fractions as source of phenolic and alkylresorcinol compounds. These fractions could be of great interest for the formulation of enriched foods.

Keywords: wheat bran, flavonoids, phenolic acids, air classification, HPLC-DAD-MS and GC-MS.

1. Introduction

Whole-grain consumption is associated with a lower risk of suffering from some types of chronic diseases as such as cardiovascular diseases[1,2], type 2 diabetes[3] and some cancers[4-6]. These beneficial properties are attributed to the content of phytochemicals in whole grains (phenolic compounds, carotenoids, vitamin E, γ -oryzanols, dietary fibers, and β-glucans)[2]. Especially, phenolic compounds in whole grain have demonstrated high antioxidant capacities and they can protect against degenerative diseases[7-15]. Phenolic compounds in cereals are concentrated in bran, which is the major by-product obtained from flour milling. Among phenolic acids, ferulic acid is the most abundant hydroxycinnamic acid found in cereal grains, whereas *p*-coumaric acid is present in the lowest amount in the centre of the grain kernel and an increasing amount towards the outer layers[16]. Alkylresorcinols are phenolic lipids, with homologues ranging from C17 to C25, they are present in high concentrations in wheat and rye whole grains, in lower concentrations in barley, and in insignificant concentrations in refined wheat flour[17].

Wheat grains contain phenolic acids such as *p*-hydroxybenzoic acid, vanillic acid, ferulic acid, syringic acid and *p*-coumaric acid, which are present in bound and in free form. Total ferulic acid represents 75-93% of total phenolic acids in the whole

wheat grains[18]. Phenolic compounds are more concentrated in wheat bran than in other parts of the grain. Wheat bran is composed by multi-layers: outer pericarp, inner pericarp, intermediate pericarp (nucellar epidermis and testa) and aleurone layer[19]. The outer pericarp and inner pericarp have a high content of ferulic acid (FA) dimers. The testa layer is a hydrophobic layer rich in lignin and lipidic compounds such as alkylresorcinols[20]. The aleurone layer represents around 50% of the wheat bran. This fraction contains esterified ferulic acid monomers[21] and flavonoids that are mainly present in conjugates form and they could be identified in whole wheat as 5,7,4'-trihydroxyflavone (apigenin) and 5,7,3'4', -tetrahydroxyflavone (luteolin). Anthocyanins have also been found in the aleurone layers of coloured wheat[18]. In addition,, lignans are also located in aleurone layer , being secoisolariciresinol the most concentrated compound from this family[21].

Aleurone is a rich source of phenolic compounds; thus, separation techniques such as air-classification and sieving, and more innovative techniques (electrostatic separation) have been developed in order to isolate aleurone particles from wheat bran[22]. Some fractionation processes utilize properties of particle size and density, using sieving and air-classification of milled bran to provide fractions rich in aleurone[21]. Besides, processes based on the electrostatic properties of the different bran layers have been used to obtain a high purity aleurone fractions[23]. Thus, the aim of this work was the determination of free and bound phenolic compounds and alkylresorcinols in two enriched aleurone bran fractions obtained by a patented technology based on dry separation that allows fractions until 90% of aleurone. Phenolic fraction of wheat was previously studied by several authors; however, a comprehensive characterization of phenolic fraction of aleurone is useful to better understand the phenolics and alkylresorcinols distribution in this fraction. For that purpose, free and bound phenolic compounds were extracted and then, they were analyzed by HPLC-ESI-TOF-MS; in addition, alkylresorcinols were determined by GC-MS. To our knowledge, the distribution of flavonoids in aleurone fractions was not previously described.

2. Materials and methods

2.1. Chemicals

HPLC-grade acetonitrile, water, methanol, acetic acid, diethyl ether, ethyl acetate, ethanol, sodium hydroxide, hexane and hydrochloric acid were purchased from Merck (Darmstadt, Germany). The standard compounds of chlorogenic acid, ferulic acid, syringic acid, apigenin, methylbehenate and nonadecylresorcinol were supplied by Sigma-Aldrich (Saint Louis, MO, USA). Pyridine was purchased from VWR Chemicals Prolabo (Fontenay-sous-Bois, France). Trimethylchlorosilane was supplied by Merck KGaA (64271 Darmstadt, Germany), and hexamethyldisilazane was purchased by Alfa Aesar GmbH & Co KG (Karlsruhe, Germany).

2.2. Samples

Aleurone fractions were obtained from wheat bran (*T. aestivum* L.) by dry milling with a patented process from Bühler AG US 2003/0175384 A1[24]. This patented process is subdivided in the following steps: 1. drying/heating, 2. separation, 3. sieving, 4. air-classification (sifting) and 5. fragmentation. Fraction named AF1 (ASP-1) presented an aleurone content of 55-70%, fraction named AF2 (ASP-2) is a fraction with high purity and presented an aleurone content of 75-90%. As reported in the data provided by Bühler, the particle size of the obtained fractions was:

AF1: < 105 µm (24.6%), < 150 µm (56.3%), < 215 µm (86.8%), < 305 µm (97.4%) < 515 µm (99.6%);

AF2: < 105 µm (6.5%), < 150 µm (22%), < 215 µm (63%), < 305 µm (95%) < 515 µm (100%).

2.3. Extraction of free and bound phenolic compounds in aleurone fraction samples

According to Verardo et al. (2011)[25], free phenolic fraction was obtained extracting 1 gram of sample three times in an ultrasonic bath (10 min) with 10 mL of ethanol/water (4:1 *v/v*). Three subsequent extractions allowed an exhaustive extraction of phenolic compounds from the matrix. The supernatants were collected, evaporated and reconstituted with 1 mL of methanol/water (1:1 v/v). After the free phenolic discharge, the residue was digested with 25 mL of NaOH 1M at room temperature for 18 h by shaking under nitrogen gas. The mixture was then brought to pH 2-3 by adding hydrochloric acid and the lipids were extracted with 250 mL of hexane. The aqueous solution was extracted three times with 50 mL of diethyl ether/ethyl acetate (1/1, *v/v*). The supernatants were collected, centrifuged at 2096 g for 10 min, evaporated and reconstituted with 1 mL of methanol/water (1:1 v/v). All the extracts were filtered through 0.22 μm RC syringe filters and stored at -18 ºC until the analyses.

2.4. Determination of phenolic compounds by HPLC-ESI-TOF-MS

HPLC analysis was performed by an ACQUITY UPLC M-Class System from Waters (Waters Corp., Milford, MA, USA) consisting of a vacuum degasser, autosampler, and a binary pump. The HPLC system was coupled to DAD and to a microTOF^{TM} (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer (oaTOF-MS), equipped with an ESI interface. MS analyses were carried out in negative ion mode in a mass range from *m/z* 50–1100. Phenolic compounds were separated in a Kinetex C18 column (4.6 x 100 mm, 2.6 μm) from Phenomenex (Torrance, CA, USA). The mobile phase and gradient program were used as previously described by Gómez-Caravaca et al. (2014)[26]. All HPLC components were controlled by Hystar 3.1 software (Bruker Daltonik, Bremen, Germany). The optimum values of the ESI-MS parameters were: capillary voltage, + 4.5 kV; drying gas temperature, 190°C; drying gas flow, 7.0 L/min; and nebulizing gas pressure, 21.7 psi. During the HPLC analyses, external instrument calibration was performed using a Cole Palmer syringe pump (Vernon

Hills, Illinois, USA) passing a solution of sodium acetate cluster containing 5 mM sodium hydroxide of 0.2% acetic acid in water/isopropanol 1:1 (v/v).

Quantification of phenolic compounds has been done using the calibration curves obtained using the standard solutions of chlorogenic, ferulic and syringic acids, and apigenin. The response of the standards can differ from the response of the compounds present in aleurone fractions, because of that the quantification of these compounds is only an estimation of their concentrations. This approach allows corroborating phenolic changes in the different wheat fractions.

Supplementary Table 1. Analytical parameters of the method

Standard	Equation	R^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Apigenin	y = 4.24E5 x - 5.33E5	0.9996	LOQ -150	0.054	0.18
Chlorogenic acid	y = 4.75E5 x - 4.89E5	0.9989	LOQ -150	0.072	0.24
Ferulic acid	y = 2.11E5 x + 2.04E5	0.9995	LOQ -150	0.033	0.11
Syringic acid	y = 2.06E5 x+ 3.18E5	0.9992	LOQ -150	0.042	0.14

The standard stock solutions were prepared at 200 µg/mL in methanol. The analytical parameters of the method were reported in the **supplementary Table 1**.

2.5. Determination of alkylresorcinols by GC-MS

Alkylresorcinols were extracted according to Ross et al. (2003)[27] using ethyl acetate as solvent (0.5 g of flour with 40 mL of ethyl acetate). The targeted compounds were silylated according to Sweeley et al. (1963)[28]. Therefore, the silylation was performed adding a reagent mixture (pyridine: hexamethyldisilazane: trimethychlorosilane 5/2/1 v/v/v) to the extract. Solution was thermostated at 40 ºC for 20 minutes. After that, silylating solution was evaporated by nitrogen and the dry extract was reconstituted in *n*-hexane. Finally, the extracts were analysed by GC-MS using a 6890 GC system (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 5975A MS detector. GC was equipped with an HP-5ms (30 m × 0.25 mm inner diameter, 0.25 µm film

thickness) from Agilent Technologies. GC separation was done according to López-Cobo et al. (2017)[29]. Methylbehenate was used as internal standard and the calibration curve was prepared using nonadecylresorcinol (LOQ = 2.04 μg/g; r^2 = 0.9999, linear range: LOQ to 150 ppm).

