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Doctorado en Ciencias de la Alimentación
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ESTUDIO DE PERFILACIÓN DE METABOLITOS SECUNDARIOS DE EXTRACTOS VEGETALES OBTENIDOS MEDIANTE TÉCNICAS Y ESTRATEGIAS NO CONVENCIONALES PARA EL DESARROLLO DE ALIMENTOS FUNCIONALES EXPLOTANDO RESIDUOS ALIMENTARIOS

STUDY OF SECONDARY METABOLITE PROFILING OF PLANT EXTRACTS OBTAINED BY NON-CONVENTIONAL TECHNIQUES AND STRATEGIES FOR THE DEVELOPMENT OF FUNCTIONAL FOODS EXPLOITING FOOD WASTE

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CERTIFY THAT

Miss Luna Pollini graduated in Pharmacy, has conducted the present work **"Study of secondary metabolite profiling of plant extracts obtained by non-conventional techniques and strategies for the development of functional foods exploiting food waste"** under their direction and authorize its presentation for applying the degree of Doctor at University of Valencia and University of Perugia.

And, for the record, they hereby issue and sign this certificate.

Dr Jordi Mañes Vinuesa

Dra Lina Cossignani

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The truth is rarely pure and never simple

Oscar Wilde

To my mom Fiorella, my dad Luciano

and my brother Leonardo

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LIST OF ABBREVIATION

AA: antioxidant activity

ABS: absorbance

ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

AP: apple pomace

ASE: accelerated solvent extraction

CA: chlorogenic acid

CHA: chlorogenic acid

DES: deep eutectic solvent

DM: dry matter

DNSA: 3,5-dinitrosalicylic acid

DPPH: 2,2-diphenyl-1-picryl-hydrazyl-hydrate

DW: dry weight

EI: electron ionization

ESI: electron spray ionization

FAPE: fresh apple pomace extract

FOSHU: food for specific health uses

FRAP: ferric reducing/antioxidant power

GAE: gallic acid equivalent

HBA: hydrogen bond acceptor

HBD: hydrogen bond donor

HPLC-DAD: high performance liquid chromatography - diode array detector

HRMS: high resolution mass spectrometry

HSD: honest significant difference

IC_{50}: inhibition capacity 50%

L/S ratio: liquid/solid ratio

LDA: linear discriminant analysis

LOD: limits of detection

LOQ: limits of quantification

MAC: maceration

MAE: microwave-assisted extraction

MHG: microwave hydrodiffusion and gravity

NADES: natural deep eutectic solvent

PCA: principal component analysis

PEF: pulsed electric field

PLE: pressurized liquid extraction

PLS:partial least squares

Q^2: goodness of predictability

QE: quercetin equivalents

Q-TOF-LC/MS: liquid-chromatography quadrupole-time-of-flight mass spectrometry

R^2: goodness of fit

RP-LC: reversed phase – liquid chromatography

RSD: relative standard deviation

RSM: response surface methodology

S/L ratio: solid/liquid ratio

SD: standard deviation

SFE: supercritical fluid extraction

SFME: solvent-free microwave extraction

TE: trolox equivalent

TFC: total flavonoid content

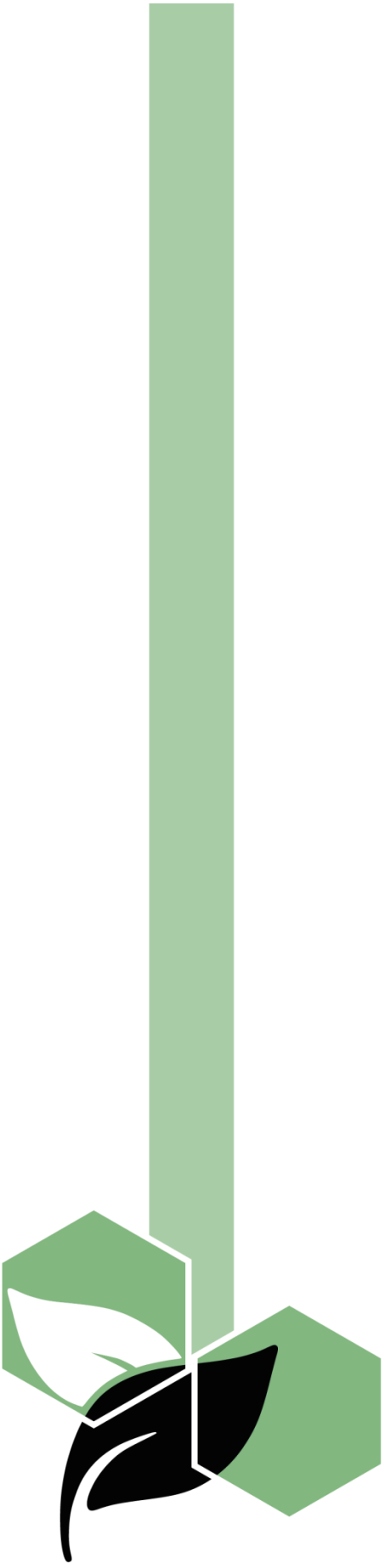
TPC: total phenolic content

TPTZ: 2,4,6-tripyridyl-s-triazine

UAE: ultrasound assisted-extraction

UHPLC-MS/MS: ultra-high performance liquid chromatography - tandem mass spectrometer

UTE: ultraturrax extraction



SUMMARY

This PhD thesis has been focused on the recovery of bioactive compounds from different vegetable waste by non-conventional extraction techniques, based on green economy and vegetable waste recycling approaches. Firstly, polyphenols were isolated from complex plant waste matrices, such as *Moringa oleifera* leaves, by Ultrasound Assisted Extraction (UAE). The experimental design approach allowed to optimize the best extraction conditions (MeOH:H_2O 50:50, *v*/*v*, 60:1 Liquid/Solid (L/S) ratio, 60 °C, 60 min) to obtain a total phenolic content (TPC) of 13.4 mg Gallic Acid Equivalent (GAE)/g dry matter (DM). Therefore, the characterization and quantification of the flavonol fraction were carried out by and UHPLC-MS/MS (Ultra High Performance Liquid Chromatography-tandem Mass Spectrometry) and HPLC-DAD (High Performance Liquid Chromatography-Diode Array Detector). The most abundant flavonols were the glycosidic forms of quercetin and kaempferol (ranging from 216.4 μg/g DM of quercetin 3-O-rhamnoside to 293.9 μg/g DM of quercetin 3-O-(6″-O-malonyl)-β-D-glucoside).

Moreover, *Lycium barbarum* leaves were considered as another interesting agricultural food waste to extract phenolic compounds, comparing UAE with Microwave Assisted Extraction (MAE), using alcoholic and hydroalcoholic solvents. The methanolic UAE resulted the most efficient extraction procedure, confirming that both the extraction technique and the solvent influenced the extraction of bioactive compounds. Therefore, *L. barbarum* and *L. chinense* leaves cultivated in central Italy were extracted by methanolic UAE technique. The antioxidant activities were investigated by *in vitro* free radical-scavenging assays such as DPPH, ABTS and ferric reducing power such as FRAP. Chromatographic analyses (HPLC-DAD) demonstrated chlorogenic acid and rutin as the most represented phenolic compounds for *L. barbarum* and chlorogenic acid and tyrosol for *L. chinense* samples. Finally, the multivariate statistical analysis was carried out to differentiate samples from different *Lycium* spp using the HPLC phenol contents.

These studies gave interesting results regarding the importance of the extraction technique to isolate phenolic compounds and they confirmed that the leaves of *M. oleifera*, *L. barbarum* and *L. chinense* represent antioxidant-rich vegetable matrices, which are commonly discarded. Therefore, these results improved the current knowledge about the recovery of

bioactive compounds from food waste to develop value-added products such as nutraceuticals and functional foods.

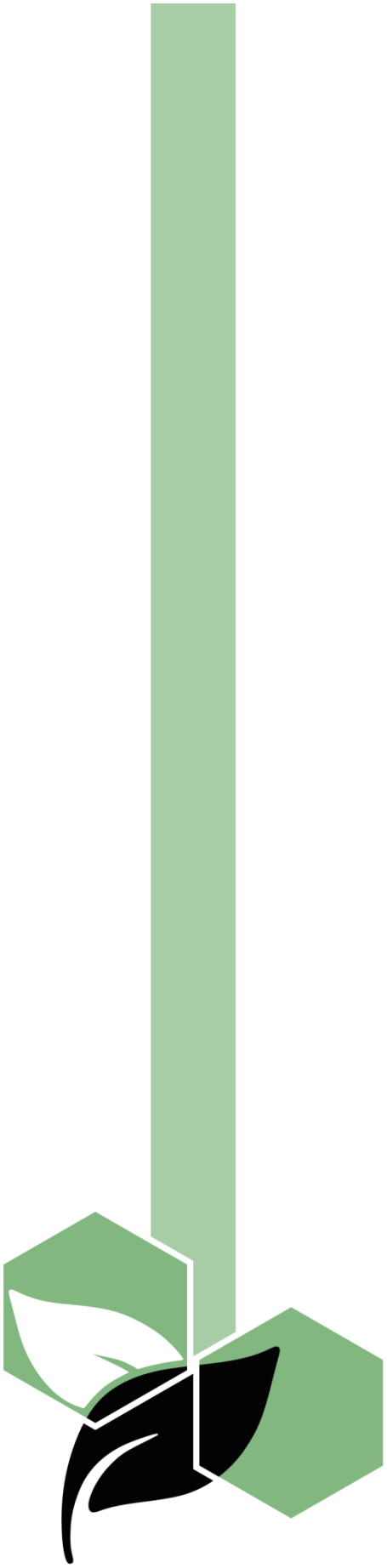
For this reason, the effect of household MAE on the phenolic content and antioxidant activity of *L. barbarum* leaf aqueous extracts was investigated to study a functional food as herbal-based beverage. An experimental design approach was carried out considering S/L ratio, irradiation time, and microwave power as independent variables on the responses of TPC, antioxidant activity, and chlorogenic acid content. The results demonstrated that the responses were positively influenced by the S/L ratio and time, while microwave power was inversely correlated with the investigated responses. This research revealed that microwave extraction conditions should be carefully monitored to obtain bioactive-rich aqueous extracts with high antioxidant activity. A comparison with household traditional methods (decoction and infusion) showed an unexpected lower phenolic content and antioxidant activity for MAE extract. In fact, it was found that *L. barbarum* leaf infusion had the best functional properties. This study contributed for raising awareness that household preparation conditions strongly affect the health properties of herbal extracts.

Nowadays, not only the antioxidant properties of bioactive compounds from vegetable waste are investigated, but also their inhibitory potential against digestive enzymes are of great interest. In this thesis work, the inhibitory activity of methanolic UAE extract from *L. barbarum* leaves on porcine pancreas α-amylase was studied. The α-amylase inhibitory activity of the constituent phenolic acids was also investigated. The phenolic acids were identified by Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) Liquid Chromatography/Mass Spectrometry (LC/MS). Chlorogenic and salicylic acids represented the most abundant phenolic acids in *L. barbarum* leaf extract. The inhibitory effect against α-amylase was determined for individual compounds (chlorogenic, salicylic and caffeic acids gave the highest inhibition) by *in vitro* assay. *L. barbarum* leaf extract showed appreciable α-amylase inhibitory effect in a concentration-dependent manner. Besides, docking studies of the considered phenolic acids into the active site of α-amylase suggested a conserved binding mode that is mainly stabilized through H-bonds and π-π stacking interactions.

In this thesis, a fruit waste such as apple pomace was also considered. It consists in the solid part of flesh, peel, seeds and petiole obtained during the apple juice or cider

production, and represents one of the most abundant food wastes produced in the world. Thus, Red Delicious apple pomace was produced in laboratory scale with a domestic blender and different non-conventional extraction techniques were used to isolate phenolic compounds, such as UAE, Ultraturrax Extraction (UTE), Accelerated Solvent Extraction (ASE) and Pulsed Electric Field (PEF) extraction pre-treatment. UAE with EtOH:H_2O (50:50, *v*/*v*) resulted the most efficient polyphenol extraction technique considering the obtained TPC values. Phloridzin, the main phenolic compound in apple, was determined by chromatographic analysis Q-TOF-LC/MS, and ASE with EtOH:H_2O (30:70, *v*/*v*) at 40°C and 50% of flush, was the most efficient technique in the recovery of phloridzin. The obtained results confirm that phloridzin could be considered a biomarker to determine the quality of numerous apple products. Moreover, the studies conducted on apple pomace represent a starting point for further investigations on its use for the development of value-added products such as functional foods or nutraceuticals.

RESUMEN



En esta tesis doctoral se desarrolló en el entorno de la economía verde y el reciclaje de residuos alimentarios estudiando diferentes técnicas de extracción no convencionales. En primer lugar, se aislaron polifenoles de matrices complejas producto del desperdicio de alimentos, como son las hojas de *Moringa oleifera*, mediante extracción asistida por ultrasonidos (UAE). El enfoque de diseño experimental permitió optimizar las mejores condiciones de extracción (MeOH:H_2O 50:50, *v/v*, proporcion 1:60 Solido/Licuido (S/L),60°C, 60 min) para obtener un contenido fenólico total (TPC) de 13,4 mg expresados como equivalentes de ácido gálico (GAE)/g de materia seca. Por lo tanto, la caracterización y cuantificación de la fracción de flavonol se llevó a cabo mediante un UHPLC-MS/MS (Cromatografía líquida de ultra alta resolución-espectrometría de masas en tándem) y HPLC-DAD (Cromatografía líquida de alta resolución-espectrofotometría de fila de diodos). Los flavonoles más abundantes fueron las formas glicídicas de quercetina y kaempferol (que van desde 216,4 μg/g de MS de quercetina 3-O-ramnosido a 293,9 μg/g de materia seca de quercetina 3-O- (6 ″ -O-malonil) -β- D-glucósido).

Además, las hojas de *Lycium barbarum* se estudiaron comparando los UAE con la extracción asistida por microondas (MAE), utilizando solventes alcohólicos e hidroalcohólicos. Los UAE metanólicos resultaron en la extracción más eficiente, confirmando que tanto el solvente como la técnica empleada influyeron en la extracción de compuestos bioactivos. Por lo tanto, las hojas de *L. barbarum* y *L. chinense* cultivadas en el centro de Italia se extrajeron con la técnica UAE con metanol. La actividad antioxidante se investigó mediante ensayos de captación de radicales libres *in vitro* como DPPH, ABTS y FRAP. Los análisis cromatográficos (HPLC-DAD) demostraron que el ácido clorogénico y la rutina son los compuestos fenólicos más importantes en *L. barbarum*, y el ácido clorogénico y el tirosol en *L. chinense*. Finalmente, se realizó el análisis estadístico multivariante para diferenciar las muestras procedentes de *Lycium* spp.

Estos estudios pusieron de manifiesto la importancia en la elección de la técnica de extracción para aislar compuestos fenólicos y también confirmaron a *M. oleifera*, *L. barbarum* y *L. chinense* como matrices vegetales ricas en antioxidantes, normalmente descartadas por la industria agrícola-alimentaria. Por tanto, estos resultados mejoraron el conocimiento actual

sobre la recuperación de compuestos bioactivos a partir de residuos alimentarios para desarrollar productos de valor añadido como nutracéuticos y alimentos funcionales.

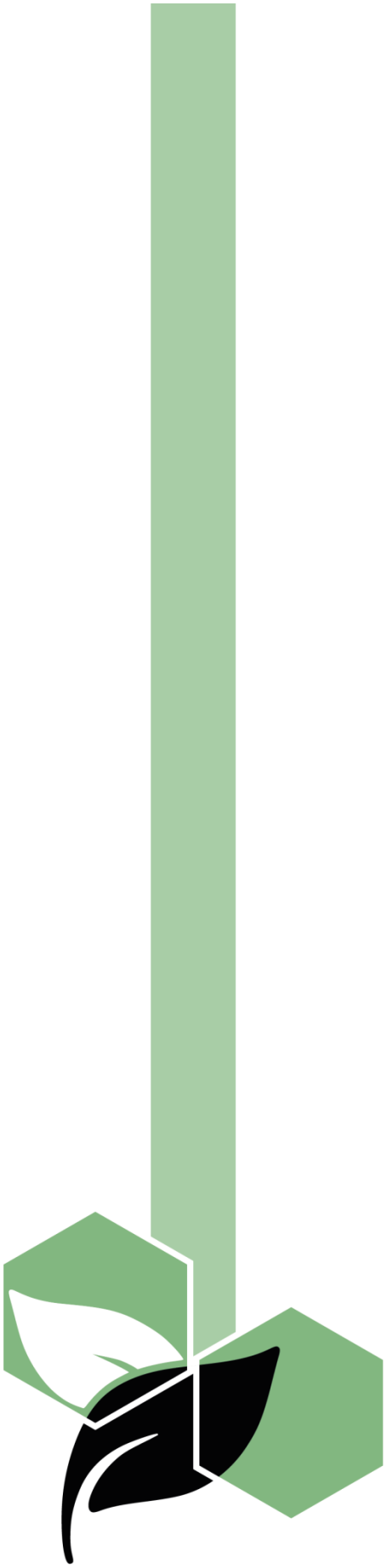
Por esta razón, se investigó el efecto del MAE doméstico sobre el contenido fenólico y la actividad antioxidante de los extractos de hojas de *L. barbarum* para estudiar un alimento funcional como las bebidas a base de hierbas. Se realizó un enfoque de diseño experimental considerando la relación S/L, el tiempo de irradiación y la potencia de microondas como variables independientes sobre las respuestas del TPC, la actividad antioxidante y el contenido de ácido clorogénico. Los resultados demostraron que las respuestas se vieron influidas positivamente por la relación S/L y el tiempo, mientras que la potencia de microondas se correlacionó inversamente con las respuestas investigadas. Esta investigación reveló que las condiciones de extracción por microondas deben monitorearse cuidadosamente para obtener extractos acuosos ricos en compuestos bioactivos con alta actividad antioxidante. La comparación con los métodos tradicionales domésticos (decocción e infusión) mostró un contenido fenólico y una actividad antioxidante inesperados al resultar más bajos para el extracto realizado con MAE. De hecho, se encontró que la infusión de hojas de *L. barbarum* tenía las mejores propiedades funcionales. Este estudio contribuyó a crear conciencia de que las condiciones de preparación de los extractos de hierbas en el hogar afectan fuertemente el estado fisológico y la salud de los consumidores.

Hoy en día es de gran interés no solo la extracción de compuestos bioactivos a partir de residuos vegetales, sino también el estudio de su potencial inhibitorio frente a las enzimas digestivas. De hecho, se estudió la actividad inhibidora del extracto metanólico de UAE de hojas de *L. barbarum* sobre la α-amilasa del páncreas porcino. También se investigó la actividad inhibidora de los ácidos fenólicos aislados. Los ácidos fenólicos se identificaron mediante cromatografía líquida/espectrometría de masas de tiempo de vuelo cuadrupolo de masa precisa (LC-Q-TOF/MS). Los ácidos clorogénico y salicílico representaron los ácidos fenólicos más abundantes en el extracto de hoja de *L. barbarum*. El efecto inhibidor frente a la α-amilasa se determinó para compuestos individuales (los ácidos clorogénico, salicílico y cafeico dieron la mayor inhibición) mediante un ensayo *in vitro*. El extracto de hoja de *L. barbarum* mostró un efecto inhibidor sobre la α-amilasa apreciable de una manera dependiente de la concentración. Además, los estudios de acoplamiento de los ácidos

fenólicos considerados en el sitio activo de la α-amilasa sugirieron un modo de unión conservado que se estabiliza principalmente a través de enlaces H e interacciones de apilamiento π-π.

En cuanto al desperdicio de frutas, el orujo de manzana es uno de los desperdicios alimentarios más abundantes que se producen a nivel mundial. Consiste en la parte sólida de la pulpa, piel, pepitas y pecíolo que se obtiene durante la elaboración del zumo de manzana o de la sidra. Así, se produjo orujo de manzana Red Delicious a escala de laboratorio y se realizaron diferentes técnicas de extracción no convencionales para aislar compuestos fenólicos, como UAE, Extracción Ultraturrax (UTE), Extracción Acelerada por Solvente (ASE) y Campo Eléctrico Pulsado (PEF). La UAE demostró ser la técnica de extracción de polifenoles más eficiente cuando se llevó a cabo con EtOH:H_2O (50:50, *v*/*v*). La floridzina, el principal compuesto fenólico en la manzana, se analizó mediante análisis cromatográfico LC-QTOF/MS, y la extracción con ASE y EtOH:H_2O (30:70, *v*/*v*) a 40°C, demostró ser la técnica más eficaz en la obtención de floridzina. Los resultados obtenidos confirman que la floridzina podría considerarse un biomarcador para determinar la calidad de numerosos productos de manzana. Por tanto, el orujo de manzana podría considerarse otro desecho de fruta interesante para desarrollar un producto de valor añadido como son alimentos funcionales y nutracéuticos.

1. INTRODUCTION



1. GENERAL INTRODUCTION

1.1. Circular economy

Nowadays, there is a growing interest in the recovery of bioactive compounds from food waste. Based on FAO assessment, about one-thirds of the total food produced (1.3 billion tons) is wasted annually all over the world (FAO, 2015). Hence, the development of optimal management strategies and policies to handle food waste is one of the main goal of industries to avoid its hazardous impact on the environment and society (Usmani et al., 2021). The concept of green economy is constantly stimulating the scientific community to reduce every kind of waste, in order to avoid pollution and increase the time as raw materials become waste. In this view, the recycle policy represents the best strategy to exploit raw materials as much as possible, and for this reason, the interest in studying food waste has been having great success. The wastes generated from the processing of various plant-based foods are rich in sugars, pectin, proteins, lipids, polysaccharides, fibres and polyphenols. These value-added products have immense value as food additives, nutraceuticals, therapeutics, cosmetics etc. and could find employment in the enrichment of functional foods (Nayak & Bhushan, 2019).

Moreover, the concept of green economy is also strictly correlated to the use of green extraction techniques of bioactive compounds from food waste, which are based on some parameters such as solvent, time and costs saving.

1.2. Recovery of bioactive compounds: polyphenols

Several researches focused on the extraction of polyphenols, some of the most representative bioactive compounds in vegetable and fruit waste, because of their health effects, among which the well-demonstrated antioxidant, antimicrobial and anticancer activities (Cai et al., 2021; Alara et al., 2021; Altemimi et al., 2017). In fact, several health benefits were investigated, such as the protection in neudegenerative, metabolic and cardiovascular diseases. The high radical scavenging ability of phenolic compounds is shown in different effects such as strengthen blood vessel walls, facilitate gastrointestinal digestion, reduce blood lipid levels, increase body immunity, and prevent arteriosclerosis and thrombus (Cai et al., 2021). On the other hand, phenolic compounds play an important

role in plant growth and they are involved in defense against ultraviolet radiation, aggression by microorganisms, in attracting pollinators or seed-dispersing animals, and in acting as stress-signaling molecules. These molecules are characterized by a phenolic structure, including flavonoids and non-flavonoids compounds with different central cores. The phenolic acids represent one of the sub-classes of non-flavonoids and they can be subdivided into hydroxybenzoic acids (e.g., gallic, protocatechuic, salicylic, syringic, and *p*-hydroxybenzoic acids) and hydroxycinnamic acids (e.g., caffeic, *p*-coumaric, synaptic, and ferulic acids). Within the flavonoids, there are six main subgroups, which are the flavanols, flavonols, flavones, flavanones, anthocyanins, and isoflavones (Weber and Passson, 2019). In figure 1 some of the phenolic compounds included in the heterogeneous polyphenols category are shown.

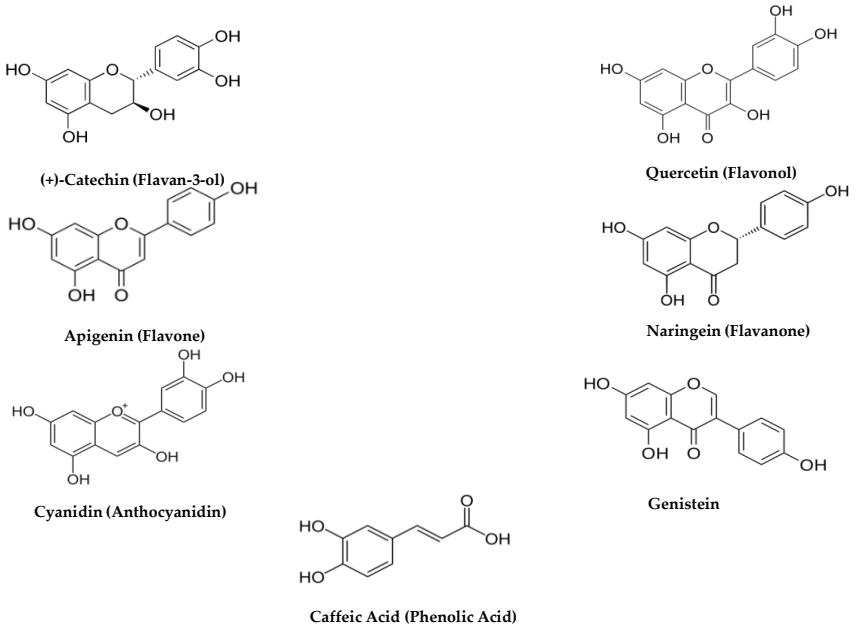


Figure 1. Structures of some phenolic compounds.

These compounds isolated from fruit and vegetable wastes have been used as natural food preservatives as they extend the shelf life and increase the antioxidant capacity of the final product (Nayak & Bhushan, 2019).

1.3. Phenolic compound extraction techniques

The optimization of the extraction technique remains one of the main steps for the isolation of phenolic compounds, due to the wide variety and differences that this class of

components shows. The choice of the solvent (alcoholic or hydroalcoholic mixture), the sample/solvent ratio, the time and temperature of the extraction are only some of the most important parameters to be taken into account, to improve the yield but at the same time to prevent the possible degradation of these compounds. So far, non-conventional extraction techniques, such as Microwave-Assisted Extraction (MAE), Ultrasound-Assisted Extraction (UAE), Supercritical Fluid Extraction (SFE), Accelerated Solvent Extraction (ASE), Pulsed Electric Field extraction (PEF) overcame the conventional extraction techniques, such as liquid-solid extractions, maceration or Soxhlet. The main advantages of these most currently used techniques are solvent, time and cost savings, while the disadvantages are related to the difficulties in scale-up, maintenance and cost of the equipment. These aspects represent other noticeable strengths in the green economy and recycle environmental policy.

1.3.1. Conventional extraction techniques

Among the conventional extraction techniques, maceration has been one of the most used for many years. In this case, the grinded sample is put in contact with an appropriate organic solvent (pure alcohol or hydroalcoholic mixtures) in a sample:solvent ratio that allows the complete coverage of the matrix, for a determined time at room temperature. Sometimes stirring during maceration is used to increase diffusion and to separate concentrated solution from the sample. It is an easy and cheap technique, and nowadays it is performed also to make a comparison with different non-conventional extraction methods. However, the extraction time and the solvent amounts are critical points of the maceration, because normally, it takes hours and the solvent waste is often high (Rocchetti et al. 2019; Ferrentino et al. 2018). Another conventional extraction technique is based on the use of the Soxhlet extractor (figure 2) that allows to extract the vegetable matrices with volatile organic solvents without direct heating of the sample. The sample is kept in a porous thimble and during operation is gradually filled with condensed fresh solvent from a distillation flask. As soon as the liquid boils, its vapors rise along a side tube to the refrigerant mounted above the extractor. The liquid obtained from the condensation of these vapors will fall drop by drop into the extraction chamber through the material contained in the thimble, filling it until it reaches the siphon placed on the side. At this point, the liquid falls into the flask below, from where it is distilled again. The cycle is repeated several times until the extraction is

considered completed. As disadvantages, Soxhlet requires long time of extraction and it cannot be used for thermolabile substances.

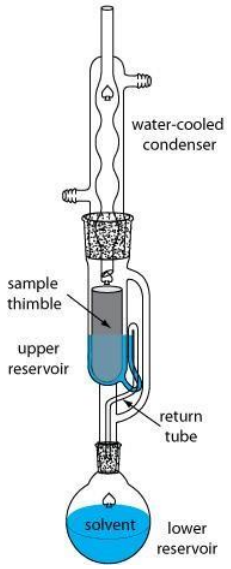


Figure 2. Soxhlet extractor.

1.3.2. Non-conventional extraction techniques

Among non-conventional extraction techniques, UAE and MAE are some of the most used techniques, because they can shorten the extraction time, decrease the solvent consumption and increase the extraction yield. On one hand, the ultrasound waves improve the permeability of the solvent on vegetable matrices, because the implosion of the cavitation bubbles generates fast adiabatic compression of gases and vapours inside the bubbles. This results in high temperatures and pressures that improves solvent penetration and increases the transport between the solid matrix and the liquid phase (Caldas et al., 2018). Moreover, the combination of water with different organic solvents (such as methanol, ethanol, acetone) improves the polyphenols extraction, due to the different polarity of these compounds. However, in comparison with other modern methods, UAE uses the highest amount of solvent and has the longest extraction time. On the other hand, in MAE the alteration and/or disruption of cells structure favors the solvent penetration. In particular, electromagnetic waves penetrate materials and interact with polar groups, causing heat generation in both the solvent and the solid (Tanase et al., 2019).

a b

Figure 3. Ultrasonic bath (a) and laboratory microwave oven (b).

Ultra-turrex extraction (UTE) is common laboratory tool for homogenization and dispersion and has recently been used to extract polyphenols or pesticides. This equipment allows for dispersing, homogenizing, mixing, and stirring thanks to a rotor-stator system in one-step as so called "one-vessel solution" (Rusu et al., 2018). In addition, UTE was also coupled to UAE to improve the extraction as reported by Torres et al. (2015) for oils, phenols and bixin recovery.

Figure 4. Ultra-turrax homogeniser.

Among other modern extraction techniques, ASE, that is often named as Pressurized Liquid Extraction (PLE), requires completely automated equipment in respect of UAE, MAE or UTE. The decrease of time analysis and solvent volume as well as the better repeatability represent the main advantages (Gomez et al., 2017). In this case, the solvent penetration into sample matrix is improved by high pressure and high temperature. Furthermore, the cycle times equilibrate time for intra- and extracellular solution. Finally, less amount of solvent was consumed with more contact time and hence more effective and faster solubilisation of the sample, under high pressure was obtained (Ahmad et al., 2020).

Figure 5. Accelerated Solvent Extraction apparatus.

Another completely automated extraction is PEF. It is considered a powerful pre-treatment to enhance the mass transfer rates in the extraction of intracellular components. This technique consists in the application of electric pulses at field strengths > 0.5 $kV \bullet cm^{-1}$, in the range from μs to ms, resulting in an increment of cell membrane permeabilization: this process is called electroporation (Maza et al., 2020). The low energy consumption and the low increasing temperature are the main advantages of this treatment. However, this equipment is not available in most laboratories. It was demonstrated that PEF treatment noticeably increased the quantity of polyphenols and their antioxidant activity (El Kantar et al., 2018).

Figure 6. Pulsed Electric Field equipment.

Another modern technology concerns the use of supercritical fluids. The use of CO_2 as an extracting solvent is advantageous due to its moderate critical conditions of temperature (31 °C) and pressure (73.8 MPa), non-toxic nature and high chemical stability. Further, CO_2 has greater diffusion coefficient and lesser viscosity and surface tension, thereby its penetration into the food matrix is faster causing reduced extraction time as compared to the use of typical organic solvent in conventional solvent extraction. Since there is minimum utilization of organic solvent, SFE is considered a green and clean technique. Moreover, CO_2 is cheap, safe and easy to recycle and this technique gives cleaner extracts

than the conventional solvent extraction. The biggest disadvantage is its high cost which limits its application on an industrial scale (Nayak & Bhushan, 2019).

Figure 7. Supercritical Fluid Extraction system.

Another extraction considered as pre-treatment is the enzyme assisted extraction, in which enzymes like cellulase, α-amylase and pectinase are used prior to solvent extraction. This technique helps to break the cell walls and hydrolyze the structural polysaccharides in order to increase the accessibility of the matrix for the solvents; enhancing the release of the bioactive compounds like antioxidants and simultaneously decreasing the extraction time. However, its major technical limitation is the difficulty in its scale up because enzymes behave differently under the changed environmental conditions (like presence of dissolved oxygen, temperature, availability of nutrient etc.) (Nayak & Bhushan, 2019).

