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Università Campus Bio-Medico di Roma

Corso di dottorato di ricerca in Bioingegneria e bioscienze
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APPLICATION OF DIFFERENT EXTRACTION TECHNIQUES AND HPLC-PDA-ESI/MS METHODS TO THE ANALYSIS OF PHENOLIC COMPOUNDS IN FOOD SAMPLES

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7 Maggio 2018

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ABSTRACT

Phenolic compounds encompass a major group of secondary plant metabolites that display a wealth of structural variety and a large diversity of significant biological activities. Over the last 15, years researchers and food manufactures have become increasingly interested in phenolic compounds. The chief reason for this interest is the recognition of their positive effects on human health, like antioxidant activity and their probable role in the prevention of various diseases associated with oxidative stress, such as cancer and cardiovascular and neurodegenerative diseases, and their great abundance in our diet.

The present thesis study was based in the development and validation of quali-quantitative analysis methods of phenolic bioactive compounds by high performance liquid chromatography (HPLC) coupled to UV-visible detector (PDA) and mass spectrometry (MS). Two food samples were investigated: Goji berry (*Lycium barbarum L.*) and Hazelnut kernels (*Coryllus avellana L.*).

Goji berry fruits (*Lycium barbarum L.*) have generated particular interest in recent times for their potential beneficial effects on human health, such as antioxidant, anti-inflammatory and antitumor activities. It is believed that phenolic compounds are responsible for these effects. The study on Goji berry fruits was divided in two sections.

A first study was based on the investigation of the effects of solvent type on the extraction of phenolic compounds through a solid-liquid method. Methanol, methanol 3% formic acid, ethyl acetate, ethyl acetate/methanol 3% formic acid (50:50 v/v) and water/methanol (90:10 v/v) were tested, in order to study their effect on the content of total phenolic compounds and on antioxidant activity, by Trolox antioxidant capacity (TEAC) assay, in Goji berry extracts. HPLC-PDA-ESI-QMS was employed for separation, identification and quantification of phenolic compounds in Goji berry samples. The analytical method was fully

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validated in terms of retention time and peak area precision, expressed as %RSD, limit of detection (LOD) and limit of quantitation (LOQ) and linearity range employing nine phenolic standard compounds belonging to phenolic acid and flavonoid classes as representative of each class of phenolics. The validated method was then applied in Goji berry extracts obtained by using different extraction solvents. Seven phenolic compounds, belonging to phenolic acid and flavonoid classes, were detected and tentatively identified in Goji berry extracts. Methanol 3% formic acid was the solvent presenting the highest amount of the seven phenolic compounds detected (18,32 μg/100 g of dried weight, DW) and the highest TEAC value (56,71 μmol TE/g DW), while ethyl acetate was the lowest one with an amount of phenolic compounds of 1,13 μg/100 g DW and a TEAC value of 0,56 µmol TE/g DW.

A second study was based on the study of pressurized-liquid extraction (PLE) method by a design of experiments (DOE) based on response surface methodology (RSM) for the extraction of phenolic compounds from Goji fruits. The global yield (% w/dw, weight/dry-weight), total phenolic content (TPC), total flavonoid (TF) and antioxidant activity (determined via ABTS assay, expressed as TEAC value) were used as response variables to study the effects of temperature (50–180 °C) and green solvent composition (mixtures of ethanol/water). The analysis of phenolic compounds were performed by HPLC-PDA-MS/MS. PLE optimum conditions (180°C and 86% ethanol in water) were obtained using the commercial sample as representative matrix. Once the experimental design was validated for commercial fruit samples, the optimum conditions were applied to three different varieties of fruit samples (*Selvatico mongolo*, *Bigol* and *Polonia*). Nine phenolic compounds were tentatively identified in these extracts, including phenolic acids and their derivatives, and flavonols. The optimized PLE conditions were compared to a conventional solid-liquid extraction (SLE), demonstrating that PLE is a useful alternative to extract phenolic compounds from Goji berry.

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Hazelnut (*Corylus avellana L.*) is one of the nuts most consumed in many countries including Turkey, Italy, Spain and United States. It is a rich source of dietary fibers and beneficial nutrients such as lipids, proteins, but also significant micronutrients like essential minerals, vitamin E, B complex vitamins and phenolic compounds, which contribute to its organoleptic properties such as astringent and bitter taste. The content of phenolic compounds may be a significant parameter in the assessment of hazelnuts quality. In fact, it depends on several factors like cultivar, geographical origin and processing condition such as roasting.

Phenolic compounds were extracted from hazelnut kernels employing two extraction techniques: ultrasound-assisted solid-liquid extraction (UA-SLE) and solid-phase extraction (SPE). Different extraction solvents were tested evaluating total phenolic content, total flavonoids and antioxidant capacity. The individual phenolic compounds in hazelnut kernels of different cultivars were analyzed by HPLC-PDA-MS/MS. The best extraction conditions in terms of the highest value of total phenolic compounds extracted together to other parameters like simplicity and cost were selected for method validation and individual phenolic compounds analysis. Different protocols were performed using commercial hazelnut kernels. The UA-SLE protocol performed using 0.1 g of defatted sample and 15 mL of extraction solvent (methanol/water/methanol 0.1 % HCOOH/acetonitrile (1:1:8:5, v/v/v/v)) was selected as best extraction conditions. The analytical method was developed and then validated using a mixture of six different phenolic acids and flavonoids standards. RSD % for intra-day e inter-day of retention time, LOD and LOQ were evaluated. The accuracy of the extraction was also assessed. Calibration curves were constructed with a good linearity and satisfactory determination coefficients R^2 for quantitative analysis. Finally, the method was applied to the analysis of phenolic compounds in three different hazelnut kernel varieties.

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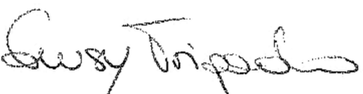

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1. INTRODUCTION

1.1. PHENOLIC COMPOUNDS

Phenolic compounds are known as secondary metabolites of plants. They represent one of the most and widely distributed groups of substances in the plant kingdom, with more than 8000 structures currently known (Bravo, 1998). The term "phenol" is a chemical term that defines a phenyl ring bearing one or more hydroxyl substituents. The term "polyphenols" could thus be used to define natural products featuring at least two phenyl rings bearing one or more hydroxyl substituents, including their functional derivatives (e.g., esters and glycosides), but in context of plant phenolic such a definition is not satisfactory, since it would include compounds such as the gossypol, the phenolic carotenoid 3-hydroxyisorenieratene, which are terpenoid in origin (Harborne, 1989). According to Quideau et al., 2011 the term "plant phenolics" should be strictly used to refer to secondary natural metabolites arising biogenetically from either the shikimate/phenylpropanoid pathway, which directly provides phenylpropanoids, or the 'polyketide' acetate/malonate pathway, and which fulfils a very broad range of physiological roles in plants.

In this chapter, it will be described the biosynthesis, chemical properties and beneficial effect on human health of phenolic compounds and, furthermore, their content in two food samples studied in this thesis will be described.

1.1.1. *Biosynthesis of phenolic compounds*

The biosynthetic pathways of phenolic compounds in plants are quite well known (Strack, 1997; Macheix et al., 1990; Haddock et al., 1982). Many phytotoxic compounds produced by higher plants are phenolic compounds. The biosynthetic pathways, from which they are derived, fall into general categories: 1) terpenoid phenolic compounds derived from five carbon isoprene units and 2)

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phenolic compounds derived from shikimic acid pathway (Figure 1.1.1.). Several compounds have been found to have profound and specific effects on certain enzymes involved in the synthesis of shikimic acid pathway-derived phenolic compounds (Duke, 1985). The biosynthesis and accumulation of secondary compounds can be endogenously controlled during developmental differentiation or it can be regulated by exogenous factors as light and temperature. Phenylalanine, produced in plants via shikimate pathway, is a common precursor for most phenolic compounds in higher plants. Similarly, hydroxycinnamic acids, and particularly their coenzyme A esters, are common structural elements of phenolic compounds, such as cinnamate esters and amides, lignin, flavonoids and condensed tannins. The phenylalanine/hydroxycinnamate pathway is defined as 'general phenylpropanoid metabolism'. It includes reactions leading from L-phenylalanine to the hydroxycinnamates and their activated forms.

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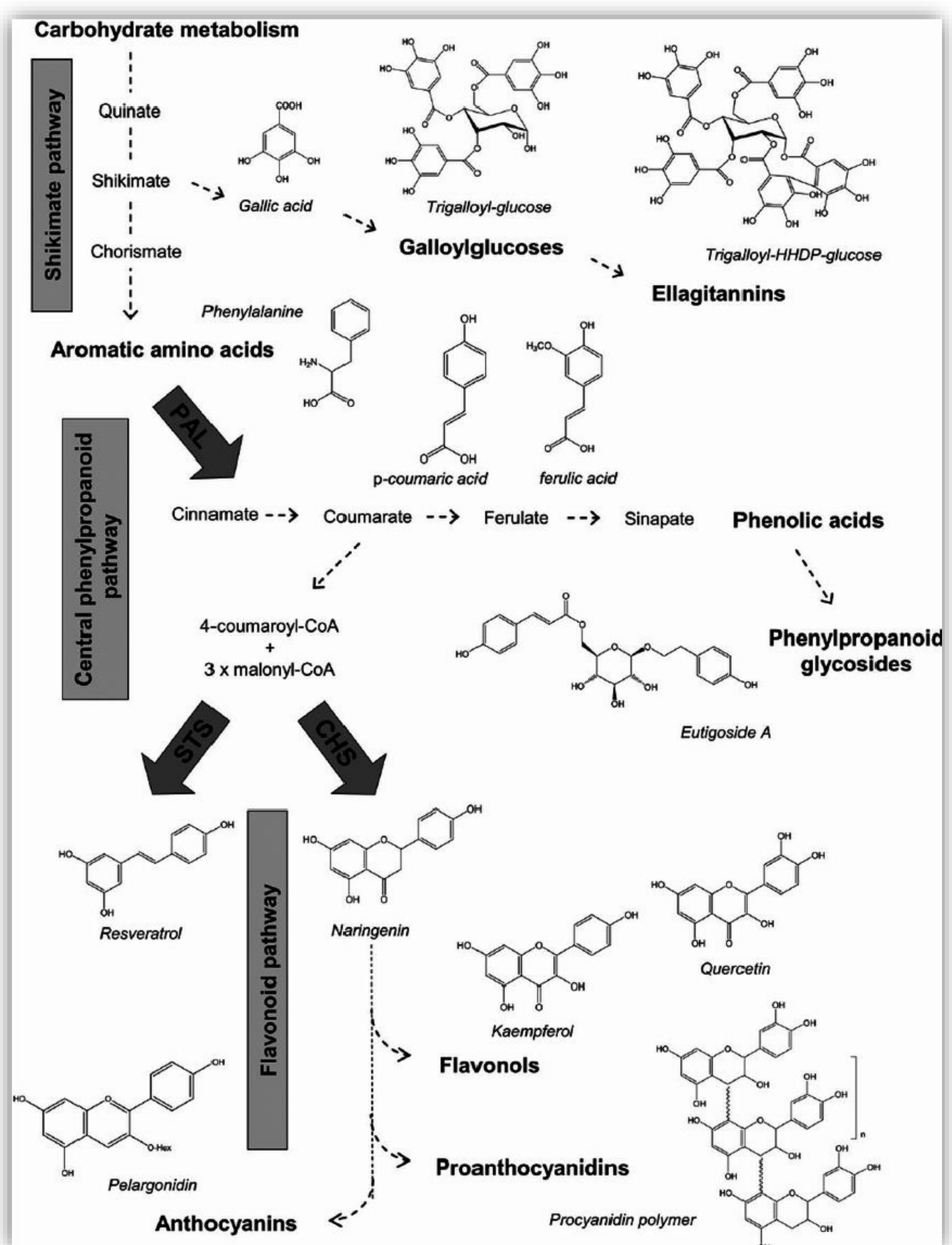

Figure 1.1.1. General phenolic flow through the phenylpropanoid pathway from the shikimic pathway.

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The enzymes catalysing the individual steps in general phenylpropanoid metabolism are phenylalanine ammonialyase (PAL), cinnamic acid 4-hydroxylase (CA4H), and hydroxycinnamate: coenzyme A ligase (C4L). These three steps are necessary for the biosynthesis of phenolic compounds. A growing body of evidence indicates that phenylpropanoid and flavonoid pathways are catalysed by several membrane-associated multienzyme complexes (Dixon and Paiva 1995, Winkel-Shirley 1999).

As representative groups of phenolic compounds, the biosynthesis of phenolic acids and flavonoids is described in more detail. Among phenolic acids, the formation of hydroxycinnamic acids (caffeic, ferulic, 5-hydroxyferulic and sinapic acids) from *p*-coumaric acid require two types of reactions: hydroxylation and methylation. Monophenol mono-oxygenase catalyses the introduction of a second hydroxyl group into *p*-coumaric acid to give the caffeic acid. Methylation of caffeic acid leads to the formation of ferulic acid which, together with p-coumaric acid, are the precursor of lignins. *O*-methyltransferase catalyses the methylation. The formation of hydroxycinnamic acids requires the formation of hydroxycinnamate-CoAs (e.g. p-coumaroyl-CoA) catalysed by hydroxycinnamoyl-CoA ligases or by the action of *O*-glycosyl transferases. The hydroxycinnamate-CoAs can conjugate with organic acids. In the biosynthesis of sugar derivatives of hydroxycinnamic acids, the transfer of glucose from uridine diphosphoglucose to hydroxycinnamic acid is catalysed by glucosyl transferase (Macheix et al., 1990; Strack, 1997).

Hydroxybenzoic acids can be directly derived from the shikimate pathway as the main route to gallic acid. However, they can also be produced by the degradation of hydroxycinnamic acids and the main intermediate are cinnamoyl-CoA esters. Hydroxybenzoates are also produced by the degradation of flavonoids. Moreover, hydroxylations and methylations of hydroxybenzoic acids are known to occur in an analogous way to the phenylalanine/hydroxycinnamate pathway (Macheix et al., 1990; Strack, 1997).

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Among the biosynthesis of flavonoids, a key step is the condensation of three molecules of malonyl-CoA with p-coumaroyl-CoA to form C_{15} intermediate 4,2,4',6'-tetrahydroxychalcone catalysed by chalcone synthase (Figure 1.1.2.).

The next step is the stereospecific isomerization of chalcone to a (2S)-flavanone, naringenin, catalysed by chalcone isomerase. Flavanones represent a branch point in the biosynthesis since they may be converted to flavones (e.g. apigenin) or to isoflavones (e.g. genistein). Additional hydroxylations can apparently occur at virtually all levels of oxidation of the flavonoid skeleton. Dihydroflavonol may enter another pathway leading to anthocyanins. An NADPH-dependent dihydroflavonol 4-reductase catalyses the formation of leucoanthocyanidin structure. The enzymes involved into oxidation and dehydration step are anthocyanidin synthases.