2.6. Statistical analyses

All chemical analyses were carried out in triplicate, and the analytical data were used for statistical comparisons. One-way analysis of variance, ANOVA (Tukey's honest significant difference multiple comparison) was evaluated using Statistica 8.0 software (2007, StatSoft, Tulsa, OK, USA). *p* values lower than 0.05 were considered statistically significant.

3. Results and discussion

The composition of fractions was previously studied and published[30]. AF2 contained the highest amount of proteins (20.8 and 16.9% d.w. for AF2 and AF1, respectively) and ashes (11.3 and 9.3% d.w. for AF2 and AF1, respectively), confirming the presence of the highest content of aleurone fraction in this wheat product. In fact as reported by several authors[21,30,31] minerals and proteins are mainly located in aleurone layer. Fat content was the same in the two fractions (5.8 g/100 g d.w.). Finally, AF1 reported the highest value of fiber content (54.1% AF1 *vs.* 47.1% AF2); AF1 also showed the highest value of insoluble dietary fiber (50% AF1 *vs.* 40% AF2) and soluble dietary fiber was 4.1% in both fractions. Dietary fiber composition was: arabinoxylans (23 and 18% in AF1 and AF2, respectively), cellulose (8 and 5% in AF1 and AF2, respectively) and β-glucans (4.3 and 4.8% in AF1 and AF2, respectively)[30].

3.1. Identification of free and bound phenolic compounds in aleurone fractions

Phenolic content of the two aleurone fractions were analysed by HPLC coupled to DAD and MS with TOF analyzer. Free and bound phenolic compounds were tentatively identified by rendering their mass spectra using the software of SmartFormula and bearing in mind the data reported in the literature and, when available, by co-elution with commercial standards. Parameters which allowed the identification of these phenolic compounds were retention time, UV max, molecular formula, *m/z* experimental and calculated, error, mSigma and m/z of fragments generated in the ionization source. Several free and bound phenolic compounds previously described in wheat whole grain and bran fractions were identified in aleurone fractions[32-34] and, to our knowledge, a feruloyl di-hexosidee, flavonoids and several ferulic derivative isomers were described for the first time in wheat aleurone.

Table 1. Free phenolic compounds identified in aleurone fractions by HPLC-E

	Free phenolic compound	RT(min)	UV max	Molecular formula	*m/z* experimental
1	*p*-hydroxybenzoic acid	5.7	254	$C_7H_6O_3$	137.0255
2	Feruloyl-dihexoside	7.8	326	$C_{22}H_{30}O_{14}$	517.1553
3	Vanillic acid	8.1	260, 292	$C_8H_8O_4$	167.0350
4	Caffeic acid	8.5	298, 322	$C_9H_8O_4$	179.0347
5	Syringic acid	9.1	276	$C_9H_{10}O_5$	197.0445
6	Apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside isomer 1	10.2	271, 338	$C_{27}H_{30}O_{15}$	593.1559
7	Apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside isomer 2	10.5	271, 338	$C_{27}H_{30}O_{15}$	593.1581
8	4-Feruloylquinic acid	10.6	238, 325	$C_{17}H_{20}O_9$	367.1021
9	Lucenin 1/3 (luteolin-6/8-C-xyloside-8/6-C-glucoside)	10.7	269, 348	$C_{26}H_{28}O_{15}$	579.1415
10	Diferulic acid	11.0	320	$C_{20}H_{18}O_8$	385.1146
11	Apigenin-6-*C*-arabinoside-8-*C*-hexoside (shaftoside/Isoshaftoside isomer 1)	11.2	272, 336	$C_{26}H_{28}O_{14}$	563.1473
12	Apigenin-6-*C*-arabinoside-8-*C*-hexoside (shaftoside/Isoshaftoside isomer 2)	11.7	272, 336	$C_{26}H_{28}O_{14}$	563.1466
13	*trans* ferulic acid	12.8	294, 322	$C_{10}H_{10}O_4$	193.0501
14	*cis* ferulic acid	13.1	310	$C_{10}H_{10}O_4$	193.0142
15	Apigenin-6-*C*-β-galactosyl-8-*C*-β-glucosyl-O-glucuronopyranoside isomer 1	14.7	272, 330	$C_{33}H_{38}O_{21}$	769.2039
16	Apigenin-6-*C*-β-galactosyl-8-*C*-β-glucosyl-*O*-glucuronopyranoside isomer 2	15.1	272, 330	$C_{33}H_{38}O_{21}$	769.2027

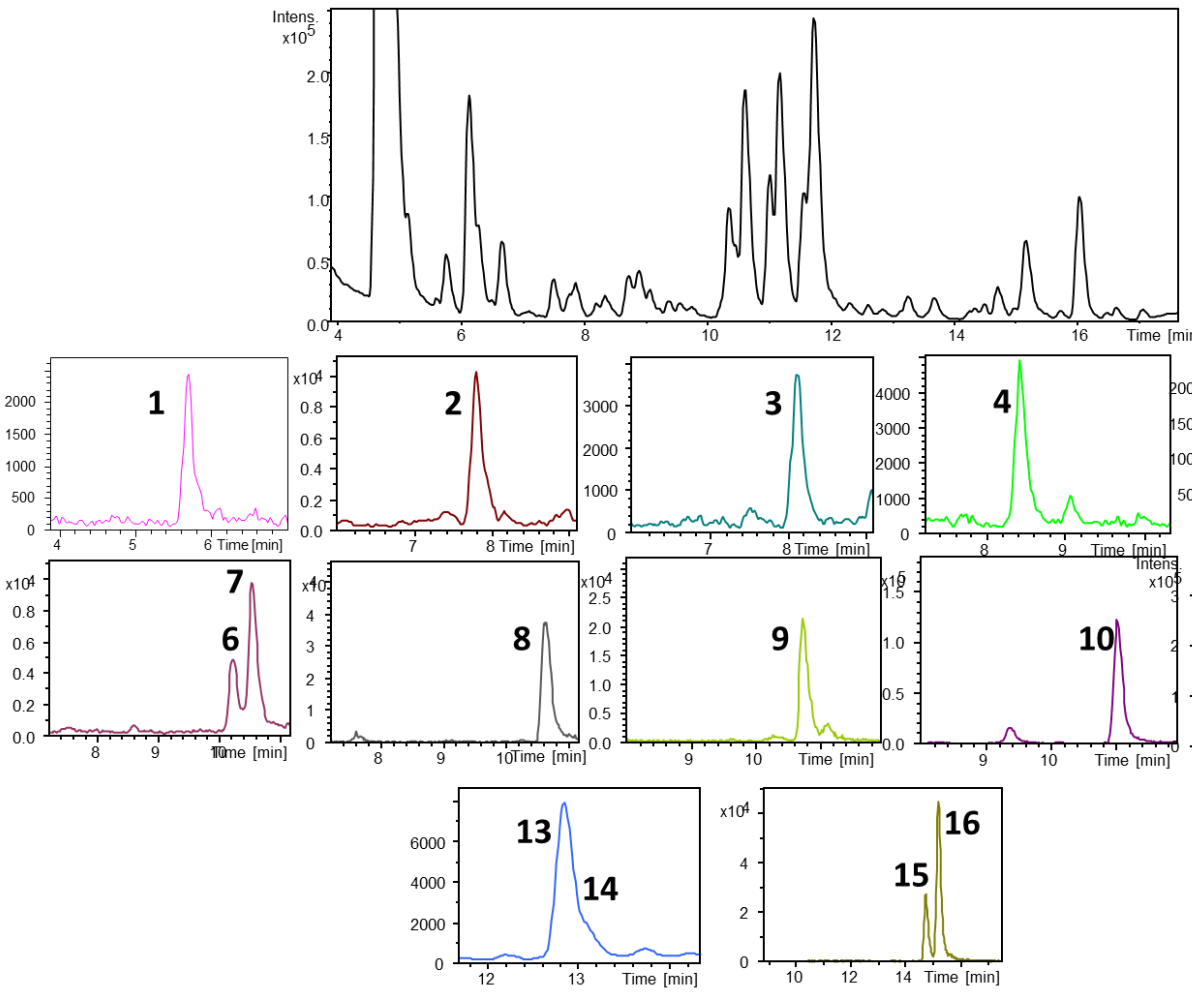

Figure S1. BPC and EICs of free phenolics in aleurone fraction determined by HPLC-ESI-TOF reported in **Table 1**)