Recently, a new trend to minimize the use of harmful and toxic solvents is developing and the solvent-free extractions have been raised increasing interest, such as solvent-free microwave extraction (SFME) or Microwave Hydrodiffusion and Gravity (MHG). In both cases, fresh plant materials are used without addition of any solvent. The principle is based on the microwave heating of the internal water present in plants that causes the rupture of cell walls and the release from inside to outside of bioactive compounds. The main advantages are the reduction of extraction time and costs, and the fact that it represents an easily scalable and safer technique thanks to the abolition of toxic solvents (Chemat et al., 2019).

Another interesting extraction technique based on the use of no toxic solvent is the Deep Eutectic Solvent (DES) extraction. These solvents are easily prepared by mixing, at a suitable temperature, a hydrogen bond acceptor (HBA), usually choline chloride, and a hydrogen bond donor (HBD), usually urea, 4-chlorophenol, ethylene glycol, glycerol, but

also alcohols, amino acids, carboxylic acids and sugars, to form a eutectic mixture, characterized by higher density and lower melting point than that of the individual constituents (Panzella et al., 2020). DESs are used for their great dissolution capacity toward bioactive compounds like polyphenols, due to their ability to donate and accept protons and electrons as well as to form hydrogen bonds. In the optical of green chemistry principles, natural sources of DES have raised great interest instead of synthetic compounds, and this new class of DES was named Natural Deep Eutectic Solvents (NADESs). They are usually organic acids (lactic, malic, citric acids, etc.), sugars (glucose, fructose, sucrose, etc.); amino acids, choline chloride, etc. They result more biodegradable and environmentally friendly, because naturally present in biological systems (Chemat et al., 2019).

1.4. Phenolic compound characterization methods

Once the extraction of phenols from natural matrices is performed, the next step consists in the characterization of the phenolic profile. In fact, the extracts are usually resuspended with an appropriate solvent and analysed by spectrophotometric methods, that determine the phenolic content and the antioxidant activity, and by chromatographic analysis, that give a detailed qualitative and quantitative profile of the bioactive compounds.

1.4.1. Spectrophotometric method: Folin-Ciocalteu' assay

One of the traditional method useful to determine the total phenolic content is Folin-Ciocalteu assay, commonly carried out in food chemistry laboratory. This spectrophotometric method is generally used to obtain a first screening of the phenolic content, although it presents a lot of issues and gives no information about which phenolic compounds are extracted or their singular quantity. Briefly, Folin-Ciocalteu assay is an *in vitro* colorimetric method based on an electron transfer mechanism. It occurs a redox reaction between the Folin-Ciocalteu' reagent and the phenolic compounds under alkaline conditions. The reaction is carried out for a predetermined time in the dark, then the absorbance is read at 750 nm. Generally, the results are expressed as Gallic Acid Equivalent.

The advantages of this traditional method are the ease to reproduce and the minimization of some interferences due to the high wavelength used, while the

disadvantages are that the lipophilic compounds are not detected, because the reaction is developed in aqueous ambient, and the interferences of reducing compounds.

1.4.2. Chromatographic analysis: targeted and untargeted metabolomics

Nowadays, advanced and powerful techniques are performed to obtain the characterization of the natural extracts, chromatographic analyses, such as reversed phase liquid chromatography (RP-LC) coupled to UV or diode array detector (DAD) or mass spectrometry (MS), are the most commonly used. Moreover, the Ultra-High-Performance Liquid Chromatography (UHPLC) is taking over traditional High-Performance Liquid Chromatography (HPLC). In fact, UHPLC is gaining an increasing interest in the recent year, especially when coupled to MS. The major advantages of UHPLC-MS over traditional technologies include high specificity and speed of analysis. Therefore, it represents one of the most powerful techniques for structure elucidation and investigation of natural compounds, for both qualitative and quantitative analysis. The main approaches carried out by UHPLC-MS are targeted analysis and untargeted metabolomics. There are also other approaches, such as ion mobility, still relatively uncommon, but with high potential, mostly in structure elucidation of a wide range of compounds (Piovesana et al., 2020).

The targeted analysis of phenolic compounds by MS represents one of the most used approaches for the quantitative analysis of these compounds in food. Through the use of pure standards, analysis can be carried out in a quantitative or semi-quantitative manner. This type of analysis can be sensitive but is restricted to known and well characterized compounds. The quantitative or qualitative analysis can be used not only for the description of a sample, but also for the discrimination between similar samples. Moreover, a significant advantage results by the use of high resolution mass spectrometry (HRMS), because the exact mass can be used to differentiate compounds with the same nominal mass, while the different fragmentation pattern can be used for tentative identification of compounds, without the need of pure standards. Furthermore, HRMS offers virtually no limitations in the number of investigated analytes and the investigation can be performed on acquired spectra as post-target retrospective analysis.

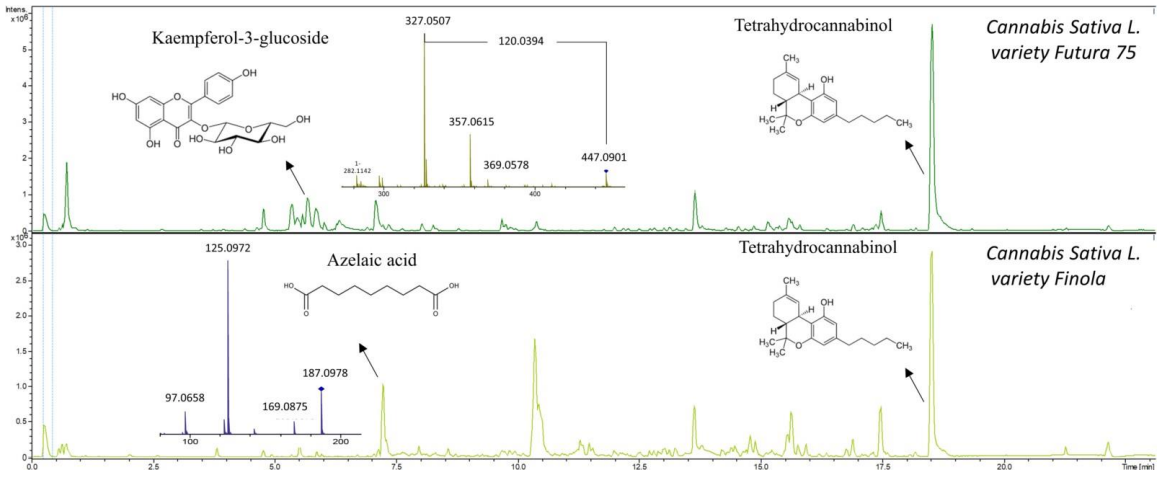


Figure 8. Targeted analysis: identification of compounds with pure standards (Nørskov et al., 2021)

On the other hand, untargeted metabolomics relies on a comprehensive analysis of all measurable analytes in a sample, including those unknowns. In fact, it allows to extend the qualitative and quantitative characterization to a wide range of compounds, even without the need for pure standards. Quality control is usually assessed by injection of the sample (that is a pool of analysed samples) preferable over synthetic mixtures of standard compounds, which cannot recreate the composition of the biological sample. Due to its comprehensive nature, the use of HRMS in untargeted metabolomics must be coupled with additional information such as the chromatographic behaviour, compound databases, additional analytical techniques (such as hyphenation with DAD), and advanced chemometric techniques, to clarify and confirm the structure of compounds. However, the use of relative quantitation should be used with care. In fact, complementary untargeted and targeted metabolomics allows for absolute quantitation. For this reason, recently it has been developed the so-called hybrid approaches to fulfil the need to expand the metabolite coverage and quantitative assays and bridge the targeted and untargeted approaches (Chen et al., 2020).

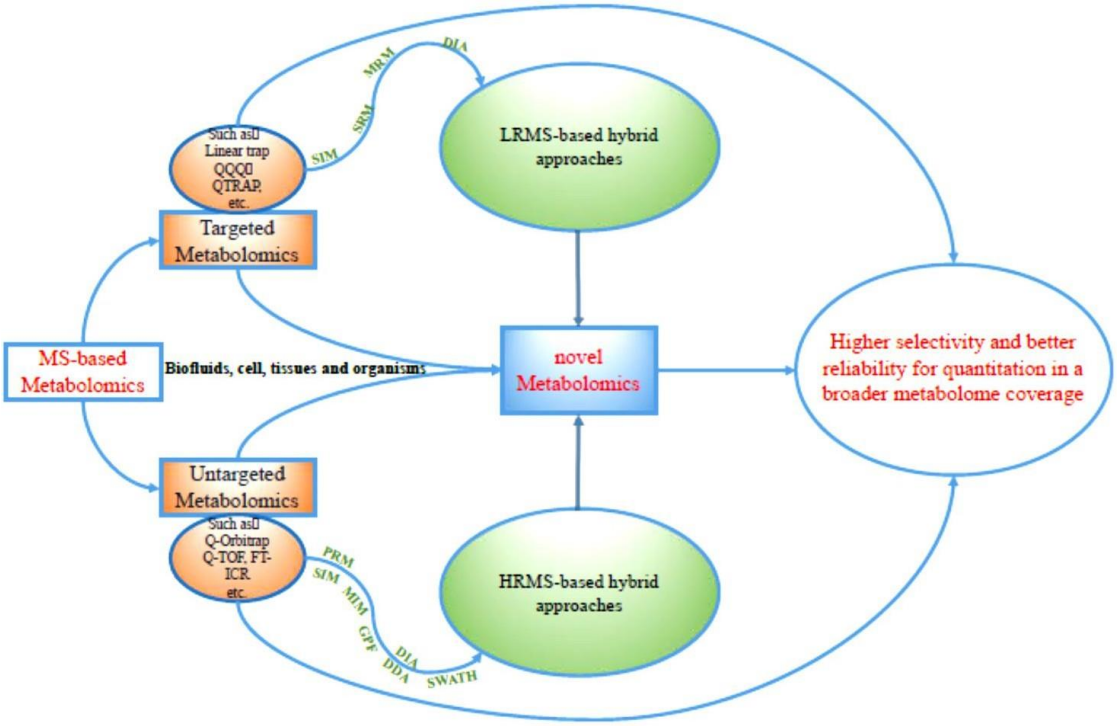


Figure 9. Workflow of novel metabolomics analysis (Chen et al., 2020).

Furthermore, the use of suitable softwares is fundamental because it allows to automate several steps for metabolite annotation, including blank subtraction, feature extraction from the $LCMS^2$ chromatograms (with retention time, peak area, accurate mass, isotopic pattern and product ions data and prediction of the elemental compositions) and database search (Chem- Spider and mzCloud databases). Moreover, the use of networking and databases, such as Global Natural Products Social Molecular Networking (GNPS; http://gnps.ucsd.edu), NIST, and Phenol-Explorer is very useful. The first one is an open-access knowledge base for community-wide organization that shares raw, processed or identified MS data of natural product compounds (Gauglitz et al., 2020). NIST mass spectral library is a fully evaluated collection of electron ionization (EI) and MS/MS mass spectra, with chemical and GC data, to identify unknown spectra. Finally, Phenol Explorer is the first comprehensive database on polyphenol content in foods, but also on polyphenol metabolism, and recently on the effects of food processing and cooking.

1.5. Fruit and vegetable waste

Fruit and vegetable waste can be used to extract and isolate potential bioactive compounds, that can be employed not only in the food industry, but also in the pharmaceutical, cosmetics, and textile industries. Therefore, the proper processing of waste materials obtained from horticultural commodities may reduce environmental issues and improve human health through foods enriched with health-enhancing substances (phenols, carotenoids, and other pigments, vitamins, dietary fibre, among several others). Although fruit and vegetable waste could be consumed raw, they should be processed using thermal or non-thermal technologies, which may affect phytochemicals. The aim is to stabilize waste from a microbiological point of view, however it should always be reached a compromise between the extent of the treatment and the possible degradation of bioactive compounds.

Among vegetable waste, leaves are considered an example of the food-agriculture waste, which could be generated during the pruning or fruit collection, as it happens during the olive production chain. In fact, tons of olive leaves are wasted during the oil production, starting from the pruning phases as well as from the olive harvest and before the olive crushing at the mill (Romani et al., 2019). Other interesting leaves to be studied are goji leaves (*Lycium* spp), that, besides the widely consumed berries, have their great potential in functional food development. In fact, leaves are rich in polyphenols and could represent an important source of bioactive compounds for the design of value-added products in the recycle view (Pollini et al., 2019).

Among fruit waste, peel has been demonstrated an interesting example for the recovery of bioactive compounds, especially for their antioxidant ability, such as mandarin peel (Nipornman et al., 2018) or pomegranate peel (Mirab et al., 2020). In most cases, studies are focused on the optimization of the phenolic compounds extraction from waste matrices and on the development of functional foods, such as the enrichment of bakery or dairy products with these health-promoting phenolic extracts. Apple pomace is another interesting fruit waste, considering it represents one of the most abundant waste obtained from fruit manufacturing industry, such as juice and cider production (about 10 million tons per year worldwide) (Alongi et al., 2019). It is the semi-solid part which derives from the fruit pressing and centrifugation and consists in flesh, skins, seeds and stalks.

1.6. Value-added products: functional foods

In the last decades, the interest in healthy diet is raising and people is continuously searching for food that, in addition to the nutritional value, can have beneficial effects on human health. For this reason, in the 80's arose for the first time this concept in Japan, where it was defined a new product category, Food for Specific Health Uses (FOSHU), as "food containing an ingredient with functions for health and officially approved to claim their physiological effects on the human body".

So far, an exhaustive legislative definition of functional food is not provided. The last one univocally recognized from UE and extra-UE countries is "natural or processed foods that contain biologically active compounds; which, in defined, effective, and non-toxic amounts, provide a clinically proven and documented health benefit utilizing specific biomarkers for the prevention, management, or treatment of a chronic disease or its symptoms", presented by the Functional Food Center (FFC) in 2018 (Alongi & Anese, 2021).

The two most relevant targets to be achieved by functional foods are reducing energy intake, for example reducing macronutrient intake or slowing down their digestion, and introducing food with healthy properties, for example introducing micronutrients with specific health benefits in the prevention or treatment of chronic diseases (Alongi & Anese, 2021).

In recent years, this concept of functional foods was applied in a circular economy view. In fact, the extraction and characterization of bioactive compounds from food waste matrices are considered as preliminary steps in order to develop new value-added products, exploiting as much as possible the good features of the raw material. For example, antioxidants could be isolated and employed in the enrichment of other food, such as bakery or dairy products to improve the shelf-life, or fibre could be used in meat product to increase the dietary fibre intake.

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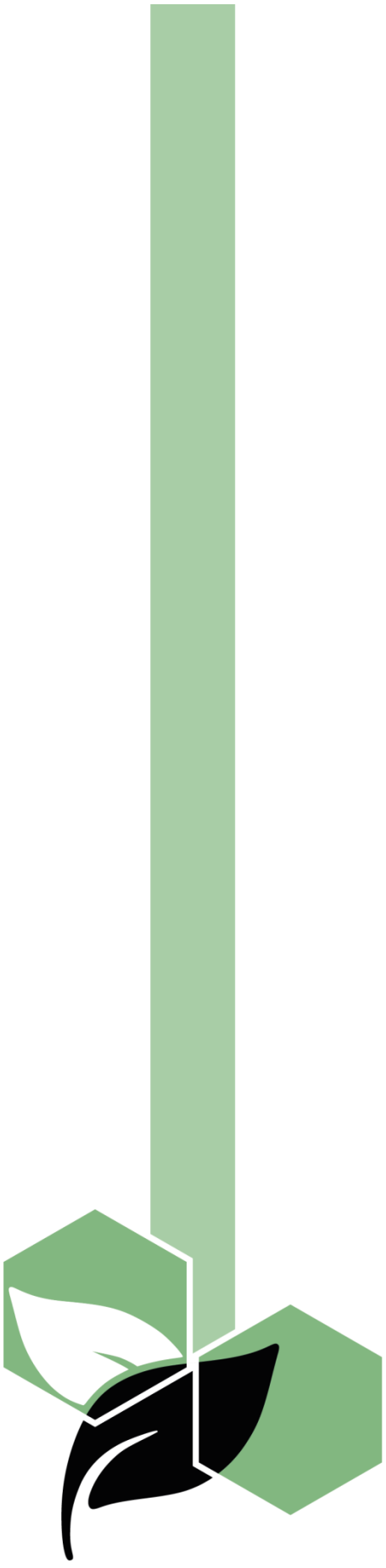


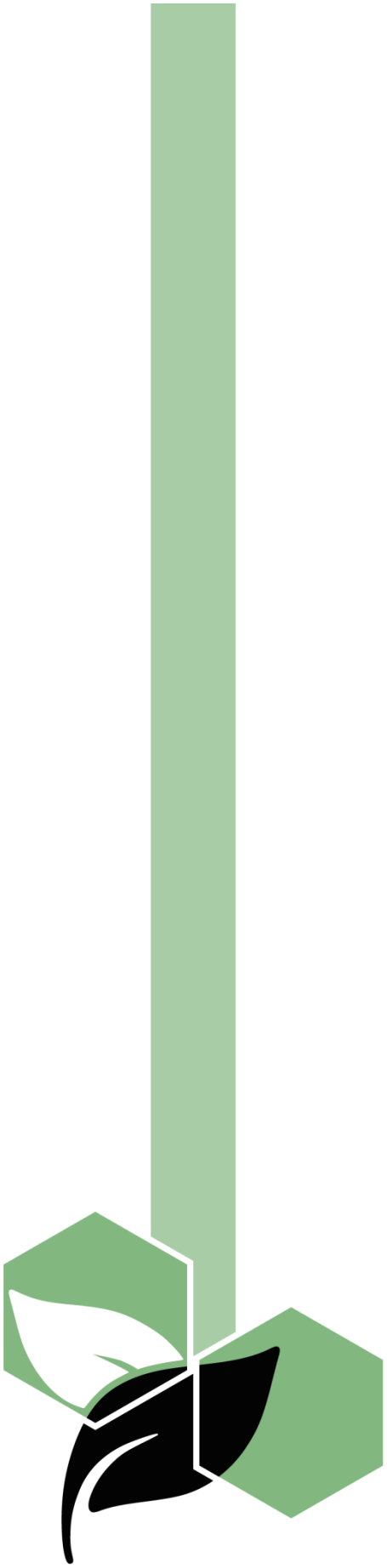
2. OBJECTIVES

The general objective of this PhD thesis is the study of secondary metabolites in some plant waste and by-products, addressing the optimization of their extraction with unconventional techniques, the characterization of the qualitative and quantitative profiles, and the evaluation of some biological properties. Therefore, the following specific objectives have been set.

1. Optimization of the UAE extraction of phenolic compounds from *Moringa oleifera* leaves through experimental design.
2. Characterization and quantification of the *M. oleifera* leaves phenolic profile by HPLC-MS/MS and HPLC-DAD, respectively.
3. Comparison of two non-conventional extraction techniques, as UAE and MAE, to recover phenolic compounds from another interesting vegetable waste: *Lycium barbarum* leaves.
4. Extraction of phenolic compounds from *L. barbarum* and *chinense* leaves and discrimination of the two *Lycium* species by multivariate statistical analysis.
5. Evaluation of the effect of *L. barbarum* leaf phenolic extract, characterized by QTOF-LC/MS, against the α-amylase enzyme, through *in vitro* and *in silico* studies.
6. Docking studies to investigate the type of bonds established in the active site of the α-amylase and evaluation of the interactions between the enzyme and some phenolic acids, present in *L. barbarum* leaf extract.
7. Study of aqueous extracts of *L. barbarum* leaves prepared with an household microwave, as potential functional beverage.
8. Comparison of total phenols, antioxidant activity and chlorogenic acid content in *L. barbarum* leaf microwave extracts and in traditional decoction and infusion preparations.
9. Comparison of different extraction techniques (UAE, UTE, ASE and PEF) to isolate polyphenols from fruit waste as apple pomace, by determination of TPC and quantification of the phloridzin concentration by QTOF-LC/MS.

El objetivo general de la presente Tesis Doctoral es el estudio de los metabolitos segundarios en algunos residuos y subproductos vegetales, abordando la optimización de su extracción con técnicas no convencionales, la caracterización de los perfiles cualitativos y cuantitativos, y la evaluación de algunas propiedades biológicas. Para ello, se han planteado los siguientes objetivos específicos:

1. Optimización de la extracción de compuestos fenólicos de hojas de *Moringa oleifera* con ultrasonidos (UAE) mediante diseño experimental.
2. Caracterización y cuantificación del perfil fenólico de las hojas de *M. oleifera* por HPLC-MS/MS y HPLC-DAD, respectivamente.
3. Comparación de dos técnicas de extracción no convencionales, UAE y extracción asistida por micotrondas (MAE), para recuperar compuestos fenólicos de hojas de *Lycium barbarum.*
4. Extracción de compuestos fenólicos desde hojas de *L. barbarum* y *L. chinense,* y discriminación de las dos especies de *Lycium* mediante análisis estadístico multivariante.
5. Evaluación del efecto inhibidor del extracto fenólico de hojas de *L. barbarum*, caracterizado por QTOF-LC/MS, sobre la enzima α-amilasa, mediante estudios *in vitro* e in silico.
6. Estudios de acoplamiento para investigar el tipo de enlaces que se establecen en el sitio activo de la α-amilasa y evaluación de las interacciones entre la enzima y algunos ácidos fenólicos presentes en el extracto de hoja de *L. barbarum*.
7. Estudio de extractos acuosos de hojas de *L. barbarum* preparados con microondas doméstico, como potencial bebida funcional.
8. Comparación de fenoles totales, actividad antioxidante y contenido de ácido clorogénico en extractos de hoja de *L. barbarum* obtenidos por microondas y en preparaciones tradicionales de decocción e infusión.
9. Comparación de diferentes técnicas de extracción (UAE, UTE, ASE y PEF) para aislar polifenoles de residuos de frutas como orujo de manzana, mediante determinación de TPC y cuantificación de la concentración de floridzina por QTOF-LC/MS



3. RESULTS

3.1. Impact of ultrasound extraction parameters on the antioxidant properties of *Moringa oleifera* leaves

1. Introduction

Consumer awareness of the nutritional and health-promoting effects of food is constantly increasing, particularly in potential antioxidant compounds in addition to classical basic nutrients. Research into antioxidant sources is justifiable because there is increasing scientific evidence that various diseases are linked to oxidative stress [1,2].

In order to recover bioactive compounds with high efficiency, conventional extraction methods are used, among which maceration and percolation are the most popular, but large solvent amounts and long extraction times are required [3].

In recent years, in order to reduce these disadvantages, many advanced extraction methods have been developed. The recovery of bioactive compounds by these extraction techniques is a promising trend in the field of nutraceuticals and functional food development. Among the non-conventional techniques, subcritical extraction, pressurized liquid extraction, microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) have been studied for the extraction of phenol compounds from *Moringa oleifera* L. [4–7].

Due to the high extraction efficiency and popularity of the ultrasonic equipment, UAE can be considered one of the most practical extraction methods for recovering bioactive compounds from plant materials, i.e., flowers, fruits, leaves, bark, seeds, and pods [3,8]. UAE can increase the rate of mass transfer of the extraction based on the cavitation generated within the material. The cell wall polymeric structure is disassembled, thereby enhancing the release of the bioactive compounds from plant material into the liquid extraction phase [9]. UAE allows for short extraction times due to the increase of the analyte's solubility in the extraction media when surface tension and solvent viscosity decrease, improving the extraction efficiency. However, in large industrial or semi-industrial scales, even though UAE requires expensive equipment and demands high energy consumption, it has been successfully used with improved bioactive compound yields for the extraction of various foods and waste, including *Moringa oleifera* leaves [7,10,11].

Moringa oleifera Lam. is the most widely cultivated species of a monogeneric family, the Moringaceae, native to the sub-Himalayan regions [12]. Despite its ancestral usefulness,

its application has been rather empirical and most of the existing information about it comes from oral tradition [13]. Only at the end of the 20th century did this tree receive deserved attention from the scientific community; in fact, numerous reports have been published on the chemical composition and nutritional properties of the different organs of the plant [12,14–16], as well as the identification of bioactive compounds and their mechanisms of action [17–19]. In oil processing, the seed pods from *M. oleifera* are used to obtain commercial products such as Ben oil, while other organs of the tree, such as the leaves, are discarded as waste. The leaves have traditionally been used in Chinese medicine due to their beneficial bioactivities for human health [14], and numerous recent studies confirm these health positive effects, such as antimicrobial, anticancer, antiulcer, analgesic, and antihypertensive properties. Recently, some authors have reviewed animal and human studies carried out on *M. oleifera* leaves to evaluate glycaemia and insulin levels [20].

The purpose of this study was to optimize the ultrasonic extraction of bioactive compounds by response surface methodology (RSM). Four parameters of extraction were investigated: solvent, liquid/solid ratio, time, and temperature. In order to evaluate the recovery of bioactive compounds, the following responses were considered: total phenol content, total flavonoid content and antioxidant capacity measured by DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric-reducing antioxidant power) *in vitro* assays. In addition, the extract obtained in the optimized conditions was analyzed by HPLC-DAD (high performance liquid chromatography-diode array detector) and UHPLC-MS/MS (ultra-high performance liquid chromatography-tandem mass spectrometry) in order to characterize the qualitative and quantitative profile of the flavonol fraction.

2. Materials and Methods

2.1. Plant Material and Chemicals

Dried leaves of *Moringa oleifera* L. were acquired from a herbalist's shop. On the label, Italian origin (Salento, South Italy) was declared. Gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH radical), 2,4,6-tripyridyl-s-triazine (TPTZ), sodium carbonate (Na_2CO_3), aluminium chloride ($AlCl_3$), iron(III) chloride hexahydrate ($FeCl_3$ $6H_2O$), Folin and Ciocalteu's phenol reagent, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), kaempferol

(≥90%), quercetin (≥95%), kaempferol-3-O-glucoside (≥95%), kaempferol-3-O-D-galactoside (≥90%), quercetin-3-D-galactoside (≥97.0%), quercetin-3-O-rhamnoside (≥78%), quercetin-3-O-D-glucoside (≥90%), quercetin 3-O-(6''-O-malonyl)-β-D-glucoside (≥85%) were from Sigma–Aldrich (Milan, Italy). HPLC grade and analytical grade solvents were acquired from VWR (Milan, Italy). Deionized water was obtained using a Milli-Q system (Millipore Corp, Billerica, MA, America).

2.2. Optimization of phenol extraction from *Moringa* Leaves by UAE

The extractions were carried out in an ultrasonic bath (AU-65; ArgoLab, Carpi, Italy), consisting of a stainless steel jug with a maximum capacity of 6500 mL. The influence of some parameters on the efficiency of the extraction of bioactive compounds from *M. oleifera* leaves and the optimization of phenol extraction conditions have been studied using the Statistical Design Package MODDE 5.0™, an experimental design software (UMETRICS, Umeå, Sweden).

The following factors have been considered:

- ✓ solvent composition: quantitative multilevel factor, with H_2O % in methanol, setting at values of 0 and 50;
- ✓ liquid/solid ratio, i.e., liquid/dry leaves, (indicated as L/S): quantitative multilevel factor, with values set at 30 and 60, expressed as liquid volume/leaf weight in grams of dry matter (mL/g DM);
- ✓ time: quantitative factor, with values set from 10 to 60 min;
- ✓ temperature: quantitative factor, with values set from 30 to 60 °C.

The following responses have been selected:

- ✓ total phenol content (TPC), expressed as milligrams of gallic acid equivalent per gram of dry matter (mg GAE/g DM);
- ✓ total flavonoid content (TFC), expressed as milligrams of quercetin equivalent per gram of dry matter (mg QE/g DM);
- ✓ free radical-scavenging activity using DPPH, expressed as percentage of antioxidant activity (AA%);

- ✓ ferric reducing antioxidant power (FRAP) assay, expressed in μmol Fe^{+2} per gram of dry matter (μmol Fe^{+2}/g DM).

Factors and responses of the experimental design are summarized in Tables 1a and 1b, together with unit, type and setting values for factors, and the unit and acronym for responses.

Table 1a. Factors set in the ultrasound-assisted extraction (UAE) experimental design.

Factor	Unit	Type	Setting
Solvent	H_2O %	quantitative multilevel	0, 50
Liquid/Solid ratio	mL/g DM	quantitative multilevel	30, 60
Time	min	multilevel	10 to 60
Temperature	°C	quantitative	30 to 60

Table 1b. Responses set in the UAE experimental design.

Response	Unit	Acronym
Total phenol content	mg GAE/g DM	TPC
Total flavonoid content	mg QE/g DM	TFC
Free radical-scavenging activity	AA%	DPPH
Ferric reducing antioxidant power	μmol Fe^{+2}/g DM	FRAP

GAE = gallic acid equivalent; QE = quercetin equivalent; DM = dry matter; AA% = % of antioxidant activity

A fractional factorial design of resolution IV, a linear model, was employed and a total of 11 experiments, named N1 to N11, was obtained (including three replicated centre points). The extractions were carried out in random order using the experimental conditions indicated in the worksheet (Table 2). The model was then fitted using the partial least squares (PLS) regression analysis.

Table 2. Worksheet with experimental conditions for UAE.

	Solvent	L/S Ratio	Time	Temperature
	H_2O %	mL/g DM	min	°C
N1	0	30	10	30
N2	50	30	10	60
N3	0	60	10	60
N4	50	60	10	30
N5	0	30	60	60
N6	50	30	60	30
N7	0	60	60	30
N8	50	60	60	60
N9	0	30	35	45
N10	0	30	35	45
N11	0	30	35	45

2.3. Determination of Total Phenol Content

The total phenol content (TPC) was determined by a spectrophotometric method, according to the Folin–Ciocalteu procedure, modified by Pagano et al. [21]. Deionized water, 20% Na_2CO_3 solution and Folin and Ciocalteu's reagent were added to an aliquot of the diluted extract. The solution was left to react in the dark for 30 min, after which the absorbance at 750 nm was measured using a Lambda 20 spectrophotometer (PerkinElmer, Inc; Waltham, Massachusetts, USA). The results were expressed as mg GAE/g DM, using a gallic acid calibration curve.

2.4. Determination of Total Flavonoid Content

The total flavonoid content (TFC) was determined by a spectrophotometric method [22]. Methanol, 10% $AlCl_3$, 1 M potassium acetate and H_2O were added to an aliquot of the appropriate diluted extract. The mixture was incubated in the dark for 30 min, after which the absorbance at 415 nm was measured. The results were expressed as mg QE/g DM, using a quercetin calibration curve.

2.5. *In vitro* antioxidant activities

2.5.1. Free Radical-Scavenging Activity using DPPH (DPPH Assay)

A DPPH assay was carried out according to the procedure reported by Blasi et al. [23]. DPPH (0.06 mM in ethanol) was added to the sample and the mixture was kept in the

dark for 30 min, after which the absorbance at 517 nm was measured. The percentage of antioxidant activity (AA%) for each sample was calculated using the following formula:

$$\text{DPPH (AA\%)} = \frac{(Absc - Abss)}{Absc} \times 100$$

where Absc is the absorbance of the control solution containing only DPPH radical and Abss is the absorbance of the DPPH solution containing the sample.

2.5.2. Ferric Reducing Antioxidant Power (FRAP) Assay

The reducing capacity of the leaf extracts was determined according to the procedure reported by Moscatello et al. [24]. The FRAP reagent, prepared by mixing acetate buffer, TPTZ and $FeCl_3 \cdot 6H2O$, was added to the leaf extracts, and then the samples were left in the dark for 30 min. The absorbance of the sample was measured at 593 nm. The results were expressed as μmol Fe^{+2}/g DM, using a calibration curve prepared with solutions of known Fe^{+2} concentrations.