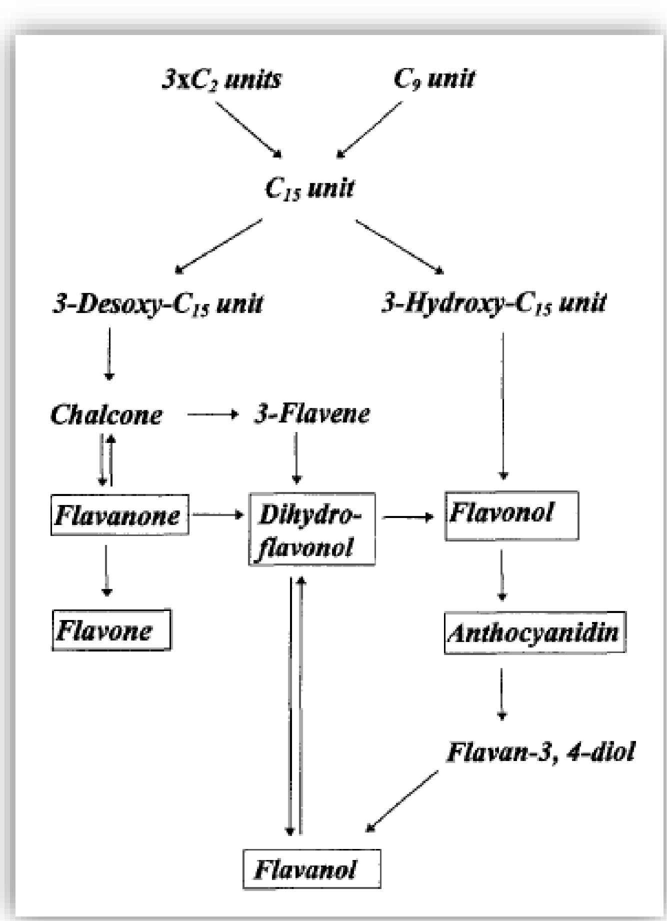

Figure 1.1.2. Schematic interconnections of flavonoid subgroups.

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Most of the flavonoids occur as glycosides in actively metabolising plant tissues. The two major types of linkages are O- and C- glycosides. The enzyme involved in this reaction is glycosyl transferase. Many flavonoids contain acylated sugars. The acyl groups are either hydroxycinnamates or aliphatic acids such as malonate. In the acylation reaction, the sugar hydroxyl and acid groups undergo esterification reaction (Harborne, 1988; Strack, 1997).

1.1.2. *Chemical classification*

Phenolic compounds may be subdivided also into different classes depending on the number of the carbon atoms (Table 1.1.1.), ranging from simple molecules, such as phenolic acids, to highly polymerized compounds, such as tannins (Harborne, 1999).

Table 1.1.1. Classes of phenolic compounds

Class	**Structure**
Simple phenolics, benzoquinones	C_6
Hydroxybenzoic acids	$C_6 - C_1$
Hydroxycinnamics acids, phenylpropanoids	$C_6 - C_3$
Naphtoquinones	$C_6 - C_4$
Xanthones	$C_6 - C_1 - C_6$
Stilbenes	$C_6 - C_2 - C_6$
Flavonoids, isoflavonoids	$C_6 - C_3 - C_6$
Lignans, neolignans	$(C_6 - C_3)_2$
Bioflavonoids	$(C_6 - C_3 - C_6)_2$
Lignins	$(C_6 - C_3)_n$
Condensed tannins	$(C_6 - C_3 - C_6)_n$

❖ Phenolic acids

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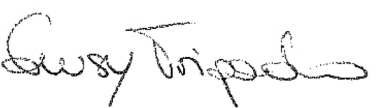
The name phenolic acid describes phenols that possess single carboxylic acid functionality. Phenolic acids can be divided into two groups: benzoic acids and cinnamic acids and derivatives thereof. A variety of structures and compounds depend by the numbers and position of the hydroxyl groups on the aromatic ring (Reis Giada, 2013; Gallardo, 2006).

Benzoic acids: have seven carbons atoms (C_6-C_1) and are the simplest phenolic acids found in nature. According to hydroxylation and methoxylation of the aromatic ring, variations can occur in the basic structure. Hydroxybenzoic acids (HBAs) can be found as free or conjugates in fruits and vegetables. They mainly occur conjugated with sugar, usually glucose. For example, gallic acid and its dimer ellagic acid may be esterified with glucose to produce the so-called hydrolysable tannins. 4-Hydroxybenzoic, protocatechuic, vanillic and syringic acids are part of this group and they also occur as esters of glucose. The most important benzoic acids are reported in Figure 1.1.3. Free and bounded phenolic acids are found in cereals. Hydroxybenzoic acid glycosides are also characteristic of some herbs and spices (Clifford & Scalbert, 2000).

Cinnamic acids: are aromatic compounds with a three carbons side chain (C_6-C_3), *trans*-phenyl-3-propenoic acids, differing in their ring substitution. The most common cinnamic acids are: caffeic (3,4-dihydroxycinnamic acid), ferulic (3-methoxy-4-hydroxy), *p*-coumaric (4-hydroxy) and sinapic (3,5-dimethoxy-4-hydroxy) acid (Figure 1.1.3.). These molecules are widely distributed as conjugates, mainly as esters of quinic acid. According to the identity, number and position of the acyl residues, this group may be divided into following subgroups: mono-esters of caffeic, p-coumaric and ferulic acid; di-, tri- and tetra-esters of caffeic acid; mixed di-esters of caffeic and ferulic acid or caffeic and sinapic acid; mixed esters of caffeic acid with dibasic aliphatic acids (Scholz, 1994). They may condense with molecules such as tartaric, rosmarinic and malic acid, aromatic amino acids, choline, mono- and polysaccharides, glycerol and different glycosides



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(Clifford, 2000). Cinnamic acids are commonly found in fruits and vegetables such as blueberries, aubergines, apples, green mate and broccoli, lettuce, spinach.

a

p-Hydroxybenzoic acid (R_2 = OH, R_1, R_3, R_4 = H);
Protocatechuic acid (R_1, R_2 = OH; R_3, R_4 = H);
Vanillic acid (R_1 = OCH_3, R_2 = OH; R_3, R_4 = H);
Gallic acid (R_1, R_2, R_3 = OH; R_4 = H);
Syringic acid (R_1, R_3 = OCH_3; R_2 = OH; R_4 = H)

b

p-Coumaric acid (R_3 = OH, R_1, R_2, R_4 = H);
Caffeic acid (R_2 = R_3 = OH; R_1, R_4 = H);
Ferulic acid (R_2 = OCH_3, R_3 = OH; R_1, R_4 = H);
Sinapic acid (R_2 = R_4 = OCH_3, R_3 = OH; R_1 = H);

Figure 1.1.3. The general basic structure and names of the main benzoic acids (a) and cinnamic acids (b).

❖ Naphthoquinones

They represent a class of quinone pigments. The basic structure is similar to naftalene (C_6 - C_4). Naphthoquinones occur in plant tissues in the reduce form, colourless and conjugated to sugar moieties. The most important higher plant families containing these molecules are *Avicenniaceae*, *Bignoniaceae*, *Boraginaceae*, *Droseraceae*, *Ebenaceae*, *Juglandaceae*, *Nepenthaceae*, and *Plumbaginaceae*.

❖ Xanthones

Xanthones (C_6 - C_1- C_6) may be classified into five major groups: simple oxygenated xanthones (this group can further be subdivided into six groups according to the degree of oxygenation), xanthone glycosides, prenylated and related xanthones, xanthonolignoids, and miscellaneous. They mainly occur in the

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leaves, roots and wood of two families of higher plants, *Gentianaceae* and *Guttiferae*.

❖ Stilbenes

This group of molecules is characterized by the C_6 - C_2- C_6 structure. They have two benzene rings divided by ethane or ethenic bridge. Stilbenes are found in liverworts, in some ferns, in gymnosperms, and in many dicotyledonous angiosperms. One of the most interesting and broadly studied stilbenes is resveratrol who is principally present in grape.

❖ Flavonoids

Flavonoids constitute the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds (Iwashina, 2000). They are found in many plant tissues, where they are present inside the cells or on the surfaces of different plant organs (Lattanzio, 2013). They are low molecular weight compounds consisting of fifteen carbons atoms, arranged in a C_6-C_3-C_6 skeleton, basic structure (Figure 1.1.4.).

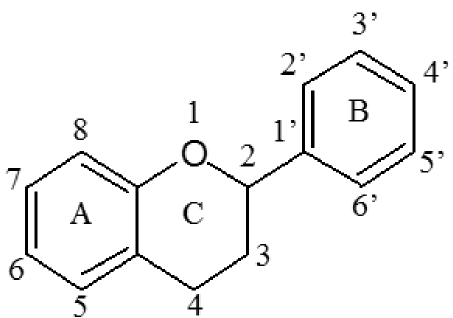

Figure. 1.1.4. Basic skeleton structure of flavonoids.

Depending on the position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety, this group of molecules may be divided into

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three classes: flavonoids (2-phenylbenzopyrans), isoflavonoids (3-benzopyrans) and neoflavonoids (4-benzopyrans). This chemical structure may differ in the saturation of heteroatomic ring C and in the overall hydroxylation patterns. These molecules may be modified according to the hydroxylation, methoxylation, or O-glycosylation of hydroxyl groups as well as C-glycosylation directly to carbon atom of the flavonoid skeleton. In addition, alkyl groups (often prenyls) may be covalently attached to the flavonoid moieties, and sometimes additional rings are condensed to the basic skeleton oh the flavonoid core (Pietta, 2000). The flavonoids may be divided into 13 subgroups, while isoflavonoids are subdivided into 11 subgroups. The neoflavonoids are structurally and biogenetically closely related to the flavonoids and the isoflavonoids (Davies & Schwinn, 2006; Panche et al., 2016).

According to the variations in substitution patterns to ring C, the major flavonoid subgroups are: flavones, flavonols, flavanols, isoflavones, anthocyanidins or anthocyanins and flavanones (figure 1.1.5.). Generally, the flavonoids occur in plants as glycosylated derivatives, and they contribute to the brilliant shades of blue and orange in leaves, flowers and fruits (Samanta et al, 2011). Apart from various vegetables and fruits, flavonoids are found in seeds, nuts, grains, spices and different medicinal plants as well in beverages, such as wine, tea and beer (Kuhnau, 1976).

Flavones: They have a double bond between position 2 and 3 and keto groups in position 4 of the ring C. Flavones are widely present in leaves, flowers and fruits as glucosides. More specifically, the flavones apigenin and luteolin are common in cereal grains and aromatic herbs (parsley, rosemary, thyme), while their hydrogenated analogues hesperetin and naringin are almost exclusively present in citrus fruits. The peels of citrus are rich in polymethoxylated flavones, tageretin, nobiletin and sinensetin (Manach et al., 2004; Pietta et al., 1995). Most of flavones of vegetables and fruits present a hydroxyl group in position 5 of the ring A, while

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the hydroxylation in other positions is common in position 7 of the ring A or 3' and 4' of the ring B, depending on the taxonomic classification of plants.

Flavonoid	**Basic structure**
Flavonols	
Flavones	
Flavanones	
Flavanols	
Anthocyanidins	
Isoflavones	

Figure 1.1.5. Chemical structures of the main classes of flavonoids

Flavonols, proanthocyanidins: they are the flavonoids with a ketone group and represent the building blocks of proanthocyanins. Flavonols have a hydroxyl group in position 3 of the ring C, which may be glycosylated. According to methylation, hydroxylation, glycosylation patterns, flavonols are a diverse subgroup of flavonoids. These molecules are predominant in fruit and vegetables, where they

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are mainly found in the skin, with the exception of onions. Lettuce, tomatoes, apples, onion, grapes and berries are rich sources of flavonols. Quercetin, kaempferol, myricetin and fisetin are the most studied flavonols (Marks, 2007). Proanthocyanidins are polymeric flavan-3-ols. The oxidative condensation occurs generally between C_4 of the ring C and carbons C_6 or C_8. Two main types of these molecules may be distinguished according to the substitution pattern of the ring B: procyanidins which are characterized by the presence of two hydroxyl groups in the 3' and 4' position of the ring B. Catechin and epicatechin are the main constituents; prodelphinidins which has three hydroxyl groups in the 3', 4' and 5' position of the ring B. The main constituent is epigallocatechin.

Flavanones, chalcones: they are also called dihydroflavones. These compounds have a presence of a chiral center at the 2-position of the ring C. The double bond between the 2- and 3-position is saturated. Flavanones may be *O*-glycosides or *C*-glycosides, but the majority of flavanones are *O*-glycosides. Naringenin, hesperitin and eriodictyol are examples of this class of flavonoids. Flavanones are also an important class which is generally present in all citrus fruits such as oranges, lemons and grapes. They are responsible for the bitter taste of the juice and peel of citrus fruits (Iwashina, 2000). Flavanones can be easily converted to isomeric chalcones in alkaline medium provided in which there is a hydroxyl substituent at 2' or 6' position of the chalcone.

Chalcones are unsatured and are characterized by the absence of the ring C of the basic flavonoid skeleton structure. Phloridzin, arbutin, phloretin and chalconaringenin are the main costituents. These molecules occur principally in tomatoes, pears, strawberries, bearberries.

Isoflavonoids: they are a class of flavonoids which differ from other flavonoid classes in having the ring B attached to the ring C in 3-position. They are subdivided into two classes: isoflavones, isoflavanol. These molecules are

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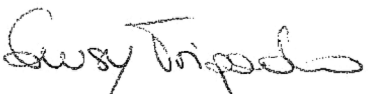
predominantly found in soybeans and other leguminous plants, and also in microbes (Matthies, 2008; Iwashina, 2000).

Flavanols: they are also called dihydroflavonols or catechins. Flavanols are the 3-hydroxy derivatives of flavanones and are a highly diversified and multisubstituted subgroup. Unlike many flavonoids, there is no double bound between 2- and 3-position. These molecules are predominantly found in bananas, apples, blueberries and pears (Rana & Bhushan, 2016).

Anthocyanins: the basic structure of anthocyanins is called flavylium, 2-phenylbenzopyrilium. The anthocyanidins represent the skeleton which are diverse for the number and position of hydroxyl and methoxyl substituents. Cyanidin, delphinidin, malvidin, pelargonidin and peonidin are the most commonly studied anthocyanidins. The anthocyanins differ for the identity, number and position of sugars which are attached to the anthocyanidin skeleton, and also for the extent of sugars acylation and the identity of the acylating agent. Glucose, galactose, rhamnose and arabinose are the most commonly encountered, usually as 3-glucosides or 3,5-diglycosides (Clifford, 2000). Anthocyanins occur predominantly in the outer cell layers of various fruits such as cranberries, black currants, strawberries, blueberries, producing blue purple, red and intermediate hues, and appear black. Their hue and structure are dependent on pH values and the presence of copigments (Muller et al., 2012).