Table 2. Bound phenolic compounds identified in aleurone fractions by HPLC-E

	Phenolic compound	RT(min)	Molecular formula	UV max	Detected ion	m/z experimental	m calcu
1	Vanillic aldehyde	4.6	$C_8H_8O_3$	258, 291	$[M-H]^-$	151.0411	151.0
2	Sinapic acid	6.0	$C_{11}H_{12}O_5$	324	$[M-H]^-$	223.0618	223.0
3	Caffeic acid	7.8	$C_9H_8O_4$	298, 322	$[M-H]^-$	179.0365	179.0
4	Vanillic acid	8.1	$C_8H_8O_4$	260, 292	$[M-H]^-$	167.0354	167.0
5	Benzoic aldehyde	8.4	$C_7H_6O_2$	280	$[M-H]^-$	121.0283	121.0
6	Syringic acid	9.1	$C_9H_{10}O_5$	276	$[M-H]^-$	197.0480	197.0
7	*p*-coumaric acid	11.7	$C_9H_8O_3$	226, 312	$[M-H]^-$	163.0417	163.0
8	*trans* ferulic acid	12.9	$C_{10}H_{10}O_4$	294, 322	$[M-H]^-$	193.0529	193.0
9	*cis* ferulic acid	13.1	$C_{10}H_{10}O_4$	310	$[M-H]^-$	193.0523	193.0
10	Disinapic acid isomer 1	13.4	$C_{22}H_{22}O_{10}$	324	$[M-H-CO_2]^-$	401.1288	401.1
11	Diferulic acid isomer 1	14.2	$C_{20}H_{18}O_8$	296, 326	$[M-H]^-$	385.0979	385.0
12	Diferulic acid isomer 2	15.4	$C_{20}H_{18}O_8$	296, 322	$[M-H]^-$	385.0992	385.0
13	Diferulic acid isomer3	16.3	$C_{20}H_{18}O_8$	320	$[M-H]^-$	385.0993	385.0
14	Disinapic acid isomer 2	17.5	$C_{22}H_{22}O_{10}$	324	$[M-H-CO_2]^-$	401.0949	401.1
15	Dehydrotriferulic acid isomer 1	18.9	$C_{30}H_{26}O_{12}$	322	$[M-H]^-$	577.1396	577.1
16	Dehydrotriferulic acid isomer 2	19.6	$C_{30}H_{26}O_{12}$	322	$[M-H]^-$	577.1464	577.1
17	Diferulic acid isomer 4	19.9	$C_{20}H_{18}O_8$	294, 326	$[M-H]^-$	385.1001	385.0

18	Diferulic acid isomer 5	21.0	$C_{20}H_{18}O_8$	290, 322	$[M-H]^-$	385.1001	385.0
19	Dehydrotriferulic acid isomer 3	21.8	$C_{30}H_{26}O_{12}$	322	$[M-H]^-$	577.1469	577.1
20	Dehydrotriferulic acid isomer 4	22.1	$C_{30}H_{26}O_{12}$	322	$[M-H]^-$	577.1468	577.1
21	Dehydrotriferulic acid isomer 5	23.6	$C_{30}H_{26}O_{12}$	322	$[M-H]^-$	577.1451	577.1

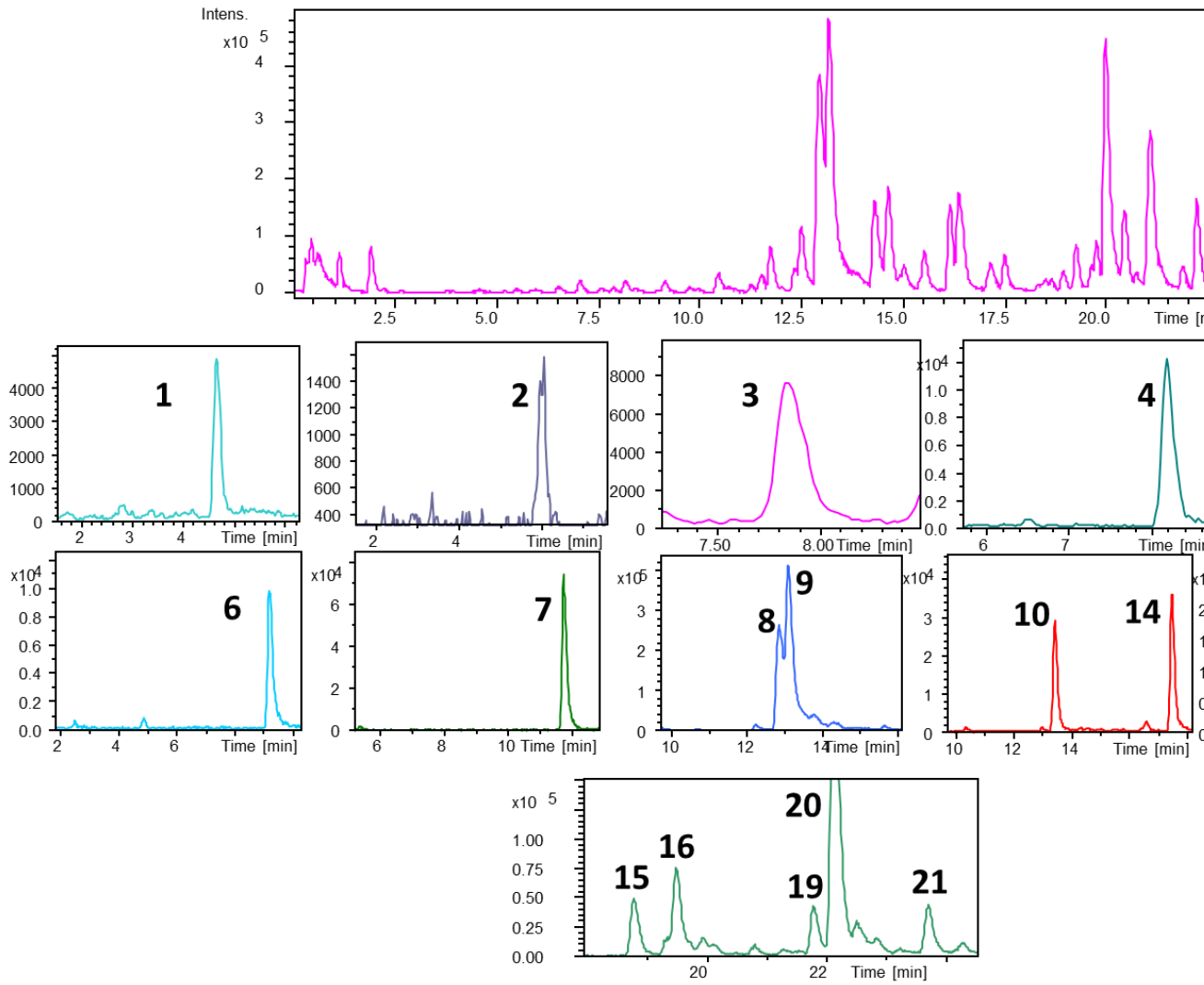

Figure S2. BPC and EICs of bound phenolics in aleurone fraction determined by HPLC-ESI-TO
reported in **Table 2**)

A total of 16 free phenolic compounds were identified in the two aleurone fractions (**Table 1.**). **Figure S1** shows the base peak chromatogram and extracted ion chromatograms EICs of free phenolics in aleurone fraction determined by HPLC-ESI-TOF-MS. Peak 1 at 5.7 min presented a deprotonated molecule at *m/z* 137.0255 and presented absorption maxima at 254 nm with the fragment at m/z 136.0145 which corresponds to *p*-hydroxybenzoic acid as previously described in wheat[35-37]. Peak 2 was found at 7.8 min with a deprotonated molecule at *m/z* 517.1553 and presented a major absorption peak at 326 nm, which was identified as feruloyl-di-hexoside, which was previously identified in rice bran[38,39] but not in wheat. Peak 3 at 8.1 min with a deprotonated molecule at *m/z* 167.0350 and presented absorption maxima at 260 and 292 nm was identified as vanillic acid[32,33,36]. Peak 4 at 8.5 min, presented a deprotonated molecule at *m/z* 179.0347 that showed a major absorption peak at 298 and 322 nm[40] and presented a fragment at m/z 135.0455 corresponding to caffeic acid as already described in wheat[34,37,41]. At 9.1 min (peak 5) with a deprotonated molecule at *m/z* 197.0445[33] and presented absorption maxima at 276 nm[36,40] was identified as syringic acid. Peaks 6 and 7, at 10.2 min and 10.5 min presented a deprotonated molecule at *m/z* 593.1559 and 593.1581 and a major absorption peak at 271 and 338 nm, thus, they were tentatively identified as isomers of apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside[33.42]. Peak 8, at 10.6 min with a deprotonated molecule at *m/z* 367.1021 with absorption maxima at 238 and 325 nm and fragments at m/z 157.0382, 187.0587, 367.1194 was identified as 4-feruloylquinic acid[37,43]. At 10.7 min (peak 9) with a deprotonated molecule at *m/z* 579.1415 and with absorption maxima at 269 and 348 nm was identified as lucenin 1/3 (luteolin-6/8-*C*-xyloside-8/6-*C*-glucoside)[33,44]. Peak 10 at 11.0 min and deprotonated molecule at *m/z* 385.1146 and with absorption maxima at 320 nm and a fragment at m/z 341.1048 was identified as diferulic acid[36,37,45]. Peaks 11 and 12, at 11.2 and 11.7 min with deprotonated molecules at *m/z* 563.1473 and 563.1466 presented absorption maxima at 272 and 336 nm, were identified as isomers of shaftoside/isoshaftoside[33,36,46]. Two isomers of ferulic acid were detected at 12.8 and 13.1 min (peaks 13 and 14) with a deprotonated molecule at *m/z* 193.0501 and

193.0542 and fragments at m/z 134, 178, 133 and 149[32,33,35,37]. *Trans* ferulic acid presented absorption maxima at 294 and 322 nm, whereas *cis* ferulic acid a mojor absorption at 310 nm[36]. Finally, peaks 15 and 16 at 14.7 and 15.1 minutes, respectively, and deprotonated molecule at *m/z* 769.2039 and 769.2027 with absorption maxima at 272 and 330 nm were proposed as isomers of apigenin-6-*C*-β-galactosyl-8-*C*-β-glucosyl-*O*-glucuronopyranoside[33,36].