2.6. Analysis of Flavonols by HPLC-DAD

The HPLC analysis was performed using a Thermo Spectraseries HPLC, coupled with the Spectra System UV6000LP DAD (Thermo Separation Products, San Jose, CA, USA), according to a previous paper [25]. The chromatographic separation of phenol compounds was carried out with a Hypersil GOLD column (150 mm 4.6 mm, 3 μm particle size). Separation was achieved using a gradient elution of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile): B increased from 5% to 20% in 30 min, and then to 95% in 5 min (flow: 1 mL/min). Standard solutions containing phenol compounds (kaempferol, quercetin, kaempferol-3-O-glucoside, kaempferol-3-O-D-galactoside, quercetin-3-D-galactoside, quercetin-3-O-rhamnoside, quercetin-3-β-D-glucoside, and quercetin 3-O-(6″-O-malonyl)-β-D-glucoside) were used to identify and quantify the analytes. Peak identification was confirmed by UHPLC-MS/MS analysis [26].

2.7. Statistical Analysis

The results of HPLC analysis were expressed as the mean and standard deviation, based on three replicates. Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA) was used for data analysis.

3. Results and Discussion

3.1. Optimization of extraction conditions of phenols from *Moringa* leaves by UAE

The optimization study was performed by experimental design, a technique for planning experiments, that allows us to use a minimum number of experiments in which several experimental parameters vary simultaneously. Based on the obtained data, a mathematical model of the studied process is created. The model can be used to understand the influence of the experimental parameters on the response and to find the optimal conditions for the process [27]. Regardless of application domain, this methodology is useful for three objectives: screening, optimization, and robustness testing. Employed at the beginning of the investigation of a new application, screening experiments are commonly designed to explore many factors in order to evaluate their effects on the responses. Response surface methodology (RSM) is an approach based on mathematical and statistical techniques that is useful in designing experiments and evaluating the effects of factors. RSM, a useful tool to build empirical models and to determine the optimum conditions for a desirable response, has been widely used for various process optimizations [28,29], including the extraction of phenol from plants [7,23,30].

To evaluate the influence of UAE conditions of *Moringa* leaves on total phenol content (TPC), total flavonoid content (TFC) and antioxidant activity (FRAP, DPPH), the software MODDE 5.0™ was used. In this work, pure methanol and hydroalcoholic solution (methanol/water 50:50, *v*/*v*) were used as extraction solvents. The solvent/dry leaves ratio (mL/g DM), indicated as the liquid/solid ratio (L/S), is also an influential parameter on extraction results and the ratios of 60:1 and 30:1 have been considered. Other important parameters affecting the extraction results were considered, such as time (ranging from 10 to 60 min) and temperature (ranging from 30 and 60°C). Selecting the screening objective and a fractional factorial design of IV resolution, the worksheet reported in the Table 2 was obtained. The experiments N1–N11 have been carried out, and the TPC, TFC and antioxidant activity (DPPH and FRAP) have been determined. The obtained results are shown in Table 3.

Table 3. Responses for fractional factorial IV resolution design.

	TPC	TFC	FRAP	DPPH
	mg GAE/g DM	mg QE/g DM	μmol Fe^{+2}/g DM	AA%
N1	3.9	3.3	99.2	13.7
N2	10.4	4.0	116.4	29.7
N3	7.7	3.4	106.5	24.8
N4	11.9	4.4	170.5	36.4
N5	6.1	2.6	45.5	30.1
N6	13.0	5.4	141.6	35.9
N7	6.8	4.2	115.7	31.0
N8	13.4	4.4	161.5	40.6
N9	6.4	3.0	79.7	25.4
N10	5.7	2.8	71.6	23.9
N11	6.2	2.9	90.3	24.3

Abbreviations are described in Tables 1 and 2.

3.2. Model Statistics

Table 4 shows the coefficients and the relative standard errors and p-values for TPC, TFC, FRAP and DPPH responses. It is possible to observe that the solvent and L/S ratio variables were significant for all the considered responses. Moreover, temperature was also significant for TPC and FRAP responses while time was variable for DPPH.

Table 4. Model coefficients (Coeff.), standard error (Std. Err.) and p-values for the responses.

	TPC			**TFC**		
	Coeff.	Std. Err.	P-value	Coeff.	Std. Err.	P-value
costant	7.8500	0.2221	**$3.41 \cdot 10^{-7}$**	3.5000	0.0944	**$2.69 \cdot 10^{-7}$**
solvent	2.3366	0.2692	**0.0003**	0.4104	0.1144	**0.0157**
L/S ratio	1.0009	0.2611	**0.0122**	0.3703	0.1110	**0.0206**
time	0.2897	0.2493	0.2976	0.0114	0.1059	0.9179
temperature	0.6537	0.2493	**0.0469**	-0.1783	0.1059	0.1532
	DPPH			**FRAP**		
	Coeff.	Std. Err.	P-value	Coeff.	Std. Err.	P-value
costant	108.9550	3.5390	**$7.80 \cdot 10^{-8}$**	28.7091	0.8509	**$4.51 \cdot 10^{-8}$**
solvent	4.8941	0.9137	**0.0017**	26.5327	3.8001	**0.0004**
L/S ratio	2.5518	0.9137	**0.0314**	17.9207	3.8001	**0.0032**
time	3.7401	0.8925	**0.0057**	-3.22185	3.7118	0.4187
temperature	0.9293	0.8925	0.3378	-11.0545	3.7118	**0.0246**

Significant p-values (≤ 0.05) are identified in bold.

The quality of the obtained mathematical model can be evaluated by two statistical criteria, goodness of fit (R^2) and goodness of predictability (Q^2). The first describes how well the model fits the experimental data, while the second describes how well the model will predict new data. When R^2 and Q^2 values are close to the unit, the model is considered a good model and it can be used for optimization and prediction [27]. Table 5 shows the R^2 and Q^2 values of the statistical models obtained with the four responses from the UAE experiments. It is possible to observe that all values are sufficiently high (R^2 values were always higher than 0.897, while Q^2 values were always higher than 0.706), and for this reason they indicate the goodness-of-fit of the obtained statistical models.

Table 5. Goodness of fit (R^2) and goodness of predictability (Q^2) coefficients of statistical models.

	R^2	Q^2
TPC	0.931	0.706
TFC	0.897	0.708
FRAP	0.943	0.795
DPPH	0.914	0.726

Figure 1a–d shows the observed vs. predicted UAE plots for the four responses, respectively. The observed vs. predicted plot for a response can be used for the estimation of the quality of a model—for a good model, all the data points will fall on a straight line. The obtained plots indicate quite good models for all responses in the UAE experimental design.

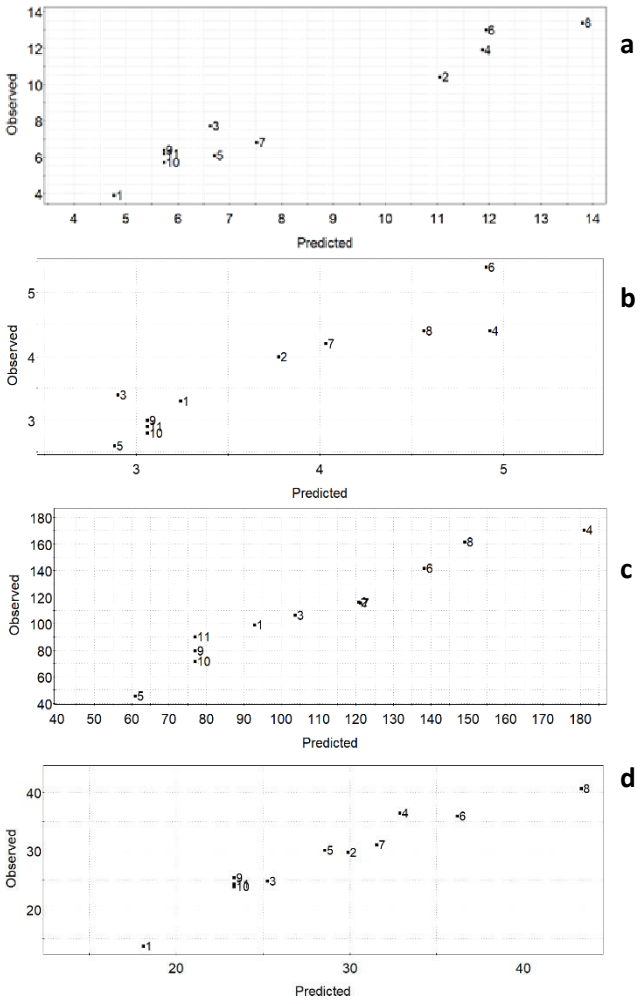


Figure 1. UAE experimental design. Observed vs. predicted plots for the responses: (a) TPC, (b) TFC, (c) FRAP, (d) DPPH.

3.3. Factor Influence on Responses

Previous research carried out on vegetable sources has shown that factors such as solvent properties and volume, extraction time and temperature, and frequency and power of the ultrasound apparatus influence the extraction efficiency, and subsequently have an impact on the bioactivity of the extract [3,9,15]. In this research, when the model was built, some relevant factors (% H_2O, L/S ratio, temperature, and time) were investigated and in Figure 2, the coefficient plots show the effect of the considered factors on the four responses: a) TPC, b) TFC, c) FRAP, d) DPPH. In all plots, it is evident that the solvent composition is the most influent factor among the considered ones. In fact, all the considered responses (TPC, TFC, FRAP and DPPH) increased when the percentage of water increased in the

solvent (50% *v*/*v*). Generally, hydroalcoholic extracts had higher TPC than methanolic ones ($P<0.01$), independently from the other variables. Among extraction conditions, the solvent has great influence on the extract composition and antioxidant activity, as also reported by various authors [4,30].

Also TFC content was significantly higher in hydroalcoholic than methanolic extracts ($p<0.05$). L/S ratio was also an influent factor, but only in the case of TFC the bars of solvent and L/S ratio were comparable, so the two parameters showed a comparable influence on the extraction.

A positive influence on the extraction results (TPC and DPPH) was also obtained for the factors time and temperature. The statistical models showed that high time (60 min) and temperature (60 °C) during extraction lead to higher TPC and DPPH values. In fact, the Optimizer function (criterion Maximize) gave the best value of TPC (13.79 mg GAE/g DM) and DPPH (43.32%) in the following conditions: 50% H_2O, 60:1 L/S ratio, 60 min and 60°C. On the other hand, it must be taken into account that high temperatures could lead to negative changes of compounds in the considered matrix.

Generally, time has very little influence on the responses, in fact, with the exception of DPPH, the coefficient is slightly positive for TPC, slightly negative for FRAP and almost null for TFC. Temperature showed a negative effect on TFC and FRAP responses. In fact, the Optimizer function (criterion Maximize) gave the best value of TFC (4.75 mg QE/g DM) in the following conditions: 50% H_2O, 60:1 L/S ratio, 32 min and 30°C, while the best value of FRAP (180.99 μmol Fe^{+2}/g DM) in the following: 50% H_2O, 1:60 L/S ratio, 10 min and 30°C.

Zhao et al. [7] reported that the best extraction conditions were ethanol 70%, 1:30 S/L ratio, 50 °C temperature and 42 min time, using an ultrasonic circulating extraction equipment at 300 W. Different optimal conditions (1:52 S/L ratio, 43 min and 76 °C) were obtained for maximizing flavonoids extraction from *M. oleifera* using an ultrasonic bath cleaner at 40 KHz and 300 W [10].

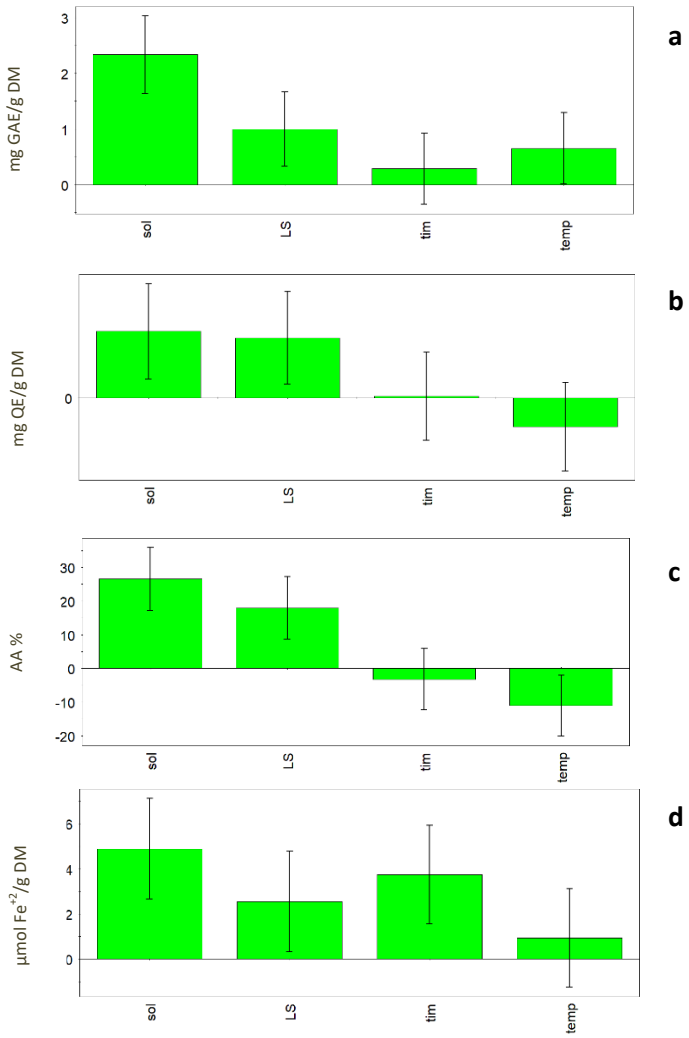


Figure 2. UAE experimental design - Coefficient plots showing the effect of solvent, liquid/solid ratio, time and temperature on the responses: a) TPC, b) TFC, c) FRAP, d) DPPH.

Figure 3 (a-d) shows the surface plots with the responses (TPC, TFC, FRAP, DPPH), as a function of two selected factors, for UAE experiments. The plots have been generated by the software, setting a constant value for the other factors. The response-surface plot is generated to get a graphical representation of the experimental region. From this plot, the most interesting area can be used to verify experiments and to plan new experiments. The colour changing from blue to red indicates an increase in the response. It is evident that, in all the considered models, an increase of H_2O % into the solvent and a higher L/S ratio (60:1) correspond to an increase in the responses, as already highlighted during the discussion of the coefficients reported in Figure 2 (a-d).

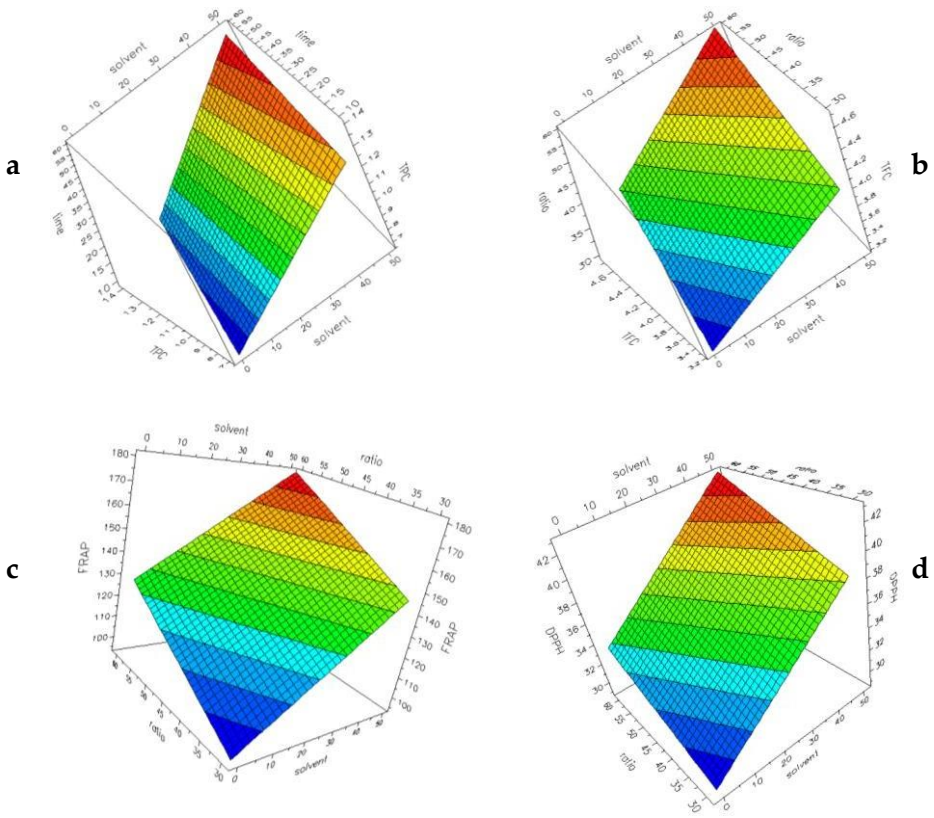


Figure 3. UAE experimental design - Surface plot showing the responses: a) TPC (60:1, 60°C); b) TFC (10 min, 30 °C); c) FRAP (10 min, 30 °C); d) DPPH (60 min, 60 °C).

3.4. Comparison with literature data (TPC and TFC)

It is known that differences in TPC, TFC and antioxidant activity in plant materials depend on cultivar, growing environment, extraction method, and other factors [15,23].

Based on the condition of UAE extraction, *M. oleifera* leaves showed TPC values ranging from 3.9 to 13.4 mg GAE/g DW (Table 3, N1 and N8, respectively). The highest TPC value was obtained in the following conditions: hydroalcoholic solvent, 60:1 ratio, 60 min and 60 °C. Generally, the samples obtained with 50% H_2O showed higher TPC values (10.4-13.4 mg GAE/g DW) than those obtained with pure MeOH (3.9-7.7 mg GAE/g DW). Other authors have studied the influence of the extraction solvent on TPC yield from *M. oleifera* leaves. For example, Rodríguez-Pérez et al. [30] have reported that the presence of water into the extraction solvent gave higher TPC results, with values of 46 mg GAE/g DW using MeOH:H_2O (50:50, *v*/*v*) and 23.2 mg GAE/g DW using MeOH, for samples collected in Madagascar. However, it is important to observe that the extracts were first obtained by maceration and then by four successive UAE extractions. The same authors [5] have

optimized the MAE extraction conditions to obtain phenol compounds from the same *M. oleifera* leaves and reported higher TPC values with 50% hydroethanolic mixture (105.70 mg GAE/g DM) than those reported for ethanolic extracts (23.69 mg GAE/g DM). It should be noted that the cited results have been obtained by MAE extraction at 180°C.

TPC values similar to those obtained in this research (Table 3) were reported by Castro-Lopez et al. [15] for *M. oleifera* leaves collected in Mexico. They used UAE with deionized water (40 kHz; 60 min; 25°C) and obtained about 3 mg GAE/g DW with 1:25 S/L ratio and about 12 mg GAE/g DW with 1:50 solid/liquid ratio.

Generally, the TPC values of *M. oleifera* leaves reported in other studies are higher, but in many cases it is difficult to carry out real comparison because very different conditions or methods are used.

TPC value of *M. oleifera* leaves collected from Greece was 29.04 mg GAE/g DM with UAE, while ranged from 24.72 to 40.24 mg GAE/g DM, when pulsed electric field at room temperature for 40 min was used [31].

Siddhuraju and Becker [32] studied *Moringa* leaf extract from different geographic origin. They reported values ranging from 7.43 to 12.33 g GAE/100 g DM (Nicaragua), from 5.25 to 8.87 g GAE/100 g DM (India), and from 6.83 to 9.76 g GAE/100 g DM (Niger), using an apparatus consisted of a round-bottom flask with an attached reflux condenser. The lower values were obtained for water extracts, while the higher ones for 80% methanol extracts.

In this work, *M. oleifera* leaves showed TFC content from 2.6 to 5.4 mg QE/g DM, based on different UAE conditions (Table 3, N5 and N6, respectively). For both experiments, time and L/S ratio were the same (60 min and 1:30), but % H_2O and temperature were different: alcoholic solvent and 60 °C for N5, hydroalcoholic solvent and 30 °C for N6. It should be noted that a good correlation was found between TPC and TFC values (R^2=0.6740)

In literature, a wide range of TFC values was reported: 10.14-14.07 gram of rutin equivalent (RE)/g DM for *M. oleifera* leaves from Central America, 3.26-5.92 g RE/g DM for samples from South Asia, 7.32-10.19 g RE/g DM for West Africa. Higher values were reported for samples from Egypt [47.04-62.53 mg QE/g DM], obtained by UAE extraction and purification of flavonoid compounds by macroporous resin [10]. The highest value of TFC

was 192.36 mg RE/g DM for young leaves of *M. oleifera* from Kenya, firstly extracted with 90% ethanol for 3 h, and then re-extracted in an ultrasonic bath (200 W, 40 KHz) for 30 min [11].

3.5. Comparison with literature data (antioxidant activity)

The interest in *Moringa* leaves is due also to their antioxidant properties [6,7]. Therefore, in this work, the antioxidant activity of *Moringa* leaves by two *in vitro* complementary tests, named FRAP and DPPH, was investigated. Because only one antioxidant mechanism did not give an overview of antioxidant potential of the bioactive compounds, hence reducing power and radical inhibiting property were analysed.

Ferric Reducing Antioxidant Potential (FRAP) method is a simple and effective procedure based on the reduction of a ferric tripyridyltriazine complex to its ferrous, coloured form in the presence of antioxidant. The FRAP assay directly measures the ability of antioxidants to act as reducing compounds, showing a reduction potential below the reduction potential of the Fe^{3+}/Fe^{2+} couple. *M. oleifera* leaves showed FRAP values from 45.5 to 170.5 μmol Fe^{+2}/g DM (Table 3), based on the different UAE conditions (N5 and N4, respectively). As already discussed for TPC, the hydroalcoholic extracts showed the highest values (from 116.4 to 170.5 μmol Fe^{+2}/g DM) in respect to extractions carried out with pure MeOH (79.7-115.7 μmol Fe^{+2}/g DM).

As regards DPPH value, the values ranged between 13.7 % for N1 and 40.6 % for N8 (Table 3); the N1 and N8 extracts were produced with very different extraction conditions: methanol, 30:1, 10 min, 30°C *vs* hydroalcoholic solvent, 60:1, 60 min, 60 °C, respectively.

A correlation study showed acceptable results, for example between TPC and DPPH values (R^2=0.7709), TPC and FRAP values (R^2=0.6749) and between TFC and FRAP values (R^2=0.7411).

Castro-Lopez et al. [15] reported values of 0.70 and 2.43 mg GAE/g DM for FRAP and values of 1.87 and 5.03 mg GAE/g DM for DPPH, by using UAE with 1:25 or 1:50 S/L ratio respectively. The same authors used other extraction methods (maceration, decoction, and MAE) and also found that 1:50 ratio gave higher FRAP and DPPH values with respect to 1:25 ratio. Xu et al. [11] studied the antioxidant activities of the crude extracts of *M. oleifera*, and

found IC_{50} value (DPPH assay) of 1.02 mg/mL and 0.99 mM Fe^{2+}/g (FRAP assay), respectively.

Bozinou et al. [31] obtained a FRAP value of 71.68 μmoL Ascorbic Acid Equivalents/g DM and 64.82 % AA (DPPH assay) for extract of *M. oleifera* from central Greece obtained with UAE at 36°C for 15 min.

3.6. Characterization of the *M. oleifera* leaf extract: flavonols

There is long-standing knowledge regarding phytochemicals present in diffierent organs of *M. oleifera*, but only recently Lin et al. [18] reviewed the current studies of the health-promoting aspects of *Moringa* flavonoids on cancer, diabetes and obesity. It has been reported that flavonols are the most common flavonoids of *Moringa*. They are in abundance linked to a wide spectrum of sugar moieties (*e.g.* acetyldihexose and hexose) and the glycon structures might greatly modulate the bioactivities of flavonoids [18]. Obviously, diffierences in the contents of bioactives are most likely due to environmental conditions, geographic origin and other factors [32-35]. In this paper, the characterization of flavonol fraction of *Moringa* leaves produced in Italy was carried out. Based on the results of optimization step, *M. oleifera* leaves have been extracted in the following experimental conditions: hydroalcoholic solvent, 60:1 L/S ratio, 35 min and 45°C. Table 6 shows the main flavonols identified and quantified in UAE extract of *M. oleifera* leaves. In Figure 4 the relative HPLC-DAD chromatogram is reported.

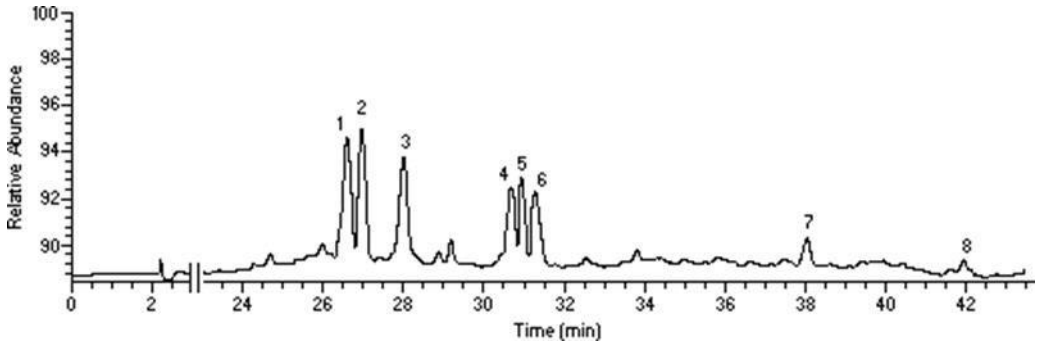


Figure 4. HPLC-DAD separation of flavonols from *Moringa* leaves. Peak assignments: 1. Quercetin 3-O-galactoside, 2. Quercetin 3-O-glucoside, 3. Quercetin 3-O-(6″-O-malonyl)-β- D-glucoside, 4. Quercetin 3-O-rhamnoside, 5. Kaempferol 3-O-galactoside, 6. Kaempferol 3-O-glucoside, 7. Quercetin, 8. Kaempferol.

Table 6. Flavonols in *M. oleifera* leaves: percentages and contents (mean value ± SD, n=3).

Compounds	UAE	
	%	μg/g DM
Quercetin 3-O-galactoside	17.1±0.3	271.8±6.7
Quercetin 3-O-glucoside	17.2±0.3	272.8±6.7
Quercetin 3-O-(6″-O-malonyl)-β-D-glucoside	18.5±0.4	293.9±4.9
Quercetin 3-O-rhamnoside	13.6±0.2	216.4±0.5
Kaempferol 3-O-galactoside	13.7±0.1	217.4±0.5
Kaempferol 3-O-glucoside	13.8±0.1	218.4±0.6
Quercetin	4.1±0.1	65.4±1.2
Kaempferol	1.9±0.0	30.1±0.3

Various glycosidic forms of quercetin were identified in *M. oleifera* leaves [11,33], but the aglyconic forms obtained after acid hydrolysis were also reported [32,36]. For example, quercetin and kaempferol contents changed respectively from 657 to 2749 and from 154 to 647 mg/100 g DM, considering samples from different origin (India, Niger and Nicaragua) and different extraction solvent [32].

In this research, kaempferol and quercetin (30.1 and 65.4 μg/g DM, respectively) are minor compounds, followed by the quercetin glycosidic forms, ranging from 216.4 μg/g DM of quercetin 3-O-rhamnoside to 293.9 μg/g DM of quercetin 3-O-(6″-O-malonyl)-β-D-glucoside.

At least fourteen flavonoids were identified by UHPLC analysis coupled with qTOF-MS (quadrupole time-of-flight mass spectrometry) in *M. oleifera* leaves, harvested in Namibia and South Africa [33]. Coppin et al. [36] identified by HPLC coupled with UV and mass spectrometer detection systems twelve flavonoids (six kaempferol derivatives and six quercetin derivatives) in *M. oleifera* leaves collected from sub-Saharan Africa; the total amount of flavonoids (quercetin plus kaempferol) ranged from 0.18 g/100 g DM for indigenous Dakar samples to 1.64 g/100 g DM for *M. oleifera* leaves originally developed in India and commercially available. Xu et al. [11] identified by LC-MS/MS five flavonoids (rutin, quercetin 3-O-glucoside, quercetin-acetyl-glycoside, kaempferol 3-O-glucoside, and kaempferol-acetyl-glycoside) from leaves, but also from root and seeds, of *M. oleifera* collected in Kenya.

4. Conclusions

In this study, an experimental design approach was successfully applied for investigating the effect of some extraction factors on UAE extraction of bioactive compounds with antioxidant activity from *M. oleifera* leaves. UAE technique, minimizing process time and temperature, is useful for the extraction of thermolabile compounds, such as phenol compounds. For these reasons, this technique represents a sustainable alternative for the use of agro-industrial residues through the efficient extraction of bioactives.

The influence of some extraction parameters on phenol and flavonoid contents and antioxidant properties was studied. The results showed that the composition of extraction solvent and the liquid/solid ratio were the most significant factors affecting the extraction of bioactives from *M. oleifera* using UAE process. The best UAE conditions were used to extract phenols from a commercial sample of *Moringa* leaves. Then the flavonol fraction was characterized by HPLC-DAD and UHPLC-MS/MS. In conclusion, the results of this study show that the optimized non-conventional extraction technique is a simple and efficient alternative for the recovery of flavonoids from *Moringa* leaves, a promising source of bioactives for functional food and nutraceutical development. Future research directions could be directed towards the purification and isolation of these bioactive compounds, also for possible exploitation on an industrial scale.

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3.2. Phenol profiling and nutraceutical potential of *Lycium* spp. leaf extracts obtained with ultrasound and microwave assisted techniques

1. Introduction

The genus *Lycium* belongs to the *Solanaceae* family and it includes numerous species which are called with the traditional name of goji. They grow in arid and semi-arid regions, such as South Africa, America, Australia, and Europe [1]. It has been suggested that the original habitat of *Lycium* spp. was located in the Mediterranean Basin [2]. Products from *Lycium barbarum* and *Lycium chinense* today are considered a "superfood". *Lycium* species are perennial shrubs or small trees, characterized by fast growth, a root system developed in depth, and good tolerance for drought and cold [3]. Recently, numerous papers reported on the phytochemical composition of goji berries [4–6]. Some studies claim that these fruits are rich in bioactives with antioxidant activity, such as carotenoids, flavonoids, and phenolic acids [7–9]. In many countries around the world, fruits are widely consumed fresh, dried, and transformed into food (as juice, in wine or tea preparation, in soups, and added to meat and vegetable dishes). Other parts of the plant (leaves, stems, flowers, and roots) are used as ethno-medicinal food [10,11]. In their origin area, leaves of *L. barbarum* and *L. chinense* are commonly used fresh, cooked, or dried for tea preparation. *L. barbarum* leaves are solitary or fasciculate, lanceolate or long elliptic. *L. barbarum* leaves are narrower than *L. chinense* leaves, which are solitary or in clusters of 2–4 at blade ovate or rhombic [1,12]. However, the phytochemical composition of *L. barbarum* leaves is less studied than *L. chinense* leaves [13]. Recently, it has been reported that *L. barbarum* leaves contain a polysaccharide–protein complex, rich in carbohydrates (including arabinose, galactose, glucose, mannose, rhamnose, ribose, and xylose), uronic acid, and calcium. This complex exhibits interesting health properties, among which are anticoagulant and antiplatelet activities [14]. As regards phenol compounds, the most abundant is rutin (quercetin-3-O-rutinoside), but also chlorogenic acid and scopoletin have been reported. Moreover, a significant level of tannins, phenolic acids, and flavonoids, among which are catechin and neohesperidin, was reported [15]. This chemical composition may change according to the type of plant: It was shown that rutin is the major component in wild and cultivated *L. barbarum* leaves, while chlorogenic acids and flavonoid glycosides are found abundantly in cultivated plants and kaempferol-3-O-rutinoside is found in wild plants [16]. Terpenoids are the most interesting compounds in *L. chinense* leaves, which also contain steroids, flavonoids, and phenolic acids, among which are rutin, quercetin, kaempferol, chlorogenic, ferulic, and *p*-coumaric acids [13]. Other

miscellaneous compounds include free amino acids, such as proline, histidine, alanine, and free sugars, among which are fructose, glucose, sucrose, and maltose. Olatunji et al. investigated the effect of *L. chinense* leaf extracts in rats with diabetic nephropathy and found that leaves are able to manage hyperglycemia and hyperlipidemia and, as a result, they could be used to treat and prevent diabetic nephropathy [17]. In recent years, agricultural and industrial wastes have attracted a lot of interest in the recovery of antioxidant compounds of potential use in food, pharmaceutical, and cosmetic industries. In this regard, recovery of phytochemicals from these products is typically achieved through different extraction techniques [18]. It has been reported that various solid-liquid extraction techniques are widely used for isolating plant antioxidants [19]. Generally, the extraction methods can be split into classical and innovative procedures [18]. The first procedure (i.e., maceration) uses conventional solvents without heat or with thermal treatment to improve the efficiency; these methods are easy to use, but have high-solvent consumption. On the contrary, innovative extraction techniques, among which are supercritical fluids, ultrasounds, and microwaves, allow us to ameliorate the extraction efficiency and/or selectivity by using processing aids/energy inputs [20]. In this research, ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) have been used with the objective to investigate the most effective technique to extract phenol compounds from a *L. barbarum* leaf sample from central Italy. A comparison with the traditional maceration (MAC) technique has been performed. Alcoholic and hydroalcoholic extracts were characterized considering their *in vitro* antioxidant properties and phenol composition. The profiling of phenol compounds isolated from *Lycium* leaves was studied by a high performance liquid chromatography-diode array detector (HPLC-DAD). The data of phenol contents have been processed by multivariate statistical techniques in order to evaluate the possible discrimination of *L. barbarum* and *L. chinense* samples.