❖ Lignans

Lignans and neolignans $(C_6\text{-}C_3)_2$ are a large and varied group of plant phenolics produced by the oxidative dimerization of two phenylpropanoid units, which occur in a wide range of plant species. When the two C_6-C_3 units are linked by a β, β'-bond (or 8,8' -bond), the parent structure lignane is used as the basis for naming the lignan. If the two C_6-C_3 units are linked by a bond other than a β, β'-bond, the parent structure, neolignane, is used as the basis for naming the neolignan. (+)-



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Pinoresinol (XV), for example, is a lignin derived by a tail-to-tail linkage in the b-position of two coniferyl alcohol residues. Related dimers, called neolignans, can be formed by other condensations between two C_6-C_3 units, for example, joining head-to-tail instead of tail-to-tail.

❖ Tannins

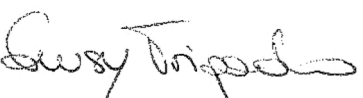
Tannins are phenolic compounds of molecular weight from intermediate to high (500-3000 Da) and can be classified into two groups: hydrolysable tannins and non-hydrolysable or condensed tannins.

The hydrolysable tannins have a center of glucose or a polyhydric alcohol partially or completely esterified with gallic acid or hexahydroxydiphenic acid, forming gallotannin and ellagitannins, respectively. These metabolites are readily hydrolyzed with acids, bases or enzymes. However, they may also be oxidatively condensed to other galoil and hexahydroxydiphenic molecules and form polymers of high molecular weight. The best known hydrolysable tannin is the tannic acid, which is a gallotannin consisting of a pentagalloyl glucose molecule that can additionally be esterified with another five units of gallic acid.

The condensed tannins are polymers of catechin and/or leucoanthocyanidin, not readily hydrolysed by acid treatment, and constitute the main phenolic fraction responsible for the characteristics of astringency of the vegetables. Although the term condensed tannins are still widely used, the chemically more descriptive term "proanthocyanidins" has gained more acceptance. These substances are polymeric flavonoids that form the anthocyanidins pigments. The proanthocyanidins most widely studied are based on flavan-3-ols (-)-epicatechin and (+)-catechin.

1.1.3. *Activity of phenolics on human health*

Cells can generate reactive species (RS) of oxygen and nitrogen from endogenous and exogenous sources. The mitochondria are a major site of RS



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production follows by other endogenous sources such as peroxisomes, fatty acid metabolism and enxymatic systems such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cytochrome P450 reductase, nitric oxide synthase (NOS). On the other side, exogenous sources include environmental agents (smoke, pollution), radiation metals (Fe and Cu) and redox cycling compounds (chlorinated compounds, barbiturates and phorbol esters). In the human body there are biological systems, endogenous antioxidant enzymes such as glutathione peroxidase (e.g. glutathione; S-transferase), non-enzymatically acting endogenous antioxidants (e.g. glutathione, GSH; lipoic acid, LA; N-acetyl cysteine, NAC; uric acid), metal binding proteins (e.g. albumin, transferrin and ferritin) and dietary antioxidants (e.g. vitamin E, vitamin C and b-carotene), that provide to maintain the balance between the RS generation and detoxification to prevent their accumulation. Oxidative and nitrosative stress may occur when cellular antioxidant defences are insufficient to remove reactive species. Reactive oxygen (ROS) and nitrogen (RNS) species are continuously generated in the body as a consequence of mitochondrial bioenergetics, oxidative metabolism and immune function. The most frequent forms of ROS include radical species such as superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($HO^{\cdot}$) and perhydroxyl radical ($HOO^{\cdot}$) and non-radical hydrogen peroxide (H_2O_2); RNS include nitric oxide ($^{\cdot}NO$) and peroxynitrite ($ONOO^-$). Oxidative and nitrosative stress is a major pathological mechanism and contributes significantly to several diseases, namely neurodegenerative and cardiovascular diseases, diabetes and cancer (Duthie et al., 2000).

Phenolic compounds, for instance phenolic acids and flavonoids, have different biological characteristics that could contribute to the prevention of disease. Phenolic compounds have generated a particular interest in the last few decades demonstrating several medicinal properties, mostly related to their antioxidant activity, which can have a positive impact for human health. There are many fruits and vegetables that contain phenolic compounds. A new diet-health ideal is evolving in the health benefits item. Foods have assumed the status of "functional

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foods", which should be capable of providing additional physiological benefit. However, their concentrations in foods may differ by the influence of several factors including species, variety, light, degree of ripeness, processing and storage (Herrmann, 1988).

Nutritional studies have pointed more attention on the protective and disease preventing potential of foods, because these compounds may have antioxidative, antiinflammatory and anticarcinogenic activities (Kaur, 2001). The antioxidant properties of phenolic compounds are owed mainly to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers.

Depending on their chemical structure, these molecules act as hydrogen donating antioxidants and also as chelators of metal ions. Many studies have reported the advantages of phenolic compounds activities. According a review by Lin et al., 2016, phenolic compounds, especially flavonoids, phenolic acids and tannins, have the important property of inhibiting α-glucosidase and α-amylase. They may modulate carbohydrate and lipid metabolism, attenuate hyperglycemia, dyslipidemia and insulin resistance, alleviate oxidative stress, stress sensitive signalling pathways and inflammatory process. Many studies have associated the increase in the consumption of fruits and vegetables containing high levels of antioxidant compounds with the reduction on the risk of certain chronic diseases, for instance diabetes and cardiovascular diseases.

Some foods such as grape, pomegranate juice and cranberry juice, have been reported to play a beneficial part in reducing cardiovascular risk elements in patients with metabolic syndrome and type 2 diabetes. Berries are considered a good source of phenolic compounds, which are considered potential molecules to prevent the development of age-related neurodegenerative diseases by inflammation and decreasing oxidative stress (Ferguson et al., 2004; Hollman, 2001). However, it is little known about the phenolic acid bioactive forms in vivo

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and the mechanisms by which they could contribute to the prevention of disease. There still is the need for further studies.

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1.2. METHODOLOGIES FOR EXTRACTION AND DETERMINATION OF PHENOLIC COMPOUNDS IN FOODS

In the last 15 years, phenolic compounds have generated a huge interest because of their beneficial positive effects on human health, like as antioxidant activity, for food industries and researchers. Phenolic compounds are widespread in plant-based foods and therefore are consumed in important amounts on a daily basis with an estimated rate of consumption of phenolic acids of 25 mg to 1 g per day, while for flavonoids can range from 50 to 800 mg a day, both depending on the diet.

It is important to consider various aspects for phenolic compounds extraction from foods, such as different bioavailability of phenolics, storage time and conditions, their possible interaction with gut microbiota as well as their chemical nature, extraction method employed and the presence of interfering substances. Furthermore, it is important to consider general aspects required nowadays such as the lower use of organic solvents, the miniaturization, the possible automation, the effectiveness and selectivity. Since all these aspects, the extraction methods range from more traditional to advanced extraction process (Herrero et al, 2012).

The sample preparation is one of the important steps of the entire analytical process, including several significant and common steps such as sampling, preservation, preparation and analysis. Most samples are not often ready after the extraction step for direct introduction into instruments. In fact, there might be several processes within sample preparation. These steps may be homogenization/size reduction, concentration or dilution, clean-up (Mitra & Brukh, 2003). The most common steps encountered are shown in Fig. 1.2.1.

Generally, the extraction of phenolic compounds is based on solvents and time consuming. An extraction method may have possible drawbacks such as the requirement of a huge amount of solvents, long extraction time, limited choice of solvents due to its food quality and the possible degradation of target compounds

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(Ajila et al., 2011). Nowadays, the several extraction methodologies may be subdivided into conventional and innovative techniques.

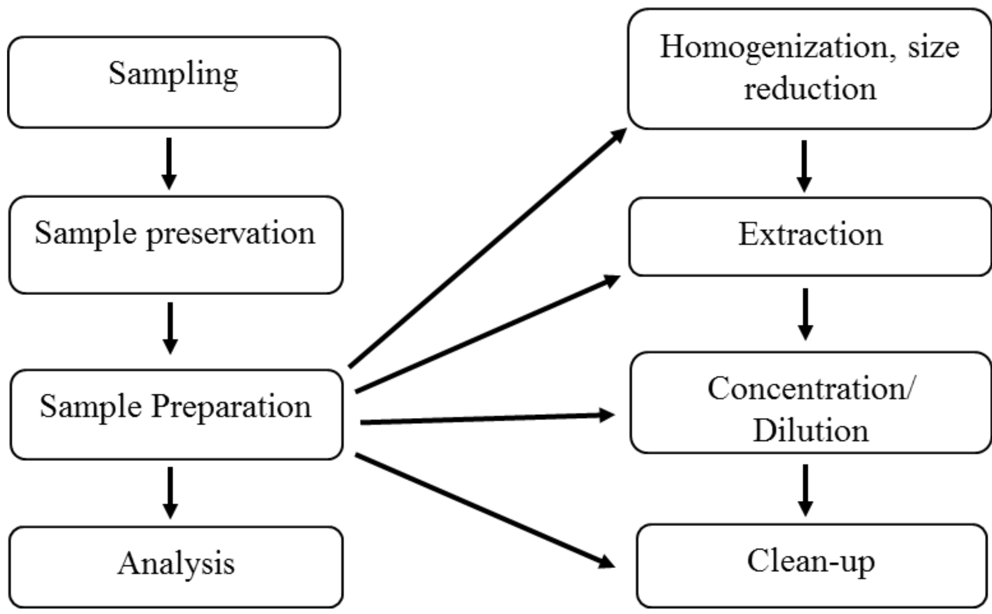

Fig. 1.2.1. Steps of analytical process. *Mitra & Brukh, 2003*.

The traditional extraction techniques, the so-called conventional extraction techniques, are still mostly used for the phenolic extraction from food. The most common techniques are liquid-liquid extraction (LLE), solid-liquid extraction (SLE) or Soxhlet extraction. These techniques are often characterized by using a large amount of damaging organic solvents, time-consuming and also based on manual protocols which are labour-intensive and highly dependent on the operator that are not perfectly reproducible. In order to overcome some of the mentioned drawbacks, new extraction processes have been developed. The advanced techniques may provide some important characteristics of an extraction process such as selectivity, automation, lower consumption of organic solvents, higher extraction efficiency. Some of these innovative techniques are supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), ultrasounds assisted

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extraction (UAE), microwave assisted extraction (MAE), solid phase extraction (SPE). Furthermore, these techniques are considered environmentally friendly since the amount of organic solvents that are used, if any, are significantly reduced (Herrero et al., 2012).

Some of common conventional and innovative extraction techniques will be discussed in this thesis.

1.2.1. *Liquid-liquid extraction (LLE) and Solid-liquid extraction (SLE)*

Liquid-liquid extraction (LLE) is based on the use of two immiscible solvents (phase A and B) by using a separatory funnel for shaking the two phases together. Commonly, separatory funnel is globe, pear or cylindrically shaped which are often shaken manually for few seconds. After shaking the two phases may be separated depending on the relative densities of the two phases. Analytes distribute themselves between the two phases, origin and extracting phase, according to the Nerst distribution law (equ. 1.2.1.), where the distribution coefficient, K_D, is equal to the analyte ratio in each phase at equilibrium (Wells, 2003).

$$K_D = C_A / C_{B;}\ C_A = \text{origin phase};\ C_B = \text{extracting phase} \qquad (1.2.1.)$$

The LLE may be useful for several aims:

- Transfer analyte from aqueous phase to immiscible organic phase. This process may occur with the use of salt solutions (*salting-out*) or adjustment of pH;
- Remove non-polar interfering from an aqueous sample by using an immiscible organic solvent;
- Separation between two immiscible organic solvents such as ethanol and hexane; for example, the separation of polar phenolic compounds in olive oil.

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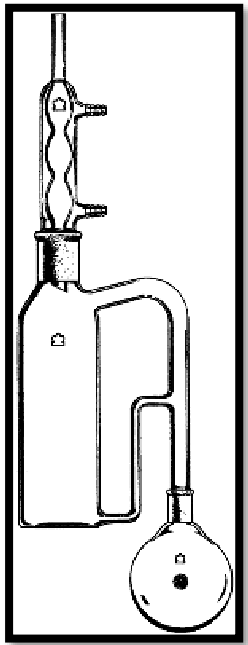

Fig. 1.2.2. Continuous liquid-liquid extraction apparatus. *Wells, 2003.*

LLE process may be repeated more times (2 or 3), especially when the distribution coefficient of the analyte between two phases is low. For huge amount of sample with low concentration of analyte, an automatic extractor system may be used such as *Soxhlet* (Fig. 1.2.2.). This apparatus performs on the principle that organic solvent cycles continuously through the aqueous phase.

In general, the extraction of phenolic compounds from food material is governed by several factors such as solubility of target compounds in the extracting solvent (polarity), degree of polymerization of phenolics, interaction with other food constituents and formation of insoluble complexes. According to the chemical nature of phenolic compounds, the solvents commonly used are water, methanol, ethanol, acetone, ethyl acetate, dimethylformamide and their combinations (Naczk & Shahidi, 2004).

For solid samples, solid-liquid extraction (SLE) has been employed. Generally, the solid sample is homogenized and extracted with a solvent or a mixture of solvents for a given time. In this case, several parameters should be considered if a complete extraction of all phenolic compounds is aimed at. Among the solid sample, some parameters are considered such as the particle size since it influences

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directly the mass transfer of the interesting compounds and their solubilisation in the organic solvents. More specific, a smaller particle size will increase the contact between sample and solvent achieving an increase on the mass transfer rate. Furthermore, sample with less humidity are preferred as well as it is important the solvent selection.

As it has been discussed previously for LLE, the solvent should have a polarity according to the type of phenolic compounds to be extracted. Common solvents are ethanol, methanol and their mixtures with different proportions of water. The addition of small proportion of an acid has been shown to have a favourable effect in some applications. Another important parameter is the temperature. In this case, the advantages will be the increase of the mass transfer and the solubility of the interesting compounds in the solvent, but the temperature should be low enough not to produce degradation of the phenolic compounds. Soxhlet extraction may be used as an automatic extractor.

1.2.2. *Solid-phase extraction (SPE)*

Solid-phase extraction (SPE) is a method used for the isolation and concentration of target analytes from sample, based on the interaction of analytes, in a liquid or gas phase, with a solid phase. The solid phase is then isolated from the sample and the analytes recovered by elution using liquid or fluid, or by thermal desorption into the gas phase.