Figure S2 shows the base peak chromatogram and extracted ion chromatograms EICs of bound phenolics in aleurone fraction determined by HPLC-ESI-TOF-MS. Twenty-one bound phenolic compounds were identified in the two aleurone fractions (**Table 2**): Peak 1, at 4.6 min with a deprotonated molecule at *m/z* 151.0411 and with absorption maxima at 258 and 291 nm was identified as vanillic aldehyde (vanillin)[33,35]. Peak 2, at 6.0 min and deprotonated molecule at *m/z* 223.0618 with fragments at m/z 193.0486, 149.0265 and presented an absorption maxima at 324 nm was identified as sinapic acid[33,36]. At 7.8 min (peak 3) was found a phenolic compound with a deprotonated molecule at *m/z* 179.0365 with a fragment at m/z 135.0497 and a major absorption peak at 298 and 322 nm that was identified as caffeic acid[32,36,37]. Peak 4, at 8.1 min with a deprotonated molecule at *m/z* 167.0354 and with a major absorption peak at 260 and 292 nm , was identified as vanillic acid[33,36]. Peak 5, at 8.4 min and deprotonated molecule at *m/z* 121.0283 and with absorption maxima at 280 nm, was identified as benzoic aldehyde (*p*-hydroxybenzaldehyde)[33,36]. Peak 6, at 9.1 min with a deprotonated molecule at *m/z* 197.0480 and with a major absorption peak at 276 nm, was identified as syringic acid[33,36]. Peak 7 was detected at 11.7 min with a deprotonated molecule at *m/z* 163.0417 with a fragment at m/z 119.0515 and with absorption maxima at 226 and 312 nm was proposed as *p*-coumaric acid[35,37,40]. Peaks 8 and 9, at 12.9 and 13.1 min, respectively, presented a deprotonated molecule at *m/z* 193.0529 and 193.0523 with fragments at m/z 134, 178, 133 and 149 and presented absorption maxima at 294 and 322 nm and 310 nm were identified as *cis* and *trans* isomers of ferulic acid. *Trans* ferulic acid is the most common and abundant phenolic acid in wheat cell walls[35,36]. Other two isomers (peaks 10 and 14) at *m/z* 401.1288 and

401.0949 were found at 13.4 and 17.5 min, with a major absorption peak at 324 nm were identified as disinapic acids[36,47]. In addition, five isomers (peaks 11, 12, 13, 17 and 18) at 14.2, 15.4, 16.3, 19.9 and 21 min, respectively, which presented a deprotonated molecule at *m/z* 385.0979 and 385.0992 with a fragment at m/z 341 and absorption maxima at 296 and 326 nm, 396 and 322 nm, 320 nm, 294 and 326 nm and 290 and 322 nm were identified as diferulic acids[33,36,37]. Finally, other five isomers at *m/z* 577 and detected 18.9, 19.6, 21.8, 22.1 and 23.6 min with a major absorption maxima at 322 nm, were identified as dehydrotriferulic acids[36,48].

3.2. Identification of alkylresorcinols in aleurone fractions

Alkylresorcinols (ARs) are another important family of phenolic compounds. ARs are amphiphilic phenolic lipids and whole wheat grain and its tissues have demonstrated to contain the ARs homologs from C17 to C25[17,20,49-51]. In this study, the homologs identified in the two aleurone fractions extracts ranged from C15 to C25. **Figure S3** shows the total ion chromatogram (TIC) and EIC (m/z 268) of alkylresorcinols in aleurone fraction determined by GC-MS. The ARs showed a base peak at 268 *m/z* proceeding from McLafferty rearrangement and a relative major fragment at 281 *m/z*.

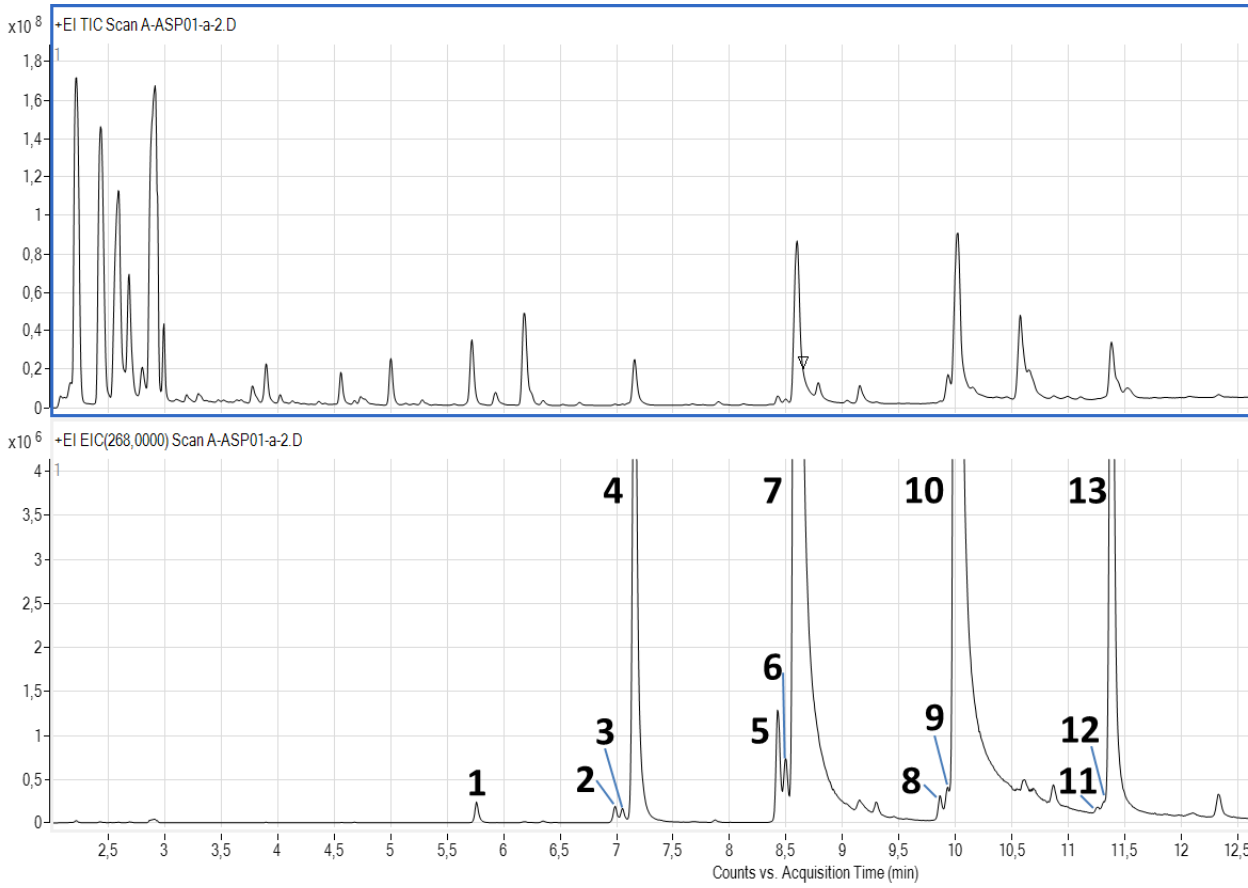

Figure S3. TIC and EIC (m/z 268) of alkylresorcinols in aleurone fraction determined by GC-MS (N
Table 5)

A compound with molecular ion 464 *m/z* (silylated molecule) was identified as pentadecylresorcinol (C15:0) according to López-Cobo et al. (2017)[29]. According to Landberg, Kamal-Eldin, Andersson, et al. (2008) and Ross et al. (2003)[20,27], the compounds with molecular ions at *m/z* 348, 376, 404, 432 and 460 were identified as C17, C19, C21, C23 and C25 saturated alkylresorcinols. Moreover, several monounsaturated alkylresorcinol isomers were also detected; C17:1, C19:1, C21:1 and C23:1 according to Knödler et al. (2008)[50] and two isomers for each one were noticed.

3.3. Quantification of phenolic compounds and alkylresorcinols in aleurone fractions

A total of 16 free phenolic compounds have been quantified in aleurone fractions: three hydroxybenzoic acids, six hydroxycinnamic acids and seven flavonoids (**Table 3**). Flavonoids were the most abundant free phenolic compounds in AF1 and AF2 and they represented 77.5 and 76.6% of total free phenolic content in AF1 and AF2 (124.5 and 116.8 µg/g d.w.), respectively. Among them, the most abundant flavonoid was shaftoside/isoshaftoside isomer 2, which value in AF1 was 10.22% higher than the obtained in AF2. The second most concentrated flavonoid was apigenin-6-*C*-β-galactosyl-8-*C*-β-glucosyl-*O*-glucuronopyranoside isomer 2, which content was 19.9 and 20.6 µg/g d.w. in AF1 and AF2, respectively, without significant differences between them. The third most abundant free phenolic compound was shaftoside/isoshaftoside isomer 1, which content in AF1 (15.4 µg/g d.w.) was 12.87% higher than the obtained in AF2 (13.4 µg/g d.w.). Therefore, the most abundant flavonoids were concentrated in AF1, being the total content of flavonoids in AF1 (96.5 µg/g d.w.) 7.25% higher than in AF2 (89.5 µg/g d.w.).