2. Materials and Methods

2.1. Plant Materials

L. barbarum and *L. chinense* fresh leaves were collected in 2017 in different areas of Umbria (central Italy). Damaged leaves were manually discarded. Undamaged intact leaves were dried in a ventilated oven (Binder, Series ED, Tuttlingen, Germany) at 40 °C for 72 h,

and in any case until a constant weight was reached. Finally, dried leaves were grounded in a blender and passed through a 250 μm sieve to obtain a fine powder (moisture 10 ± 1%). These samples were stored in amber glass containers away from light and humidity at room temperature, until extraction. One sample of *L. barbarum* leaves was used for the comparison of different extraction methods, while four different *L. barbarum* leaf samples (1B–4B) and *L. chinense* leaf samples (1C–4C) have been analyzed to characterize the phenol fraction and evaluate the possible discrimination of *Lycium* spp.

2.2. Reagents

2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH radical), Folin and Ciocalteu's phenol reagent, gallic acid, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-s-triazine (TPTZ), chlorogenic acid (≥95%), *p*-cumaric acid (≥98%), ferulic acid (99%), kaempferol-3-O-glucoside (≥95%), rutin (≥95%), and tyrosol (98%) were from Sigma–Aldrich (Milan, Italy). Ultrapure water, formic acid, and acetonitrile were Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS) grade and were purchased from Carlo Erba Reagents (Milan, Italy). The other solvents were purchased from VWR (Milan, Italy).

2.3. Extraction methods of goji (*Lycium*) leaves

- Ultrasound-Assisted Extraction (UAE)

Dried *Lycium* leaf samples (330 mg) were extracted with 20 mL of pure methanol (UAE 1) and methanol/water 50:50 *v*/*v* (UAE 2) for 30 min at 45°C using UAE (sonication bath mod. AU-65, ArgoLab, Carpi, Italy). The ultrasonic power was 180W. The extract was then filtered through paper filter (MN 615, Macherey–Nagel, Düren, Germany), collected in amber glass vials, and kept at -20 °C until further analysis. The extraction was repeated three times.

- Microwave-Assisted Extraction (MAE)

Dried *Lycium* leaf samples (330 mg) were extracted with 20 mL of pure methanol (MAE 1) and methanol/water 50:50 *v*/*v* (MAE 2) for 30 min at 45 °C using a closed vessel

system microwave (Model Initiator 2.0, version 2.3, Biotage AB, Uppsala, Sweden) under controlled conditions. The temperature was the preferred controlled variable to avoid degradation of the target compounds and to achieve the maximum efficiency. The other parameters were directly dependent on the temperature, such as the magnetron power (maximum 40 W) and pressure (maximum 5 bar). At the end of the treatment, the vessel used was cooled to room temperature. The extract was filtered through paper filter (MN 615, Macherey–Nagel, Düren, Germany), collected in amber glass vials, and kept at -20°C until further analysis. The extraction was repeated three times.

- Maceration (MAC)

Dried *Lycium* leaf samples (330 mg) were extracted for 4 h min at room temperature while being stirred, using a dynamic maceration with 20 mL of pure methanol (MAC 1) and methanol/water 50:50 *v*/*v* (MAC 2). The extract was filtered through paper filter (MN 615, Macherey–Nagel, Düren, Germany), collected in amber glass vials, and kept at -20 °C until further analysis. The extraction was repeated three times.

UAE, MAE and MAC extracts were evaporated to dryness by a rotary evaporator (Buchi Rotavapor R-114 system) and the percentage of leaf extraction (Yield %) was calculated using the following equation:

$$\text{Yield \% (g/100 g)} = (W1 \times 100)/W2$$

where W1 is the weight of the extract residue obtained after solvent removal; W2 is the initial weight of leaf powder.

2.4. Determination of Total Phenol Content (TPC)

The total phenol content (TPC) was determined spectrophotometrically according to the method of Singleton and Rossi (1965), as modified by Pagano et al. [21]. Folin and Ciocalteu's phenol reagent was used and the absorbance was measured at 765 nm. The TPC was expressed as mg of gallic acid equivalents (GAE) per gram of dry leaves (mg GAE/g).

2.5. *In Vitro* Antioxidant Activities

- Free Radical-Scavenging Activity Using DPPH (DPPH Assay)

DPPH assay was carried out according to the procedure described by Blasi et al. [22]. DPPH methanolic solution was added to each extract. The change in the absorbance of the sample extract was measured at 515 nm after 30 min. The percentage of antioxidant activity (AA%) for each sample was calculated using the following formula:

$$AA\% = (ABSC - ABSS/ABSC) \times 100$$

where ABSC is absorbance of the control solution containing only DPPH and ABSS is absorbance of the DPPH solution containing the sample. The extract concentration that gave 50% inhibition (IC_{50}) was calculated using the regression equation obtained by plotting the AA% against the extract concentration. IC_{50} was expressed as mg/mL of the leaf extract.

- Free Radical-Scavenging Activity Using ABTS (ABTS Assay)

ABTS assay was performed according to the procedure described by Urbani et al. [23]. A freshly prepared ABTS+ solution was added to the sample extracts and the absorbance was measured at 734 nm after 10 min. The antioxidant capacity of each sample was expressed as mg Trolox equivalents (TE) per gram of dry leaves (mg TE/g).

- Ferric Reducing Antioxidant Power (FRAP) Assay

The reducing capacity of the leaf extracts was determined according to the procedure reported by Rocchetti et al. [19]. A ferric reducing antioxidant power (FRAP) reagent was added to the leaf extracts, and then the samples were left in the dark for 30 min. The absorbance of the sample was measured at 593 nm. Aqueous solutions of known Fe^{+2} concentrations (2–5 mM) were used for calibration, and results were expressed as μmol Fe^{+2} per gram of dry leaves (μmol Fe^{+2}/g).

2.6. HPLC-DAD Analysis of Phenol Compounds

The HPLC analysis of leaf samples was performed according to a previous paper [24]. A pump Thermo Spectraseries, coupled with the Spectra System UV6000LP DAD (Thermo Separation Products, San Jose, CA, USA), was used. The chromatographic separation of polyphenols was carried out with a C18 Hypersil GOLD column (150 × 4.6 mm, 3 m particle size). The mobile phase solvents were: (A) 0.1% (*v*/*v*) formic acid in water; (B) 0.1% (*v*/*v*) formic acid in acetonitrile. For the analytical separation of the compounds, a gradient profile

was employed: Phase B increased from 5% to 20% in 30 min, and then to 95% in 5 min. The mobile phase flow rate was 1 mL/min, while the injection volume was 20 μL. UV detection was performed, scanning between 280 and 360 nm. The chromatograms were acquired and the data was handled using Xcalibur software version 1.2 (Finnigan Corporation 1998–2000, San Jose, CA, USA). A standard solution containing phenol compounds (tyrosol, *p*-coumaric acid, ferulic acid, rutin, kaempferol-3-O-glucoside, and chlorogenic acid) was used to identify and quantify the analytes. Calibration curves were obtained by three injections of four different concentrations, ranging from 1.5 to 117.2 μg/mL.

2.7. UHPLC-MS/MS Analysis of Phenol Compounds

The ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analysis of leaf samples was performed according to Simeoni et al. [25]. An UHPLC system Nexera XR (Shimadzu, Tokyo, Japan) coupled with a 4500 Qtrap mass spectrometer (Sciex, Toronto, ON, Canada) equipped with a heated Electrospray Ionization (ESI) source (VTM source) was used. The ion source parameters were set as follows: Negative ionization mode; ion spray voltage −4.5 kV; air as nebulizer gas at 40 psi, nitrogen as turbo gas at 40 psi; and temperature at 500 °C. The chromatographic separation of polyphenols was carried out with an Excel 2 C18-PFPcolumn (2 μm, 10 cm × 2.1 mm ID-ACE, Aberdeen, UK). The mobile phase was made with the following solvents: (A) Aqueous 0.1% formic acid and (B) acetonitrile. For the analytical separation of the compounds, a gradient profile was employed: 5% phase B was increased up to 100% in 5 min, held for 1 min, and switched back to 5% in 3 min (total time 9 min). The flow rate was 0.3 mL/min. The chromatograms were acquired and the data was handled using MultiQuant 3.0.2 software (AB Sciex, Concord, ON, Canada).

2.8. Statistical Analysis

All analytical determinations were performed in triplicate, and the results, expressed as the mean ± standard deviation, were reported on dry leaves. Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA) was used for data analysis. Principal component analysis (PCA) and linear discriminant analysis (LDA) were performed using the

Excel-based "XLSTAT" V2006.06 package (Addinsoft, Inc., New York, NY, USA) and the phenol contents obtained by HPLC-DAD analysis were used as variables.

3. Results and Discussions

In this research, *Lycium* leaves were extracted by UAE, MAE and MAC methods and a comparison among them has been carried out. In addition, pure methanol or methanol/water (50:50 *v/v*) have been comparatively tested to evaluate the impact of the solvent on the recovery of the bioactive phenols from goji leaf powder. Other extraction conditions, as ratio, temperature and time, were selected taking into consideration previous papers [19,22]. Table 1 shows the results of the characterization of the different extracts, regarding the extraction yield, total phenol contents and *in vitro* antioxidant activities.

Table 1. Yield of extraction, total phenol content (TPC) and *in vitro* antioxidant activities.

		Yield	**TPC**	**DPPH**	**ABTS**	**FRAP**
	Extraction solvent	%	mg GAE/g	IC50*	mg TE/g	μmol Fe^{+2}/g
UAE 1	MeOH	26.04 ± 0.13	10.02 ± 0.23	2.53 ± 0.18	20.51 ± 1.62	112.65 ± 2.86
UAE 2	MeOH:H_2O, 50:50	31.95 ± 0.76	8.13 ± 0.06	9.45 ± 0.84	14.65 ± 0.75	92.17 ± 6.64
MAE 1	MeOH	22.17 ± 0.79	6.65 ± 0.35	3.42 ± 0.25	17.67 ± 0.81	140.74 ± 0.58
MAE 2	MeOH:H_2O, 50:50	31.48 ± 1.75	5.68 ± 0.38	5.68 ± 0.63	13.14 ± 0.69	140.88 ± 0.61
MAC 1	MeOH	24.34 ± 0.86	9.52 ± 0.25	3.92 ± 0.52	14.63 ± 0.92	121.23 ± 3.69
MAC2	MeOH:H_2O, 50:50	29.82 ± 0.62	5.07 ± 0.06	23.35 ± 3.27	11.68 ± 0.86	55.19 ± 0.53

Data are reported as mean ± standard deviation of three independent measurements (n=3) and are expressed on dry weight; *mg/mL.

As regards phenol content, it is possible to observe that the UAE extraction technique with methanolic solvent was the most effective. In fact, the values were 10.02 and 8.13 mg GAE/g, respectively for methanolic (UAE1) and hydro-alcoholic (UAE2) extract. Also for MAE and MAC techniques, the methanolic extracts showed better results than hydroalcoholic mixture (6.65 mg GAE/g for MAE1 vs 5.68 mg GAE/g for MAE2; 9.52 mg GAE/g for MAC1 vs 5.07 mg GAE/g for MAC2).

Recently, the interest in phenol compounds characterizing goji leaves is due to their health-promoting properties, such as their antioxidant potential [2,10]. Therefore, in this work, the *in vitro* antioxidant activity of goji leaves was investigated by means of three complementary tests, namely DPPH, ABTS and FRAP (Table 1). The results obtained for DPPH radical-scavenging assay, expressed as IC_{50}, ranged from 2.53 of UAE1 to 23.35 mg/mL

of MAC2. Higher antiradical activity was found in methanolic extracts (2.52 for UAE1, 3.42 for MAE1 and 3.92 for MAC1), with respect to the hydroalcoholic extracts (9.45 for UAE2, 5.68 for MAE2 and 23.35 for MAC2), independently from extraction technique. It should be observed that IC_{50} values are inversely proportional to the antiradical activity, as IC_{50} represents the sample concentration that give 50% inhibition of DPPH radical. Table 1 also shows that ABTS values ranged from 11.68 mg TE/g of MAC2 to 20.51 mg TE/g of UAE1, with higher values in methanolic than hydroalcoholic extracts, independently from the extraction techniques. The results of antiradical activity are in agreement with TPC values. In fact, methanolic UAE had the highest antiradical activities and TPC values. Similar findings have been previously reported by Rocchetti et al. [19], who investigated traditional and innovative extraction methods to obtain phenols from *Moringa oleifera* leaves.

Moreover, the FRAP values of the extracts, expressed as μmol Fe^{+2}/g, ranged from 55.19 in MAC2 up to 140.88 in MAE2. Considering the methanolic extracts, the highest value was obtained for MAE1, while the lowest for UAE1, but generally these values are higher than values obtained with hydroalcoholic mixture, with the exception of MAE extracts, where the values are very similar between them (140.74 and 140.88, for MAE1 and MAE2, respectively). An interesting correlation between FRAP and DPPH results (R^2 = 0.7819) has been obtained. These data confirm that the *in vitro* antioxidant activities were significantly affected by the extraction method.

In this research, HPLC-DAD and UHPLC-MS/MS procedures were performed in order to characterize the phenol components of goji extracts, with the objective to further evaluate the most efficient extraction technique. In particular, UHPLC-MS/MS technique was carried out for the identification of the analytes while HPLC-DAD was performed in order to quantify them.

Table 2 shows the parameters obtained from HPLC-DAD analysis of the phenol compounds, used as reference standards.

Table 2. Range, regression equation, R^2, RSD Intradie and Interdie, limits of detection (LOD), and limits of quantification (LOQ) of standard compounds from high performance liquid chromatography-diode array detector (HPLC-DAD) analysis.

Standard	Range	Regression Equation		R^2	RSD* Intra-day	RSD* Inter-day	LOD	LOQ
	μg/mL	Slope	Intercept		%	%	μg/mL	μg/mL
Tyrosol	7.31 – 29.24	1.88E+06	-9.18E+05	0.9993	1.12	4.98	1.51	4.82
Kaempferol-3-*O*-Glu[a]	14.80 – 59.00	6.06E+06	7.29E+06	0.9998	1.13	3.57	0.71	2.10
Chlorogenic acid	1.50 – 117.20	3.38E+06	-5.20E+05	0.9996	0.98	3.71	0.68	2.17
***p*-Coumaric acid**	1.78 – 7.10	1.89E+07	1.58E+06	0.9999	0.79	4.80	0.06	0.19
Ferulic acid	1.86 – 7.45	1.68E+07	1.20E+06	0.9997	1.72	4.55	0.64	2.05
Rutin	11.10 – 44.30	5.51E+06	-1.24E+06	0.9997	1.32	4.13	1.68	5.34

*RSD, relative standard deviation (n=4); [a]kaempferol-3-O-glucoside

Tyrosol, kaempferol-3-O-glucoside, chlorogenic acid, *p*-coumaric acid, ferulic acid, and rutin with different concentrations (ranging from 1.5 to 117.2 μg/mL) were tested in order to determine the linearity using DAD for this chromatographic method. For each concentration level, injections were performed in triplicate and the average value was used for the external standard calibration curves. For all compounds, the value of R^2 was good (from 0.9993 of tyrosol to 0.9999 of *p*-coumaric acid). In this research, for intra-day RSD were considered one-day measures of four sample replicates (intra-day precision or repeatability), whereas for inter-day RSD, samples were analyzed for four consecutive days (inter-day precision or with in laboratory reproducibility); it is possible to observe that both intra-day and inter-day precisions were acceptable. The limits of detection (LOD) and limits of quantification (LOQ) were calculated according to the following equations [26]:

$$LOD = 3.3 \times SD/B$$

$$LOQ = 10 \times SD/B$$

where SD is the standard deviation of the curve and B is the slope of the curve.

The values of LOD and LOQ (Table 2) show a good sensitivity of the analytical procedure used to determine phenol compounds in *Lycium* leaves.

Table 3 reports the values of the content, expressed as mg/g goji leaves, of phenol compounds of the different extracts (UAE, MAE, MAC), using methanol or hydroalcoholic

mixture. It can be noted that chlorogenic acid, followed by rutin, is the main phenol compound, with the exception of MAC2 where the extraction was carried out with MeOH:H_2O, 50:50 *v*/*v*. In fact, for MAC2 the main compounds were chlorogenic acid (732.71 mg/g) and tyrosol (577.18 mg/g), followed by rutin (452.41 mg/g). The content of chlorogenic acid ranged from 732.71 mg/g of MAC2 to 2,991.55 of UAE1. It can be noted that two isomers of chlorogenic acid were also detected (neochlorogenic and cryptochlorogenic acids) and their concentrations were determined by the same regression equation of chlorogenic acid.

Table 3. Content of phenol compounds of *L. barbarum* extracts, obtained with different extraction methods: Ultrasound-assisted extraction (UAE); microwave-assisted extraction (MAE); maceration (MAC).

	UAE 1	UAE 2	MAE 1	MAE 2	MAC 1	MAC 2
Neochlorogenic acid	594.04±45.60	412.50±23.11	462.37±25.74	597.91±43.91	450.11±58.40	21.26±2.34
Tyrosol	660.61±37.13	640.80 ±30.73	476.10±34.91	598.19±46.92	501.30±6.40	577.18±40.42
Chlorogenic acid	2,991.55±46.62	1,728.83±110.08	2,656.66±150.23	2,059.65±104.72	2,692.46±166.06	732.71±35.81
Cryptochlorogenic acid	375.76±30.55	374.88±4.34	323.91±24.71	346.87±21.94	227.69±28.05	378.29±20.35
***p*-Coumaric acid**	83.81±8.26	81.20±8.42	88.08±7.83	80.20±7.41	33.14±1.55	42.08±3.84
Rutin	1,678.68±61.74	1,197.55±95.89	1,328.50±74.91	705.06±34.92	1,330.61±184.12	452.41±24.75

1 and 2 mean pure methanol and methanol/water 50:50 *v*/*v* extraction solvent used, respectively.

Data are reported as mean ± standard deviation of three independent measurements (n=3) and are expressed on a dry weight basis.

Our findings are in agreement with the previous investigation of Rocchetti et al. [19], who reported that the extractions carried out with pure methanol were more effective than hydroalcoholic mixture for phenol compounds recovering from *Moringa oleifera* leaves. Total polyphenol and flavonoid contents of *Olea europaea* leaf, harvested at the two different stages, were significantly higher in the methanol extract than in less polar fractions [27]. In addition to the analytical procedure, it is well known that the antioxidant capacity of leaf extracts is influenced by several pedoclimatic and agronomic factors, among which harvesting period and cultivar [22].

Considering the results reported in Tables 1 and 3, UAE was more effective than MAE and MAC, and *Lycium* leaves extracted with methanol had more phenol compounds and antioxidant activities than the hydroalcoholic solution extracts. For this reason, in the next step of the research, the UAE methanolic extraction was applied to eight goji leaf samples (four leaf samples of *L. barbarum* and four leaf samples of *L. chinense*) from Umbria (central Italy) region. Table 4 shows yield, total phenol content and antioxidant properties of *Lycium* leaf samples.

Table 4. Yield of extraction, total phenol content (TPC) and *in vitro* antioxidant activities of *Lycium* leaf samples.

	Yield	**TPC**	**DPPH**	**ABTS**	**FRAP**
	%	mg GAE/g	IC_{50}*	mg TE/g	μmol Fe^{+2}/g
1B	16.67±0.54	14.31±0.12	1.01±0.06	30.18±1.32	194.40±6.85
2B	23.84±0.84	8.95±0.08	5.30±0.26	21.91±0.45	138.08±1.15
3B	22.78±0.76	6.35±0.14	14.95±1.53	15.46±0.86	76.34±7.92
4B	27.34±0.95	19.12±0.26	0.40±0.02	34.27±1.19	272.26±4.94
1C	21.12±0.86	12.68±0.51	2.21±0.22	25.62±0.23	165.60±2.36
2C	21.36±0.98	14.37±0.24	1.93±0.12	26.79±0.87	222.57±3.82
3C	16.32±0.72	13.54±0.18	1.33±0.09	24.23±0.64	210.19±8.38
4C	19.07±0.92	10.78±0.23	2.05±0.21	21.41±1.21	158.89±0.75

Data are reported as mean ± standard deviation of three independent measurements (n=3) and are expressed on a dry weight basis; *mg/mL; B = *L. barbarum* leaf samples; C = *L. chinense* leaf samples.

It can be observed that the yield of extraction ranged from 16.67 % to 27.34 % for *L. barbarum* samples, and from 16.32 % to 21.36 % for *L. chinense* samples. As regards TPC, the lowest value has been obtained for 3B sample (6.35 mg GAE/g), while the highest for 4B sample (19.12 mg GAE/g). The *L. chinense* leaf samples showed more homogenous TPC values, which varied in a small range (from 10.78 mg GAE/g of 4C to 14.37 mg GAE/g of 2C).

The results obtained for *L. chinense* samples showed small ranges of values for DPPH (1.33 mg/mL of 3C - 2.21 mg/mL 1C), FRAP (158.89 μmol Fe^{+2}/g of 4C - 222.57 μmol Fe^{+2}/g of 2C) and ABTS (21.41 mg TE/g of 4C - 26.79 mg TE/g of 2C). On the contrary, greater variability was observed for *L. barbarum* samples, with 4B sample showing the lowest DPPH value (0.40 mg/mL) and the highest values of FRAP (272.26 μmol Fe^{+2}/g) and ABTS (34.27 mg TE/g). Correspondingly, the 4B sample had also the highest value of yield and TPC. On the basis of these results, a correlation study has been carried out considering all the samples. Interestingly, it has been observed that phenol content well correlated with DPPH (R^2=0.6494), ABTS (R^2=0.9197) and FRAP (R^2=0.9509) values.

In order to obtain the complete profile of phenol compounds in *Lycium* leaf samples, HPLC-DAD and UHPLC-MS/MS have been performed. In Table 5, the UV-VIS and MS spectral data used for the identification of the ten phenol compounds in *Lycium* leaf samples are shown.

Table 6 shows the results of the quantitative analysis of the ten phenol compounds identified in *L. barbarum* and *L. chinense* leaves by HPLC-MS/MS. Quercetin-3-O-rutinoside-7-O-glucoside (quercetin-3-O-Rut-7-O-Glu) and quercetin-3-O-sophoroside-7-O-rhamnoside (quercetin-3-O-Soph-7-O-Rha) have been quantified using the regression equation of rutin, while that of kaempferol-3-O-glucoside has been used for kaempferol-3-O-rutinoside-7-O-glucoside (kaempferol-3-O-Rut-7-O-Glu). As already observed for the *L. barbarum* sample extracted with the different methods, chlorogenic acid and rutin were generally the most represented compounds. These results have been also confirmed by Lopatriello et al. [16] for Italian *L. barbarum* leaves and flowers. However, further considerations should be made since more complex profiles have been obtained, showing remarkable differences both for samples of different species and for samples of each species. For example, rutin was not very represented in 1C, 2C and 4C, while tyrosol was more abundant than rutin in 2B, 3B, 2C and 4C samples. The main difference between *L. barbarum* and *L. chinense* leaves concerns quercetin-3-O-Rut-7-O-Glu and quercetin-3-O-Soph-7-O-Rha, compounds detected only in *L. chinense* leaves. Moreover, cryptochlorogenic acid was found in all *L. barbarum* samples but in none of the *L. chinense* ones. As regards phenolic acids, ferulic acid was quantified in all *L. chinense* samples (198.96 for 2C - 1201.89 for 4C μg/g) while it was detected in small concentrations only in 3B (14.58 μg/g) and 4B (10.53 μg/g). Moreover, *p*-coumaric acid was

not detected in *L. chinense* samples, while it was quantified in three *L. barbarum* sample (from 24.55 μg/g in 3B to 585.47 μg/g in 4B).

Table 5. UV-VIS and MS spectral data of the identified phenol compounds.

	R_t (min)	λmax (nm)	$[M+H]^+$	MS fragments (m/z)
Neochlorogenic acid	8.1	296sh; 324	377$[M+Na]^+$	191 $[M-H\text{-caffeoyl}]^-$; 179 $[M-H\text{-quinic}]^-$; 707 $[2M-H]^-$
Tyrosol	8.9	231; 275	137	137 $[M-H]^-$; 93 $[M-H-CO_2]^-$
Quercetin-3-*O*-Rut-7-*O*-Glu[a]	12.3	255; 266sh; 354	773	611 $[M-H\text{-glucose}]^+$; 465 $[M-H\text{-rutinose}]^+$;
Quercetin-3-*O*-Soph-7-*O*-Rha[b]	12.8	255; 266sh; 354	773	627 $[M-H\text{-rhamnose}]^+$; 465 [M-H-rhamnose; M-H-sophorose]$^+$;
Kaempferol-3-*O*-Rut-7-*O*-Glu[c]	13.3	265; 340	757	611 $[M-H\text{-glucose}]^+$; 449 $[M-H\text{-rutinose}]^+$
Chlorogenic acid	13.8	244; 296sh; 320	355	191 $[M-H\text{-caffeoyl}]^-$; 179 $[M-H\text{-quinic}]^-$ 707 $[2M-H]^-$
Cryptochlorogenic acid	14.3	244; 296sh; 326	377$[M+Na]^+$	191 $[M-H\text{-caffeoyl}]^-$; 179 $[M-H\text{-quinic}]^-$; 707 $[2M-H]^-$
***p*-Coumaric acid**	20.2	312	163	147 $[M-H-H_2O]^-$; 119 $[M-H-CO_2]^-$
Ferulic acid	23.7	238; 290sh; 322	193	193 $[M-H]^-$; 178 $[M-H-CH_3]^-$
Rutin	26.6	256; 266sh; 354	611	303 $[M-H\text{-rutinose}]^+$; 1243 $[2M+Na]^+$

[a] quercetin-3-O-rutinoside-7-O-glucoside, [b] quercetin-3-O-sophoroside-7-O-rhamnoside, [c] kaempferol-3-O-rutinoside-7-O-glucoside

Mocan et al. studied the phenol compounds in *L. barbarum* and *L. chinense* leaves and reported the presence of three hydroxycinnamic acid derivates, namely ferulic, chrologenic and *p*-coumaric acids in *L. barbarum* leaves [10]. They found high amount of chlorogenic acid in the leaves of both *Lycium* spp., with higher content in *L. chinense* than in *L. barbarum* leaves. Mocan et al. identified also isoquercitrin, rutin, quercitrin, and quercetin. Ferulic acid and kaempferol were detected only in the ethanolic extract of *L. chinense* leaves [10]. Liu et al. studied the compositions of phenolic acids and flavonoids in leaves and stems of the three varieties of *L. chinense* and found that neohesperidin and catechin were the major flavonoids in the leaves while ferulic, *p*-coumaric and p-hydroxybezoic acid were the major phenolic acids [15]. According to the results obtained in this work, rutin is generally the main flavonoid detected in *Lycium* leaves [2,10].

In order to compare the samples of different varieties (*L. barbarum* or *L. chinense*) a statistic approach was applied to the profiling phenol dataset obtained by HPLC-DAD analysis (data shown in Table 6). Among multivariate statistical data analyses, the most used methods are represented by principal component analysis (PCA) and linear discriminant analysis (LDA). In order to highlight the influence and correlations between variables, phenol results were elaborated by PCA, that reduces the number of potentially correlated variables into a smaller number of uncorrelated factors (principal components). Moreover, LDA is a useful statistical method to examine differences between samples of different groups and to determine the most useful variables for their discrimination. In previous research, lipid analysis and LDA have been applied with success to animal and vegetable foods for authentication of species or cultivar [28-30].

Table 6. Content of phenol compounds in *L. barbarum* and *L. chinense* leaves.

	1B	**2B**	**3B**	**4B**
	μg/g	μg/g	μg/g	μg/g
Neochlorogenic acid	466.43 ± 9.72	8,655.31 ± 266.61	324.82 ± 3.11	508.14 ± 8.88
Tyrosol	513.51 ± 18.71	1,921.88 ± 19.14	1,105.65 ± 24.58	716.23 ± 60.40
Quercetin-3-*O*-Rut-7-*O*-Glu[a]	nd	nd	nd	nd
Quercetin-3-*O*-Soph-7-*O*-Rha[b]	nd	nd	nd	nd
Kaempferol-3-*O*-Rut-7-*O*-Glu[c]	610.30 ± 38.4	108.25 ± 2.66	99.45 ± 8.51	nd
Chlorogenic acid	6,354.36 ± 204.81	3,048.82 ± 13.93	1,353.13 ± 12.24	3,139.02 ± 132.54
Cryptochlorogenic acid	492.43 ± 65.23	230.46 ± 2.35	161.93 ± 1.17	429.92 ± 6.80
***p*-Coumaric acid**	nd	49.49 ± 0.23	24.55 ± 0.27	585.47 ± 8.80
Ferulic acid	nd	nd	14.58 ± 0.44	10.53 ± 1.80
Rutin	5,756.65 ± 340.5	1,808.75 ± 19.37	743.50 ± 4.13	5,233.17 ± 264.88
	1C	**2C**	**3C**	**4C**
	μg/g	μg/g	μg/g	μg/g
Neochlorogenic acid	439.58±13.80	325.54±10.91	432.58±7.24	423.96±9.50
Tyrosol	596.37±29.25	2,057.51±30.74	118.40±1.54	1,303.28±43.07
Quercetin-3-*O*-Rut-7-*O*-Glu[a]	195.39±2.33	939.49±21.03	268.94±11.07	205.60±3.04
Quercetin-3-*O*-Soph-7-*O*-Rha[b]	1,946.70±38.95	1,011.92±27.54	344.02±5.58	1,271.42±16.21
Kaempferol-3-*O*-Rut-7-*O*-Glu[c]	380.72±3.07	91.77±4.83	170.21±10.04	366.13±10.04
Chlorogenic acid	3,811.85±41.41	7,721.47±130.84	6,056.74±149.80	2,153.11±187.22
Cryptochlorogenic acid	nd	nd	nd	nd
***p*-Coumaric acid**	nd	nd	nd	nd
Ferulic acid	670.72±16.53	198.96±5.39	451.57±12.85	1,201.89±24.12
Rutin	506.21±8.47	432.63±6.40	1,029.45±57.52	375.60±14.95

Data are reported as mean ± standard deviation of three independent measurements (n=3) and are expressed on a dry weight basis; [a]quercetin-3-O-rutinoside-7-O-glucoside, [b]quercetin-3-O-sophoroside-7-O-rhamnoside, [c]kaempferol-3-O-rutinoside-7-O-glucoside. Not detected (nd).

Table 7 shows eigenvalue, percentage of variance, and cumulative percentage of the principal components, obtained using HPLC-DAD results as variables, relative to the phenol compositions of *Lycium* spp. leaves.

Table 7. Principal component analysis (PCA): Eigenvalue, percentage of variance, and cumulative percentage.

	F1	F2	F3	F4	F5	F6	F7
Eigenvalue	5.235536	2.186099	1.160795	0.671911	0.579965	0.115526	0.050168
Variability %	52.35536	21.86099	11.60795	6.719115	5.799649	1.155262	0.50168
Cumulative %	52.35536	74.21635	85.82429	92.54341	98.34306	99.49832	100

The first two components explained 74.2% of the total variance. Figure 1 shows the variable correlation circle (axes F1 and F2); it represents a projection of the initial variables in the factors space.