The first applications through SPE have been developed in the seventies (Subden et al., 1978) for determination of histamines in wines using a C18 (octadecyl-group) packaging. This method has been introduced as an advanced alternative to traditional liquid-liquid extractions, since it allows to obtain some benefits such as reduced analysis time, reduced cost, reduced labour, reduced organic solvent consumption, reduced analyst exposure to organic solvents.

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Generally, SPE is used to prepare liquid samples and extract semivolatile or non-volatile analytes, but can also be used with solids that are pre-extracted into solvents. Typical cartridge devices consist of short columns, generally an open syringe barrel, containing a sorbent with a nominal particle size between 20 and 60 μm, packed between porous plastic or metal frits (Fig. 1.2.3.). Several sorbents are available today providing for the diverse application base of modern SPE.

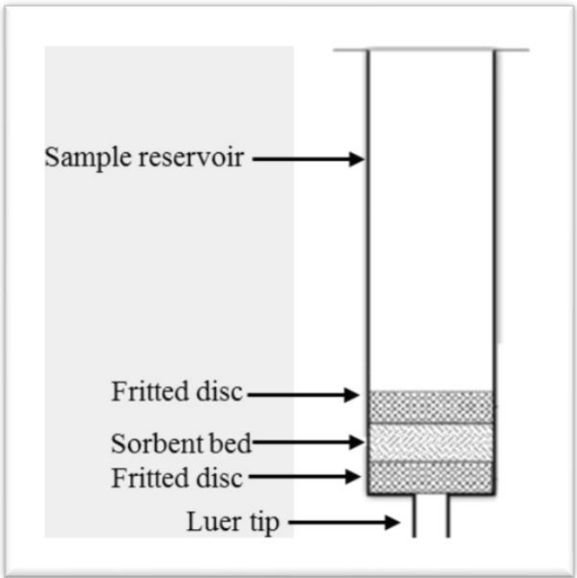

Fig. 1.2.3. Schematic diagram of the typical construction of a solid-phase extraction cartridge.

According to the nature of material, the sorbents may be divided into inorganic oxides, polymer and carbon-based form. According to the interaction between sorbent and analytes, the sorbents may be divided into polar, polymeric, bonded silica, graphitized as well as functionalized polymeric resins, ion-exchange, controlled-access, immunoaffinity and molecularly imprinted polymeric sorbents.

SPE procedure consists of four steps: column preparation or prewash, sample loading (retention or sorption), column post-wash, and sample desorption (elution or desorption) (Figure 1.2.4.). All four steps are required for the reversed phase, normal phase or ion-exchange SPE procedures, while for some sample clean-up

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procedures, only the first two steps may apply. Three mechanisms may be exploited for separating the target analytes from the interfering (Sigma-Aldrich, 1998): 1) the selected components or the interfering ones are retained during the sample loading; 2) the interfering ones may be extracted through a selective washing avoiding the analytes elution; 3) the analytes may be extracted through a selective washing avoiding the interfering elution.

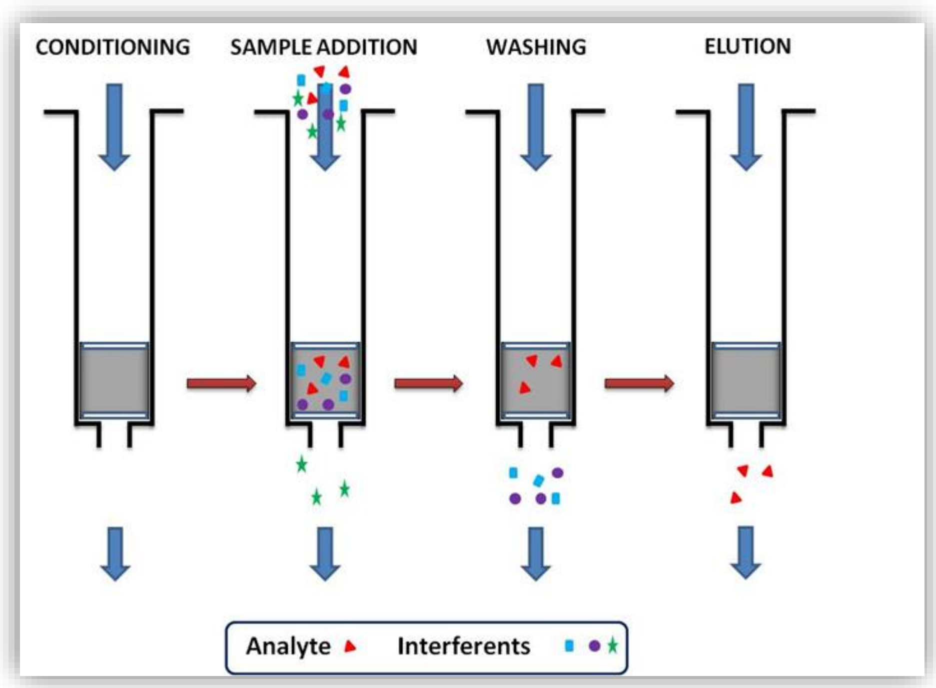

Fig. 1.2.4. Four steps SPE process. *Lucci et al., 2012.*

Solvents can be passed through SPE sorbents by pressure, hand pumping or can be pulled through by vacuum. During the past decade, SPE has become automated. High-throughput 96-well workstations and extraction plates are commercially available and allow numerous samples to be processed simultaneously. According to Rossi and Zhang (Rossi & Zhang, 2000), the automation of SPE procedure

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allows to obtain some advantages such as timesaving, high throughput with serial sample processing, improving precision and accuracy, reduced tedium and possibility of automated method development. Anyway, in addition to the advantages, the disadvantages should also be considered like the systematic errors that can occur undetected, decreased precision and sample stability issues (Wells, 2003).

Recent advances in SPE have displayed that the combination of extraction, clean-up, separation and detection operations may occur continuously and be fully automated.

SPE can be an interface between two operations, e.g., LC-SPE-NMR and supercritical fluid extraction (SFE)-SPE-LC, or used as a collection trap in preparative chromatography (Wang et al, 2004; Stevens et al, 2007). Solid-phase extraction has been used for phenolic compounds extraction or for the cleanup of the polar fraction from foods in most applications. The first study was carried out by Papadopoulos and Tsimidou (Papadopoulos & Tsimidou, 1992) focused in a comparative study of two SPE system and a liquid-liquid one. Ruiz-Gutiérrez and Pérez-Camino (Ruiz-Gutiérrez & Pérez-Camino, 2000) presented an application for phenolic compounds from olive oil.

1.2.3. *Supercritical fluid extraction (SFE)*

Supercritical fluid extraction (SFE) is a valid alternative extraction method to traditional extraction techniques. It is based on the use of solvents at temperatures and pressure above their critical points. In 1822, Charles Cagniard de la Tour was the first proposing the concept of a critic point for each substance. According to Cagniard de la Tour's experiment, for each substance exists a temperature above the liquid phase disappears despite the increment of pressure (Moret & Conte, 2014). In 1879, Hannay and Hogarth introduced the possibility of a new solvent medium due to the observations of the dissolution of solutes in supercritical fluid

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(SCF). However, it was not until around 1960 that this extraction method was accurately examined (Steytler, 1996).

A pure compound is considered to be a supercritical fluid (ScF) when its temperature and pressure are greater than its critical temperature and pressure, which are represented as T_c and P_c, respectively. The critical temperature is defined as the highest temperature at which a gas can be converted to liquid by an increase in pressure. The critical pressure is the highest pressure at which a liquid can be converted to a gas by an increase in temperature. These properties characterize the critical point (CP). Figure 1.2.5. shows a general phase diagram (Cavalcanti & Meireles, 2012). Several supercritical fluids can be used as extraction solvents in SFE such as CO_2, ethane, butane, pentane, nitrous oxide, ammonia, trifluoromethane and water. CO_2 is the most used solvent due to its low critical temperature (T_c = 31 °C). Despite its high critical pressure (P_c = 72 bar), CO_2 is attractive as extraction medium because of its high diffusivity combined with its high solvent strength; it is gaseous at room temperature and ambient pressure, which makes product recovery fairly simple; it can be recycled as the solutes dissolved in it will precipitate upon depressurization; it is non-toxic and low cost. One of disadvantages is that CO_2 is non-polar and it is not good for polar compounds, e.g., phenolic compounds. In this case, the disadvantage may be overcome by using an organic co-solvent, namely methanol, ethanol, acetonitrile or acetone enhancing the solvating power of CO_2 and the yield of extraction (Murga et al., 2000; Louli et al., 2004).

SFE is simply performed by pumping the SF through a vessel filled with a sample, and further down the line depressurize the SF for collection of extracted components.

A SFE instrument (Figure 1.2.6.) is equipped by one or two high-pressure pumps for delivery of SF and, if necessary, also a polar co-solvent; a high-pressure vessel for holding the sample; a restrictor and a collection device. The sample vessel is placed in an oven for control of extraction temperature. An advantage of

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SFE is the possibility of performing on-line coupling to gas-chromatography (GC), high performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC). Compared to off-line techniques, the on-line techniques provide to offer some advantages as incrementing sensibility due the direct transfer of extracted analytes to chromatography column and the low manipulation of sample that allows obtaining low extraction time. Method development in SFE is important in terms of temperature and pressure of SF, extraction time, flow rate, addition of co-solvent (type and amount) and collection mode (Turner, 2006).

Considering several studies among phenolic compounds extraction from foods by using the supercritical fluid, SFE has proved a good valid alternative extraction method for phenolic compounds (Mendiola et al., 2007; Murga et al., 2003).

1.2.4. *Pressurized liquid extraction (PLE)*

Pressurized liquid extraction (PLE) is also known as pressurized fluid extraction (PFE), enhanced solvent extraction (ESE), high-pressure solvent extraction (HPSE) or accelerated solvent extraction (ASE) (Nieto et al., 2010). PLE was introduced for the first time in 1996 (Richter et al., 1996). This novel technique is based on the combination of high temperatures and pressures which uses organic solvents above their normal boiling point. The increment of temperature provides to promote the kinetics of extraction, mass transfer, while, the increment of pressure provides to maintain the solvents in its liquid state above their boiling point which can promote higher analytes solubility.

The first PLE instrument was employed and commercialized by Dionex, known as ASE (Accelerated Solvent Extraction). Generally, a solid sample is packed in an extraction cell and extracted with a suitable solvent under elevated temperature (40-200 °C) and pressure (500-3000 psi) for short periods time (5-15 min), and the purged sample extract will be collect into a collection vial by compressed gas. A typical PLE instrument is shown in figure 1.2.7.

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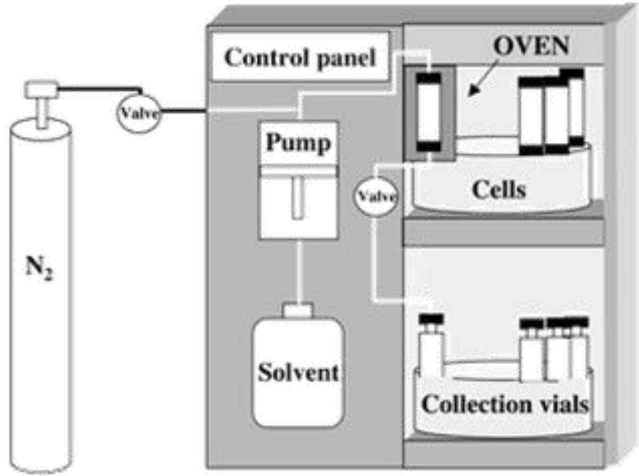

Figure 1.2.7. Schematic of a PLE instrument. *Camel, 2001.*

In a typical PLE extraction some parameters should be optimized (Herrero et al., 2012). Solvent selection is one of the most important aspects. Several solvents and mixtures of them, such as ethanol, methanol and mixtures in different proportions of them with water, have been employed to extract and analyse phenolic compounds from food. Considering the chemical structure of phenolic compounds, it has been demonstrated how the solvent selection can influence the extraction of different phenolics even contained in the same sample. The acidification of solvents may also keep to good results. Other parameters are extraction temperature, extraction time, sample particle size, packing of the sample inside the extraction cell, number of static extraction cycles or extraction solvent flow rate. Since phenolic compounds are highly labile, particular attention should be put on the maximum extraction temperature. Typical temperatures employed for phenolic compounds extraction are usually above 100 °C. The particle size can influence the extraction outcome. Since a very small particle size can produce the clogging of the system, the use of packing material as diatomaceous earth, sea sand and other supporting materials can be employed to avoid this problem. These materials can help the correct dispersion of the sample, to avoid the formation of preferential paths through the sample. Another important effect is given by the extraction time. It is possible to work under static or dynamic conditions. In the first case, the

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sample remains in contact with the extraction solvent maintaining the system in equilibrium. In the second case, the extraction time refers to the time a particular flow rate of extraction solvent passes through the extraction cell.

When PLE uses only water as extraction solvent, it is commonly known as subcritical water extraction (SWE) or superheated water extraction (SHWE). In this case, it is interesting since it may be considered as environmentally friendly extraction technique. For phenolic compounds with high polarity, the use of this technique may produce good results, instead, for less polar phenolic compounds, different solvent mixtures can be employed, including a small amount of an organic solvent.

1.2.5. *Ultrasound-assisted extraction (UAE)*

Ultrasound-assisted extraction (UAE) is an advanced extraction technique that dramatically reduces extraction time, increasing yields. For many years, the use of ultrasound energy has been applied in food-processing and sample treatment. UAE is based on the use of mechanical waves that need an elastic medium to spread. Sound and ultrasound differ for the frequency of the wave, 16 Hz to 16-20 kHz and 20kKz to 10 MHz respectively. Ultrasound applications, characterized by sound power (W), sound intensity (W/m^2) or sound energy density (W/m^3), may be subdivided into two groups: high intensity low frequency (20 kHz $\leq f \leq$ 100 kHz) and low intensity high frequency ($f >$ 100 kHz). Low-intensity ultrasounds do not alter the physical or chemical properties of the material through which the ultrasonic wave propagates. High intensity ultrasounds generate intense pressures and temperature gradient due to the bubble cavitation producing a disruption effect within matrix. The UAE process provides for a creation of longitudinal waves when a sonic wave meets a liquid medium. In this way, it is created a region of alternating compression and rarefaction waves induced on the molecules of the medium. In these regions of changing, cavitation occurs and gas bubbles are

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formed. These bubbles have a larger surface area during the expansion cycle increasing the diffusion of gas. In heterogeneous phases, these hot areas create micro-jets and shock waves that create the erosion, fragmentation or disaggregation in the solid phase of the sample to be investigated (Picó, 2013).