Table 3. Free phenolic compounds (µg/g d.w.) quantified in aleurone fractions by HPLC-ESI-TOF-MS.

Compounds	**AF1**	**AF2**
p-hydroxybenzoic acid[1]	4.46 ± 0.002[a]	4.81 ± 0.11[a]
Feruloyl-sucrose[2]	2.28 ± 0.01	<LOQ
Vanillic acid[1]	4.86 ± 0.05[a]	4.68 ± 0.026[a]
Apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside isomer 1[3]	8.68 ± 0.51[a]	6.88 ± 0.16[b]
Caffeic acid[1]	0.36 ± 0.01[a]	0.34 ± 0.01[a]
Syringic acid[1]	5.85 ± 0.04[a]	5.61 ± 0.05[b]
Apigenin-6/8-*C*-pentoside-8/6-*C*-hexoside isomer 2[3]	7.28 ± 0.08[a]	7.46 ± 0.10[a]
3-Feruloylquinic acid[4]	2.09 ± 0.13[a]	2.11 ± 0.07[a]
Lucenin 1/3[3]	5.80 ± 0.31[a]	4.77 ± 0.26[b]
Diferulic acid[2]	4.99 ± 0.15[a]	2.81 ± 0.19[b]
Shaftoside/Isoshaftoside isomer 1[3]	15.44 ± 0.49[a]	13.45 ± 0.11[b]
Shaftoside/Isoshaftoside isomer 2[3]	29.35 ± 0.54[a]	26.35 ± 0.29[b]
trans ferulic acid[2]	2.60 ± 0.21[b]	6.31 ± 0.12[a]
cis ferulic acid[2]	0.47 ± 0.01[b]	0.70 ± 0.03[a]
Apigenin-6-*C*-B-galactosyl-8-*C*-β-glucosyl-*O*-glucuronopyranoside isomer 1[3]	10.06 ± 0.20[a]	9.98 ± 0.21[a]
Apigenin-6-*C*-β-galactosyl-8-*C*-B-glucosyl-*O*-glucuronopyranoside isomer 2[3]	19.91 ± 0.69[a]	20.63 ± 0.09[a]
Hydroxybenzoic acids	15.18 ± 0.09[a]	15.10 ± 0.32[a]
Hydrocinnamic acids	12.80 ± 0.22[a]	12.23 ± 0.37[a]
of them		
Hydroxycinnamic acid monomer	7.81 ± 0.07[b]	9.43 ± 0.19[a]
Hydroxycinnamic acid dimer	4.99 ± 0.15[a]	2.81 ± 0.59[b]
Flavonoids	96.52 ± 0.06[b]	89.52 ± 0.25[a]
Total	124.50 ± 0.36[a]	116.85 ± 0.95[b]

LOQ: Limit of quantification = 0.024 µg/mL.

[1] µg/g syringic acid. [2] µg/g ferulic acid. [3] µg/g apigenin. [4] µg/g chlorogenic acid.

Different letters (a–b) in the same line indicate significant differences ($p < 0.05$).

The content of free hydroxycinnamic monomers represented 6.27% in AF1 and 8.07% in AF2 regarding total free phenolic compounds (**Table 3**). Furthermore, the content of hydroxycinnamic monomers obtained in AF2 was 17.18% higher than in AF1; in particular, *trans* and *cis* ferulic acid concentrations were 58.79% and 32.86% higher in AF2 than in AF1, respectively. Conversely, the highest concentration of diferulic acid was obtained in AF1, 43.69% higher than the obtained in AF2. Therefore, fraction with a higher percentage of aleurone

contained more hydroxycinnamic monomers, whereas the fraction with a lower content of aleurone was mainly composed by diferulic acid. This fact can be explained because wheat grain outer layers contain more oligomers than the inner layers[23].

Comparing the results of free hydroxycinnamic acids in AF1 and AF2 with previous studies, concentrations of ferulic acid and caffeic acid were in the same range that the previously data reported in aleurone enriched-bread and in aleurone durum wheat by other authors (0.18-0.2 µg/g d.w. and 2.92-4.1 µg/g d.w)[52,53].

Concerning hydroxybenzoic acid content, no significant difference was observed between the two aleurone fractions (15.2 and 15.1 µg/g d.w. in AF1 and AF2, respectively). However, the content of *p*-hydroxybenzoic present in aleurone fractions was 89.69 and 90.44% higher than the obtained by a previous study in aleurone (0.46µg/g d.w.)[53]. Besides, the content of *p*-hydroxybenzoic acid and syringic acid in AF1 and AF2 was around 80.15 and 74.87% higher, respectively, than the reported in coarse bran[32].

As for the bound phenolic compounds, a total of 21 compounds were quantified: 16 hydroxycinnamic acids and 5 hydroxybenzoic acids (**Table 4**). These bound phenolic compounds represented 79.26-83.85% of the total phenolic compounds. The family of hydroxycinnamic acids was the most concentrated and it constituted 92.73 and 94.19 % of total bound phenolic compounds in AF1 and AF2, respectively. Hydroxycinnamic acids monomers in AF1 and in AF2 represented 69.02% and 81.36% of the total bound phenolic content, whereas hydroxycinnamic acid dimers/trimers were 23.22 and 12.58% of the total bound phenolic compounds in AF1 and AF2, respectively.

Table 4. Bound phenolic compounds (µg/g d.w.) quantified in aleurone fractions by HPLC-ESI-TOF-MS.

Compounds	**AF1**	**AF2**
Vanillic aldehyde[1]	4.45 ± 0.02[a]	4.49 ± 0.21[a]
Sinapic acid[2]	4.58 ± 0.05[b]	5.28 ± 0.10[a]
Caffeic acid[2]	<LOQ	0.01 ± 0.004
Vanillic acid[1]	8.02 ± 0.24[a]	7.31 ±0.54[a]
Benzoic aldehyde[1]	7.98 ± 0.17[a]	7.41 ± 0.78[a]
Syringic acid[1]	9.94 ± 0.09[a]	10.74 ± 0.48[a]
p-coumaric acid[2]	5.96 ± 0.26[b]	9.99 ± 0.55[a]
trans ferulic acid[2]	303.81 ± 1.6[b]	447.55 ± 3.43[a]
cis ferulic acid[2]	18.67 ± 0.27[b]	35.95 ± 0.42[a]
Disinapic acid isomer 1[2]	0.88 ± 0.10[a]	0.36 ± 0.05[b]
Diferulic acid isomer 1[2]	6.44 ± 0.34[a]	3.90 ± 0.21[b]
Diferulic acid isomer 2[2]	1.95 ± 0.05[a]	1.35 ± 0.10[b]
Diferulic acid isomer 3[2]	15.24 ± 0.50[a]	8.90 ± 0.35[b]
Disinapic acid isomer 2[2]	1.47 ± 0.08[a]	1.16 ± 0.06[a]
Dehydrotriferulic acid isomer 1[2]	2.65 ± 0.02[a]	1.36 ± 0.13[b]
Dehydrotriferulic acid isomer 2[2]	2.46 ±0.06[a]	1.41 ± 0.08[b]
Diferulic acid isomer 4[2]	23.81 ± 0.42[a]	17.06 ± 0.12[b]
Diferulic acid isomer 5[2]	47.02 ± 0.41[a]	35.43 ± 1.09[b]
Dehydrotriferulic acid isomer 3[2]	7.63 ± 0.19[a]	5.28 ± 0.32[b]
Dehydrotriferulic acid isomer 4[2]	2.07 ± 0.003[a]	0.77 ± 0.02[b]
Dehydrotriferulic acid isomer 5[2]	1.19 ± 0.08[a]	0.82 ± 0.13[a]
Hydroxybenzoic acids	34.98 ± 0.43[a]	35.22 ± 1.49[a]
Hydroxycinnamic acids of which	441.26 ± 0.06[a]	571.32 ± 3.86[a]
Hydroxycinnamic monomers	328.44 ± 2.17[b]	493.49 ± 3.20[a]
Hydroxycinnamic Dimers/Trimers	110.47 ± 2.05[a]	76.29 ± 0.57[b]
Total	475.84 ± 0.37[b]	606.55 ± 5.35[a]

LOQ: Limit of quantification = 0.024 µg/mL.
[1] µg/g syringic acid, [2] µg/g ferulic acid.

Different letters (a–b) in the same line indicate significant differences ($p < 0.05$).