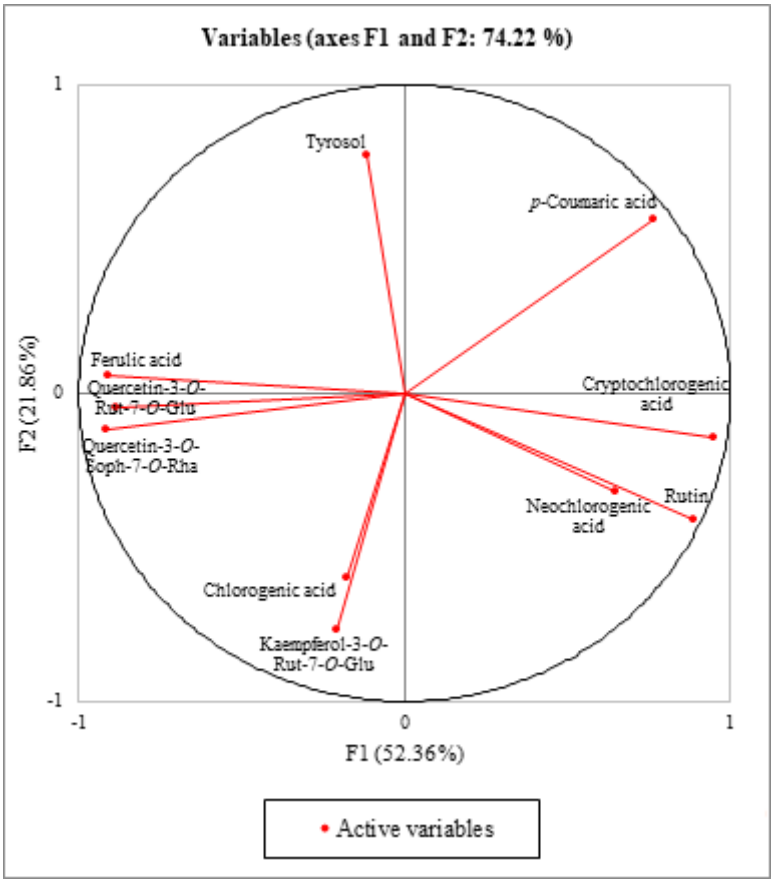

Figure 1. Loading plot for principal components F1 and F2.

The correlation circle is useful in interpreting the meaning of the axes. In fact, the horizontal axis is linked with ferulic acid, quercetin-3-O-Rut-7-O-Glu, quercetin-3-O-Soph-7-O-Rha and cryptochlorogenic acid, and the vertical axis with tyrosol, chlorogenic acid and kaempferol-3-O-Rut-7-O-Glu. Moreover, it is possible to observe that the vectors of the variables, chlorogenic acid/kaempferol-3-O-Rut-7-O-Glu and neochlorogenic acid/rutin, are grouped, indicating that they are positively correlated.

In Figure 2, the distribution of goji leaf samples in the plane defined by the values of the two principal components, according to phenol content. Two main clusters were obtained when considering the different goji leaf extracts. The first cluster, on the right of the plot, was characterized by B1-B4 (methanolic extract of *L. barbarum*), while all the other C1-C4 samples (methanolic extract of *L. chinense*) were included in the second cluster, on the left of the plot.

The multivariate parametric LDA technique was also used in order to classify and discriminate goji leaf samples from *L. barbarum* and *L. chinense* species. The selection of the most significant variables was performed by stepwise analysis. The Wilks' Lambda test allows to test if the vector of the means for the various groups are equal or not. A p value equal to 0.000 indicates that the difference between the means vectors of the groups is significant. XLSTAT software selected rutin and cryptochlorogenic acid as variables for *Lycium* samples discrimination.

Figure 3 shows the plot of the observations on the discriminant function axis, using phenol content data. It allows to confirm that the species are very well discriminated on the factor axis extracted from the original explanatory variables. In fact, it is possible to observe that goji samples are well discriminated according to their species (on the right *L. barbarum* samples and on the left *L. chinense* samples). The centroid coordinate on F1 axis were 4.929 and -4.929 for B and C group, respectively.

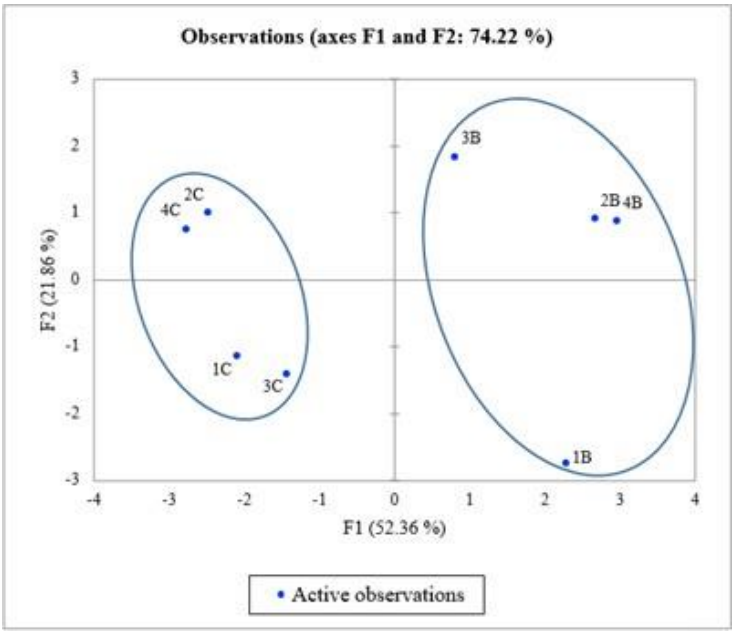

Figure 2. Goji leaf samples on the principal components of the F1/F2 score plot.

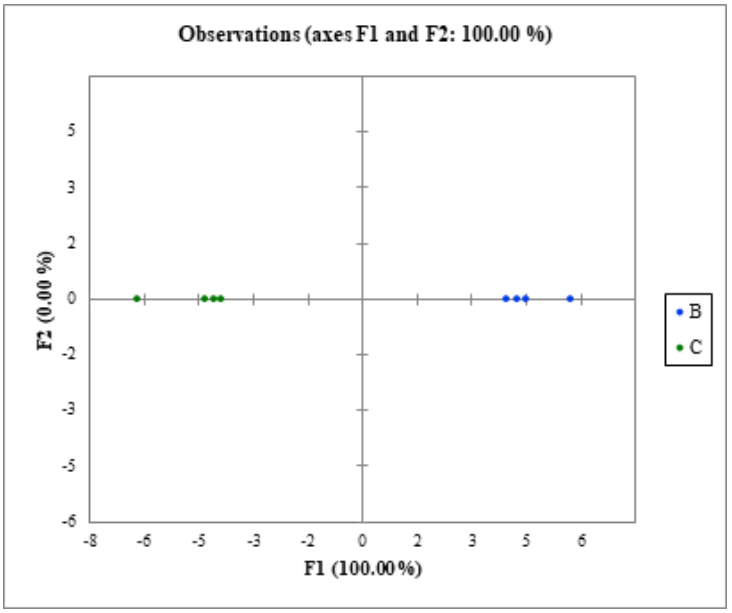

Figure 3. Goji leaf samples on the discriminant function F1 score plot.

Table 8 summarizes the reclassification of the observations, showing for each observation the factor scores (the coordinate of the observations in the new space), the probability to belong to each group, and the squared Mahalanobis distances to the centroid of each of the classes. Each observation is classified into the group for the which the probability of belonging is the greatest. It is possible to observe that all the samples have been correctly reclassified.

Table 8. PCA: prior and posterior classification, membership probabilities, scores and squared distances.

Observation	Prior	Posterior	Pr(B)	Pr(C)	F1	D^2(B)	D^2(C)
Obs1	B	B	1.000	0.000	6.134	4.188	125.126
Obs2	B	B	1.000	0.000	4.793	2.181	96.680
Obs3	B	B	1.000	0.000	4.565	3.633	93.645
Obs4	B	B	1.000	0.000	4.225	3.258	86.562
Obs5	C	C	0.000	1.000	-4.626	92.706	1.487
Obs6	C	C	0.000	1.000	-4.347	87.470	1.758
Obs7	C	C	0.000	1.000	-6.613	134.879	4.499
Obs8	C	C	0.000	1.000	-4.131	83.530	2.087

B = *L. barbarum* leaf group; C = *L. chinense* leaf group.

The results of classification, reported in Table 9, showed that 100 % of original grouped cases were correctly classified. Additionally, to verify the power and the stability of the model, a leave-one-out cross-validation discriminant analysis was performed. From the cross-validation results, it can be observed that 100.0 % of cross-validated group cases were correctly classified.

Table 9. Linear discriminant analysis (LDA) classification results.

Classification results (training sample)				
From/to	**B**	**C**	**Total**	**% correct**
B	4	0	4	100.00%
C	0	4	4	100.00%
Total	4	4	8	100.00%
Classification results (cross-validation)				
From/to	**B**	**C**	**Total**	**% correct**
B	4	0	4	100.00%
C	0	4	4	100.00%
Total	4	4	8	100.00%

B = *L. barbarum* leaf group; C = *L. chinense* leaf group.

The obtained results show that phenol compounds represent useful markers and fingerprinting components for assessing authenticity of goji leaves.

4. Conclusions

In this work, the impact of different extraction technologies (UAE and MAE) was evaluated in terms of recovering and profiling of the phenol compounds from goji leaves, by using both alcoholic and hydroalcoholic solvents. The results of phenol contents and antioxidant capacity showed that methanolic UAE was the most efficient extraction method, compared with traditional maceration and non-conventional MAE technique. The obtained results confirm that the extraction technique and solvent have a deep impact on the efficiency of the analytical procedure. These findings are relevant considering that the potential use of ultrasound extraction is promising for extraction of antioxidants on an industrial scale.

Moreover, in this work, the developed analytical procedure, based on methanolic ultrasound extraction, allowed to study the phenol profile of leaves from different *Lycium* species. Goji leaf phenol contents, obtained by HPLC-DAD analysis, were processed by PCA and LDA, and the results highlighted the possibility of distinguishing leaves from different *Lycium* spp., despite the limited number of samples. These findings confirm that phenol profile has a discriminating power for plant-based products and by-products from different species. This approach can be implemented for quality control and authentication of goji leaf-containing foods.

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3.3. Phenolic acids from *Lycium barbarum* leaves: *in vitro* and *in silico* studies of the inhibitory activity against porcine pancreatic α-amylase

1. Introduction

Despite the large number of synthetic pharmaceuticals, interest into natural products is increasing. The agricultural field offers a great opportunity thanks to the large quantities of by-products of plant food processing, a promising source of biologically active compounds. In this context, food waste as leaves are a cheap and available source of naturally-occurring bioactives, potentially useful for the development of functional foods and food supplements [1,2]. Starting from plant materials, numerous and different processes are used to obtain single bioactive substances or compounds belonging to the same chemical category. The recovery of high added-value components from food wastes can be carried out by traditional or innovative technologies. Among the latter, ultrasound-assisted extraction (UAE) can be considered a very successful and efficient extraction process for bioactive extraction [2].

Previous studies have reported the phytochemical composition of *Lycium* spp. berries and the availability of their bioactive compounds, including carotenoids, polyphenols, phytosterols, and phenolic acids [3-6]. *Lycium* leaves are interesting by-products with health properties, due to the presence of polyphenols [7,8]. Nowadays, these bioactives are considered functional ingredients in the composition of different phytopharmaceuticals and dietary supplements due to numerous biological properties, among which antioxidant, anti-inflammatory, anti-hypertensive [9]. In addition to these activities, it has been reported that chlorogenic acid (CHA) shows an inhibitory effect on α-amylase [10]. This enzyme, together with other digestive enzymes (e.g. α-glucosaccharase, transglycosylase), is involved in the *in vivo* hydrolysis of starch into glucose, that, transported into blood, leads to postprandial hyperglycaemia. In particular, α-amylase is the key digestive enzyme that acts on starch to release dextrin, disaccharides or other low molecular-weight carbohydrates, which will be then broken down into glucose [11]. Since high blood glucose level can be associated with diabetes or obesity, the inhibition of α-amylase might be an effective way to reduce starch digestibility and thereby relieve postprandial glycaemia [11].

It has been reported in the literature that the extracts from leaves of various origin possessed inhibitory activity against α-amylase [12-14]. Abdelli et al. [15] evaluated the inhibitory activity against α-amylase of tannic acid, catechin, gallic acid, quercetin and epicatechin by *in silico* approach, revealing that tannic acid can be used as alternative drug

for the regulation of postprandial hyperglycaemia. Demir et al. [16] reported that genistein, tangeretin, pelargonidin, formononetin, and delphinidin showed antidiabetic properties. *L. barbarum* leaves showed also inhibition activity against various enzymes, among which cholinesterase, α-amylase, and α-glycosidase [8].

In this work, *L. barbarum* leaves were extracted with methanol by UAE and the phenolic acid profile was investigated by ultra-high-performance liquid chromatography quadrupole-time-of-flight tandem mass spectrometry (UHPLC-Q-TOF MS/MS) analysis. In fact, this phenolic class represents one of the most abundant in *Lycium* leaves, in particular chlorogenic acid. The identified phenolic acids were studied for α-amylase inhibitory activity by *in vitro* approach. The objective of this research was also to assess a putative binding mode of active phenolic acids to human pancreatic α-amylase. To the best of our knowledge, this is the first time that the evaluation of the inhibitory effect of individual phenolic acids, identified in the leaf extract, against porcine pancreatic α-amylase is performed.

2. Materials and Methods

2.1. Plant sample

Intact fresh leaves from *L. barbarum*, harvested in 2019 in Umbria (central Italy), were dried to constant weight in a ventilated oven (Binder, Series ED, Tuttlingen, Germany) at 40 °C for 72 h. Then the dried leaves were ground to obtain a fine powder and stored in amber glass vial at room temperature in the dark, until extraction.

2.2. Reagents

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS; ≥98%), 2,2-diphenyl-1-picrylhydrazyl (DPPH radical), Folin & Ciocalteu's phenol reagent, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; 97%), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ; ≥98%), CHA (≥95%), salicylic acid (≥99%), *p*-coumaric acid (≥98%), sinapic acid (≥98%), syringic acid (≥95%), caffeic acid (≥98%), vanillin (99%), vanillic acid (≥97%), gallic acid (97.5-102.5%), starch from potato, 3,5-dinitrosalicylic acid (DNSA; ≥98%), sodium chloride (≥99.5%), and α-amylase from porcine pancreas (Type VI-B, ≥5 units/mg solid) were acquired from Sigma-Aldrich (Milan, Italy). Acetonitrile, formic acid and ultrapure water from Carlo Erba Reagents (Milan, Italy) were UPLC–MS grade.

2.3. Ultrasound-Assisted Extraction (UAE) of *L. barbarum* leaves

Dried *L. barbarum* leaves (330 mg) were extracted with pure methanol (20 mL) using an ultrasonic bath (mod. AU-65, ArgoLab, Carpi, Italy) with ultrasonic power of 180 W. The extraction was carried out for 30 min at 45°C, then the extract was filtered and collected in amber glass vials until the further analysis. The extraction was repeated three times.

The extract was evaporated to dryness by a rotary evaporator (Buchi Rotavapor R-114 system) and the percentage of leaf extraction (Yield %) was calculated using the following equation:

$$\text{Yield \% (g/100 g)} = (W1 \times 100)/W2$$

where W1 is the weight of the extract residue obtained after solvent removal; W2 is the initial weight of leaf powder.

2.4. Determination of Total Phenol Content (TPC)

The TPC was determined spectrophotometrically according to the method previously reported *17+, based on redox reaction of phenols with Folin & Ciocalteu's reagent. Lambda 20 spectrophotometer (PerkinElmer, Inc; Waltham, Massachusetts, USA) was used to measure the absorbance at 765 nm. The TPC was expressed as mg of gallic acid equivalents (GAE) per gram of dry leaves (mg GAE/g).

2.5. *In vitro* Antioxidant Activities

2.5.1. Free Radical-Scavenging Activity using DPPH Assay

The measurement of the scavenging ability of phenolic extract towards the stable DPPH radical was performed by DPPH assay, using the procedure previously reported [18]. An aliquot (3.9 mL) of a 0.0634 mM DPPH solution in methanol (95%) was added to *L. barbarum* leaf extract (100 μL) and vortexed for about 5–10 sec. After 30 min, the change in the absorbance was measured at 515 nm using the spectrophotometer reported in 2.4 paragraph. The DPPH antiradical capacity was expressed as mg Trolox Equivalents (TE) per gram of dry leaves (mg TE/g).

2.5.2. Free Radical-Scavenging Activity using ABTS Assay

ABTS assay was carried out following the procedure described by Urbani et al. [19]. The ABTS stock solution was prepared by mixing 7 mM ABTS and 2.45 mM of potassium persulphate as the oxidant agent. The sample extract (200 μL) was added to $ABTS^{+\cdot}$ solution (1800 μL) and after 10 min the absorbance at 734 nm was measured using the spectrophotometer reported in 2.4 paragraph. The ABTS antiradical capacity was expressed as mg TE/g.

2.5.3. Ferric Reducing Antioxidant Power (FRAP) Assay

The reducing capacity of leaf extracts was determined using a modified method of the FRAP assay [20]. Freshly prepared FRAP reagent (2.0 mL) was mixed with *L. barbarum* extract (100 μL) and distilled water (900 μL), then the sample was left away from light at room temperature. After 30 min the absorbance at 593 nm was measured using the spectrophotometer reported in 2.4 paragraph. Distilled water was used as blank. The FRAP value was expressed as mg TE/g.

2.6. α-Amylase Inhibition Activity Assay

The inhibitory activity of α-amylase was carried out according to the procedure reported by Wu et al. [21]. *L. barbarum* extract solutions were mixed with α-amylase and maintained at 30 °C for 10 min. Then, soluble starch solution was added as substrate and the hydrolysis was carried out at 30°C. After 3 min, DNSA reagent was added, and the mixture was placed in boiling water bath at 80°C for 15 min. Afterwards, the solutions were diluted with water, and then the absorbance at 540 nm (Abs extract) was measured using a Lambda 20 spectrophotometer. For each concentration of the *L. barbarum* extract, a blank incubation was prepared by replacing the enzyme solution with distilled water, to correct for absorbance due the *L. barbarum* extract (Abs blank extract). A control incubation, representing 100% enzyme activity, was carried out by replacing *L. barbarum* extract with the vehicle, and Abs control was measured. The same test was also carried out without the enzyme and Abs blank control was registered. The α-amylase inhibitory activity was calculated as percentage inhibition using the following formula:

$$\% \text{ inhibition} = *((\Delta Abs \text{ control} - \Delta Abs \text{ extract}))/(\Delta Abs \text{ control} + \times 100)$$

where

$$\Delta Abs \text{ control} = Abs \text{ control} - Abs \text{ blank control}$$

$$\Delta Abs \text{ extract} = Abs \text{ extract} - Abs \text{ blank extract}$$

The concentration of the extract able to inhibit the α-amylase activity by 50% (IC_{50}) was calculated by regression analysis. Single phenolic acids were also tested for α-amylase inhibitory activity and their IC_{50} values were determined as above described. Acarbose was also analyzed as a known α-amylase inhibitor.

2.7. Analysis of Phenolic Acids by Q-TOF-LC/MS

The ultra-high-performance Accurate-Mass Q-TOF-LC/MS analysis was performed using an Agilent Technologies (Santa Clara, CA, USA) 1200 Infinity Series LC coupled with an Agilent Technologies 6540 UHD Accurate-Mass Q-TOF-LC/MS. This device was equipped with an electrospray ionization Agilent Technologies Dual Jet Stream ion source (Dual AJS ESI). Chromatographic separation was carried out with an Agilent Infinity lab Poroshell 120 EC-C18 (3 x 100 mm, 2.7 μm) column. The injection volume was 10 μL. The mobile phase consisted of 0.1 % formic acid in water milli-Q (solvent A) and acetonitrile (solvent B), at a flow rate of 0.5 mL/min, with the following gradient: 0–10 min, 5% B; 10–13 min, 95% B; 13-15 min, 95% B. The Q-TOF-MS conditions were the following: drying gas flow (N_2), 12.0 L/min; nebulizer pressure, 45 psi; gas drying temperature, 370 °C; capillary voltage, 3500 V; fragmentor voltage,110 V; skimmer voltage 65 V and octopole RF peak, 750 V. Dual AJS ESI interface was used in negative ionization mode and negative ions were acquired in the range of 100–1100 m/z for MS scans, and 50–600 m/z for auto MS/MS scans, at a scan rate of 5 scans/s for MS and 3 scans/s for MS/MS, respectively. Automatic acquisition mode MS/MS were carried out using the following collision energy values: m/z 20 eV; m/z 30 eV and 40 eV. Internal mass correction was enabled, by using two reference masses at 121.0509 and 922.0098 m/z. Instrument control and data acquisition were performed using Agilent MassHunter Workstation software B.08.00. All the MS and MS/MS data of the validation standards were integrated by MassHunter Quantitative Analysis B.10.0 (Agilent Technologies).

2.8 Molecular Docking

The crystal structure of human pancreatic α-amylase (PDB ID: 5KEZ) was downloaded from the RCSB PDB (https://www.rcsb.org/) [22], removing the peptide inhibitor from the active site. The optimization procedure was performed using the Protein Preparation Wizard tool implemented in Maestro (Maestro, Version 12.1, Schrödinger, LLC, New York (NY), 2019). In particular, this procedure included the refinement of the hydrogen bond network that was performed using the default setting. The OPLS3e force field was used for the energy minimization of the protein structure, restraining atomic coordinates of heavy atoms. Atomic coordinates of chemical structures were retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) [23]. LigPrep (v.8.4) was then used to add hydrogens, and Epik (Epik, Version 2.5, Schrödinger, LLC, New York, NY, 2013) to generate tautomeric and protonation states at pH 7.0 ± 2.0. Next, energy minimization of chemical structures was carried out with the OPLS3e force field until reaching an energy gradient convergence criteria of 0.05 kJ/Å mol. Docking simulations were performed using Glide (v.8.4) [24,25]. Specifically, a grid box was defined with its center located on the center of mass of binding site residues in the reference structure 5KEZ. These residues included Ala128, Pro130, Tyr131, Ser132, Tyr151, Asp197, Lys200, His201, Glu233, Ile235, Asp300, and His305. The inner grid box was sized 10x10x10Å. Docking studies were carried out using the standard precision (SP) method and the Gscore scoring function. The top ten scored binding poses were stored for each molecule. The binding mode for each compound was selected in terms of the best docked pose showing the highest number of ligand/target interactions (electrostatic interactions, hydrogen bonds, π-stacking/cation interactions, hydrophobic contacts) and lowest Gscore (kcal/mol). Experimental or calculated acidic constants (pKa) of phenolic acids were retrieved from DrugBank [23,26].

2.9. Statistical Analysis

Each analytical procedure was carried out in triplicate and the results, reported on dry leaves, were expressed as mean ± standard deviation (SD). For data analysis Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA) was used.

3. Results and Discussion

3.1. Characterization of *L. barbarum* Leaf Extract

In this study, *L. barbarum* leaf extract was obtained by non conventional UAE using methanol as solvent. This extraction process has been chosen on the basis of the results obtained in a recent paper [7], which showed that UAE is a very successful and efficient technique for extracting phenols from *L. barbarum* leaves, also in comparison with microwave-assisted extraction. The UAE methanolic extraction yield was 20.39% ± 1.08. The obtained residue was first characterized by evaluating the phenol content and antioxidant activity, then the phenolic acid profile was determined by Q-TOF-LC/MS analysis. The total phenolic content, determined according to the Folin-Ciocalteu method, was 7.75 mg GAE/g ± 0.43; similar findings have been previously reported in other studies regarding Italian and Romanian *L. barbarum* leaves [7,8]. Mocan et al. reported values of 11.14 mg GAE/g dw (dry weight) and 11.98 mg GAE/g dw for Bigligeberry and Erma cultivars respectively, while for wild-growing *L. barbarum* leaves lower amount (2.49 mg GAE/g dw) was reported [8].

The evaluation of the antioxidant activity, based on different complementary assays, is a routine analysis commonly carried out to characterize vegetable extracts. The antioxidant capacity of *L. barbarum* leaf extract was measured using three spectrophotometric methods, DPPH and ABTS assays determining antiradical activity, and FRAP assay determining reducing power; the results are shown in Figure 1.

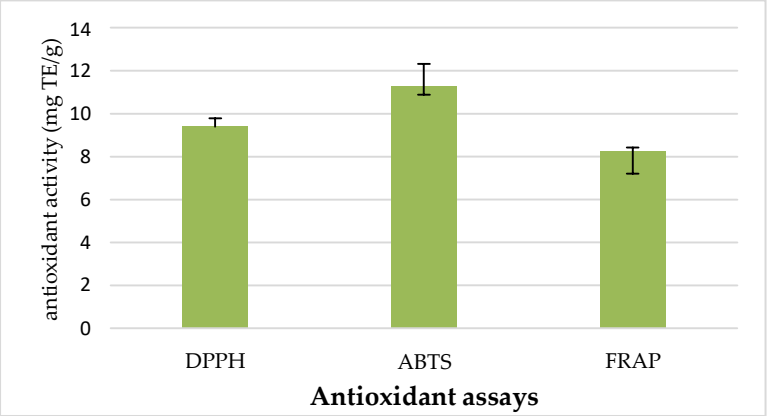

Figure 1. Values of *in vitro* antioxidant activities of *L. barbarum* leaf extracts, evaluated by DPPH, ABTS, and FRAP assays. The error bars represent the standard deviation (n=3).

The results obtained for the DPPH and ABTS radical-scavenging assays were 9.39 mg TE/g and 11.28 mg TE/g, respectively, while 8.25 mg TE/g was obtained by FRAP assay. The lower value obtained for DPPH assay in respect to ABTS assay can be attributed to the steric hindrance effects and the chemical characteristics of the phenolic compounds. Mocan et al. [8] reported different antioxidant values obtained by ABTS assay for leaves of two cultivars of Romanian *L. barbarum* and from wild-growing plants. Other authors studied the antioxidant activity of another plant source of chlorogenic and neochlorogenic acids, plum fruits of three different species, and found lower ABTS values [27]. In another paper, Mocan et al. [28] studied the antioxidant capacity of *L. barbarum* and *L. chinense* leaves from Romania using DPPH, ABTS and hemoglobin ascorbate peroxidase activity inhibition assays and found higher values for *L. chinense* than *L. barbarum* leaves. ABTS values similar to those obtained in this work were obtained for other vegetable waste, such as olive leaves harvested in different season from different *Olea europaea* cultivar [1]. However, it is important to highlight that it is not always possible to make comparisons with antioxidant data reported in the literature, in particular for DPPH and FRAP data, since the results of the same assay can be expressed with different measure unit. In this regard, it is known that antioxidant activity of leaf extracts is strongly dependent on the harvesting season, cultivar, and extraction conditions [1, 20]. In this study, a Q-TOF-LC/MS analysis was carried out to investigate the qualitative and quantitative profile of phenolic acids in the UAE methanolic extract of *L. barbarum* leaves, considering that the main objective of this paper was the *in vitro* and *in silico* study of the inhibitory activity of this category of phenolic compounds against porcine pancreatic α-amylase. Figure 2 shows the chemical structures of the main phenolic acids identified in the methanolic extract of *L. barbarum* leaves, while Table 1 shows their content.

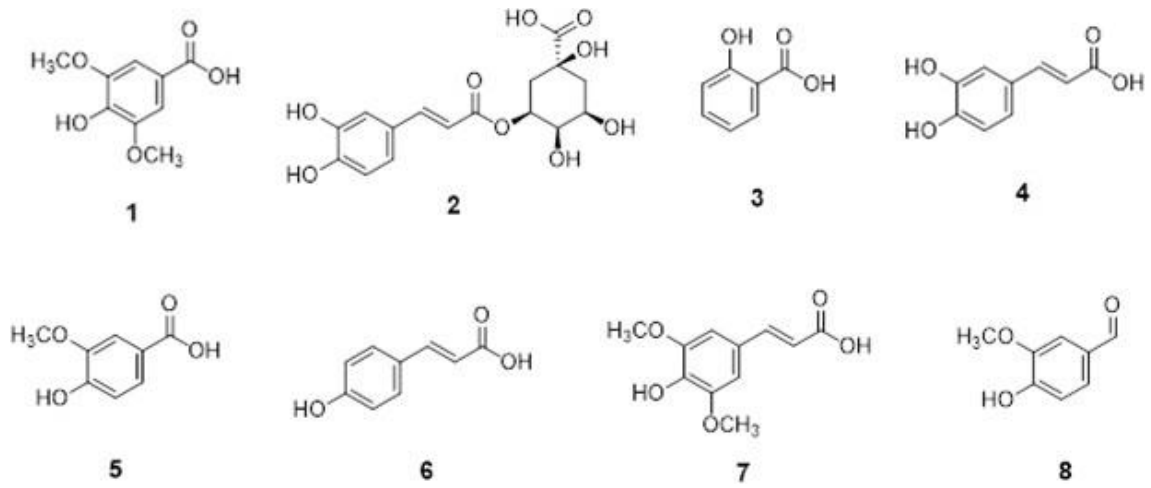

Figure 2. Chemical structures of the main phenolic acids and vanillin identified in the *L. barbarum* leaf extract. 1: syringic acid; 2: chlorogenic acid; 3: salicylic acid; 4: caffeic acid; 5: vanillic acid; 6: *p*-coumaric acid; 7: sinapic acid; 8: vanillin.

Table 1. Phenolic acid content (μg/g ± SD) of *L. barbarum* leaf extract.

n.	Compounds	Concentration (μg/g)	Rt (min)	Mass calculated (m/z, M-H$^-$)	MS fragments (m/z, M-H$^-$)	Error (ppm)	Score
1	Syringic acid	0.76 ± 0.000	4.39	197.0458	123.0083/ 147.8891/95.0138	−1.22	98.26
2	Chlorogenic acid	358.34 ± 0.004	4.69	354.0955	191.0543/127.0397/85.0301	−1.22	98.72
3	Salicylic acid	239.02 ± 0.005	5.13	137.0247	93.0351/65.0405	−1.23	99.41
4	Caffeic acid	0.07 ± 0.000	5.67	180.0432	135.0447/107.0506/89.0404	−1.10	97.22
5	Vanillic acid	9.46 ± 0.002	6.39	167.0350	108.0218/123.0458/91.0196	−1.34	97.11
6	*p*-Coumaric acid	0.84 ± 0.000	6.58	223.0619	164,0483/119.0497/93.0349	−1.50	97.23
7	Sinapic acid	2.36 ± 0.000	6.69	283.0833	224.0696/149.0232/93.0350	−1.71	99.34
8	Vanillin	8.62 ± 0.003	4.47	197.0457	146.7437/136.016/76.9614	−1.67	96.44

Data are reported as mean ± SD of three independent measurements (n = 3) and are expressed on dry leaves.

CHA was the main phenol compound (358.34 μg/g), followed by salicylic acid (239.02 μg/g). These two molecules are also reported in two systematic *Lycium* reviews [29, 30]. CHA was previously described as dominant in the leaves of wild and cultivated *L. barbarum* [8,28,31,32], but also in *L. chinense* leaves [28, 32]. Caffeic, chlorogenic, *p*-coumaric, and vanillic acids were also certainly identified by Inbaraj et al. [31]. It is widely reported in the literature that the phenolic profile of leaves may strongly change according to the type of plant, seasonal conditions, extraction method [1,7,20].

3.2. α-Amylase Inhibition of *L. barbarum* Extract and Constituent Phenolic Acids

In order to evaluate the capacity of the *L. barbarum* extract to inhibit the α-amylase, and therefore the potential hypoglycemic effect, an *in vitro* assay based on the

spectrophotometric evaluation of the hydrolytic activity of porcine pancreatic α-amylase was carried out. The linear regression equations, obtained plotting α-amylase % inhibition versus concentration of the standard phenolic acids and UAE methanolic extract are reported in Table 2. The R^2 values and the IC_{50} values are shown in the same Table. The IC_{50} value for Acarbose was 0.1 mg/mL, while Min and Han [33] reported a value of 0.45 mg/mL. The results revealed that the percentage of α-amylase inhibition increased for chemical standards in a dose-dependent manner, with a linear trend, as also reported in other papers [10,34], with good R^2 values (from 0.9987 of CHA to 0.9999 of sinapic acid).