According to Herrero et al. (2012), the most important parameters involved in the extraction of phenolic compounds from food are extraction time, extraction temperature, power, solvent composition and amount of sample. Among temperature, it is important to be safe and to avoid phenolics thermodegradation. In UAE, the temperature should not exceed 40 °C. The power of the energy as well as its frequency depend on the instrument employed. Several studies have reported a power values from 25 to 200 W. For instance, to extract anthocyanins from grape seeds by UAE, were used power and frequency values of 250 W and 40 kHz respectively (Ghafoor et al., 2009); to obtain flavanone glycosides from orange peel, were used power and frequency of 150 W and 25 kHz respectively (Khan et al., 2010).

The amount of sample and its physical state is also important, in particular, the particle size. Smaller particles allow a good penetration of the solvent. One of the advantageous of UAE is that it is able to provide higher extraction efficiencies using less solvent and faster than using traditional procedures as LLE. Besides, several studies have been directly compared the UAE to traditional technique obtaining good results by using UAE (Tatke & Rajan, 2013).

1.2.6. *Microwave-assisted extraction (MAE)*

Microwave-assisted extraction (MAE) is an efficient and fast advanced technique. It is based on the use of microwave radiation heating solvents in contact with solid samples and to favour the solubilisation of analytes from the sample into the solvent. The first MAE application was in 1986 (Ganzler et al., 1986). Since then it has broadly been applied for extraction of different samples as biological

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(serum, tissue and hair) and foods (fat, organic contaminants, bioactive compounds and nutrients).

Microwave energy is a non-ionizing electromagnetic wave of frequency between 300 MHz to 300 GHz and it lies between the X-ray and infrared rays in the electromagnetic spectrum. The principle of MAE is mainly based on the direct effect of microwaves on molecules by ionic conduction and dipole rotation. The ability of a solvent to absorb microwave energy and pass it on in the form of heat to other molecules will partly depend on the dissipation factor (tan δ) according to the following equation (equ. 1.2.2.)

$$\tan \delta = \varepsilon'' / \varepsilon' \qquad (1.2.2.)$$

where ε'' is the dieletric loss, a measure of the efficiency of converting microwave energy into heat, and ε' is the dieletric costant, a measure of the polarizibility of a molecule in an electric field. Polar molecules Polar molecules and ionic solutions (usually acids) will absorb microwave energy strongly because they have different a permanent dipole moment that will be affected the microwaves. However non-polar solvents such as hexane will not heat up when exposed to microwaves. The extraction heating process may occur by a number of mechanisms: the sample could be immersed in a single solvent or mixture of solvents that absorb microwave energy strongly; the sample could be extracted in a combined solvent containing solvents with both high and low dieletric losses mixed in various proportions; samples that have a high dieletric loss can be extracted with a microwave transparent solvent. A typical MAE equipment consists of magnetron tube, an oven where the extraction vessels are set upon a turntable, monitoring devices for controlling the temperature and pressure, and a number of electronic components (Figure 1.2.8.) (Eskilsson & Björklund, 2000).

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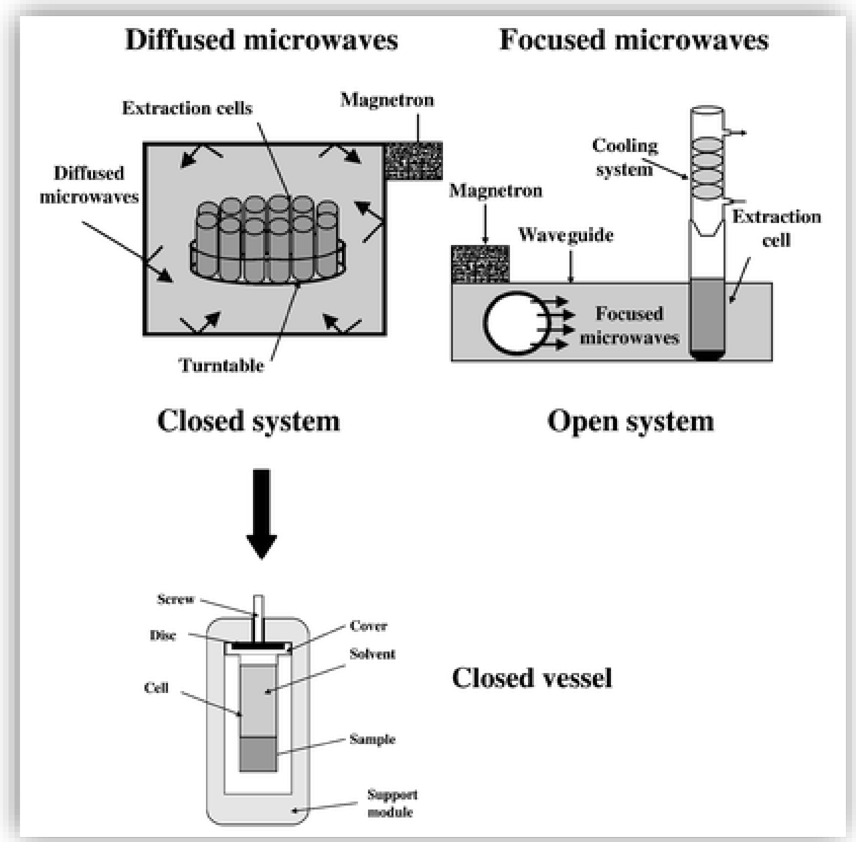

Figure 1.2.8. Main components of a diffused microwave system. *Camel, 2001.*

The main advantages of MAE are the possibility of reducting both extraction time and solvent consumption. It is important to carefully select some parameters for extraction of phenolic compounds from food. Firstly, the solvent composition is very important. The solvent extraction has to be tuned to the sample to be extracted. Generally, ethanol, methanol, water and their mixtures are the most used solvents for the MAE of phenolic compounds. Typical extraction times for phenolic compounds are around 2 minutes and it can be considered a very fast extraction method (Ballard et al., 2010). Another important parameter is extraction temperature that is correlated to the microwave power. It has been demonstrated that powers up to 600-700 W and two different controlled temperatures (100 °C and 80 °C) were useful for diverse phenolic compounds extraction (Nkhili et al., 2009).

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The MAE is considered a valid alternative extraction technique to conventional extraction technique, as SLE or Soxhlet, in terms of solvent consumption, extraction efficiency and speed for the extraction of phenolic compounds from food (Pedroza et al., 2015; Beejmohun et al., 2007).

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1.3. SEPARATION CHROMATOGRAPHIC TECHNIQUES FOR DETERMINATION OF PHENOLIC COMPOUNDS IN FOODS

In the last decades, nutritional science, due to the globalisation and the development of new technologies, has significantly been developed. The molecular characterization of food is becoming more essential, especially when the foods are functional.

The functional foods have generated broad interest, being characterized by molecules which possess positive beneficial human effects, such as antioxidant activity, anti-inflammatory and anti-carcinogenic activities. These molecules are known as "bioactive compounds" and are widely distributed in the vegetal kingdom. Main families of compounds with proved antioxidant activity are phenolic compounds, carotenoids and tocopherols which are easily available in vegetable kingdom.

The interest in finding natural sources of antioxidants is increasing. Liquid chromatography, more specifically, high performance liquid chromatography (HPLC) is one of the most powerful separation techniques, used in the food fields for the analysis of components of both raw and processed products. HPLC is widely applied for qualitative and quantitative analysis of phenolic compounds in foods. In this chapter, the main phenolics techniques used in the present thesis will be presented.

1.3.1. *High performance liquid chromatography (HPLC)*

The liquid chromatography was discovered for the first time by M.S. Tswett in 1903, who separated pigments adsorbed on filter paper using non-polar solvent mixtures and then using columns containing sorbents for pigments separation. Since then, more researchers have provided contributes to chromatography technique.

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Liquid chromatography (LC) represents one mode to achieve the separation of a mixture of diverse compounds, based on their different solubility between two phases, namely stationary and mobile. The stationary phase can be either spread on a flat support (thin layer) or enclosed in a column while appropriate solvents or a mixture of them are the mobile phase. The column packing material used in conventional LC is based on porous inorganic oxide or porous polymers with 30-200 μm particles. The most used stationary phases are based on silica particles because they are resistant to pressure and can be easily modified using different chemical reactions. According to the nature of the mobile phases, liquid chromatographic techniques can be subdivided in normal-phase (NP) and reversed phase (RP).

NP-chromatography has a polar stationary phase and a non-aqueous mobile phase. The retention mechanism is favourable for hydrophilic compounds, while the high non-polar ones are eluted first. Silica gel is the most used stationary phase in NP.

RP-chromatography has a non-polar stationary phase and an aqueous, moderately polar mobile phase. It is the widely used for analysis of phenolic compounds due to their chemical nature. One common stationary phase is silica, which has been treated with RMe2SiCl, where R is a straight-chain alkyl group such as C8 or C18 chains. Less polar molecules will have a longer retention time, while polar molecules will elute more readily. The retention of analytes can be decreased by adding a less polar solvent such as methanol, acetonitrile. Generally, using a gradient elution in which the polarity of the mobile phase changes automatically during the analysis procedure, this effect is achieved. Most methods use buffering agents to control the pH due it can change the hydrophobicity of the analytes (Fanali et al., 2012).

The high-performance liquid chromatography (HPLC), also known as high pressure liquid chromatography, is a technique broadly used to separate, identify and quantify phenolic compounds from foods. HPLC utilizes a column that holds

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the stationary phase, a pump that moves the mobile phase through the column, and a detector that generates a signal proportional to the amount of compound present in the sample mixture and a processor, which uses a software interface to control the instruments and provides data analysis (Figure 1.3.1.).

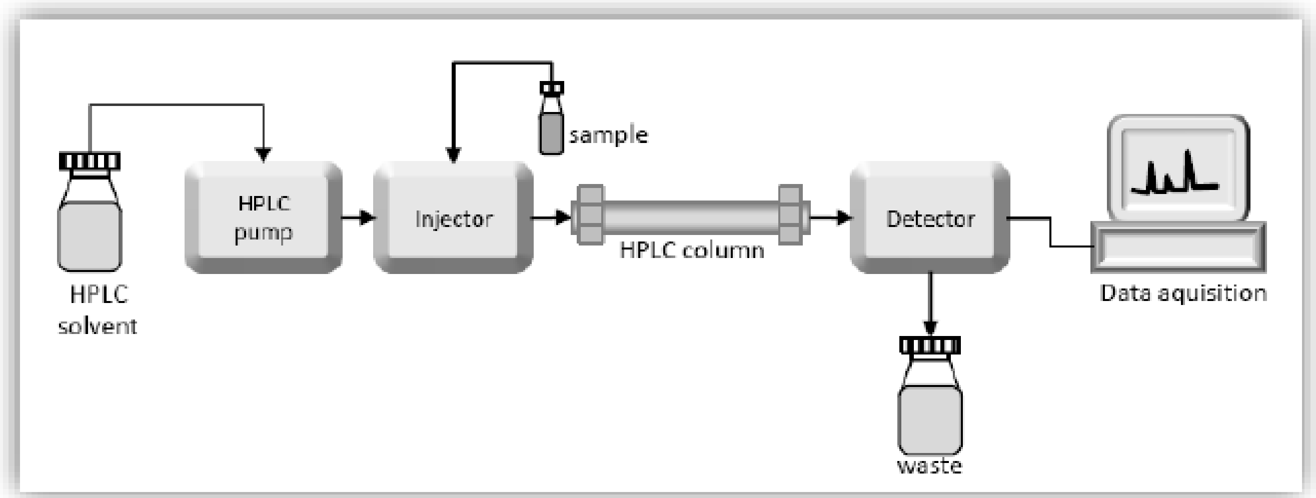

Figure 1.3.1. Scheme of an HPLC instrumentation used for chromatographic separations.

The solvents (mobile phase) are contained into reservoirs made of glass or polymeric material and pumped by systems controlled manually or by computers. Three types of pumps are known: single-head reciprocating pump, double-head reciprocating pump and syringe pumps. Generally, the most employed pumps are the double-head reciprocating pumps because it allows maintaining a flow rate constant. The elution can be isocratic or gradient mode based. By isocratic elution, the percentage of mobile phase is constant during all the time of data analysis. Often it not allows obtaining a complete separation of analytes. Very often a gradient elution, in which the composition of mobile phase changes during the data analysis, is necessary to separate co-eluting compounds or to reduce analysis time for those analytes that are more strongly retained into the column.

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1.3.2 *Detectors coupled to HPLC*

Once the analytes are separated into the chromatographic column, they are detected online using different detector types. The choice of the ideal detector for each application is based on the physical-chemical properties of the analytes to be determined and the sensitivity required and what information is sought. For the detection of phenolic compounds, the most detection systems widely used in several applications are spectroscopic UV-visible and mass spectrometry detector (MS). Generally, the UV-visible detectors give no structural information and cannot unequivocally identify compounds, when there is no pattern. It can be useful in determining phenolic compounds in order to limit the family to which the analytes belong. To overcome this limit, a coupling to mass spectrometry is widely exploited since the MS detector provides structural information.

1.3.2.1. *UV-Visible detector*

The UV-visible absorbance detector is the most common HPLC detector in use today for phenolic compounds, since they absorb in the UV (or visible) region, from 190 to 800 nm. A reference signal is measured and the absorbance calculated according to the Lambert-Beer law:

$$A = \log (I_0/I) = \varepsilon bc$$

where A is absorbance, I_0 is the incident light intensity, I is the intensity of the transmitted light, ε is the molar extinction coefficient of the sample, b is the path length of the cell in cm, and c is the molar sample concentration. Most aromatic compounds as phenolic compounds exhibit a higher or lower absorption in the UV or UV/VIS region depending on the intrinsic existence of conjugated double and aromatic bonds. Phenolic acids with benzoic acid carbon framework have their maxima in the 200-290 nm range. The cinnamate derivatives, due to the additional

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conjugation, show a broad absorbance band from 270-360 nm. Flavonoids have two absorption bands. Band II, with a maximum in the 240-285 nm range, due to the ring-A. Band II, with a maximum in the 300-350 nm range, presumably arises from the ring-B (Merken & Beecher, 2000). Anthocyanins are the most unique subclasses because they absorb visible light near 520 nm.

There are three different types of UV detectors: fixed wavelength detectors, variable wavelength detector (dispersion detector), and photodiode array detectors (PDA). The first type relies on distinct wavelengths. It is cheap and simple, but is in limited use today because of it is possible to select one wavelength. Variable wavelength detectors can be tuned to operate at the absorbance maximum of an analyte or at a wavelength that provides more selectivity. In variable wavelength detectors, light from broad spectrum (for UV deuterium is common, tungsten for visible) lamp is directed through a slit to a diffraction grating that spreads the light out into its constituent wavelengths. PDA detectors have an optical path similar to variable wavelength detectors except the light passes through the flow cell prior to hitting the grating, allowing it to spread the spectrum across an array of photodiodes. One of the advantages of PDA is that multiple signals in the same chromatographic data can be easily collected.