The content of bound hydroxycinnamic acids monomers in AF2 (493.49µg/g d.w.) was 33.44% higher than the obtained in AF1 (328.44 µg/g d.w.). Monomers of hydroxycinnamic acids quantified in aleurone fractions were *trans* and *cis* ferulic acid, *p*-coumaric acid, sinapic acid and caffeic acid. The most concentrated hydroxycinnamic acid was the monomer *trans* ferulic acid, which content in AF2 (447.55 µg/g d.w.) was 32.12% higher than in AF1 (303.81 µg/g d.w.). This result

totally agree with the data reported by Zhou and co-workers[54] in ASP-1 and ASP-2 samples; moreover, it was in concordance with a previous work that reported that the content of ferulic acid in aleurone was twice higher than in wheat bran[55]. Barron et al. (2007)[56] reported the content of ferulic acid in aleurone in different cultivars (7980-8140 µg/g), which average was 40% higher than the obtained in intermediate layers and 60 % higher than the obtained in the outer pericarp[56]. In addition, Antoine et al. (2004) reported that the content of ferulic acid in aleurone was 27.54% higher than the obtained in intermediate layers[57]. The second most abundant hydroxycinnamic monomer in AF2 was *cis* ferulic acid (35.9 µg/g), which was 48.07% higher than in AF1. Therefore, total ferulic acid content in AF2 was 33.30% higher than in AF1. This result agrees with a previous study that reported that total ferulic content in aleurone-rich fraction was 39.40% higher than the obtained in pericarp-rich fraction[58]. Besides, the content of *p*-coumaric acid was 9.99 µg/g in AF2, 40.34% higher than the obtained in AF1.This result is in concordance with the reported by previous works, where *p*-coumaric content in aleurone was from 52 to 94% higher than the obtained in the outer pericarp and in intermediate layers[23,56,57]. Spaggiari et al. (2020) also reported a higher concentration of *p*-coumaric acid in aleurone (14.52 µg/g d.w.) with respect to the obtained in bran (13.50µg/g d.w.) in durum wheat[53]. Therefore, it has been shown that aleurone contains a higher concentration of phenolic acids monomers than the other wheat grain layers. Because of that, it can be explained that AF2 contains monomers in a greater proportion with respect to AF1, since AF2 is composed by aleurone in a high proportion (75-90%).

Conversely, total content of bound hydroxycinnamic dimers/trimers in AF1 (110.47 µg/g d.w.) was 30.93% higher than in AF2 (76.3 µg/g d.w.). Among them, the most abundant were diferulic acids, which total content in AF1 (94.5 µg/g d.w.) was 29.52 % higher than the obtained in AF2 (66.6 µg/g d.w.); whereas the content of dehydrotriferulic acids was 16.0 µg/g in AF1, 40 % higher than the obtained in AF2 (9.6 µg/g d.w.). These results agree with the reported by previous works. Barron et al. (2007) reported a content of ferulic acid dimer in outer pericarp and intermediate layers, which average value was 85% and 44% higher than in

aleurone layers, whereas the content of dehydrotriferulic acids in outer pericarp and in intermediate layer was 97% and 67% higher than in aleurone[56]. Besides, other works reported that the content of ferulic acid dehydrodimer and dehydrotrimer in outer pericarp was 79 and 91% higher than the obtained in the aleurone layer, whereas in intermediate layers obtained a lower concentration than aleurone[23,57]. Therefore, the highest content of oligomers is found in the outer pericarp[21]. AF1 contained the outer pericarp and intermediate layers in 30-45%, whereas AF2 had only 10-25% of these layers. For this reason, AF1 contained higher concentration in oligomers.

Regarding bound hydroxybenzoic acids, their total content was 35.0 µg/g d.w. and 35.2 µg/g d.w. in AF1 and AF2, respectively, so no significant differences were found between them.

Similar trend was noticed for disinapic acids; their content in AF1 sample was 35.32 % higher than in AF2.

Finally, the total phenolic compounds content in AF2 (723.4 µg/g d.w.) was 17 % higher than in AF1 (600.34 µg/g d.w.). This is because the major compound was ferulic acid that is present in high concentration in AF2.

Sum of free and bound ferulic acid monomers in AF2 (490.51 µg/g d.w.) was 33.63% higher than the obtained in AF1 (325.55 µg/g d.w.), whereas total ferulic acid oligomers in AF1 (115.45 µg/g d.w.) was 33.9 % higher than the obtained in AF2 (76.28 µg/g d.w.). According to these results, the aleurone layer showed the highest content of monomers, whereas the outer pericarp layer contained the highest concentration in ferulic acid oligomers.

Table 5. Alkylresorcinols (µg/g d.w.) quantified in aleurone fractions by GC-MS.

	Compounds	**M^+ and qualifier fragment**	**AF1**	**AF2**
1	C15:0	464, 268	19.17 ± 0.64[a]	15.91 ± 0.32[b]
2	C17:1	490, 268	13.52 ± 0.67[a]	11.63 ± 0.30[b]
3	C17:1	490, 268	13.48 ± 0.73[a]	11.94 ± 1.01[b]
4	C17:0	492, 268	774.00 ± 10.22[a]	653.37 ± 8.36[b]
5	C19:1	518, 268	95.10 ± 1.99[a]	77.92 ± 0.62[b]
6	C19:1	518, 268	57.46 ± 1.30[a]	50.02 ± 2.99[b]

7	C19:0	520, 268	4450.26 ± 82.16[a]	4093.45 ± 62.13[b]
8	C21:1	546, 268	18.45 ± 0.93[a]	16.49 ± 0.69[b]
9	C21:1	546, 268	18.11 ± 0.85[a]	16.16 ± 0.69[b]
10	C21:0	548, 268	4521.96 ± 119.39[a]	4070.19 ± 32.21[b]
11	C23:1	574, 268	4.91 ± 0.24[a]	4.99 ± 0.06[a]
12	C23:1	574, 268	6.11 ± 0.34[a]	5.23 ± 0.50[a]
13	C23:0	576, 268	896.39 ± 17.31[a]	757.59 ± 7.67[b]
14	C25:0	604, 268	184.54 ± 4.04[a]	147.58 ± 3.94[b]
	Total		11073.44 ± 178.2[a]	9932.45 ± 174.6[b]

Different letters (a–b) in the same line indicate significant differences ($p < 0.05$)

Finally, a total of 14 alkyresorcinols were quantified (**Table 5)**. An increase on its total content of 10.30% from AF2 (9932.4 µg/g d.w.) to AF1 (11073.4 µg/g d.w.) was observed. These values are in the same range of magnitude as other studies that reported the highest content of ARs in intermediate layers (hyaline, testa and inner pericarp), which was 16200-16400 µg/g d.w., whereas the content in aleurone was very low 27-30 µg/g d.w[20,23]. Therefore, ARs are mainly located in intermediate layers, concretely in a cuticle at the surface of testa, and not in the aleurone layer[20,21]. The fact that AF1 and AF2 contained a high content of these ARs could be because these fractions contained testa in a high proportion. Particularly, AF1 contained more quantity of this layer. Besides, the most concentrated ARs were the ones with saturated chain, that represent about the 98 % of its total content in both fractions. C19:0 and C21:0 were the most abundant alkylresorcinols that represented the 41 % of total alkylresorcinol fraction according to previous works[20,27]. Therefore, the results confirmed that the technology used in this work is valuable to obtain aleurone fractions enriched in alkylresorcynols.

4. Conclusions

In conclusion, two aleurone enriched fractions have been obtained using dry separation technology and they have shown to be a good source of phenolic compounds. New information could be added thank to the use of advanced analytical platforms; HPLC-ESI-TOF-MS permit to identify and quantify 16 free and 21 bound phenolic compounds; GC-MS analyses showed the presence of 14 alkylresorcinols identifying also some isomers of monounsaturated compounds.

To our knowledge, feruloyl di-hexoside and several flavonoids were determined for the first time in aleurone fractions. Moreover, several monounsaturated isomers of alkylresorcinols have been determined. Concretely, AF1 with a proportion of 55-70% of aleurone was rich in alkylresorcynols, free flavonoids and free and bound oligomers of hydroxycinnamic acids, specifically, diferulic acids. AF2 composed by 75-90% of aleurone was particularly rich in free and bound monomers of hydroxycinnamic acids, being *trans* ferulic acid the most concentrated compound. Therefore, these results confirm that aleuronic layer is rich in monomers whereas the outer pericarp is rich in oligomers and the intermediate layers are particularly rich in alkylresorcinols.

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Conflict of interest

The authors have no conflicts of interests.

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CONCLUSIONS

✓ HPLC-ESI-TOF-MS platform allowed the identification and quantification of 42 and 41 phenolic compounds in 'Hojiblanca' olive leaves extracts obtained by pressurized liquid extraction (PLE) and microwave assisted extraction (MAE), respectively. The model established by Box-Behnken design was suitable for the optimization of the extraction parameters (temperature, % ethanol/water (v/v) and extraction time), which proved significant effects on the concentration response. On the one hand, the optimal conditions for PLE were 105 ºC, 100% ethanol and 5 min, and the total phenolic content obtained was 158.7 ± 0.4 mg/g d.w. On the other hand, the optimal conditions for MAE were 123 °C, 100% ethanol and 23 min, obtaining 86.7 ± 0.4 mg/g d.w as total concentration of phenolic compounds. Total phenolic content obtained by PLE was 45% higher than that obtained by MAE. In addition, PLE provided a shorter extraction time than MAEthat allows lower energy consumption, which could be economically beneficial. Therefore, PLE has demonstrated to be an efficient and valuable extraction technique for the recovery of phenolic compoundsfrom olive leaves and that it could be implemented at industrial scale.