Table 2. Linear regression equations, R^2 values and IC_{50} values of phenolic acids and *L. barbarum* leaf extract for α-amylase inhibition activity.

n.	Compounds	Regression Equation		R^2	IC_{50}
		Slope	Intercept		mg/mL
1	Syringic acid	46.4	-228.28	0.9925	6.0
2	Chlorogenic acid	178.56	-37.986	0.9987	0.5
3	Salicylic acid	384	-633.93	0.9930	1.8
4	Caffeic acid	15.923	-5.7764	0.9995	3.5
5	Vanillic acid	12.512	-27.403	0.9996	6.2
6	*p*-Coumaric acid	10.627	- 9.4865	0.9989	5.6
7	Sinapic acid	8.8633	-23.288	0.9999	8.3
8	Vanillin	4.8082	-1.8485	0.9990	10.8
	Acarbose				0.1
	L. barbarum leaf extract	2.1847	-5.52	0.9977	25.4

Higher α-amylase inhibitory activity was found for CHA, salicylic and caffeic acids than the other constituents. On the other hand, the inhibitory activity of the *L. barbarum* methanolic extract was lower than the individual phenolic acids, probably due to the complexity of the chemical composition of vegetable matrices, as leaves. However, comparable results were reported by other authors [35], which found an IC_{50} value of 23.7 mg/mL for ethanolic extract of dried *Garcinia schomburgkiana* leaves. Antidiabetic properties of phenolic compounds were reported in other papers, regarding phenolic compounds from leaves [8], fruits [36], and plants [37]. It has been reported that natural compounds, such as phenolics, may bind by hydrogen bonding the amino acid residues in the active sites of digestive enzyme. In this way the hydrolysis reaction of digestive enzymes on carbohydrates may be inhibited and consequently slowed down their absorption [21]. Only few data on enzyme inhibition potential of *Lycium* leaves are reported, as for example Mocan et al.

reported similar results for amylase inhibitory activity (0.24-0.26 mmol Acarbose equivalents/g extract) for *L. barbarum* considered samples [8].

Abdelli et al. *15+ reported that tannic acid showed a stronger α-amylase inhibitory capacity (IC_{50} = 3.46 μg/mL) in comparison with Acarbose (IC_{50} = 10.41 μg/mL). Demir et al. *16+ found that phenolic compounds exhibit potential inhibitor properties for α-amylase, α-glycosidase and aldose reductase. For α-amylase assay, the authors reported IC_{50} values from 601.56 nM of delphinidin chloride to 2067.78 nM of pelargonidin, while 10.000 nM for Acarbose was reported.

The investigation of the amylase inhibitory activity of the individual phenolic acids showed that some molecules (chlorogenic acid, CHA, **2**; caffeic acid, **4**; salicylic acid, **3**) had inhibitory activity against the key carbohydrate digestive enzyme, while other molecules (syringic acid, **1**; *p*-coumaric acid, **6**; vanillic acid, **5**; sinapic acid, **7**; vanillin, **8**) showed lower inhibition potency. These results confirmed that CHA (**2**) has high inhibitory activity against α-amylase. It has been reported that this should be due to its ability to form quinones, rather than semiquinones [34]. Sinapic and caffeic acids are less active than CHA (**2**), as also reported by Funke and Melzig [34]. Notably, in this work the inhibition mechanism of the main phenolic compounds toward this key digestive enzyme was investigated by molecular docking analysis, in order to provide a better understanding of how the considered phenolic acids inhibit α-amylase activity.

3.3. Docking Studies

In order to infer a putative binding mode of active phenolic acids (**1**-**8**) to the enzyme, docking studies were carried out using the human pancreatic α-amylase that shares 90% of sequence similarity with the porcine isoform (Figure 3).

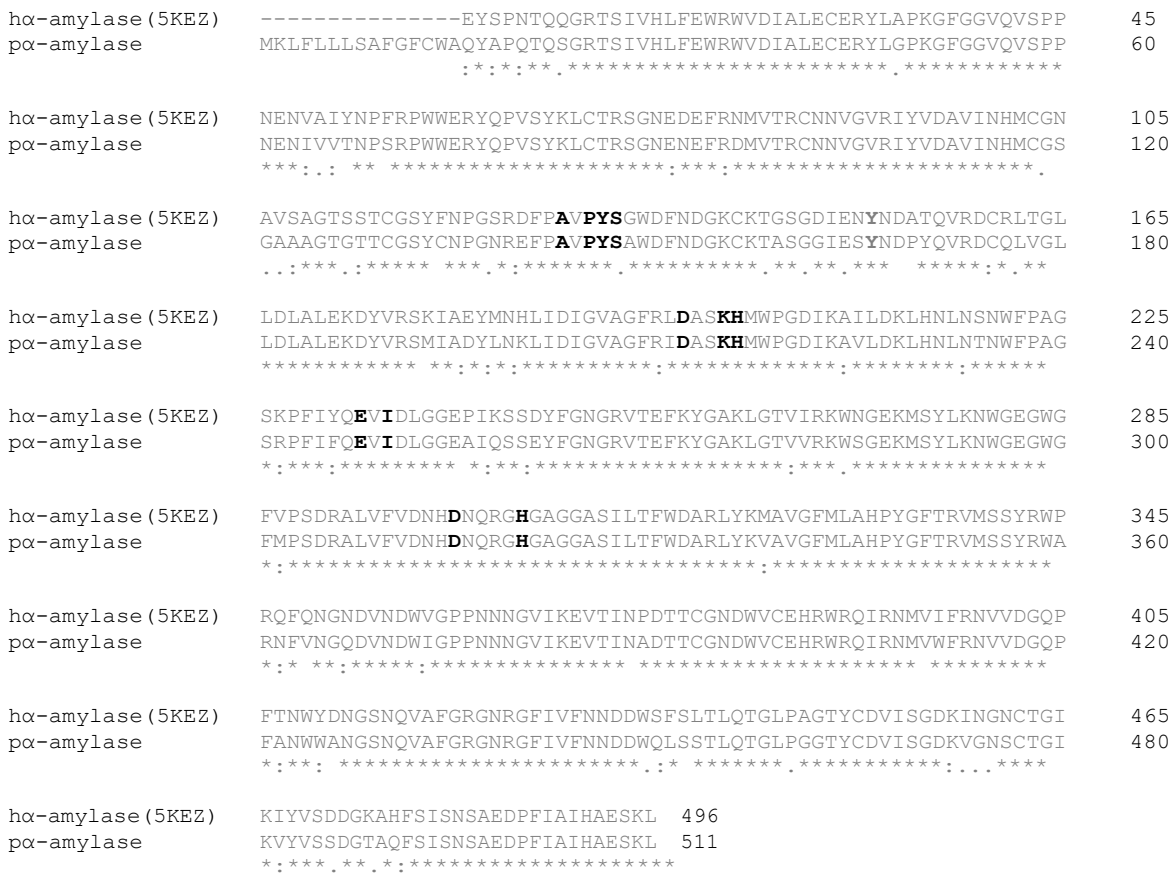

Figure 3. Sequence alignment between human pancreatic α-amylase (hα-amylase; 5KEZ) and porcine pancreatic α-amylase (pα-amylase) as generated with Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/). Binding site residues selected for docking studies are bold typed. Symbols at the bottom indicate identity (*), high (:) or moderate (.) similarity.

In particular, the crystal structure of the enzyme in complex with a peptide inhibitor (pdb code: 5KEZ) as reference structure was used [38]. Firstly, CHA (**2**, $_{calc}$pKa = 3.3) was docked into the enzyme and the resulting binding mode is shown in Figure 4. CHA forms hydrogen bonds between the three hydroxyl groups of the cyclohexane ring and the side chains of Asp197, Glu233 and Asp300. An additional hydrogen bond is observed between the *para* hydroxyl group of the caffeic acid moiety and the side chain of Glu240, whereas the phenyl ring engages Lys200 with a π-cation interaction (Figure 4).

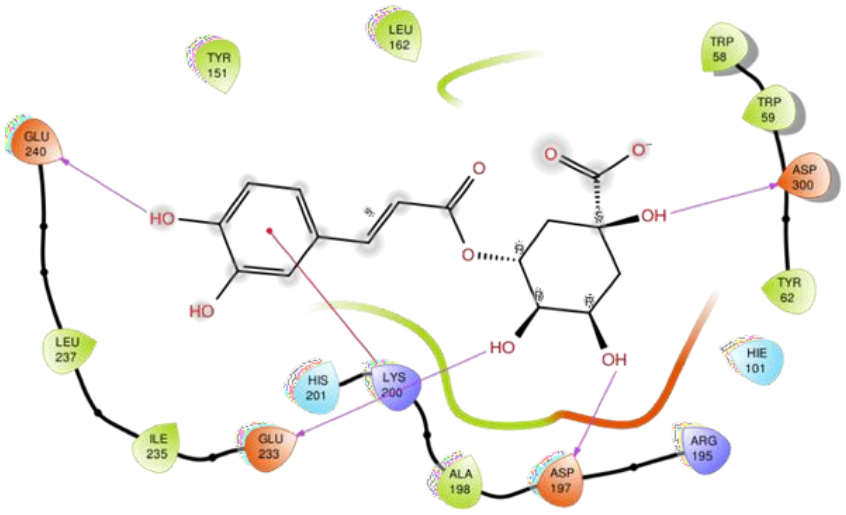

Figure 4. Binding mode of CHA (**1**) to human pancreatic α-amylase as resulting from the docking study (Gscore = -4.10 kcal/mol). Hydrogen bonds are shown with magenta arrows. π-Cation interaction is shown with red line. Polar and hydrophobic contacts between ligand and enzyme residues are displayed with red and green faded tick lines, respectively.

Caffeic acid (**4**, $_{exp}$pKa = 4.6) is a structural fragment of CHA. The docking study of this compound into the crystal structure of human pancreatic α-amylase yields a binding mode wherein the carboxylic group engages the side chain of Lys200 with a salt bridge interaction, while making a hydrogen bond with the backbone amide group of Ile235 (Figure 5A). The shift of the molecule within the active site of the enzyme with respect to the ester fragment of CHA is driven by the electrostatic interaction of the free carboxylic group with Lys200.

Differently from CHA, such a shift places the *meta* hydroxyl moiety in a complementary position for the formation of a hydrogen bond with Glu233. *p*-Coumaric acid (**6**, $_{calc}$pKa = 3.8) lacks the *meta* hydroxyl moiety while keeping the *para* hydroxyl group of caffeic acid. As a result of the docking study, this compound adopts a binding mode akin to caffeic acid, keeping the interactions with Lys200 and Ile235 while losing the hydrogen bond with Glu233 (Figure 5B). This binding mode is also found in the docking study of sinapic acid (**7**, $_{calc}$pKa = 3.4) to human pancreatic α-amylase, wherein a further π-stacking interaction is also observed between the substituted aromatic moiety and His201 (Figure 5C).

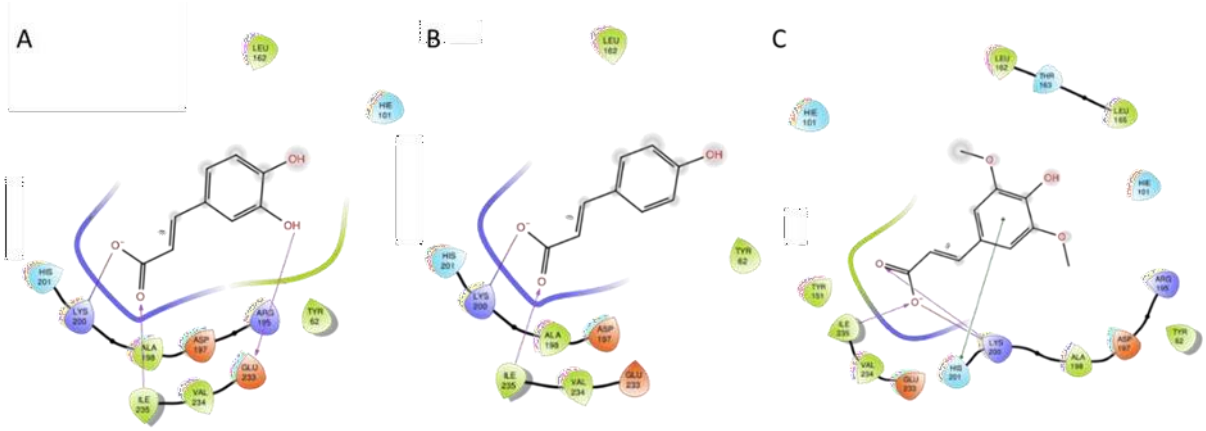

Figure 5. Binding mode of caffeic acid (A; **4** Gscore = -4.02 kcal/mol), *p*-coumaric acid (B; **6** Gscore = -3.56 kcal/mol) and sinapic acid (C; **7** Gscore = -3.89 kcal/mol), to human pancreatic α-amylase. Salt bridge interaction is depicted with a continuous blue/red faded line. Hydrogen bonds are shown with magenta arrows. π-Stacking interaction is plotted with a green capped line. Polar and hydrophobic contacts between ligand and enzyme residues are displayed with blue and green faded tick lines, respectively.

Since a fraction of vanillin (**8**, $_{exp}$pKa = 7.4) exists in the anionic form at the experimental pH of the inhibition assay (pH = 6.9), this compound was docked using both the neutral form and negatively charged form. These forms adopt a binding mode that is markedly affected by the electrostatic interaction with Lys200 (Figure 6). In the anionic form, the hydroxylate group of vanillin is placed nearby Lys200, forming a salt bridge interaction with this residue and a hydrogen bond with the backbone amide group of Ile235. A further hydrogen bond is observed between the methoxy group and the side chain of Lys200, whereas a π-stacking interaction is found between the imidazole ring of His201 and the aromatic moiety of vanillin. No specific interactions are observed for the aldehyde group of the anionic form of vanillin. In the neutral form, only the π-stacking interaction with His201 is kept, while the aldehyde group engages the backbone carbonyl group of Ile235 with a hydrogen bond.

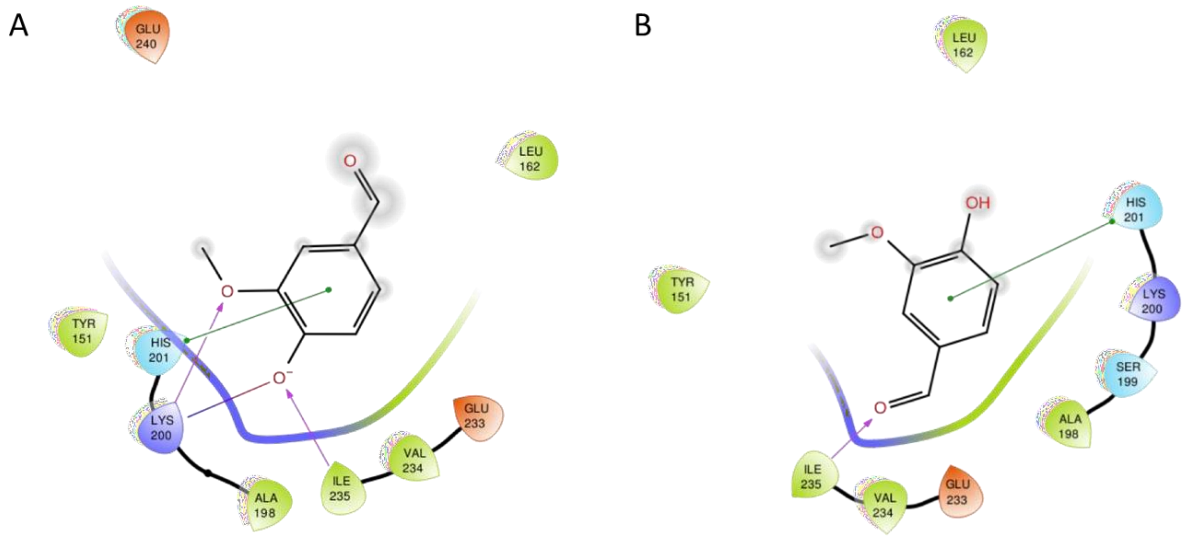

Figure 6. Binding mode of vanillin (**8**) to human pancreatic α-amylase according to its anionic (A; Gscore = -3.70 kcal/mol) and neutral (B; Gscore = -5.59 kcal/mol) form. Salt bridge interaction is depicted with a continuous blue/red faded line. Hydrogen bonds are shown with magenta arrows. π-Stacking interaction is plotted with a green capped line. Polar and hydrophobic contacts between ligand and enzyme residues are displayed with blue and green faded tick lines, respectively.

Akin to the anionic form of vanillin, vanillic acid (**5**, $_{exp}$pKa = 4.5) adopts a binding mode featured by a conserved salt bridge interaction with Lys200 and a π-stacking interaction with His201 (Figure 7A). A further hydrogen bond is established between the *para* hydroxyl group of this compound and Glu233.

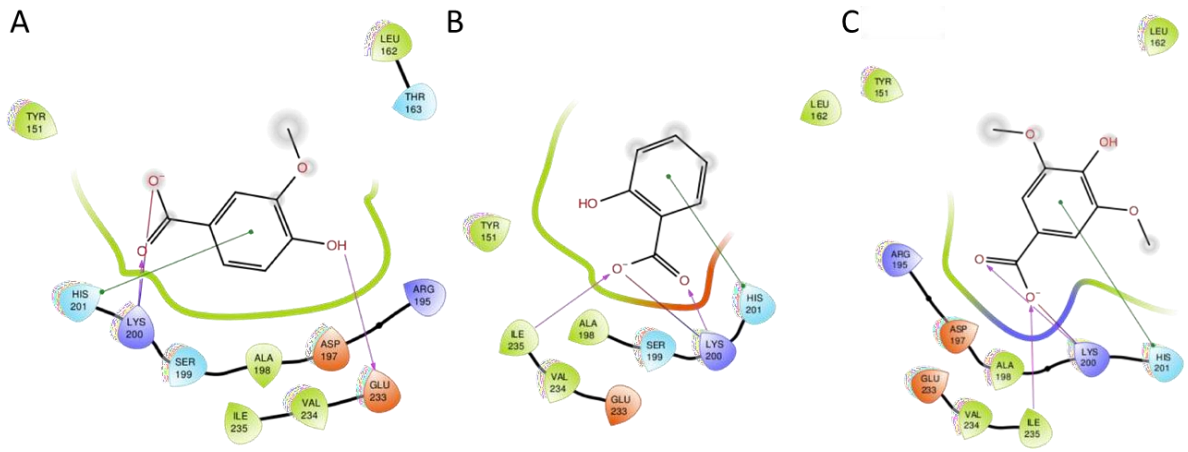

Figure 7. Binding mode of vanillic acid (A; **5** Gscore = -4.65 kcal/mol), salicylic acid (B; **3** Gscore = -4.84 kcal/mol) and syringic acid (C; **1** Gscore = -4.32 kcal/mol), to human pancreatic α-amylase. Salt bridge interaction is depicted with a continuous blue/red faded line. Hydrogen bonds are shown with magenta arrows. Polar and hydrophobic contacts between ligand and enzyme residues are displayed with blue and green faded tick lines, respectively.

Two additional analogues of vanillic acid are salicylic acid (**3**, $_{exp}$pKa = 2.97) and syringic acid (**1**, $_{calc}$pKa = 3.9). Docking studies of these compounds reveal conserved binding modes in which both compounds engage Lys200 and His201 with salt bridge and π-stacking interactions, respectively (Figures 7B-C). A further hydrogen bond interaction is observed between the backbone amide group of Ile235 and carboxyl group of salicylic acid and syringic acid. At odds with vanillic acid, no hydrogen bond is made by the *para* hydroxyl group of syringic acid, which is due to the steric shield provided by the two methoxy groups in *meta* positions of the benzene ring. Overall, results of docking studies allow drawing relationships among chemical structure, pKa property, binding mode and inhibition activity of tested compounds. Specifically, the conserved salt bridge or π-cation interaction between the anionic carboxylate or aromatic ring and Lys200 is found important for the inhibition activity. Indeed, vanillin (**8**, IC_{50} = 10.8 mg/mL) shows a significant drop of activity being in part present as neutral form at the experimental pH for its poor acidic constant ($_{exp}$pKa = 7.4). The higher inhibition activity of CHA (**2**, IC_{50} = 0.5 mg/mL) with respect to caffeic acid (**4**, IC_{50} = 3.5 mg/mL) suggests that the binding interaction of the quinic acid moiety of CHA is important for the inhibition potency, making the hydrogen bond interactions between its three hydroxyl groups and the acidic residues Asp197, Glu233 and Asp300. Likewise, the hydrogen bond interaction of the *meta* hydroxyl group of caffeic acid (**4**) with Glu233 favours

the inhibition activity against pancreatic α-amylase. Indeed, removal or methylation of this group determines a decrease of the inhibition activity in *p*-coumaric acid (**6,** IC_{50} = 5.6 mg/mL) and sinapic acid (**7**, IC_{50} = 8.3 mg/mL), respectively. This trend is also observed in the shorter homologues of caffeic acid (**4**), namely vanillic acid (**5**, IC_{50} = 6.2 mg/mL) and syringic acid (**1**, IC_{50} = 6.0 mg/mL). Conversely, salicylic acid (**3**, IC_{50} = 1.8 mg/mL) keeps a similar inhibition activity to caffeic acid, albeit its *ortho* hydroxyl group is not involved in any hydrogen bond interaction with the enzyme binding site. Hence, the better inhibition activity of salicylic acid (**3**, IC_{50} = 1.8 mg/mL) than vanillic acid (**5**, IC_{50} = 6.2 mg/mL) and syringic acid (**1**, IC_{50} = 6.0 mg/mL) may likely be explained by the lowest acidic constant of this compound (**3**, $_{exp}pKa$ = 2.97) that favours a stronger electrostatic salt bridge interaction with Lys200.

4. Conclusions

In the present work, for the first time several phenolic acids, identified in *L. barbarum* leaf extract, have been studied for their inhibitory activity against porcine pancreatic α-amylase by *in vitro* and *in silico* approaches. The results obtained by *in vitro* assay indicated that phenolic acids had interesting α-amylase inhibitory activity and showed that chlorogenic acid as the most active compound, followed by caffeic and salicylic acids with one-fold lower inhibition activity. The high sequence similarity between porcine and human isoforms of the enzyme (90%) suggests that such results may apply in both species. Results of docking studies identifies the interaction with Lys200 as important for the inhibition activity of tested extracts from *L. barbarum* leaves. Although further studies are needed to investigate the nutraceutical potential of these natural extracts for the management of hyperglycemia related disorders, findings of this study support the α-amylase inhibitory properties of phenolic acids, commonly represented in plant extracts.

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3.4. Is the household microwave recommended to obtain antioxidant-rich extracts from *Lycium barbarum* leaves?

1. Introduction

Functional herbal-based beverages, the most consumed drink category in the world, play an important role in daily life and contribute to nutritional well-being. The daily use of functional food can be considered a complementary approach for the prevention of some systemic diseases. Their daily intake has been associated with health benefits due to the presence of important bioactive molecules such as vitamins, minerals, and phenols [1]. Moreover, nowadays there is a great deal of interest in the use and preparation of plant-based aqueous beverages using waste and by-products, interesting sources of bioactives. As an example, numerous papers report on leaf infusions [2,3] and decoctions [4,5]. *Lycium* spp. leaves are interesting wastes of the goji berry production for their traditional use as herbal drugs and the actual potentiality as functional tea and dietary supplements [6]. In fact, even if *Lycium* leaves are less studied in respect to most used berries [7,8], some authors reported valuable biological activities, including antioxidant, antimicrobial, enzyme inhibitory, and antinflammatory activities [9–13]. These activities were associated with the presence of numerous bioactive compounds, including lipopolysaccharides and phenolic compounds, mainly represented by flavonols and phenolic acids. It was reported that chlorogenic acid (CA) was the main phenolic acid in *Lycium* leaves in alcoholic and hydroalcoholic extracts [9,11–12].

Extraction is the first essential process for obtaining herbal-based products [14]. Recently, in the food and nutraceutical industries, new techniques have been developed and applied in the extraction of various phytochemicals [15,16]. For example, supercritical fluid extraction, ultrasound, and microwave-assisted extraction (MAE) are replacing the conventional extraction techniques (i.e. maceration and Soxhlet).

Regarding functional herbal-based beverages, to the best of our knowledge, no study was carried out on the use of *Lycium* leaves to prepare a drink with potential health properties. For the first time, the effect of household MAE on the antioxidant properties and CA content of *L. barbarum* leaf extracts was investigated. The objectives of the present study are (i) to investigate, using experimental design, the effect of MAE extraction conditions on phenolic content and antioxidant properties of *L. barbarum* leaf extracts; (ii) to make a comparison with traditional home beverages (decoction and infusion).

2. Materials and Methods

2.1. Materials

L. barbarum dried leaves were collected in 2019 in Umbria (Perugia, Italy). Diammonium 2,2'-azino bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS; ≥98%), Folin-Ciocalteu's phenol reagent, gallic acid (97.5–102.5%), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; 97%), and chlorogenic acid (CA; ≥95%) were purchased from Sigma-Aldrich (Milan, Italy). High Performance Liquid Chromatography (HPLC) grade, acetonitrile, formic acid, and ultrapure water, were from Carlo Erba (Milan, Italy).

2.2. Household microwave extraction of *L. barbarum* leaves

2.2.1. Sample treatment

The extractions were carried out in a household microwave oven (1350 W max power; 2450 MHz frequency; Samsung-G633C; cityLondon, UK). Dried whole *L. barbarum* leaves were placed in 150 mL room-temperature oligomineral water (fixed residue: 276 mg/L) in a 300 mL glass backer and located in the oven in different conditions, reported in the following section (Section 2.2.2). Then, the aqueous extracts were placed on ice to cool to room temperature, and filtered through paper filters (MN 615, Macherey-Nagel, Düren, Germany). Finally, the final volume was checked and, if evaporation occurred, water was added to have the same initial volume (150 mL). The filtered samples were collected in an amber glass bottle and promptly subjected to analysis.

2.2.2. Experimental Design

An experimental design software (UMETRICS, Umeå, Sweden), named Statistical Design Package MODDE 5.0TM, was employed to evaluate the influence of three inde-pendent microwave parameters (solid/liquid ratio, time, microwave power) on the phe-nolic content and antioxidant activity of MAE extracts from *L. barbarum* leaves. Table 1 shows the considered factors affecting the extraction efficiency; the independent variables were ratio (X1), time (X2), and microwave power (X3).

Table 1. Independent variables, unit and values of experimental design.

Independent variables	Unit	Values
Ratio (X_1)[1]	g/150 mL	1; 3; 5
Time (X_2)	Min	1; 3; 5
Microwave Power (X_3)	Watt	1; 3; 5

[1]Solid/liquid ratio, i.e., dry leaves/water, expressed as leaf weight in grams of dry matter/liquid volume (g DM/mL).

As dependent variables, the following responses were selected:

- Total phenolic content (TPC), reported as milligrams of gallic acid equivalent per gram of dry matter (mg GAE/g DM);

- Free radical-scavenging activity by ABTS assay, reported as milligrams of Trolox equivalents per gram of dry matter (mg TE/g DM);

- Ferric reducing antioxidant power (FRAP) assay, reported as micromoles of Fe^{+2} per gram of dry matter (μmol Fe^{+2}/g DM);

- CA content, reported as micrograms per gram of dry matter (μg/g DM).

A total of 15 experiments, including three replicated center points, were obtained setting the screening objective and a D-optimal design. Table 2 shows the worksheet with the experimental conditions of the extractions, which were carried out in random order. Multiple linear regression analysis was used to fit the model.

Table 2. Worksheet and responses of the experimental design.

Experimental Number	ratio	time	power
1	1	1	300
2	5	1	300
3	1	3	300
4	1	5	300
5	3	5	300
6	5	5	300
7	3	3	400
8	1	5	400
9	1	1	500
10	5	1	500
11	1	5	500
12	5	5	500
13	5	5	500
14	5	5	500
15	5	5	500

2.3. Infusion and Decoction Preparations

The infusion and decoction were obtained by using 2 g of *L. barbarum* dried whole leaves (corresponding to the weight of herbal material in a teabag) and 150 mL of room temperature oligomineral water (corresponding to the volume of a tea cup) in a 300 mL glass backer.

The infusion was prepared by adding the herbal material in boiling water and allowing it to repose for 5 min. The decoction was prepared by adding cold water on herbal material and boiling for 5 min.

Both infusion and decoction herbal beverages were placed on ice to cool to room temperature and filtered through paper filters. Finally, the final volume was checked and, if evaporation occurred, water was added up to the initial volume (150 mL). The filtered samples were collected in an amber glass bottle and promptly subjected to analysis.

2.4. Determination of total phenolic content (TPC)

The TPC was determined according to the spectrophotometric method reported by Pagano et al. (2017) *17+. The assay involved the reduction of Folin and Ciocalteu's reagent, and the absorbance was measured at 765 nm. The TPC was reported as mg GAE/g DM.

2.5. *In vitro* antioxidant activities

2.5.1. ABTS Assay

ABTS assay was carried out following the procedure described by Rocchetti et al. (2020) *18+. Sample was added to ABTS+· reagent and, after 10 min, the absorbance was measured at 734 nm. The antioxidant capacity of each sample was reported as mg TE/g DM.

2.5.2. FRAP Assay

The reducing capacity of the extracts was determined using the FRAP assay [19]. The sample was added to FRAP reagent and, after 30 min, the absorbance was measured at 593 nm. The calibration curve was obtained using aqueous solutions of known Fe^{+2} concentrations (2–5 Mm). The reducing capacity was expressed as μmol Fe^{+2}/g DM.

2.6. HPLC-DAD analysis of phenolic compounds

The HPLC analysis of leaf extracts was carried out according to a previous paper [9]. A pump Thermo Spectraseries, a diode array detector Spectra System UV6000LP (Thermo Separation Products), and a Hypersil GOLD column (3 μm, 150 × 4.6 mm) were used The mobile phase consisted of 0.1% (*v*/*v*) formic acid in water (A) and 0.1% (*v*/*v*) formic acid in acetonitrile (B), with the following gradient: phase B increased from 5% to 20% in 30 min, and then to 95% in 5 min. The flow rate was 1.0 mL/min. Xcalibur software version 1.2 (Finnigan Corporation 1998–2000, San Jose, CA, USA) was used for chromatogram and data acquisition.

2.7. Statistical analysis

All the analytical procedures were carried out in triplicate, and the results were expressed as mean ± standard deviation on dry leaves. Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA) was used for data analysis.

One-way ANOVA (Analysis of Variance) was used to assess the differences among the means of the investigated groups performing the post-hoc Tukey's HSD (Honest Significant Difference) methodology (with a confidence level of 95%). The statistical analyses

were performed using Originpro 2021 software (OriginLab Corporation, Northampton, MA, USA).

3. Results and Discussion

3.1. MAE extraction of *L. barbarum* leaves

Nowadays, microwave ovens are ubiquitous in homes and workplaces because irradiation is a time-saving and energy-efficient cooking method, useful to prepare herbal beverages. In the present study, the optimization of the conditions of household microwave extraction on some bioactive properties of *L. barbarum* leaf extracts was studied.

Table 2 shows the worksheet with the extraction conditions of the experiments, designed around a mathematical model with the highest G-efficiency using the software MODDE™ (MKS Umetrics, Umeå, Sweden). Among the considered responses, in addition to TPC, two complementary antioxidant assays were used: ABTS to evaluate the radical scavenging ability of the MAE extracts and FRAP to evaluate their reducing capacity. CA was chosen because, on the basis of previous results [9,20,11], it was the main phenolic acid detected in *L. barbarum* leaf extracts. Table 3 shows the experimental values obtained for TPC, FRAP, ABTS, and CA. In order to estimate the coefficients of the terms in the model, representing the relationship between the response (Y) and the factors (X1, X2, <), multiple linear regression analysis was used.

Table 3. Experimental values of TPC, ABTS, FRAP, and CA of MAE extracts.