1.3.2.2. *Mass spectrometry (MS)*

Mass spectrometry (MS) is a powerful analytical technique used to quantify target analytes, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules. Generally, MS detector is coupled to gas chromatography (GC) or LC systems, GC/MS or LC/MS respectively. The principle of mass spectrometry is based on the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ions (m/z) and relative abundances. Multiple

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ions from the sample, under investigation, are separated according to their specific mass-to-charge ratio (m/z).

A mass spectrometer should always perform the following processes:

1) Produce ions from the sample in the ionization source;
2) Separate these ions according to their mass-to-charge ratio in the mass analyser;
3) Fragment the selected ions and in a tandem system analyse the fragments in a second analyser,
4) Detect the ions emerging from the last analyser and measure their abundance with the detector that converts the ions into electrical signals;
5) Process the signals from the detector that are transmitted to the computer and control the instrument through feedback.

A typical mass spectrometer consists of three main parts: the ion source, the mass analyser and the detector (de Hoffmann & Stroobant, 2007).

Ion source: In the ion sources, the analysed samples are introduced, by a syringe pump (direct infusion) or as eluent from a separation technique such as LC, for ionization prior to analysis in the mass spectrometer. A variety of ionization techniques can be used. Some ionization techniques are very energetic (hard) and cause extensive fragmentation such as electron ionization (EI), also known as electron impact. Other techniques are softer (soft) and only produce ions of the molecular species. The pressure atmospheric ionization (API) is the most ionization technique used to ionize the molecules from the liquid-phase. The main pressure atmospheric ionization sources are: electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI) and atmospheric-pressure photoionization (APPI).

ESI is one of the most versatile ionization sources that provide a simple, real-time means of analysing a wide range of polar molecules (100-200,000 Dalton range). It is the preferred choice for detecting polar compounds separated by liquid chromatography, due to its very high sensitivity and to easy coupling to HPLC system, μHPLC or capillary electrophoresis. ESI is based on the applying a strong

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electric field, under atmospheric pressure, to a liquid passing through a capillary tube with a weak flux. The electric field is obtained by applying a potential difference of 3-6 kV between this capillary and the counter-electrode, separated by 0.3-2 cm, producing electric fields of the order of 10^6 V m^{-1}. This field induces a charge accumulation at the liquid surface located at the end of the capillary, which will break to form highly charged droplets. A gas injected coaxially at a low flow rate allows the dispersion of the spray to be limited in space. These droplets then pass either through a curtain of heated inert gas, most often nitrogen, or through a heated capillary to remove the last solvent molecules. Ions are formed at atmospheric pressure and pass through a small aperture into the high vacuum at the mass analyser. The samples arrive to the spray chamber as a fine mist of droplets or spray. A voltage gradient between the tip of the spray needle and the entrance to transfer capillary, as well as a pressure difference from atmospheric pressure to vacuum encourages appropriately charged ions to move into the capillary and on towards the skimmers. Pressure is reduced by pumping most of the vaporized sample and mobile phase to waste; only a tiny fraction of the sample is drawn into the MS itself. As the solvent evaporates, the droplet shrinks until it reaches the point that the surface tension can no longer sustain the charge (the Rayleigh limit) at which point a "Coulombic explosion" occurs and the droplet is ripped apart.

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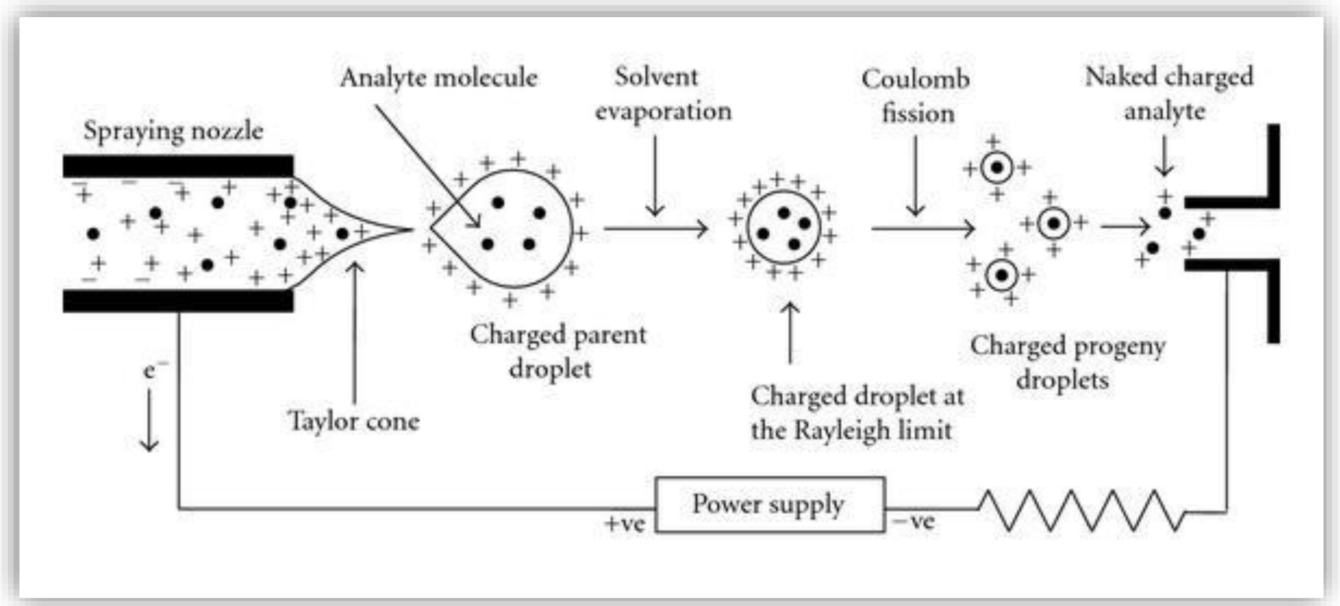

Figure 1.3.2. Electrospray ionization theory scheme. *Banerjee & Mazumdar, 2011.*

This phenomenon produces smaller droplets that can repeat the process as well as naked charged analyte molecules. These charged analyte molecules may be singly or multiply charged. This is a soft method of ionization and the generation of multiply charged molecules enables high-molecular-weight components such as protein to be analysed, since the mass range of the mass spectrometer is greatly increased. One of the major disadvantage of ESI technique is that very little fragmentation occurs, although this may be overcome through the use of tandem mass spectrometric technique such as MS/MS or MS^n.

Mass analyser: Once the gas-phase ions have been produced, they need to be separated according to their masses, which must be determined. The physical property of ions that is measured by a mass analyser is their mass-to-charge ratio (m/z) rather than their mass alone. As there are a great variety of sources, several types of mass analysers have been developed. All mass analysers use static or dynamic electric and magnetic fields that can be alone or combined. Most of the basic differences between the various common types of mass analyser lie in the manner in which such fields are used to achieve separation.

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Each mass analyser has its advantages and limitations. Analysers can be divided into two broad classes on the basis of many properties. Scanning analysers transmit ions of different masses successively along a time scale. They are either magnetic sector instruments with a flight tube in the magnetic field, allowing only the ions of a given mass-to-charge ratio to go through at a given time, or quadrupole instruments.

However, other analysers allow the simultaneous transmission of all ions, such as the dispersive magnetic analyser, the TOF mass analyser and the trapped-ion mass analysers that correspond to the ion traps, the ion cyclotron resonance or the orbitrap instruments. Analysers can be grouped on the basis of other properties, for example ion beam versus ion trapping types, continuous versus pulsed analysis, low versus high kinetic energies (Westman-Brinkmalm & Brinkmalm, 2009; Smith, 2004).

The **Quadrupole Mass Analyser** (QMS) was realized by W. Paul (shared Nobel Prize in Physics, 1989). A quadrupole mass spectrometer (figure 1.3.3.) is widely used and consists of four electrical poles (usually called rods) that are held in strict alignment with one another. Opposing poles are connected in pairs to both radio frequency (RF) and direct current (dc) generators, bathing ions in a combined electric and RF field during their passage through the analyser. Ions emerging from the source, typically accelerated over a potential of 5 to 20 V, enter the analyser region between the rods and travel parallel to the rods. At given values of the DC and RF potentials and the RF frequency, only ions within a certain narrow *m*/*z* range will have stable trajectories through the quadrupole. The m/z range for ions allowed passing through depends on the ratio between the DC and RF potentials. Ions that do not have a stable trajectory will collide with the rods, never reaching the detector. The motion of an ion traveling in the quadrupole field is described by the Mathieu equation (McLachlan, 1947). Qualitatively, the heavy (more inert) ions mainly respond to the DC component of the field while the lighter (quicker) ions also respond to the alternating RF component. One pair of rods will act as a

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high pass filter and force the heavy ions to the middle between the electrodes. Low mass ions respond faster when, once every cycle, the net force from the DC and RF components is attractive for a short time. Then, if the mass of the ion is low enough, the ion will be accelerated towards one of the electrodes and hit it before the force changes direction again. The opposite pair of rods will instead act as a low pass filter. High mass ions experience an attractive force and will hit one of the electrodes. The low mass ions also experience an attractive force most of the time, but they will, once every cycle, respond to the repulsive force and be pushed back towards the middle between the electrodes. A mass spectrum is acquired by scanning the ratio of the DC and RF potentials and monitoring the abundance of the detected ions. The mass selectivity (i.e., resolution) of the quadrupole is dependent on the number of cycles an ion undergoes while it is in the analyser. Thus, the resolution is negatively affected by an increased ion velocity and decreased RF frequency. Quadrupole mass filters are not, however, typically chosen to achieve high mass accuracy and resolution, but rather for maintaining good speed and sensitivity.

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Recently, however, with better machined parts and better electronics, commercially available instruments can perform quite well. A mass accuracy ≤ 5 ppm can be obtained with internal calibrants (Westman-Brinkmalm & Brinkmalm, 2009; Smith, 2004).

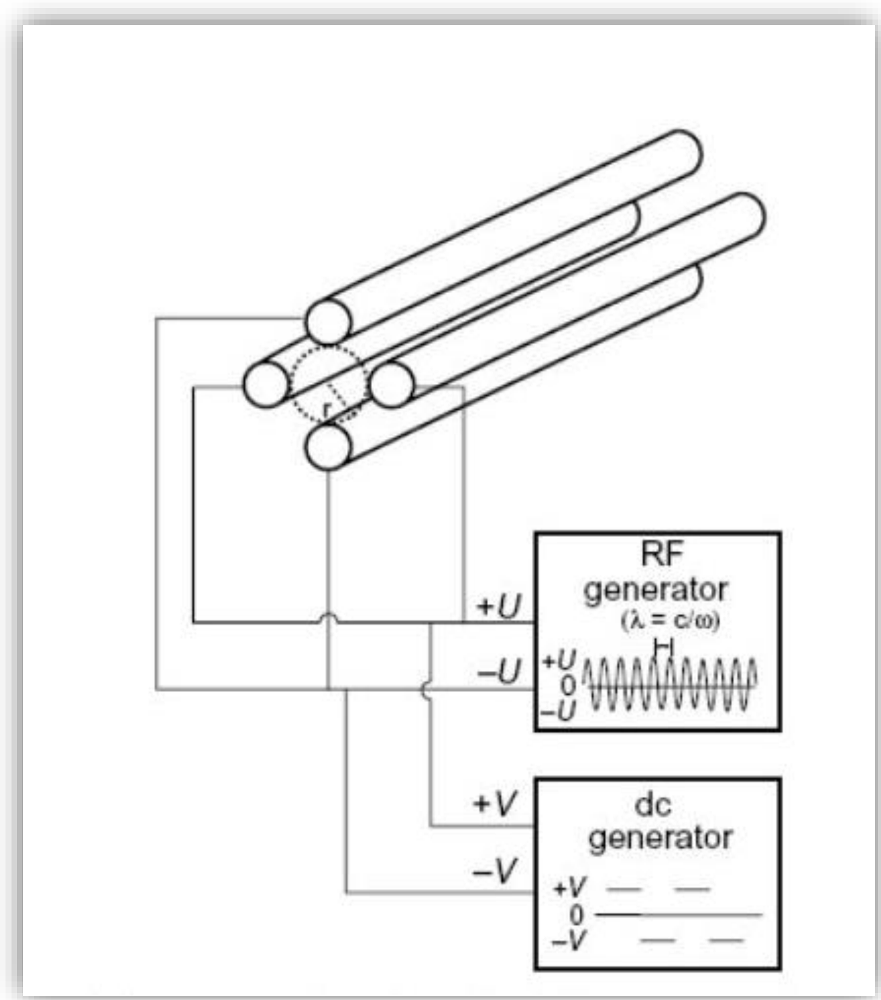

Figure 1.3.3. Quadrupole mass analyser scheme. *Smith, 2004.*

The **Quadrupole Ion Trap Mass Spectrometer** (QITMS) functions not only as a mass spectrometer of high sensitivity and high specificity but also as an ion store in which gaseous ions can be confined for periods of some hundreds of milliseconds.

While other mass spectrometers operate at pressures $<10^{-6}$ Torr, the ion trap operates at a pressure of 1 mTorr of helium buffer gas. With advent of new methods by which ions can be formed in the gas phase and introduced

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subsequently into an ion trap, the range of applications of the quadrupole ion trap is now considerable. The coupling of liquid chromatography (LC) with ESI and with MS in the early 1980s, together with the rapid advancement in ion trap technology, have led to the development of new ion trap instruments for the analysis of non-volatile, polar, and thermally labile compounds.

Ion trap (figure 1.3.4.) can be imagined as a quadrupole bent in on itself in order to form a closed loop. The quadrupole ion trap consists essentially of three electrodes. Two of the three electrodes are virtually identical and, while having hyperboloidal geometry, resemble small inverted saucers; these saucers are called end-cap electrodes and are distinguishable by the number of holes in the centre of each electrode. The third electrode is also of hyperboloidal geometry and is called the ring electrode. The ring electrode is positioned symmetrically between the two end-cap electrodes. In this case, the principle is different.