✓ HPLC-ESI-IT-MS allowed the quantification of 36 phenolic compounds in 'Koroneiki' olive leaves extracts. after their extraction by sonotrode ultrasound assisted extraction using a Box-Benhken design. It was observed that extraction parameters (% ethanol/water (v/v), the amplitude (%) and the extraction time) considerably influence the response of the total phenolic content. The optimal conditions were ethanol/water (55/45; v/v), 100% amplitude and 8 minutes, in which a total phenolic content of 40.9 ± 0.2 mg/g d.w. was obtained. Total phenolic content was similar to that obtained by conventional extraction by ultrasonic bath after three consecutive extractions with ethanol/water (80/20; v/v) and 20 minutes. Therefore, it could be affirmed that sonotrode ultrasonic assisted extraction allows the phenolic recovery in shorter extraction times than the conventional extraction by ultrasonic bath in olives leaves. Furthermore, this ultrasound sonotrode technology is industrially scalable.

✓ The study of free and bound phenolic compounds in brewers spent grain previously treated with pulsed electric field (PEF) and determined by HPLC-ESI-MS has proven that PEF is an effective pretreatment in enhancing phenolic recovery in brewers spent grains. The extraction models were established by Box-Behnken design. PEF parameters such as electric field intensity, frequency, and total treatment time were optimized for free phenolic compounds extraction and they were found to significantly affect to the recovery of free phenolic compounds, flavan-3-ols, flavonoids and phenolic acids. The optimal PEF conditions were 2.5 kV/cm, 50 Hz and 14.5 seconds, obtaining extracts with 101±2 µg/g d.w of free phenolic compounds. In addition, bound phenolic compounds were determined at the optimal conditions established for free phenolic compounds, their total content was 536.46 ± 2.89 µg/g d.w. According to these results, the total phenolic recovery obtained with PEF was 2.7 and 1.7 times higher than that obtained without treatment with PEF for free and bound phenolic compounds, respectively. This fact could be due to the increase of the solvent permeability through the cell membrane, which would improve the mass transfer of bioactive compounds. Therefore, PEF is a promising pretreatment that could be coupled with other extraction techniques in order to reduce extraction times and improve phenolic recovery in brewers spent grains.

✓ NP-HPLC-FLD-MS platform has been used for the determination of proanthocyanidins in brewers spent grains for the first time. Proanthocyanidins were extracted by sonotrode ultrasound assisted extraction using a Box-Behnken experimental design. The effect of parameters such as acetone/water ratio (% (v/v)), time of extraction, and potency was evaluated and it was observed that they significantly affected to the total concentration of PAs. The optimal conditions were 80%/20% acetone/water (v/v), 55 min and 400W, obtaining extracts with 1.01 mg/g d.w of total proanthocyanidins. This proanthocyanidins (PAs) concentration was around 58% higher than the obtained by a conventional ultrasound bath. NP-HPLC-FLD-MS analyses allowed the identification and quantification of 11 PAs according to their degree of polymerization and the mass

spectra obtained. Thus, BBD of sonotrode ultrasound assisted extracts has demonstrated to be suitable for the optimization of PAs extraction from brewers spent grains.

✓ The study of phenolic compounds in buckwheat flours: middling flour, bran meal, light flour, and whole meal by HPLC-ESI-Q-MS after milling process allowed considering some fractions as flours enriched in phenolic compounds that could be used to elaborate food with health benefits. HPLC-ESI-Q-MS platform allowed the quantification of 25 free and 24 bound phenolic compounds in the different buckwheat flours. Roller milling in dehulled buckwheat flour appeared as a profitable technology to obtain different buckwheat flour fractions with different phenolic contents: bran meal (1932.30 mg/kg d.w.), middling flour (1605.57 mg/kg d.w.), and light flour (254.57 mg/kg d.w.). Middling flour and bran meal contained the highest phenolic concentration due to the outer layers of grain are richer in many phenolic compounds than the inner parts of the grain (endosperm).

✓ Sieving has proven to represent a suitable dry green technology in order to produce functional buckwheat flours. Sieving allowed the obtention of buckwheat flour at 215 µm, 160 µm, 85 µm, and 45 µm of particle size with different chemical composition in phenolic compounds, protein, starch and ashes. HPLC-ESI-Q-MS was used to perform the analyses and it permitted the determination of 25 free and 26 bound phenolic compounds. According to the results, buckwheat fraction with 215 µm (GS215) was the most enriched fraction in free and bound phenolic compounds, protein and ashes. The content in phenolic compounds decreased as the particle size diminished. In fact, the concentration of rutin was 40 mg/kg d.w. in dehulled buckwheat flour, whereas it increased in GS215 (174 mg/kg d.w.). At the same time, GS215 fraction reported protein and ashes amounts twice higher than the GSTQ flours. This could be because the most enriched fraction contains bran that could be lost with sieving at lower particle sizes, providing a fine fraction which is composed mainly of endosperm. Therefore, sieving has shown to be a

suitable dry fractionation technology to obtain enriched buckwheat flour fractions in phenolic compounds.

✓ Air classification has been used for the first time in dehulled buckwheat grains and it has been observed that could be a valuable technology in order to obtain flour fractions enriched in phenolic compounds. Two different fraction named coarse (CF) and fine (FF) fraction have been obtained. Briefly, air-classification provided the following flours: 30% and 20% of coarse fraction and 70% and 80% of fine fraction. Free and bound phenolic compounds were identified and quantified by high-performance liquid chromatography coupled to electrospray ionization-time of flight mass spectrometry (HPLC-ESI-TOF-MS). In addition, the antioxidant capacity by ferric reducing antioxidant power (FRAP) and 2,2-difenil-1-picrylhydrazyl (DPPH) essays were carried out to compare the antioxidant activities of different buckwheat flour fractions. Total phenolic content in coarse fractions CF20 and CF30 was 1.74 and 1.40 times greater than in the whole flours. Indeed, CF20 and CF30 showed an increase of most free and bound phenolic compounds with regards to whole buckwheat flour and total phenolic content in CF20 and CF30 was twice higher than in their fine fractions. Antioxidant activity evaluated by FRAP and DPPH assays were higher in the coarse flour fractions than in the fine flour fractions and dehulled buckwheat flours. In addition, antioxidant activities showed positive correlations total phenolic content confirming the potential bioactivity of the obtained fractions.

✓ The dry separation technologies showed to be valuable and suitable tools to obtain flour fractions enriched of phenolic compounds. Thus, dry separation technologies such as sieving followed by air-classification were used to obtain wheat bran fractions enriched of aleurone. Thanks to these technologies, two fractions (AF1, 55-70% aleurone and AF2, 75-90% aleurone) were obtained. Free and bound phenolic compounds present in both fractions have been determined by HPLC-DAD-ESI-TOF-MS; GC-MS was used for the determination of alkylresorcinols. Using these analytical platforms, feruloyl di-hexoside, and

several flavonoids and monounsaturated isomers of alkylresorcinols were determined for the first time in wheat aleurone. The results proved that the content of alkylresorcynols, free flavonoids and free and bound oligomers of hydroxycinnamic acids, specifically, diferulic acids in AF1 was higher than AF2. Instead, AF2 reported higher amounts of free and bound monomers of hydroxycinnamic acids, being trans ferulic acid compared to AF1. Therefore, these results confirmed as aleurone layer has demonstrated to be rich in monomers, whereas the outer pericarp (that is contained in higher amounts in AF1) is rich in oligomers and alkylresorcinols.

CONCLUSIONES

✓ La plataforma HPLC-ESI-TOF-MS ha demostrado ser una técnica potente que ha permitido la identificación y cuantificación los compuestos fenólicos en extractos de hojas de olivo 'Hojiblanca' obtenidos mediante extracción líquida presurizada (EPL) (un total de 42 compuestos) y extracción asistida por microondas (EAM) (41 compuestos), respectivamente. El modelo establecido mediante el diseño experimental Box-Behnken permitió realizar la optimización de los parámetros de extracción (temperatura, % etanol/agua (v/v) y tiempo de extracción), los cuales mostraron efectos significativos sobre la concentración total de compuestos fenólicos obtenida. Por un lado, las condiciones óptimas para ELP fueron 105 ºC, etanol 100% y 5 min, obteniéndose un contenido fenólico total de 158,7 ± 0,4 mg/g p.s. Por otro lado, las condiciones óptimas para AEM fueron 123 °C, 100% etanol y 23 min, obteniendo 86,7 ± 0,4 mg / g p.s como concentración total de compuestos fenólicos. El contenido fenólico total obtenido por ELP fue un 45% superior al obtenido por MAE. Además, con ELP el tiempo de extracción fue más corto que con MAE, lo cual permite un menor consumo de energía siendo económicamente más beneficioso. Por lo tanto, ELP ha demostrado ser una técnica de extracción eficaz y valiosa para la recuperación de compuestos fenólicos de la hoja de olivo que podría implementarse a escala industrial.