Experimental Number	TPC	ABTS	FRAP	CA
	mg GAE/g	mg TE/g	μmol Fe^{+2}/g	μg/g
1	3.4	8.4	34.7	1.5
2	4.4	8.4	43.3	13.2
3	4.8	12.4	57.7	7.5
4	5.0	13.2	61.4	43.5
5	6.2	16.3	71.9	72.0
6	5.5	11.9	60.4	195.3
7	5.9	13.5	69.0	7.0
8	5.3	13.9	62.5	48.0
9	2.8	9.5	29.2	1.5
10	4.6	16.1	43.3	0.3
11	5.7	13.0	63.0	6.0
12	5.9	11.3	72.6	144.9
13	5.5	11.4	66.5	130.5
14	5.8	8.3	64.4	120.0
15	5.8	10.9	73.3	115.8

The values are expressed as dry weight.

The factor interactions were entered for a better comprehensive understanding of the factor dynamics. In the following, the obtained equations (1–4) for TPC, ABTS, FRAP, and CA are shown:

$$TPC = 4.81127 + 0.385671X_1 + 0.864694X_2 - 0.0420571X_3 - 0.267204X_1X_2 + 0.0510864X_1X_3 \quad (1)$$

$$ABTS = 13.5331 + 2.1559X_1 - 1.00171X_2 - 0.17909X_3 - 3.15727X_1X_2 - 0.15509X_1X_3 \quad (2)$$

$$FRAP = 53.3367 + 3.17606X_1 + 13.1511X_2 - 0.61149\ X_3 - 1.87349X_1X_2 + 2.14145X_1X_3 \quad (3)$$

$$CA = 424.573 + 326.841X_1 + 436.109X_2 - 114.033X_3 - 177\ 292.127X_1X_2 - 690.515X_1X_3 \quad (4)$$

The best summary of fit of the model is given by the R^2 and Q^2 values (Figure 1).

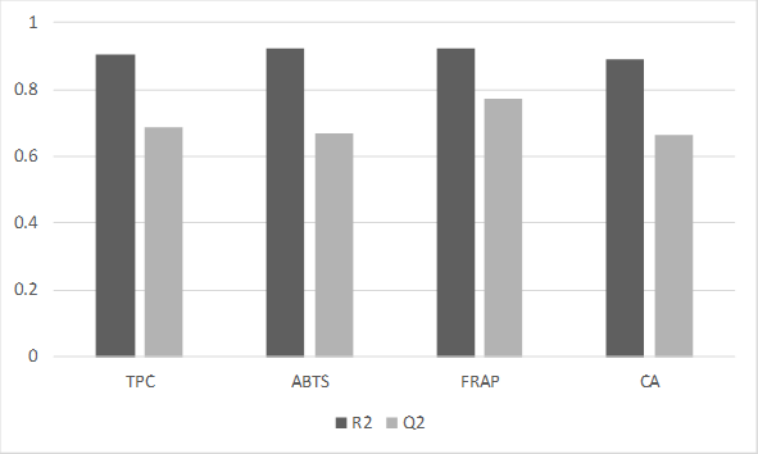

Figure 1. Statistic of the model - R^2 and Q^2 values of the four responses (TPC, ABTS, FRAP, CA).

The R^2 values ranged from 0.9057 for TPC to 0.9234 for FRAP, meaning that the model fitted the data very closely. The Q^2 values ranged between 0.6670 for ABTS and 0.7739 for FRAP, indicating a high predictive accuracy.

As regards the influence of the different factors, it can be observed from the obtained equations (1–4) that all responses are positively influenced by the X1 factor (weight of herbal material/150 mL of water), while an inverse correlation was found for the X3 factor (microwave power). The X2 factor (time) showed a high positive correlation with TPC, FRAP, and CA. Interesting interactions are represented by ratio × time (inverse correlations except for CA) and L/S ratio × power (positive for TPC and FRAP, negative for ABTS and CA).

As shown in Table 3, the values of the TPC of MAE extracts ranged from 2.8 mg GAE/g (of the sample obtained with ratio 1, irradiation time 1 min, microwave power 500 W) to 6.2 mg GAE/g of the sample, obtained with a lower microwave power (300 W), but with a higher ratio (3) and irradiation time (5 min). It was also observed that high irradiation time corresponded to high FRAP value and that TPC values were well correlated to FRAP values (R^2=0.9192). As regards CA, the main phenolic acid in *L. barbarum* leaves, it was observed that its content was higher (195.3 μg/g DM) when the power of the household microwave was lower (300 Watt), the extraction time was longer (5 min), and the ratio was higher (5 g/150 mL). The results obtained for CA confirm those reported for TPC, and it can be deduced that microwave power could lead to the modification of phenolic structure.

Figure 2 shows the surface plots of the responses TPC (a), FRAP (b), and ABTS (c) as a function of time and ratio factors, maintaining the power at 300 Watt, and of CA response (d) as a function of ratio and power factors, maintaining the time at 5 min. It is possible to observe that high TPC and FRAP values were relative to a long time, while a short time and high ratio positively influenced ABTS and CA responses.

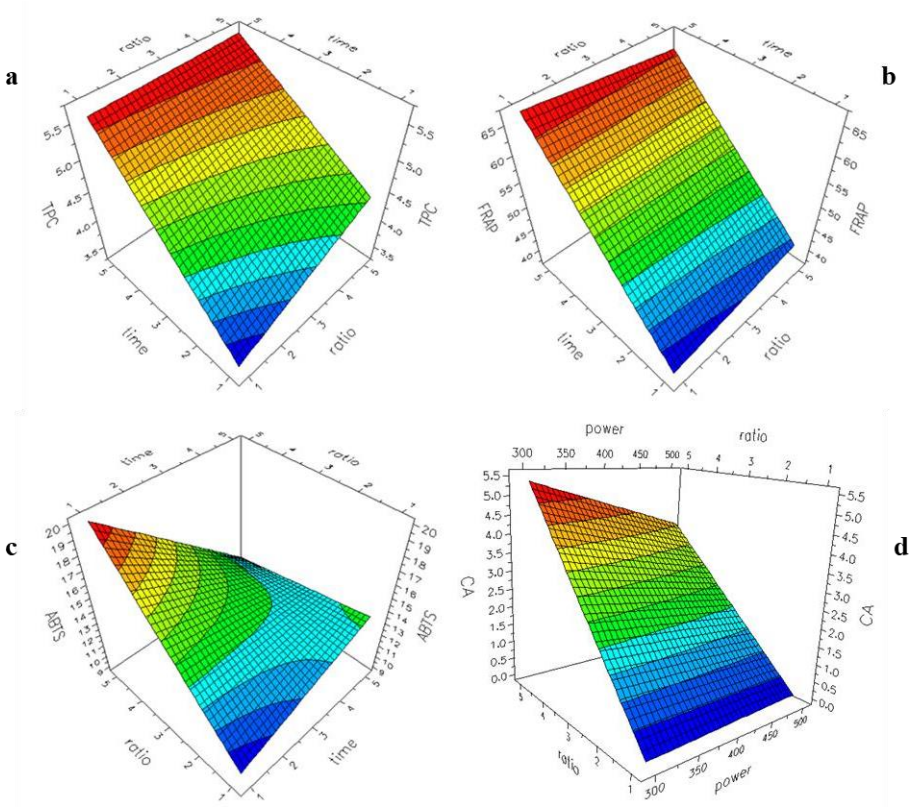

Figure 2. Surface plot showing the responses: (a) TPC (300 Watt), (b) FRAP (300 Watt), (c) ABTS (300 Watt), (d) CA (5 min).

To make a comparison of the phenolic content and *in vitro* activities of the MAE extract with classic home preparations, infusions and decoctions were prepared and analyzed. Since two grams of *L. barbarum* leaves were used to prepare infusions and decoctions (150 mL), the prediction function of the software provided the values of TPC, FRAP, ABTS, and CA for a MAE extract from two grams of leaves in 150 mL, irradiated for 5 min at 300 W. Figure 3 shows the values of TPC, FRAP, ABTS, and CA content of *L. barbarum* leaf extracts obtained by MAE, decoction, and infusion.

The results of post-hoc Tukey's HSD test showed that the difference of the means was significant at the 0.05 level for all the analytical assays among MAE, decoction, and infusion treatments. Unexpectedly, lower values were obtained for the MAE extract compared to both

infusion and decoction for all parameters studied; in particular, the CA content was strongly reduced when the MAE treatment was performed. Finally, *L. barbarum* leaf infusion had the highest values for the analytical assays, thus preserving bioactive compounds of the extract and its related properties.

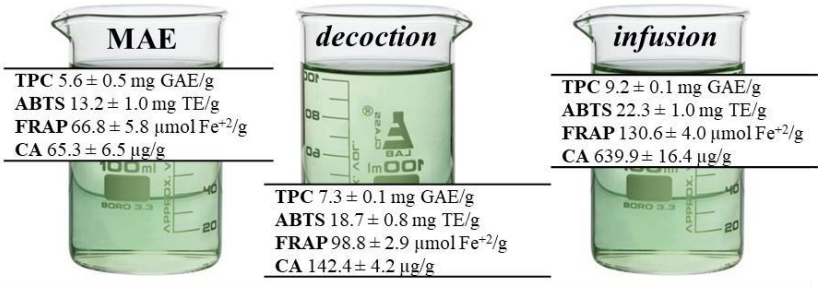

Figure 3. TPC, ABTS, FRAP, and CA content of *L. barbarum* leaf MAE, decoction, and infusion extracts.

3.2. Comparison with literature data

In a previous paper, unconventional extraction techniques such as ultrasound-assisted extraction and MAE have been used to extract *L. barbarum* leaves [9]. The TPC and ABTS values of MAE extracts were similar with the highest values obtained in this paper; however, it should be observed that alcoholic and hydroalcoholic solvents were used. In fact, to the best of our knowledge, this is the first study on household microwave extraction for obtaining *L. barbarum* leaf extract, so it is difficult to make a comparison with the literature data. Other authors studied the microwave assisted extraction of different herbal materials, in particular, green tea leaves [16,21–23+. Taşkın and Özbek have investigated the microwave conditions for the extraction of bioactives from green tea and found that the TPC value increased with an increase in microwave power up to 440 W, while it showed a steady decrease when the power was higher, up to 600 W [16]. It was concluded that the use of the right combination of some extraction variables (moderate microwave power-long irradiation time) is recommended for obtaining bioactives from waste without thermal deterioration. Vuong et al. (2012) investigated the effect of household microwave on catechins, caffeine, and theanine extraction from commercial teabag [21]. They found that the extracted amounts were lower than those obtained using the manufacturer's instructions (classic decoction), a result similar to what observed in this work. Upadhyay, Ramalakshmi, and Rao (2012) have instead studied the microwave extraction of green coffee bean to obtain chlorogenic acids

and reported that the yields of MAE under optimum conditions (water, 800 W, 5 min, and 50 °C) were higher than those from the conventional aqueous extraction at 5 min and 50 °C [23].

Finally, on the basis of the good results obtained in this paper for traditional infusion, a comparison can be made with other plant-based infusions. Among these, Li et al. (2013) studied the properties of 233 medicinal plants, including powdered *L. barbarum* berries [24] and found similar TPC value to those reported in this paper. Higher TPC values were instead reported for dried goji berry infusions [8].

5. Conclusions

In this research, for the first time the investigation of MAE parameters on extraction efficiency of bioactives from *L. barbarum* leaves was carried out. The results showed that ratio and time had a positive influence on the functional properties of leaf extract, while the increase in power corresponded to lower phenolic content and antioxidant properties. The combination of moderate microwave power with long time and high ratio provided higher CA content in MAE extract. Unexpected results were obtained taking into consideration classic household preparations; in fact, the investigated analytical parameters provided lower values for MAE extracts in respect to decoction and infusion. The traditional infusion has proved to be the best procedure for extracting bioactives from *L. barbarum* leaves and obtaining a functional beverage. Further studies are underway to deepen the knowledge on the effect of microwave extraction on the chemical stability of phenolic compounds in aqueous solutions, with particular regard to the modification of chlorogenic acid due to irradiation power. The control of chemical stability of bioactive compounds from plant materials is mandatory for pilot and large-scale extraction processes.

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3.5. Extraction of phenolic compounds from fresh apple pomace by different non-conventional techniques

1. Introduction

In recent years, apple has been considered one of the mostly wide consumed fruit in the world, due to widespread geographical and seasonal availability. Besides, the consumers of apples are of a large range of age, starting from infants, which eat them as apple purée or compote, to adolescents, as mainly apple juice, and to adults as fresh fruit.

The well-known health benefits of fruit against cardiovascular diseases are due to polyphenols, considered the main compounds responsible of this cardioprotective, anticancer and chemopreventing effects [1]. In fact, the main phenolic compounds in apple, phloridzin and chlorogenic acid, have antioxidant properties that contribute to prevent inflammation and hyperglycaemia and many diseases affecting vascular function, blood pressure and hyperlipidemia [2]. Moreover, the important role against diabetes of the phenolic compounds of the apple, for their anti-hyperglycemic effect, has also been reported *3+. These authors claimed the ability of young apple's polyphenols to retard the postprandial blood glucose and insulin levels in mice for both the acute and 1-week intervention trials. In fact, phloridzin is a well-known competitive inhibitor of glucose transporters (SGLT1 and SGLT2) through the binding of the glucose moiety to the Na+/glucose co-transporter [4].

The main phenolic compounds found in apple were hydroxycinnamic acids, flavan-3-ol monomers, flavan-3-ol polymers also called procyanidins, dihydrochalcones, flavonols and anthocyanidins. Among them, the most represented are chlorogenic acid as hydroxycinnamic acid, catechin and epicatechin as flavan-3-ol monomers and phloridzin as dihydrochalcone [5]. It is also important to consider the different distribution of phenolics in different part of the fruit, such as flesh, seeds, leaves, and skin. For example, dihydrochalcones concentrate in the core and the seeds, while the skin is also rich in flavonols and anthocyanins. Besides, different cultivars, area of cultivation, maturity, storage, extraction procedures, analytical techniques and pre- or post-harvest factors could affect the amount of polyphenols found in apples [6].

Recently, the green economy and the recycling trends are having a great impact on industries, in particular food industries, where the amount of waste represents an important management problem. The main common challenge is to recover bioactive compounds, in

order to reuse the food waste and develop functional foods using these value-added products with beneficial effects on human health [7].

Apple pomace is one of the most abundant waste, in fact 10 million tons are produced every year worldwide. It consists of pulp, peels, seeds and stalks obtained from apple juice production [8]. The use of apple pomace for fortification purposes has been investigated in the bakery products such as cakes, muffins, cookies, bread, biscuits, crackers, and extruded snacks, in the enrichment of dairy products, such as yoghurt and ice cream, apple juice and meat products such as chicken patty and beef jerky [9,10].

Regarding the recovery of phenolics from vegetable waste, one of the most important phases is the extraction technique. Nowadays, non-conventional extractions such as Ultrasound Assisted-Extraction (UAE), Microwave Assisted-Extraction (MAE), Supercritical Fluid Extraction (SFE), Pressurised Liquid Extraction or Accelerated Solvent Extraction (PLE or ASE) are increasingly used, showing several advantages over conventional extractions such as maceration, decoction or Soxhlet. In particular, the main advantages of non-conventional in respect of conventional techniques are the better yield and the time-, cost- and solvent savings, due to the better penetration of solvent in vegetable matrices, while the main disadvantage is the possible degradation of phenolic compounds, due to the heat generation during the extraction [7]. However, considering the structural differences of the numerous categories of phenols present in a plant product, it is necessary to develop the extraction method that guarantees high extraction yields for the different phenols and at the same time, it should be fast, economical and environmentally friendly [11,12].

The aim of this research was to investigate the extraction of phenolic compounds from Red Delicious apple pomace, with different non-conventional extraction techniques such as UAE, UTE, ASE and PEF, using EtOH:H_2O (50:50, 70:30 and 30:70, *v*/*v*), in order to establish the most efficient and powerful extraction method. Therefore, the determination of total phenolic content was carried out using a colorimetric method based on Folin-Ciocalteu' reagent. Afterwards, the analysis of phloridzin, one of the main phenolic compounds in apple, was performed by ultra-high-performance liquid chromatography quadrupole time of flight coupled to mass spectrometry (Q-TOF-LC/MS).

2. Materials and Methods

2.1. Reagents

Folin and Ciocalteu's phenol reagent was from Sigma-Aldrich (St. Louis, MO, USA), while sodium carbonate anhydrous was from Merck KGaA (Darmstadt, Germany). Deionized water was used throughout and obtained using a Milli-QPLUS system (Merck). Ethanol absolute, HPLC grade, Sharlau (Barcelona, Spain) and methanol for UV, IR, HPLC, ACS, PanReac Applichem (Darmstadt, Germany) were used for the extractions, while methanol OPTIMA LC/MS grade, Fisher Chemical (Madrid, Spain) for HPLC analysis. Catechin, chlorogenic acid, quercetin, *p*-coumaric acid, gallic acid and phloridzin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The individual stock solutions of phenolic compounds were prepared in MeOH:H_2O (70:30, v/v) at 1000 μg/mL and maintained at -20 ºC in the dark. Matrix-matched calibration curves at concentrations between 0.05 and 21 μg/mL were used to quantify the phenols in samples.

2.2. Preparation of apple pomace

Apple Red Delicious sample was acquired in supermarket. The apples were cultivated by Ulla from Girona region in Spain. Therefore, the apple pomace was obtained, after separating seeds and petioles, with a domestic blender (Habitex Style, SC-650N blender). Aliquots were stored at -20 °C till other analyses were performed.

2.3. Extractions of phenolic compounds

2.3.1. Ultrasound Assisted Extraction (UAE)

UAE was carried out for fresh apple pomace samples according to Pollini et al. [13], with slight modifications. The following extraction conditions were used: solid/liquid (S/L) ratio of 1:10 (g/mL), in an ultrasonic bath (Ultrasonic Cleaner, VWR) at 60 °C for 60 min, with EtOH:H_2O at different ratio (50:50, 70:30 and 30:70, *v*/*v*). After centrifugation at 3000 rpm for 5 min, the obtained extract was filtered with folded qualitative filter paper (particle retention 10-20 μm, VWR), and the solvent was evaporated with gentle nitrogen flow in TurboVap Zymark at 40 °C. Then, the extracts were stored at -20 °C and resuspended in H_2O for further analysis.

2.3.2. Ultraturrax Extraction (UTE)

UTE was performed according to Rusu et al. [14]. Apple pomace samples were homogenised through Ultraturrax IKA T 18 with S/L ratio of 1:10 (g/mL), using the mixture EtOH:H_2O at different ratio (50:50, 70:30 and 30:70, *v*/*v*) for 1 min at 9500 rpm and then 1 min at 13500 rpm. After vortex, the extracts were centrifuged at 3000 rpm for 15 min and filtered with folded qualitative filter paper (particle retention 10-20 μm, VWR). The solvent was evaporated with gentle nitrogen flow in TurboVap Zymark at 40 °C. Then, the extracts were stored at -20 °C and redissolved with H_2O for further analysis.

2.3.3. Accelerated Solvent Extraction (ASE)

ASE (Dionex ASE 200) was carried out mixing the apple pomace samples with inert diatomaceous earth, in order to absorb water from the sample, in a sample:inert earth ratio (2:1, *w*/*w*). According to Franquin-Trinquer et al. [5], two sets of extraction, one at a temperature of 25 °C and the other at 40 °C, were performed using EtOH:H_2O at different ratio (50:50, 70:30 and 30:70, *v*/*v*), with 3 cycles, at 1500 psi, setting 25 or 50% of flush percentage. The solvent was evaporated with gentle nitrogen flow in TurboVap Zymark at 40 °C. Then, the extracts were stored at -20 °C and redissolved with H_2O for further analysis.

2.3.4. Pulsed Electric Field treatment (PEF)

PEF-Cellcrack III (German Institute of Food Technologies (DIL)) equipment (ELEA, Quakenbrück, Germany) was used to treat apple pomace. Samples were prepared as follow, 28.7 g of apple pomace were mixed with 200 g of tap water, and the gap between the electrodes was set at 10 cm. Two experiments were performed inspired by Lohani et al. [15]: the first one with a specific energy input of 100 kJ/kg and the second one of 17 kJ/kg; the number of pulses was 115 and 9 pulses, depending on the voltage applied (field strength 2 or 3 kV/cm, respectively). These conditions derive from the fact a minimum of 1 kV/cm field strength is required to produce changes in cells and 3-4 kV/cm for the electroporation [16]. For this reason, only two experiments were carried out in order to compare a treatment of moderate intensity and short duration (3 kV/cm and 9 pulses with an energy per pulse of 450 J) with another one of low intensity and long duration (2 kV/cm and 115 pulses with an energy per pulse of 200 J). Before and after treatment, the temperature and conductivity were

measured in the sample with a portable conductivity meter ProfiLine Cond 3310 (WTW, Xylem Analytics Weilheim in Oberbayern, Germany). The PEF treated samples were freeze-dried and then phenolics were extracted using a S/L ratio of 1:10 (g/mL), EtOH:H_2O at different ratio (50:50, 70:30 and 30:70, *v*/*v*) low shaking for 1 h, followed by a centrifuge at 4500 rpm for 10 min and a filtration with folded qualitative filter paper (particle retention 10-20 μm, VWR). The solvent was evaporated with gentle nitrogen flow in TurboVap Zymark at 40°C. Then, the extracts were stored at -20 °C and redissolved with H_2O for further analysis.

2.4. Determination of the total phenolic content (TPC)

The spectrophotometric determination of TPC of apple extracts was carried out according to Moscatello et al. [17], using 20% Na_2CO_3 water solution and Folin and Ciocal-teu's reagent 2 N. After 30 min of reaction in the dark the absorbance at λmax=750.0 nm was read using a UV-Vis Cecil Super Aquarius CE 9500 spectrophotometer. Different gallic acid solutions were used to create a calibration curve and the results were ex-pressed as microgram of gallic acid equivalent per gram of fresh apple pomace (μg GAE/g of fresh apple pomace AP).

2.5. Q-TOF-LC/MS analysis of phenolic compounds

The chromatographic ultra-high-performance Accurate-Mass Q-TOF-LC/MS analysis was performed using an Agilent Technologies (Santa Clara, CA, USA) 1200 Infinity Series LC coupled with an Agilent Technologies 6540 UHD Accurate-Mass Q-TOF-LC/MS. This device was equipped with an electrospray ionization Agilent Technologies Dual Jet Stream ion source (Dual AJS ESI). Chromatographic separation was carried out with an Agilent Infinity lab Poroshell 120 EC-C18 (3 × 100 mm, 2.7 μm) column. The injection volume was 20 μL. The mobile phase consisted of 0.1 % formic acid in water milli-Q (solvent A) and 0.1 % formic acid in acetonitrile (solvent B), at a flow rate of 0.5 mL/min, with the following gradient: 0–10 min, 5% B; 10–13 min, 95% B; 13-15 min, 95% B. The Q-TOF-MS conditions were the following: drying gas flow (N_2), 12.0 L/min; nebulizer pressure, 45 psi; gas drying temperature, 370°C; capillary voltage, 3500 V; fragmentor voltage, 110 V; skimmer voltage 65 V and octopole RF peak, 750 V. Dual AJS ESI interface was used in negative ionization mode and negative ions were acquired in the range of 100–1100 m/z for MS scans, and 50–600 m/z for auto MS/MS scans, at a scan rate of 5 scans/s for MS and 3 scans/s for MS/MS,

respectively. Automatic acquisition mode MS/MS were carried out using the following collision energy values: m/z 20 eV; m/z 30 eV and 40 eV. Internal mass correction was enabled, by using two reference masses at 121.0509 and 922.0098 m/z. Instrument control and data acquisition were performed using Agilent MassHunter Workstation software B.08.00. All the MS and MS/MS data of the validation standards were integrated by MassHunter Quantitative Analysis B.10.0 (Agilent Technologies).

2.6. Standard Preparation, Calibration Curves, Limits of Detection and Quantification

A methanol stock solution containing all the six reference standards (gallic acid, catechin, chlorogenic acid, quercetin, *p*-coumaric acid and phloridzin) was prepared by dissolving the reference standards in methanol to final concentration of 1,000 μg/mL for each reference standard, then diluted the mixture stock solution to an appropriate concentration to establish calibration curves. The matrix-matched calibration solutions (3.12, 6.25, 12.5, 25, and 50 μg/mL) were prepared by combining the working solution with blank matrix solution. All of the solutions were stored at −20 °C before use. All calibration curves were constructed from peak areas of reference standards versus their concentrations. The lowest concentration of working solution was diluted with methanol to yield a series of appropriate concentrations. According the International Conference on Harmonization (ICH, 2005), the limit of detection (LOD) and limit of quantification (LOQ) were calculated based on matrix-matched calibration as the concentration for which S/N were 3 and 10, respectively. Quantifier ions were $^{*}M-H^{+-}$ for negative mode with a mass accuracy window of 5 ppm. Figure 1 shows the chromatographic separation of each analysed phenolic compound in a standard solution of 3 μg/ml.

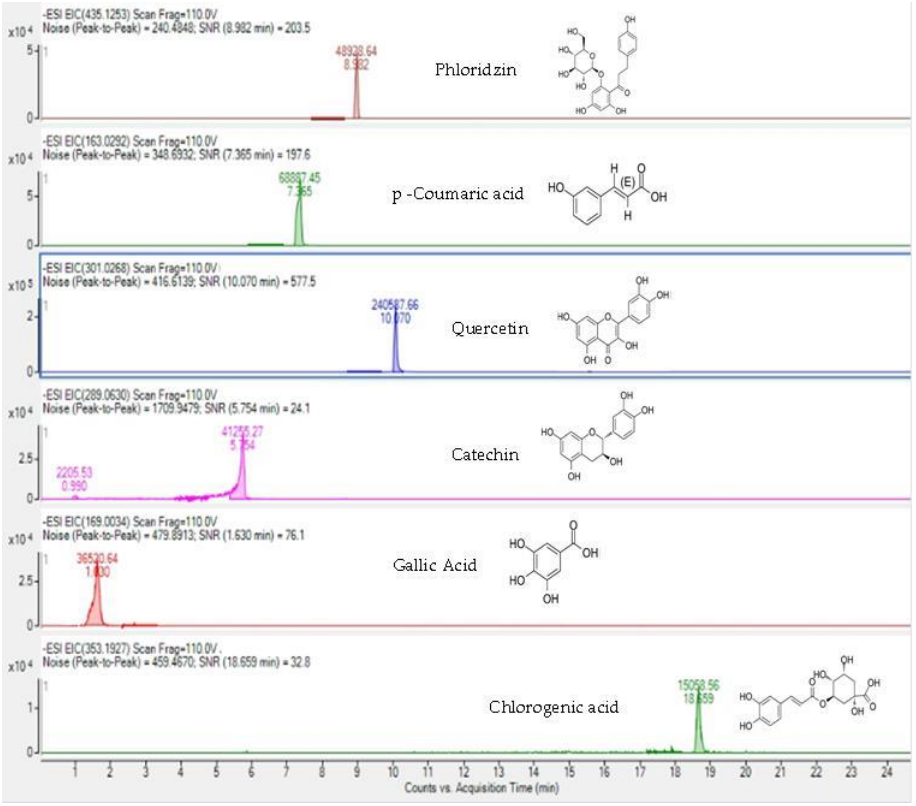

Figure 1. Chromatographic separation of each analysed phenolic compound in a standard solution of 3 μg/ml by LC-q-TOF/MS.

2.7. Statistical Analysis

The results were reported as mean values, based on two replicates, ± standard deviation. Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA) was used for data analysis.

3. Results and discussion

3.1. Total Phenolic Content of UAE, UTE, ASE and PEF extracts from fresh apple pomace

To investigate the efficiency of different non-conventional extraction techniques, fresh apple pomace extracts (FAPE) were subjected to analytical procedures for determining the phenolic compounds. Firstly, the spectrophotometric determination with Folin-Ciocalteu' reagent was performed to quantify the TPC through a redox reaction. Figures 2-4 show the content of TPC, expressed as μg GAE/g of fresh apple pomace, for the different extraction techniques (UAE, UTE, ASE and PEF). The results are reported in the graphs as mean ±

standard deviation of two replicates. Considering the TPC values, UAE (Figure 2) gave the highest amount, in particular the FAPE extracted with EtOH:H_2O (50:50, *v*/*v*) showed 1062.9 μg GAE/g of fresh AP. Lower TPC content was observed for EtOH:H_2O (70:30, *v*/*v*) extract and even lower when EtOH:H_2O (30:70, *v*/*v*) was used as extraction solvent. Nowadays, UAE is one of the most frequent technique carried out for bioactive compounds extraction, due to the fact it uses a common laboratory equipment. Moreover, it is well-known the ability of ultrasound waves to disrupt membrane cell of vegetal tissues, improving the recovery of bioactive compounds from plant foods and by-products [18].

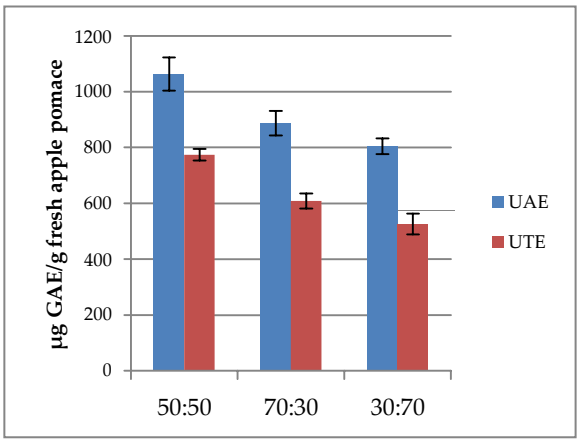

Figure 2. TPC results of UAE and UTE extracts. Error bars represent the SD.

The results obtained in this study were quite in accordance with the values (150-1200 mg GAE/100 g DM) reported by Wang et al. [19], which isolated phenolic compounds from flesh and peel of Granny Smith and Red Delicious apple through ultrasound-assisted aqueous extraction, using a titanium ultrasonic probe at different specific energy inputs. Figure 2 showed also that FAPE obtained with Ultraturrax had lower TPC content than UAE extracts, with values ranging from 525.7 to 774.6 μg GAE/g of fresh apple pomace and showing the same trend observed for UAE extract, regarding the ethanol:water ratio of extraction mixture. Ultraturrax is a homogenizing, emulsifying and suspending tool, but it is also used in the extraction of plant material. In literature has been described a successful extraction coupling UTE with UAE (UT-UAE), improving yield and decreasing extraction time [20,21]. Furthermore, UTE is suitable for the analysis of pesticides too, as reported by Sturm et al. [22].

Santarelli et al. [23] reported higher TPC studying UTE extracts from pulp of organic Golden Delicious apples, considering the pre-treatment with dipping and vacuum

impregnation in lemon juice solution, and different storage freezing or frozen (3.8-6.3 mg GAE/g DM). However, it should be observed that their results were expressed on dry weight, while in the present work on fresh matrix.

In this investigation, ASE was used as alternative non-conventional technique to extract phenolic compounds from apple pomace. ASE is a non-conventional time-saving extraction technique, where pressure is one of the main important parameter. In fact, the high pressure allows to increase the penetration power of the solvent into the matrix, increasing the time of contact between sample and solvent. ASE has been used to extract wide variety of compound and different samples, such as herbal samples, dietary supplement and nutraceuticals saving great amount of time and solvent [24]. The elevated temperatures improve the extraction performance due to the increase in mass transfer effect and the destruction of surface equilibrium [25].

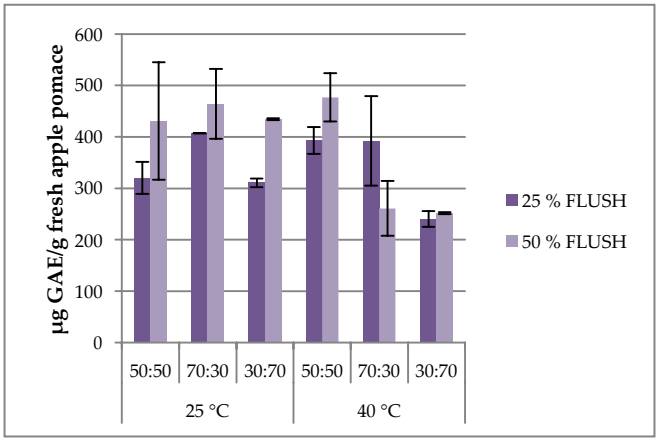

Figure 3. TPC results of ASE extracts. Error bars represent the SD.