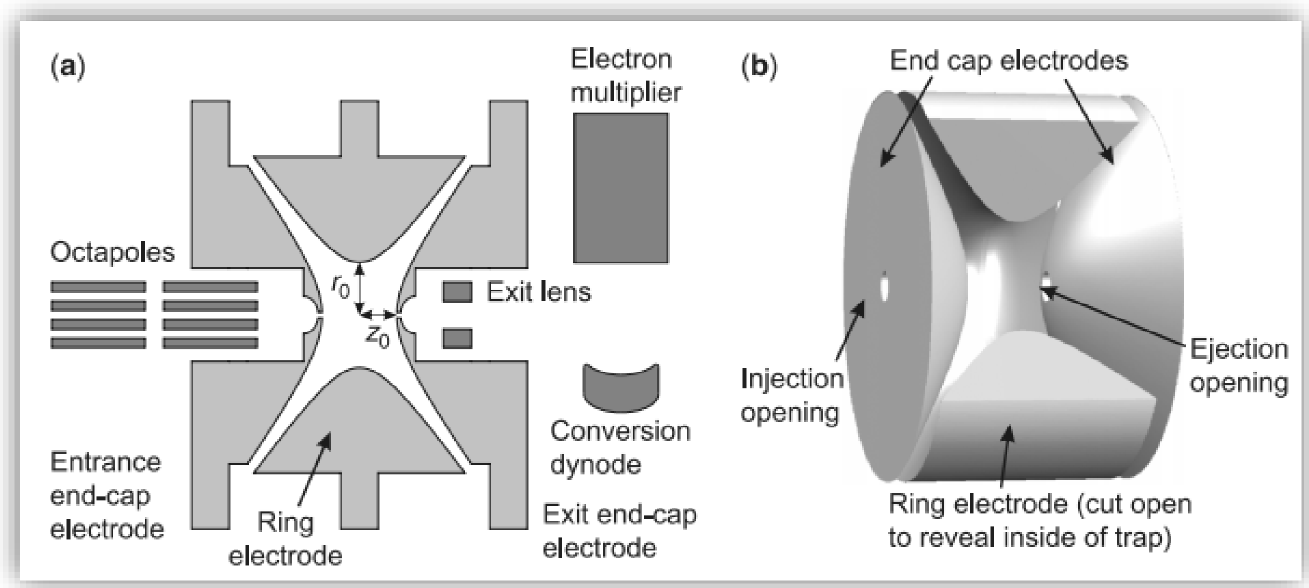

Figure 1.3.4. A cross-section schematic of a quadrupole ion trap mass spectrometer (a), with a three-dimensional perspective view of the quadrupole ion trap (b). *Smith, 2004.*

Ions of different masses are present together inside the trap, and are expelled according to their masses so as to obtain the spectrum. Most instruments have an

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external source. Ions produced in the source are focused through a skimmer and two RF-only octapoles. Differential pumping ensures the vacuum in the trap, while the source is at atmospheric pressure. A gating lens limits the number of injected ions to an acceptable limit. Most commercial traps operate by applying radio-frequency (RF) voltage to the ring electrode. Its frequency is constant, but its amplitude *V* can be varied. Additional RF voltages of selected frequencies and amplitudes can be applied to the end caps. Other ion traps allow the application of DC voltages too.

There are several ways to perform tandem mass spectrometry in an ion trap. Time-dependent rather than space-dependent tandem mass spectrometry occurs in the trap. The general sequence of operations is as follows:

1. Select ions of one m/z ratio, by expelling all the others from the ion trap. This can be performed either by selecting the precursor ion at the apex of the stability diagram or by resonant expulsion of all ions except for the selected precursor.

2. Let these ions fragment. Energy is provided by collisions with the helium gas, which is always present. This fragmentation can be improved by excitation of the selected ions by irradiation at their secular frequency.

3. Analyse the ions by one of the described scanning methods: stability limit or resonant ejection.

4. Alternatively, select a fragment in the trap, and let it fragment further. This step can be repeated to provide MS^n spectra.

The quadrupole ion trap still suffers from mass accuracy problems. The mass range depends on the settings of the main RF voltage and frequency. Unfortunately, selecting a lower frequency in order to extend the m/z range means sacrificing resolution. Typical m/z ranges are 15 to 3000 Th but can be extended with reduction in other performance parameters. As stand-alone instruments they are nowadays of benchtop size and, together with quadrupole mass filters, are considered to be standard low-cost devices, which are commonly coupled to LC systems.

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Detectors: The role of the detector is to convert the energy of incoming particles into a current signal that is registered by the electronic devices and transferred to the computer of the acquisition system of the mass spectrometer. When an incoming particle strikes the detector the energy from the impact causes emission of secondary particles, for example, electrons or photons. To increase the detection sensitivity the ions are often post-accelerated before they strike the detector. A detector preferably should have:

- high efficiency for converting the energy of the incoming ion to electrons or photons;
- a linear response;
- low noise;
- short recovery time;
- minimal variations in transit time.

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2. **AIMS OF THE STUDY**

The general aim of this doctoral thesis was devoted to the analysis of phenolic compounds in two different food samples: Goji berry (*Lycium barbarum L.*) and Hazelnut kernels (*Coryllus avellana L.*).

The works presented here had different defined objectives for each study. In particular, the focus of my thesis was to develop, optimize and validate analytical methods for phenolic bioactive compounds analysis. Special emphasis was given to the optimization of phenolic compounds extraction methods by using different techniques: solid-liquid extraction (SLE), solid-phase extraction (SPE) and pressurized-liquid extraction (PLE), as well as to their chromatographic separation and identification techniques by using HPLC coupled with two different detection systems: UV-visible and mass spectrometry analysers.

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3. **EXPERIMENTAL SECTION**

3.1. *Gogji berry (Lycium barbarum L.) phenolic compounds analysis and extraction*

The Goji berry is the fruit of *Lycium barbarum* (*L. barbarum*) and *Lycium chinense* (*L. chinense*), planta that belong to the family of Solanaceae ranging from tomato and potato to eggplant. The fruit is an ellipsoid orange-red berry with a sweet-and-tangy flavour. *L. barbarum* and *L. chinense* are native to Asia, primarily in the central north region called Ningxia Hui Autonomous Region, spreading also in Chinese regions, Japan, Korea and Taiwan.

L. barbarum has been one of the most important traditional Chinese medicinal plant species for more than 2000 years due to their chemical compositions and health benefits, such as antioxidant, anti-inflammatory and antitumor properties (Kulczynski & Granza-Michalowska, 2016). Extensive research has been carried out concerning the beneficial properties of this fruit, using different methods, particularly, studies in animals and in *vitro* have been shown to have interesting antioxidant, immune-enhancing, radio-protective, anti-aging. For this reason, nowadays, goji berry is known as "superfruits" being very rich in nutrients such as carotenoids, vitamin C and phenolic compounds with high antioxidant capacity (Ionica et al., 2012; Telang et al., 2014; Tang et al., 2012).

According to a review by Qian et al. (2017) the chemical composition of goji berry is characterized by several nutrients. Polysaccharides are the most important group of constituents in goji berry with a molecular weight range of 8-241 kDa and comprise 5-8 % of the dried fruits. Various classes such as phenylpropanoids, carotenoids, alkaloids, phenolic compounds, vitamin C, essential minerals, fatty acids, organic acids and peptides represent the small molecules.

Phenolic compounds are one of the groups responsible of beneficial effects on human health. The interest to elucidate the role of antioxidant phenolic compounds has promoted the research into the characterization of this fruit. In several studies,

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the extraction of Goji berry phenolic compounds has been reported by using conventional SLE (Forino et al., 2016; Magiera & Zareba, 2015; Bondia-Pons et al., 2014; Le et al., 2007) and only one by an innovative extraction technique like MAE (Carvalho et al., 2016). Furthermore, chromatography separation is one of the principal techniques used for the determination of these molecules, particularly, HPLC. The coupling with UV and MS was the most used to accurately determine the concentration of phenolic compounds in Goji berry of *L. barbarum L.* (Dong et al., 2009; Inbaraj et al., 2010; Le et al., 2007; Qian et al., 2004).

A total of 28 phenolic compounds were detected in previous recent studies. The most important classes found are phenolic acids, flavonoids and their derivatives. Among flavonoids, the most common are quercetin and kaempferol derivatives such as quercetin-3-*O*-rutinoside (rutin) (Carvalho et al, 2016; Forino et al., 2016; Magiera & Zareba, 2015; Mikulic-Petkovsek et al., 2012). Myricetin and isorhamnetin-3-*O*-rutinoside have been also reported (Bondia-Pons et al, 2014). Among phenolic acids, cinnamic and benzoic acids have been detected. The most common detected acids are caffeic, vanillic, ferulic, chlorogenic, caffeoylquinic, *p*-coumaric and gallic acids with some derivatives, (Carvalho et al, 2016; Forino et al., 2016; Magiera & Zareba, 2015; Bondia-Pons et al, 2014). As described previously, SLE technique was the most used for phenolic compounds extraction. The sample preparation process was generally characterized by lyophilisation, grinding and then a storage at - 20 °C in the darkness (Mikulic-Petkovsek et al., 2012; Le et al., 2007) and the use of methanol, ethanol, water, mixture at different percentage of them and, furthermore, with some percentage of acid, as extraction solvents (Forino et al., 2016; Bondia-Pons et al., 2014; Mikulic-Petkovsek et al., 2012). Magiera & Zareba (2015) proposed in their study the use of a solid phase extraction for a purification-step after a SLE process, testing two different sorbents to improve clean-up conditions. Twenty phenolic compounds, phenolic acids and flavonoids, were identified and quantified. The analytes were isolated from the

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fruits *L. barbarum L.* by ultrasound-assisted extraction,using 50 % methanol, in conjunction with a purification step by reversed phase SPE cartridge.

Recently, MAE was employed for qualitative and quantitative analysis of bioactive Goji berries compounds. Temperature, proportion of methanol in the solvent mixture and time were studied. Results of this study revelated the presence of 9 phenolic acids and 5 flavonoids (Carvalho et al., 2016). Although there are reports describing the chemical composition of Goji extracts, very little attention has been paid to the optimization of the extraction method. To my knowledge, only one study reported the optimization of phenolic compounds extraction method from Goji berry (Magiera & Zareba, 2015).

This thesis's work was focused on two different studies of phenolic compounds in Goji berry. The first study was based on the evaluation of the effect of the solvent type as the only parameter considered for the optimization of SLE method in Goji berry. The second study was focused on the use, for the first time, of an innovative extraction method, PLE, for the analysis of phenolic compounds in Goji berry. The two studies will be described below.

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3.1.1. Development of a method based on solvent extraction and high performance liquid chromatography for phenolic compounds analysis in Goji berry (*Lycium barbarum L.*)

In this work, the purpose was to investigate the effects of solvent type on the extraction of antioxidant phenolic compounds through a SLE method. Methanol, methanol 3% formic acid, ethyl acetate, ethyl acetate/methanol 3% formic acid (50:50 v/v) and water/methanol (90:10 v/v) were tested, in order to study their effect on the content of total phenolic compounds and on antioxidant activity, by Trolox antioxidant capacity (TEAC) assay, in Goji extracts. HPLC-PDA-ESI-Q-MS was employed for separation, identification and quantification of phenolic compounds in Goji berry samples.

3.1.1.1. Materials and methods

Chemicals

Solvents employed for the extraction procedure and for HPLC-MS analyses were, methanol, ethyl acetate, water, acetonitrile and formic acid and were purchased from Sigma-Aldrich (Milan, Italy). The standard compounds and reagents namely gallic acid, 3,4-dyhydroxybenzoic acid, caffeic acid, vanillic acid, syringic acid, p-coumaric acid, (-) epicatechin, quercetin, kaempferol, potassium persulfate, ABTS(2,2-Azinobis-(3-ethylbenzothiazoline-6-sulfonic) diammoniumsalt), Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2 carboxylic acid) and were purchased from Sigma-Aldrich (Milan, Italy).

Goji berry fruit samples

Fresh fruits, variety *Polonia*, were obtained from an Italian local producer (Lazio).

Goji phenolic compounds solid-liquid extraction (SLE)

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Before extraction, all fruit samples were freeze-dried, ground in a mortar and stored at -20°C in darkness until phenolic compounds extraction. Goji berry samples were subjected to solvent extraction considering the solvent type as the only variable. The extraction method was performed on 1 g of dried fruit sample, extracted with 20 mL of solvent in an ultrasonic bath for 10 minutes at room temperature and then centrifuged at 12100 x g for 10 minutes. The extraction procedure was repeated three times and the obtained liquid phase portions were collected and brought to dryness in a rotary evaporator at 40 °C. The extraction method was carried out testing different solvent or mixture of them, in particular, pure methanol, methanol 3% formic acid, ethyl acetate, ethyl acetate/methanol 3% formic acid (50:50 v/v) and water/methanol (90:10 v/v). The obtained extracts were solubilized in 1 mL of water/methanol (50:50 v/v), centrifuged 12100 x g and filtered on a 0.45 μm pore size syringe filter.

HPLC-PDA-ESI-QMS

The analyses were carried out on Shimadzu Prominence LC-20A instrument (Shimadzu, Milan, Italy) equipped with two LC-20 AD XR pumps, SPD-M10Avp PDA detector, SIL-10ADvp, CTO-20 AC column oven and DGU-20 A_3 degasser. For MS analyses was used a mass spectrometer detector (LCMS-2010, Shimadzu) equipped with ESI interface in negative ionization mode. MS data were acquired by Shimadzu Lcsolution Ver. 3.7 software (Shimadzu).

Goji extracts were analysed at the following analytical conditions. HPLC separations were carried out using a guard column (Ascentis C18 2 cm x 2.1 mm I.D., 3 µm d.p., Supelco) and a column (Ascentis® C18 150 x 2.1 mm I.D., 3 µm d.p., Supelco). Elution was conducted at 35 °C with H_2O/0.1 % HCOOH (solvent A) and methanol (solvent B) at a constant flow of 0.2 mL/min. The gradient mode was applied: 0-20 min 10-15% B, 20-50 min 15-20% B, 50-53 min 20-60%B, 53-58 min 60-90% B, 58-63 min 60-90% B. The injection volume was 2 μL. Data

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were acquired using a PDA in the range 210-400 nm and the chromatograms were extracted at 280, 325 and 370 nm.

Chromatograms were acquired in the MS instrument using ESI as interface in negative ionization mode at the following parameters: nebulizing gas flow (N_2): 1.5 mL/min; Event time: 1 sec; mass spectral range: 100-800 m/z; scan speed: 1000 amu/sec; detector voltage: 1.5 kV; Interface temperature: 250 °C; CDL temperature: 300 °C; Heat Block: 300 °C; Interface Voltage: - 3.50 kV; Q-array: 0.0 V; Q-array RF: 150.0 V.

HPLC-PDA method validation

Standard compounds were chosen, according to the phenolic composition of goji berry fruit, as representative of each class of phenolics. A mixture of nine phenolic standard compounds (gallic acid (GA), 3,4-dyhydroxybenzoic acid (3,4-DHBA), vanillic acid (VA), caffeic acid (CA), syringic acid (SYA), epicatechin (EC), *p*-coumaric acid (*p*-CA), quercetin (Q) and kaempferol (K)) was employed for quantitative analysis and method validation.