✓ HPLC-ESI-IT-MS permitió la cuantificación de 36 compuestos fenólicos en extractos de hojas de olivo ‘Koroneiki’ extraídos mediante extracción asistida por sonda de ultrasonidos (sonótrodo) utilizando para ello un diseño Box-Benhken. Los diferentes ensayos demostraron que los parámetros de extracción (% etanol/agua (v/v), la amplitud (%) y el tiempo de extracción) influían considerablemente sobre contenido fenólico total. Las condiciones óptimas fueron etanol/agua (55/45; v/v), 100% de amplitud y 8 minutos, con las cuales se obtuvo un contenido fenólico total de 40,9 ± 0,2 mg/g p.s.. Dicho contenido fenólico total fue similar al obtenido mediante extracción convencional por baño de ultrasonidos, el cual se llevó a cabo mediante tres extracciones consecutivas con etanol/agua (80/20; v/v) durante 20 minutos. Por lo tanto, se podría afirmar que la extracción asistida por sonda de ultrasonidos permite una alta recuperación fenólica en

tiempos de extracción más cortos que la extracción convencional por de ultrasonidos en hojas de olivo. Esto conlleva la gran ventaja de que esta tecnología de sonda de ultrasonidos es escalable a nivel industrial.

✓ El estudio llevado a cabo acerca de los compuestos fenólicos libres e hidrolizables presentes en bagazo de cerveza, previamente tratado mediante campos eléctricos pulsados (CEP), y determinados por HPLC-ESI-MS ha demostrado que CEP es un pretratamiento efectivo para la mejora de la recuperación fenólica en bagazo de cerveza. Los modelos experimentales de extracción fueron establecidos mediante un diseño Box-Behnken. Se optimizaron los parámetros CEP (intensidad del campo eléctrico, frecuencia y tiempo total de tratamiento), observándose que estos afectan significativamente en la recuperación de los compuestos fenólicos libres, flavan-3-oles, flavonoides y ácidos fenólicos. Las condiciones óptimas encontradas fueron 2,5 kV/cm, 50 Hz y 14,5 segundos, obteniendo así un extracto con 101 ± 2 µg/g p.s. de compuestos fenólicos libres. Además, los compuestos fenólicos hidrolizables también se determinaron empleando las condiciones óptimas establecidas para los compuestos fenólicos libres y su contenido total fue de 536,46 ± 2,89 µg/g p.s. De acuerdo con estos resultados, la recuperación fenólica total obtenida con CEP fue 2,7 y 1,7 veces superior a la obtenida sin tratamiento con PEF para los compuestos fenólicos libres e hidrolizables, respectivamente. Este hecho podría deberse al aumento de la permeabilidad del disolvente a través de la membrana celular, lo cual mejoraría la transferencia de masa de los compuestos bioactivos. Por lo tanto, se puede afirmar que CEP es un pretratamiento prometedor que podría combinarse con otras técnicas de extracción con la finalidad de reducir los tiempos de extracción y mejorar la recuperación fenólica en el bagazo de cerveza.

✓ La determinación de proantocianidinas en bagazo de cerveza se ha llevado a cabo por primera vez mediante NP-HPLC-FLD-MS. Las proantocianidinas se extrajeron mediante extracción asistida por sonótrodo utilizando para ello un

diseño experimental Box-Behnken. Los experimentos del diseño permitieron evaluar el efecto de diferentes parámetros de extracción como la relación acetona/agua (v/v), el tiempo de extracción y la potencia; observándose que afectaban significativamente en la concentración total de proantocianidinas. Las condiciones óptimas fueron acetona/agua (80/20; v/v), 55 min y 400W, obteniendo extractos con 1.01 mg/g p.s. de proantocianidinas totales. Esta concentración de proantocianidinas (PA) fue aproximadamente un 58% mayor a la obtenida mediante extracción convencional con baño de ultrasonidos. Los análisis mediante NP-HPLC-FLD-MS permitieron identificar y cuantificar 11 PA de acuerdo a su grado de polimerización y a los espectros de masas obtenidos. Por lo tanto, el diseño Box-Benhken ha demostrado ser adecuado para la optimización de la extracción de PA de bagazo de cerveza mediante sonda de ultrasonidos.

✓ El estudio acerca de la determinación de compuestos fenólicos en harinas de trigo sarraceno obtenidas mediante un proceso de molienda (harinilla, harina de salvado, harina refinada y harinas integrales) mediante HPLC-ESI-Q-MS, permitió establecer algunas de las harinas como fracciones enriquecidas en compuestos fenólicos, que podrían utilizarse como ingredientes en la elaboración de alimentos que aporten beneficios para la salud. La plataforma HPLC-ESI-Q-MS permitió la cuantificación de 25 compuestos fenólicos libres y 24 hidrolizables en las diferentes harinas de trigo sarraceno. La molienda del trigo sarraceno descascarillado se presenta como una tecnología rentable para la obtención de diferentes fracciones de harina de trigo sarraceno con diferentes contenidos fenólicos: harina de salvado (1932,30 mg/kg p.s.), harinilla (1605,57 mg/kg p.s.) y harina refinada (254,57 mg/kg de peso seco)). La concentración fenólica más alta se encontró en la harinilla y la harina de salvado debido a que las capas externas del grano son más ricas en compuestos fenólicos que las partes internas del grano (endospermo).

✓ El tamizado ha demostrado ser una tecnología verde de fraccionamiento en seco adecuada para producir harinas de trigo sarraceno funcionales. El uso del tamizado

permitió la obtención de harina de trigo sarraceno con tamaño de partícula de 215 µm, 160 µm, 85 µm y 45 µm, y por ello, con diferente composición química en compuestos fenólicos, proteína, almidón y cenizas. La determinación de los compuestos fenólicos se realizó mediante HPLC-ESI-Q-MS y esto permitió la identificación y cuantificación de 25 compuestos fenólicos libres y 26 hidrolizables. Según los resultados obtenidos, la fracción de trigo sarraceno con 215 µm (GS215) fue la fracción más rica en compuestos fenólicos libres e hidrolizables, proteínas y cenizas. El contenido en compuestos fenólicos disminuyó a medida que disminuía el tamaño de partícula; de hecho, la concentración de rutina fue de 40 mg/kg p.s. en la harina de trigo sarraceno descascarillado (GSTQ), mientras que esta aumentó hasta 174 mg/kg p.s. en la fracción GS215. Al mismo tiempo, la fracción GS215 presentó cantidades de proteína y cenizas dos veces más altas que las harinas GSTQ. Esto podría deberse a que la fracción GS215 contiene más salvado que las fracciones con menor tamaño de partícula. Por lo tanto, el tamizado ha demostrado ser una tecnología de fraccionamiento en seco adecuada para obtener fracciones de harina de trigo sarraceno enriquecidas en compuestos fenólicos.

✓ La clasificación por aire se ha utilizado por primera vez como tecnología prometedora para la obtención de distintas fracciones de harina enriquecidas en compuestos fenólicos a partir de trigo sarraceno descascarillado. Con esta tecnología se obtuvieron dos fracciones distintas: una gruesa (CF) y una fina (FF). En este estudio, a partir de la harina integral, y dependiendo de la forma de llevar a cabo la clasificación por aire, se obtuvieron dos grupos de harinas: 30% de fracción gruesa (CF30) y 70% de fracción fina (FF70) y, 20% de fracción gruesa (CF20) y 80% de fracción fina (FF80). La determinación de los compuestos fenólicos libres e hidrolizables en las distintas fracciones se realizó mediante HPLC-ESI-TOF-MS. El contenido fenólico total en las fracciones gruesas CF20 y CF30 fue 1,74 y 1,40 veces mayor, respectivamente, que en la harina de partida. De hecho, CF20 y CF30 mostraron un aumento de la mayoría de los compuestos fenólicos libres e hidrolizables con respecto a la harina de trigo sarraceno integral,

y el contenido fenólico total fue dos veces mayor que en sus respectivas fracciones finas. La actividad antioxidante evaluada mediante los ensayos FRAP y DPPH fue mayor en las fracciones de harina gruesa que en las fracciones de harina fina y en la harina de trigo sarraceno descascarillado. Además, se encontraron correlaciones positivas entre el contenido fenólico y la actividad antioxidante demostrando la potencial bioactividad de las fracciones obtenidas.

✓ Las tecnologías de separación en seco demostraron ser unas herramientas valiosas y adecuadas en la obtención de fracciones enriquecidas en compuestos fenólicos. Por ello el tamizado seguido de un proceso de clasificación por aire se utilizaron para obtener fracciones de salvado de trigo enriquecidas en aleurona. Con este proceso se produjeron dos fracciones (AF1, 55-70% de aleurona y AF2, 75-90% de aleurona). Los compuestos fenólicos libres e hidrolizables presentes en ambas fracciones se determinaron mediante HPLC-DAD-ESI-TOF-MS, mientras que los alquiresorcinoles fueron identificados y cuantificados mediante GC-MS. Gracias al uso de estas plataformas analíticas, se determinaron por primera vez en aleurona de trigo el feruloil di-hexósido, diferentes flavonoides y varios isómeros monoinsaturados de alquilresorcinoles. Los resultados demostraron que la fracción AF1 presentaba un mayor contenido de alquilresorcinoles, flavonoides libres y oligómeros libres e hidrolizables de ácidos hidroxicinámicos (ácidos diferúlicos) comparada con la fracción AF2. Esta última presentaba una mayor cantidad de monómeros de ácidos hidroxicinámicos libres e hidrolizables, siendo el ácido *trans*-ferúlico el compuesto más concentrado. Por lo tanto, estos resultados han confirmado como la capa de aleurona ha demostrado ser rica en monómeros, mientras que el pericarpio externo (mayormente presente en la fracción AF1) es rico en oligómeros y alquilresorcinoles.