The results obtained for ASE extracts from apple pomace reported in Figure 3, showed quite homogeneous TPC values. Higher efficiency (from 310.7 to 464.2 µg GAE/g of fresh apple pomace) were obtained at 25°C with 50 % or 25 % of flush and with all solvents and proportions proved, however at 40°C, for EtOH:H_2O (70:30 and 30:70, *v*/*v*) the extracts presented less extractive effectiveness. The 50:50 hydroalcoholic mixture confirmed to be the best extraction solvent, as visible for the extract obtained with EtOH:H_2O (50:50, *v*/*v*) at 40 °C and 50 % flush (476.8 µg GAE/g of fresh apple pomace), even if a similar TPC content was determined in the extract obtained with EtOH:H_2O (70:30, *v*/*v*) at 25 °C with 50 % of flush (464.2 µg GAE/g of fresh apple pomace). Franquin-Trinquier et al. [5] performed ASE experimental design using different solvents (methanol, acetone:water 70:30, or both) on

freeze-dried powder of Braeburn apple pulp, and reported values ranging from 2240.5 to 3348.5 mg catechin/g fresh fruit, amounts of TPC higher than our results.

Lastly, PEF was also tested in this study as pre-treatment to extract phenolic compounds from apple pomace. PEF is a processing technology that consists in the application of short electric field pulses of high intensity to the matrix between the two electrodes. The induction of a transmembrane potential difference can result in the electroporation of the cell membrane, increasing the permeability of the cytoplasmatic membranes that results in the easier release of the intracellular contents [15]. The main advantages of this treatment are the low energy consumption as well as the low increasing temperature. It has been proven that PEF treatment noticeably increased the amount of polyphenols extracted and their antioxidant activity [26]. TPC results obtained for PEF pre-treated samples (181.4-223.5 μg GAE/g of fresh apple pomace) are showed in Figure 4.

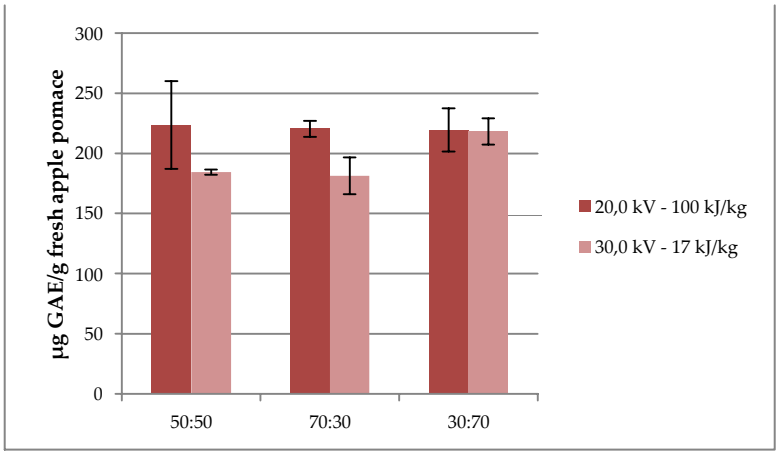

Figure 4. TPC results of PEF pretreated extracts. Error bars represent the SD.

It can be observed the values were very similar considering the two conditions set (20,0 kV - 100 kJ/kg and 30,0 kV - 17 kJ/kg), regardless the hydroalcoholic mixture used as solvent extraction. These results are lower than those obtained by Lohani & Muthukumarappan [15], who carried out PEF pre-treatment on fermented apple pomace powder, to release the bound phenolics (402.7 mg GAE/100 g dry weight). However, it should be taken into account that the results of this study were reported on fresh weight.

Considering all the obtained results, it could be noticed the highest TPC value of FAPE was with EtOH:H_2O 50:50 (*v*/*v*), in all technique carried out, except for PEF pre-treated

(30,0 kV - 17 kJ/kg), which EtOH:H_2O (30:70, *v*/*v*) was the solvent with the best recoveries of TPC.

3.2. Phloridzin quantification in fresh apple pomace extracts by Q-TOF-LC/MS

Phloridzin, a dihydrochalcone with antioxidant, anti-cardiovascular diseases and anti-diabetes effects, represents one of the most abundant and prerogative phenolic compound in apple so that it could be used as a biomarker of authentication [27]. For this reason, the phloridzin content in these hydroalcoholic FAPE was evaluated, to show the different efficiency of the extraction and to compare the different extraction methods by Q-TOF-LC/MS analysis. The observed chromatogram and spectrum of phloridzin in standard solution (3 μg/ml) (a) and UAE extract (b) it is shown in Figure 5. The LOD and LOQ values of phloridzin were 0.04 μg/mL and 0.15 μg/mL, respectively. It has been reported the phloridzin content, expressed as μg/g of fresh apple pomace (AP), in different FAPE obtained with UAE and UTE (Table 1) PEF (Table 2) and ASE (Table 3).

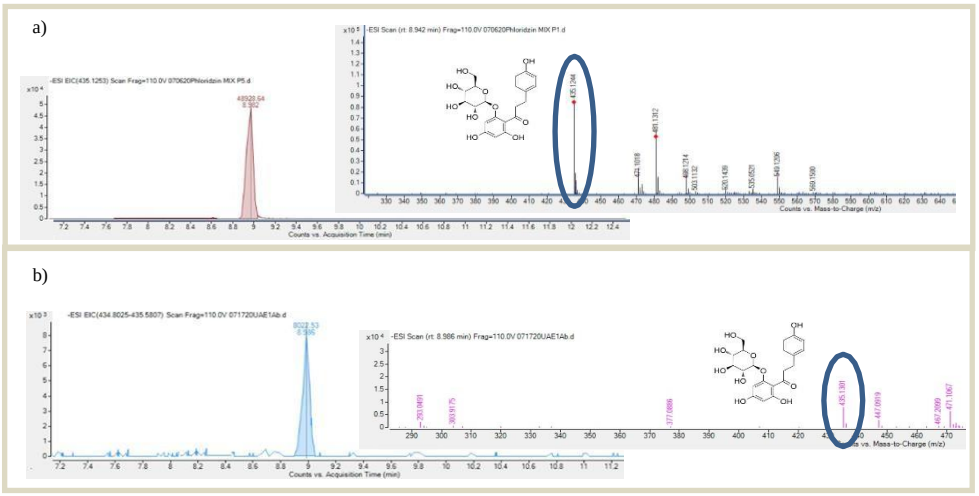

Figure 5. The observed chromatogram and spectrum of phloridzin in standard solution (3 μg/ml) (a) and UAE extract (b) with LC-q-TOF/MS.

As regards UAE and UTE FAPE (Table 1), content of phloridzin was very similar, despite the UAE results were slightly higher than the UTE results, ranging from 55.86 to 71.19 and from 58.39 to 64.43 of μg/g fresh apple pomace, respectively. Li et al. [28] found slightly lower contents of phloridzin extracting polyphenols by UAE treatment from different varieties of apple pulps without core and peel, ranging from 11.40 to 40.91 μg/g of

fresh pulp, while Santarelli et al. [23] found lower phloridzin content carrying out UTE technique on freeze-dried apple pulp, ranging from 39.9 to 77.0 μg/g of dry weight.

Table 1. Phloridzin content (μg/g fresh AP, mean±SD n=2) obtained with UAE and UTE.

Conditions	**UAE**	**UTE**
EtOH:H_2O (*v*/*v*)	60 °C, 60 min	1 min at 9500 rpm, 1 min at 13500 rpm
50:50	71.19±26.34	64.43±23.84
70:30	63.63±23.76	62.23±23.21
30:70	55.86±21.62	58.39±21.90

It could be observed in Table 2 that the highest value was obtained pre-treating the fresh apple pomace with PEF at low intensity and long duration (2 kV/cm and 100 kJ/kg), using EtOH:H_2O (70:30, *v*/*v*). Furthermore, the lowest phloridzin contents were observed in both the PEF extracts when EtOH:H_2O (30:70, *v*/*v*) was used as solvent mixture, followed by the EtOH:H_2O (50:50, *v*/*v*) FAPE obtained with moderate intensity and short duration (3 kV/cm and 17 kJ/kg).

Table 2. Phloridzin content (μg/g fresh AP, mean±SD n=2) obtained with PEF.

Conditions	**PEF**	
EtOH:H_2O (v/v)	20.0 kV - 100 kJ/kg	30.0 kV - 17 kJ/kg
50:50	65.21±2.11	17.56±0.63
70:30	753.84±26.38	208.53±7.30
30:70	9.29±0.34	11.49±0.43

In Table 3 more homogeneous results obtained performing ASE technique were reported, in particular the ones extracted at 40 °C were higher than the other at 25°C, both when 25 % or 50 % of flush was applied, except for the EtOH:H_2O (50:50, *v*/*v*) samples extracted with 25 % of flush, for which the opposite occurred. The temperature of 40°C was chosen because it represents a good compromise between the increment of the extraction and the risk to degrade phenolic compounds. Moreover, ASE performed with EtOH:H_2O (30:70, *v*/*v*), at 40°C and 50 % of flush was the most efficient extraction technique that gave the highest content of phloridzin (938.33 μg/g fresh AP). PEF extracts obtained with EtOH:H_2O (70:30, *v*/*v*) and ASE extracts, especially obtained at 40 °C, showed phloridzin contents higher than the results obtained by Fernandes et al. [29], who determined polyphenols by UHPLC-DAD after thioacidolysis (0.14 g/kg of dry apple pomace), and very similar to the ones

reported by Garcia et al. [30] who found contents ranging between 0.6 and 1.5 g/kg of dry weight.

The phloridzin contents determined on Red Delicious fresh apple pomace by Q-TOF-LC/MS and TPC values were very different among them, because the method used for TPC exclusively measures the capacity of FAPE to reduce the Folin-Ciocalteu's reagent and thus it is an index that measures the reducing power of the extract [15].

Table 3. Phloridzin content (μg/g fresh AP, mean±SD n=2) obtained with ASE.

Conditions	**25 % FLUSH**		**50 % FLUSH**	
EtOH:H_2O (v/v)	25 °C	40 °C	25 °C	40 °C
50:50	654.10±7.85	401.98±16.14	533.37±48.00	782.84±13.31
70:30	393.86±4.21	513.42±20.54	314.23±29.16	388.06±6.21
30:70	271.07±2.98	653.63±26.15	140.20±12.44	938.33±15.60

4. Conclusions

Based on the obtained results it is possible to assess that UAE is the best extraction technique to evaluate the TPC using EtOH:H_2O (50:50, *v*/*v*), while ASE at 40 °C was the most efficient extraction method to recovery phloridzin. Therefore, ASE could be considered a powerful tool to isolate and QTOF-LC-MS/MS to identify and quantify phloridzin as an important biomarker for apple pomace in food industry and food quality. In the context of circular economy, it is interesting to investigate the most efficient extraction techniques to isolate phenolic compounds from food waste and give them new life in the development of a value-added product. This work is as an important starting point to valorise apple pomace, a very cheap and common by-product, that is obtained in tons during juice production. Furthermore, apple pomace could be considered a re-valuable waste as a source of numerous bioactive compounds, such as polyphenols for the development of functional food or nutraceuticals.

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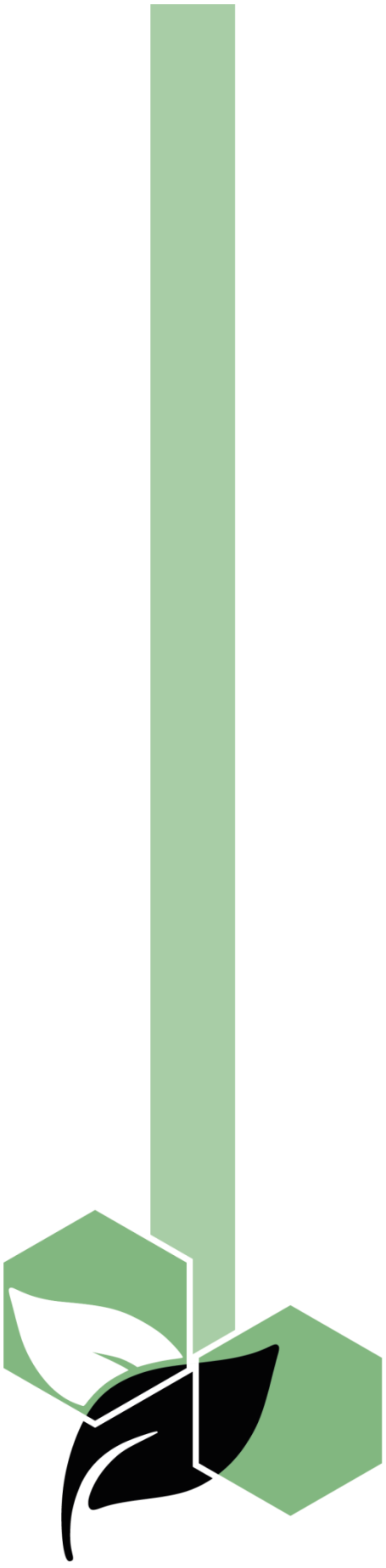


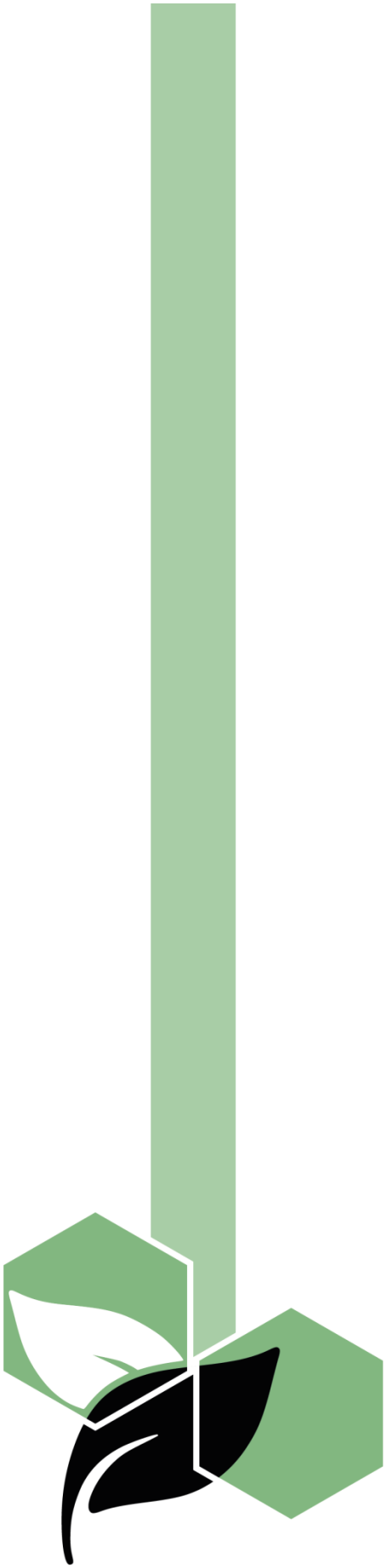
4. CONCLUSIONS

The conclusions of this PhD thesis were the following:

1. Experimental design approach resulted very useful to optimize the UAE extraction conditions (MeOH:H_2O 50:50, *v/v*, in 60:1 L/S ratio, 60 °C for 60 min) to recover polyphenols from *Moringa oleifera* leaves, in particular solvent and liquid/solid ratio were the most significant factors affecting the extraction.
2. HPLC-MS/MS and HPLC-DAD allowed to characterize and quantitate the phenolic profile of *M. oleifera* leaves extract, respectively, in particular the flavonol class (glycosidic forms of quercetin and kaempferol).
3. Methanolic UAE demonstrated to be the most efficient non-conventional extraction technique to recover phenolic compounds from *Lycium barbarum* leaves, comparing UAE and MAE techniques and alcoholic and hydroalcoholic solvent.
4. UAE methanolic extraction was used to extract phenolic compounds from two different *Lycium* spp (*L. barbarum* and *chinense)* leaves and the multivariate statistical analysis represented a powerful tool to discriminate samples from the two species.
5. QTOF-LC/MS proved to be an efficient analytical technique to characterize the phenol profile of vegetable waste as *L. barbarum* leaves.
6. *In vitro* studies of the inhibitory activity against α-amylase of *L. barbarum* leaf extract, and of the constituent phenolic acids, demonstrated the inhibition in a concentration-depent manner, while molecular docking studies were able to identify Lys200 as important for inhibition activity while H-bonds and π-π stacking represented the main stabilization interactions for a conserved binding mode.
7. Experimental design approach proved very useful to optimize the preparation of herbal-based beverages, using household microwave, considering *L. barbarum* leaveas extract as a potential functional beverage. However, the traditional preparation of herbabl-based beverage infusion proved to be the best procedure to obtain a functional beverage.
8. Apple pomace has proven to be an interesting fruit waste, for the presence of bioactive compounds as polyphenols. Among different non-conventional extraction techniques, such as UAE, UTE, ASE and PEF, UAE gave the highest value of TPC using EtOH:H_2O (50:50, *v/v*), while ASE gave the highest value of phloridzin content using EtOH:H_2O (30:70, *v/v*) at 40°C. QTOF-LC/MS allowed to quantitate the phloridzin content in fresh apple pomace, which represents the most important and characteristic phenolic compound of apple.

Las conclusions de esta tesi doctoral son las siguientes:

1. El enfoque del diseño experimental resultó muy útil para optimizar las condiciones de extracción mediante ultrasonidos con MeOH:H_2O 50:50, *v/v*, en una proporción de 1:60 S/L, a 60°C durante 60 min, para recuperar los polifenoles de las hojas de *Moringa oleífera*. En particular, el solvente y la relación S/L fueron los factores más significativos que afectaron la extracción.
2. La cromatografía líquida acoplada a espectrometría de masas y fila de diodos permitieron caracterizar y cuantificar el perfil fenólico del extracto de hojas de *M. oleifera*, respectivamente. En particular la clase flavonol (formas glucosídicas de quercetina y kaempferol).
3. La extracción metanólica con ultrasonidos demostró ser la técnica de extracción no convencional más eficiente para recuperar compuestos fenólicos de las hojas de *Lycium barbarum*, frente a la extracción con microondas.
4. La extracción metanólica con ultrasonidos se utilizó para aislar compuestos fenólicos de hojas de *Lycium* spp (*L. barbarum* y *L. chinense*) y el análisis estadístico multivariante representó una herramienta poderosa para discriminar muestras de las dos especies.
5. La cromatografía líquida acoplada a QTOF-MS demostró ser una técnica analítica eficiente para caracterizar el perfil de fenoles de residuos vegetales, en concreto hojas de *L. barbarum*.
6. Los estudios *in vitro* de la actividad inhibitoria frente a la α-amilasa del extracto de hojas de *L. barbarum* y de los ácidos fenólicos constituyentes demostraron la inhibición de una manera dependiente de la concentración, mientras que los estudios de acoplamiento molecular permitieron identificar a Lys200 como importante para la inhibición, mientras que los enlaces H y π-π representaron las principales interacciones de estabilización para un modo de unión conservado.
7. El enfoque de diseño experimental resultó muy útil para optimizar la preparación de bebidas a base de hierbas, utilizando microondas domésticos, considerando el extracto de hojas de *L. barbarum* como una potencial bebida funcional. Sin embargo, la preparación tradicional de infusión de bebidas a base de hierbas demostró ser el mejor procedimiento para la obtención de una bebida funcional.
8. El orujo de manzana ha demostrado ser un desecho de fruta de interés, debido a la presencia de compuestos bioactivos como los polifenoles. Entre diferentes técnicas de extracción no convencionales, como UAE, UTE, ASE y PEF, la extracción con ultrasonidos proporcionó el valor más alto de polifenoles totales usando EtOH:H_2O (50:50, *v/v*), mientras que la extracción asistida con disolventes generó el valor más alto de contenido de floridzina usando EtOH:H_2O (30:70, *v/v*) a 40°C. La cromatografía líquida acoplada a QTOF-MS permitió cuantificar el contenido de floridzina en pulpa de manzana fresca, que representa el compuesto fenólico más importante y característico de la manzana.



5. ANNEX

5.1. ANNEX 1

antioxidants

MDPI

Article

Impact of Ultrasound Extraction Parameters on the Antioxidant Properties of *Moringa Oleifera* Leaves

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Abstract: Recently, much interest has been focused on *Moringa oleifera* L., a highly versatile and sustainable plant. In addition to its nutritional properties, numerous bioactive compounds have been identified in *M. oleifera* leaves, for which healthy properties have been reported. In the present research, the impact of ultrasound-assisted extraction (UAE) on the recovery of the bioactive compounds from leaves was investigated. Firstly, an experimental design approach has been used to highlight the influence of some extraction parameters (solvent, solvent/dry leaves ratio, temperature, time) on phenol compound recovery and antioxidant activity. Solvent composition was the most influential factor; in fact, the presence of water in the solvent (50:50, v/v) corresponded to an increase in the extraction performance. The liquid/solid ratio (L/S) also influenced the extraction process; in fact, the total phenol content reached 13.4 mg gallic acid equivalent (GAE)/g dry matter (DM) in the following UAE conditions: 50% water, 60:1 L/S ratio, 60 °C, 60 min. In order to quantify flavonols, hydroalcoholic extracts were analysed by HPLC-DAD (high performance liquid chromatography-diode array detector). In the flavonol class, the glycosidic forms of quercetin and kaempferol were mainly detected. Their content ranged from 216.4 μg/g DM of quercetin 3-O-rhamnoside to 293.9 μg/g DM of quercetin 3-O-(6″-O-malonyl)-β-D-glucoside. In summary, the leaves of *M. oleifera* are a potential natural source of bioactive compounds, proving to be very promising for the development of health-promoting food supplements.

Keywords: *Moringa oleifera* leaves; UAE; phenol compounds; optimization; liquid chromatography

1. Introduction

Consumer awareness of the nutritional and health-promoting effects of food is constantly increasing, particularly in potential antioxidant compounds in addition to classical basic nutrients. Research into antioxidant sources is justifiable because there is increasing scientific evidence that various diseases are linked to oxidative stress [1,2].

In order to recover bioactive compounds with high efficiency, conventional extraction methods are used, among which maceration and percolation are the most popular, but large solvent amounts and long extraction times are required [3].

In recent years, in order to reduce these disadvantages, many advanced extraction methods have been developed. The recovery of bioactive compounds by these extraction techniques is a promising trend in the field of nutraceuticals and functional food development. Among the non-conventional

5.2. ANNEX 2

antioxidants

MDPI

Article

Phenol Profiling and Nutraceutical Potential of *Lycium* spp. Leaf Extracts Obtained with Ultrasound and Microwave Assisted Techniques

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Abstract: In recent years, agricultural and industrial residues have attracted a lot of interest in the recovery of phytochemicals used in the food, pharmaceutical, and cosmetic industries. In this paper, a study on the recovery of phenol compounds from *Lycium* spp. leaves is presented. Ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) have been used with alcoholic and hydroalcoholic solvents. Methanolic UAE was the most successful technique for extracting phenols from *Lycium* leaves, and we used on leaves from *L. barbarum* and *L. chinense* cultivated in Italy. The extracts were then characterized as regards to the antioxidant properties by in vitro assays and the phenol profiling by a high performance liquid chromatography-diode array detector (HPLC-DAD). Chlorogenic acid and rutin were the main phenol compounds, but considerable differences have been observed between the samples of the two *Lycium* species. For example, cryptochlorogenic acid was found only in *L. barbarum* samples, while quercetin-3-O-rutinoside-7-O-glucoside and quercetin-3-O-sophoroside-7-O-rhamnoside only in *L. chinense* leaves. Finally, multivariate statistical analysis techniques applied to the phenol content allowed us to differentiate samples from different *Lycium* spp. The results of this study confirm that the extraction is a crucial step in the analytical procedure and show that *Lycium* leaves represent an interesting source of antioxidant compounds, with potential use in the nutraceutical field.

Keywords: antioxidant activity; food waste; *Lycium* leaves; phenol profiling; extraction methods

1. Introduction

The genus *Lycium* belongs to the Solanaceae family and it includes numerous species which grow in arid and semi-arid regions, such as South Africa, America, Australia, and Europe [1]. It has been suggested that the original habitat of *Lycium* spp. was located in the Mediterranean Basin [2].

Products from *Lycium barbarum* and *Lycium chinense* today are considered a "superfood". *Lycium* species are perennial shrubs or small trees, characterized by fast growth, a root system developed in depth, and good tolerance for drought and cold [3].

Recently, numerous papers reported on the phytochemical composition of goji berries [4–6]. Some studies claim that these fruits are rich in bioactives with antioxidant activity, such as carotenoids, flavonoids, and phenolic acids [7–9]. In many countries around the world, fruits are widely consumed

5.3. ANNEX 3

processes

MDPI

Article

Phenolic Acids from *Lycium barbarum* Leaves: In Vitro and In Silico Studies of the Inhibitory Activity against Porcine Pancreatic α-Amylase

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Abstract: Nowadays, bioactive compounds from vegetable food and waste are of great interest for their inhibitory potential against digestive enzymes. In the present study, the inhibitory activity of methanolic extract from *Lycium barbarum* leaves on porcine pancreas α-amylase has been studied. The α-amylase inhibitory activity of the constituent phenolic acids was also investigated. The leaves were extracted by ultrasound-assisted method, one of the most efficient techniques for bioactive extraction from plant materials, and then the phenolic acids were identified by Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) Liquid Chromatography/Mass Spectrometry (LC/MS). Chlorogenic and salicylic acids were the most abundant phenolic acids in *L. barbarum* leaf extract. The inhibitory effect against α-amylase, determined for individual compounds by in vitro assay, was higher for chlorogenic, salicylic, and caffeic acids. *L. barbarum* leaf extract showed an appreciable α-amylase inhibitory effect in a concentration-dependent manner. Docking studies of the considered phenolic acids into the active site of α-amylase suggested a conserved binding mode that is mainly stabilized through H-bonds and π-π stacking interactions.

Keywords: hypoglycaemic activity; molecular docking; goji leaves; bioactives; antioxidant activity; food waste; UAE

1. Introduction

Despite the large number of synthetic pharmaceuticals, interest into natural products is increasing. The agricultural field offers a great opportunity thanks to the large quantities of byproducts of plant food processing, a promising source of biologically active compounds. In this context, food waste such as leaves are a cheap and available source of naturally-occurring bioactives, potentially useful for the development of functional foods and food supplements [1,2]. Starting from plant materials, numerous and different processes are used to obtain single bioactive substances or compounds belonging to the same chemical category. The recovery of high added-value components from food wastes can be carried out by traditional or innovative technologies. Among the latter, ultrasound-assisted extraction (UAE) can be considered a very successful and efficient extraction process for bioactive extraction [2].

Previous studies have reported the phytochemical composition of *Lycium* spp. berries and the availability of their bioactive compounds, including carotenoids, polyphenols, phytosterols,

5.4. ANNEX 4

processes

MDPI

Communication

Is the Household Microwave Recommended to Obtain Antioxidant-Rich Extracts from *Lycium barbarum* Leaves?

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Abstract: Nowadays, much interest is devoted to the extraction of plant materials, considering also their waste and by-products, to obtain antioxidant-rich products. The effect of household microwave-assisted extraction (MAE) on the phenolic content and antioxidant activity of *Lycium barbarum* leaf extracts was investigated. An experimental design approach was adopted considering solid/liquid ratio (1, 3, and 5 g of leaves in 150 mL water), irradiation time (1, 3, and 5 min), and microwave power (300, 400, and 500 W) as independent variables. These three factors and their interactions were studied to evaluate the effect of MAE conditions on the responses of total phenolic content, antioxidant activity, and chlorogenic acid content. The results showed that the analytical parameters were positively influenced by the solid/liquid ratio and time. On the contrary, microwave power was inversely correlated with the investigated responses. This research revealed that microwave extraction conditions should be carefully monitored to obtain bioactive-rich aqueous extracts with high antioxidant activity. A comparison with household traditional methods showed an unexpected lower phenolic content and antioxidant activity for MAE extract in respect to the decoction and infusion. In fact, it was found that *L. barbarum* leaf infusion had the best functional properties, regarding the investigated characteristics. The outcome of this study has implications for raising awareness that household preparation conditions strongly affect the health properties of herbal extracts.

Keywords: *Lycium barbarum* leaves; aqueous infusion; chlorogenic acid; optimization; microwave-assisted extraction

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1. Introduction

Functional herbal-based beverages, the most consumed drink category in the world, play an important role in daily life and contribute to nutritional well-being. The daily use of functional food can be considered a complementary approach for the prevention of some systemic diseases. Their daily intake has been associated with health benefits due to the presence of important bioactive molecules such as vitamins, minerals, and phenols [1]. Moreover, nowadays there is a great deal of interest in the use and preparation of plant-based aqueous beverages using waste and by-products, interesting sources of bioactives. As an example, numerous papers report on leaf infusions [2,3] and decoctions [4,5]. *Lycium* spp. leaves are interesting wastes of the goji berry production for their traditional use as herbal drugs and the actual potentiality as functional tea and dietary supplements [6]. In fact, even if *Lycium* leaves are less studied in respect to most used berries [7,8], some authors reported valuable biological activities, including antioxidant, antimicrobial, enzyme inhibitory, and antinflammatory activities [9–13]. These activities were

5.5. ANNEX 5

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Extraction of Phenolic Compounds from Fresh Apple Pomace by Different Non-Conventional Techniques

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Abstract: Red Delicious apple pomace was produced at laboratory scale with a domestic blender and different non-conventional extraction techniques were performed to isolate phenolic compounds, such as ultrasound-assisted extraction (UAE), ultraturrax extraction (UTE), accelerated solvent extraction (ASE) and pulsed electric field (PEF) extraction pre-treatment. Total phenolic content (TPC) was determined by Folin–Ciocalteu assay. Phloridzin, the main phenolic compound in apples, was determined by chromatographic analysis Q-TOF-LC/MS. The results obtained with these techniques were compared in order to identify the most efficient method to recover polyphenols. The highest value of TPC (1062.92 ± 59.80 μg GAE/g fresh apple pomace) was obtained when UAE was performed with EtOH:H_2O (50:50, *v*/*v*), while ASE with EtOH:H_2O (30:70, *v*/*v*) at 40 °C and 50% of flush was the most efficient technique in the recovery of phloridzin. The concentration of the main phenolic compounds ranged from 385.84 to 650.56 μg/g fresh apple pomace. The obtained results confirm that apple pomace represents an interesti-ng by-product, due to the presence of phenolic compounds. In particular, phloridzin could be considered a biomarker to determine the quality of numerous apple products. Therefore, this research could be a good starting point to develop a value-added product such as a functional food or nutraceutical.

Keywords: apple pomace; polyphenols; phloridzin; non-conventional extractions; UAE; UTE; ASE; PEF; Q-TOF-LC/MS

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1. Introduction

In recent years, apple has been considered one of the most widely consumed fruits in the world, due to its widespread geographical and seasonal availability. Moreover, the consumers of apples cover a large age range, starting from infants, who eat them as apple purée or compote, to adolescents, as mainly apple juice, and to adults as fresh fruit.

The well-known health benefits of fruit against cardiovascular diseases are due to polyphenols, considered the main compounds responsible for their cardioprotective, anti-cancer and chemopreventing effects [1]. In fact, the main phenolic compounds in apple, phloridzin and chlorogenic acid, have antioxidant properties that contribute to the prevention of inflammation and hyperglycaemia and many diseases affecting vascular function, blood pressure and hyperlipidemia [2]. Moreover, the important role against diabetes of the phenolic compounds of the apple, for their anti-hyperglycemic effects, has also been reported [3]. These authors claimed the ability of young apple's polyphenols to retard the postprandial blood glucose and insulin levels in mice for both acute and 1-week intervention trials. In fact, phloridzin is a well-known competitive inhibitor of glucose transporters (SGLT1 and SGLT2) through the binding of the glucose moiety to the Na+/glucose co-transporter [4].

The main phenolic compounds found in apple are hydroxycinnamic acids; flavan-3-ol monomers; flavan-3-ol polymers, also called procyanidins; dihydrochalcones; flavonols,