The analytical method was validated considering linearity, repeatability (intra-day precision) and reproducibility (inter-day precision), limit of detection (LOD) and limit of quantification (LOQ).

Linearity was determined by the calibration curves obtained from the HPLC analysis of the standard solutions. Stock standard solutions of each compound were prepared at concentration 1000 mg/L in methanol. External standard calibration curve was constructed by using five dilutions. Evaluation of each points was conducted in five replicates and the calibration curve was fitted by linear regression. LOD and LOQ were calculated based on the signal (S)-to-noise (N) ratio by the following equations (3.1.1.1. and 3.1.1.2.):

$$LOD = 3\ S/N \qquad (3.1.1.1.)$$

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LOQ = 10 S/N (3.1.1.2.)

Precision of the method was determined in terms of relative standard deviation percentage (%RSD) for intra-day and inter-day repeatability, related to retention time and peak area. Six injections of standards compounds mixture for a day and for three consecutive days were conducted and then the %RSD of retention times and peak area were calculated.

Trolox equivalent antioxidant capacity assay (TEAC)

The antioxidant capacity was assessed by TEAC method described by Re and co-workers (Re et al., 1999) with some modifications. ABTS$^{\cdot +}$ radical cation was generated by reacting 7 mM ABTS solution and 2.45 mM potassium persulfate solution in the dark at room temperature for 16 h. Subsequently, ABTS$^{\cdot +}$ solution was diluted in ethanol to rich an absorbance of 0.70 ± 0.05 at λ = 734 nm. The reacting mixtures were prepared mixing 10 μL of each extracted sample, opportunely diluted, with 190 μL of ABTS$^{\cdot +}$ solution in a 96-multiwell insert system (Greiner Bio-one, Germany). After 10 min of incubation in darkness, absorbance was recorded at 734 nm by a multifunctional microplate reader (InfiniteM, 200 PRO, Tecan, Italy) in a sample dispensed in triplicate. Trolox was used as reference standard and TEAC data were calculated from the Trolox standard curve (50-600 μmol/L). The antioxidant capacity of goji fruit was expressed as Trolox equivalent antioxidant capacity (TEAC) and reported as μmol of Trolox equivalent per g of dried fruit sample.

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3.1.1.2. Results and Discussion

Chromatographic method development and validation

The HPLC method was validated through a mixture of nine standard compounds belonging to phenolic compounds classes representative of Goji berry reported from literature. Chromatographic separation of phenolic acids and flavonoids was carried out on a C18 stationary phase column (150 x 2.1 mm I.D., 3 μm d.p.), considering the chemical properties of the studied analytes. To optimize the separation, in terms of resolution of analytes, a gradient elution system was developed and the mobile phase composition was studied. Methanol and acetonitrile as organic solvent, also with percentage of formic acid, were evaluated. Best conditions were obtained with water/0.1% formic acid as solvent A and methanol as solvent B. A temperature of 35 °C was selected as compromise between the best resolution and a moderate pressure. In figure 3.1.1. is reported the HPLC-PDA separation of standard phenolic compounds obtained using the best analytical conditions.

The validation was based on the evaluation of precision, expressed as RSD (%) of retention time (t_R) and peak area, limit od detection (LOD), limit of quantification (LOQ) and linearity range (Table 3.1.1.1.).

Retention time and peak area precision of the method was studied by analysing the standard mixture of phenolic compounds six times on the same day and in two different days (n = 12), obtaining a RSD% values of retention times in the range 0.02 - 0.40 %, and RSD % values of area in the range 1.17 - 4.32%.

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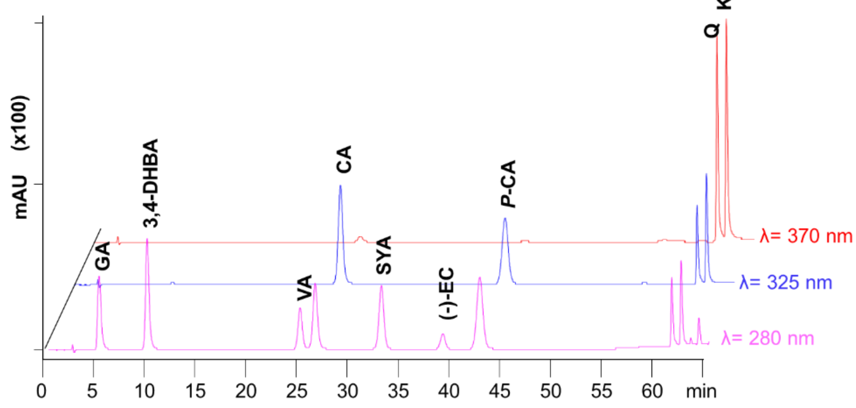

Figure 3.1.1.1. HPLC-PDA chromatograms of standard phenolic compounds.

For quantification of the real sample, calibration curves of gallic acid, 3,4-dyhydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, epicatechin, p-coumaric acid, quercetin and kaempferol were constructed under the same analytical conditions. Good correlation coefficients, R^2, between 0.991 to 0.998 were obtained, without the employment of an internal standard. The linearity of the method was calculated for each standard: 0.20-50 mg/L for gallic acid, caffeic acid and syringic acid; 0.25-50 mg/L for 3,4-dyhydroxybenzoic acid and *p*-coumaric acid; 0.50-50 mg/L for vanillic acid and epicatechin; 1.00-50 mg/L for quercetin and kaempferol. LOD and LOQ values were calculated for all standard compounds in a range of 0.10-0.30 (mg/L) and 0.20-1.00 (mg/L) respectively.

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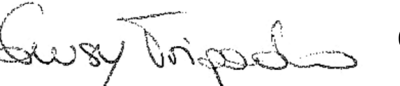
Table 3.1.1.1. Calibration curves, correlation coefficients (R^2), retention time and area precision (RSD, %), limit of detection (LOD) and limit of quantification (LOQ) retention time (t_R ± standard deviation) for each phenolic compound standard injected by HPLC-PDA method.

Compound	t_R (min)	UV/Vis λ max (nm)	Calibration curve	R^2	t_R precision (RSD, %)	Area precision (RSD, %)	LOD (mg/L)	LOQ (mg/L)
Gallic acid	5.03±0.02	280	y = 25309x - 18880	0.996	0.40	2.77	0.10	0.20
3,4-dyhydroxybenzoic acid	9.84±0.02	280	y = 38208x - 47781	0.996	0.20	3.04	0.10	0.25
Vanillic acid	25.10±0.05	280	y = 17043x +8731,8	0.991	0.20	1.79	0.15	0.50
Caffeic acid	26.62±0.05	280	y = 26897x + 45500	0.991	0.19	2.61	0.10	0.20
Syringic acid	33.22±0.06	280	y = 28552x + 16690	0.995	0.18	1.17	0.10	0.20
(-) Epicatechin	39.39±0.08	280	y = 7036x - 5201	0.996	0.20	3.06	0.15	0.50
p-Coumaric acid	42.94±0.07	325	y = 41784x - 27458	0.998	0.16	1.93	0.10	0.25
Quercetin	61.32±0.01	370	y = 38612x - 83600	0.995	0.02	3.63	0.30	1.00
Kaempferol	62.25±0.01	370	y = 40968x - 91314	0.996	0.02	4.36	0.30	1.00

Analysis and optimization of extraction of Goji berry fruits phenolic compounds

Chromatographic separation of phenolic compounds was carried out on the C18 column. Due to the wide range of polarity of molecules, a gradient elution system was developed. Methanol, instead acetonitrile, and water 0.1% formic acid were found to improve the resolution of phenolic acids and flavonoids. Analytes identification in Goji extracts was based on the DAD and mass spectra characteristics, by comparison with data reported in literature, and by comparison of retention time of commercial standards, when available. UV/Vis spectra allowed distinguishing among phenolic compounds belonging to the different classes.



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Additionally, the identity of analytes of my interest was confirmed by HPLC-ESI/MS.

The MS data were achieved in SIM (single ion monitoring) negative mode, comparing m/z of molecules detected with standard compounds and data already reported in literature. Once HPLC-PDA was validated, it was then applied to Goji berry extracts from different extraction solvents for qualitative and quantitative analysis.

A total of seven phenolic compounds belonging to two different classes, 4 phenolic acids and 3 flavonoids, were detected and tentatively identified in analysed samples (table 3.1.1.2.), namely:

Table 3.1.1.2. Identified phenolic compounds in Goji berry extracts.

Peak	**Compound**	**λ_{max} (nm)**	**m/z [M-H]$^-$**
1	3,4-dyhydroxybenzoic acid	280	153
2	chlorogenic acid	325	353
3	caffeic acid	280	179
4	*p*-coumaric acid	325	163
5	quercetin3-*O*-rutinoside (rutin)	280	609
6	quercetin	370	301
7	kaempferol	370	285

Phenolic acids. Peak 1 (Rt: 9.8 min, λ_{max}: 280 nm) was identified as 3,4-dyhydroxybenzoic acid on the basis of the molecular ion ([M-H]$^-$) at m/z 153. This compound was corroborated comparing UV-visible spectrum, retention time and mass spectra after injecting standard compound. Peak 2 (Rt: min, λ_{max}: 325 nm) was tentatively identified as chlorogenic acid on the basis of elution order, UV-visible spectra and MS data, showing of the molecular ion ([M-H]$^-$) at m/z 353.

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Peak 3 (Rt: 26.6 min, λ $_{max}$: 280 nm) was identified as caffeic acid on the basis of the molecular ion ($[M-H]^-$) at m/z 179. This compound was corroborated comparing UV-visible spectrum, retention time and mass spectra after injecting standard. Peak 4 (Rt: 43.0 min, λ $_{max}$: 325 nm) was identified as *p*-coumaric acid on the basis of the molecular ion ($[M-H]^-$) at m/z 163. This compound was corroborated comparing UV-visible spectrum, retention time and mass spectra after injecting standard.

Flavonoids. Peak 5 (Rt: min, λ $_{max}$: 280 nm) was tentatively identified as quercetin-*O*-rutinoside (rutin) on the basis of elution order, UV-visible spectra and MS data, showing a molecular ion ($[M-H]^-$) at m/z 609. Peak 6 (Rt: 61.3 min, λ $_{max}$: 370 nm) was identified as quercetin comparing the retention time and mass spectra to standard compound, showing a molecular ion ($[M-H]^-$) at m/z 301. Peak 7 (Rt: 62.2 min, λ $_{max}$: 370 nm) was identified as kaempferol on the basis of the molecular ion ($[M-H]^-$) at m/z 285. This compound was corroborated comparing UV-visible spectrum, retention time and mass spectra after injecting standard.

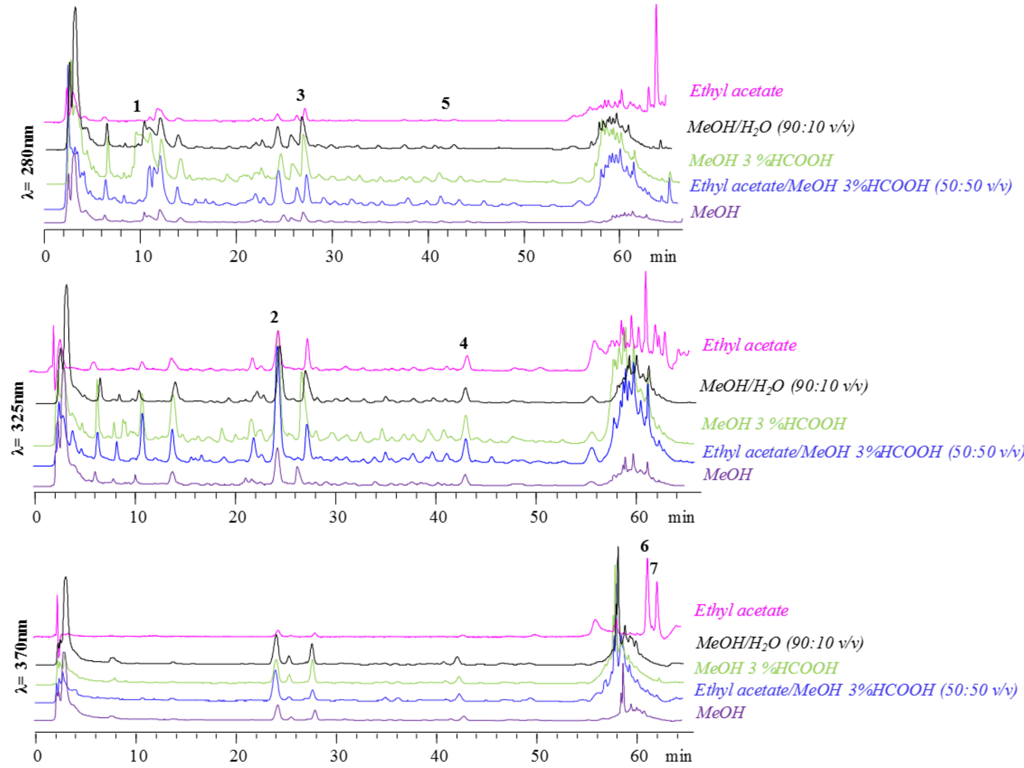

Figure 3.1.1.2. HPLC-PDA chromatograms of the different phenolic compounds extracts.

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Seven phenolic compounds identified in Goji berry, variety *Polonia*, were mainly rich in phenolic acids and flavonoids, in good agreement with previous published works (Carvalho et al., 2016; Forino et al., 2016; Bondia-Pons et al., 2014; Mikulic-Petkovsek et al., 2012). Figure 3.1.1.2. shows HPLC chromatograms of all extracts obtained at 280, 325 and 370 nm. Every colour corresponds to a solvent used for phenolic compounds extraction. As it can be seen, the qualitative profile changes depending on the solvent used for the extraction. Subsequently, it was possible to observe the different quantitative profile of the five extracts. The 7 detected phenolic compounds were quantified by HPLC-DAD through the interpolation of peak area value with calibration standard curve. Each phenolic compound was expressed with the respective standard when it was available. When this was not possible, the phenolic compounds were expressed with a representative and available standard compound of each group. Quantitative results are shown in table 3.1.1.3. Concentration values were calculated as mean value of analysis in triplicate and the content of total phenolic compounds was calculated as the sum of all compounds detected for each extract sample. Phenolic compounds concentration was expressed as μg per 100 g of dried fruit weight.

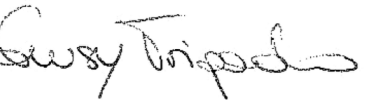

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Table 3.1.1.3. Amount of the seven identified phenolic compounds according the different extraction solvent extraction.

		Content of phenolic compounds (µg/100 g DW)				
N°	**Compound**	**Ethyl acetate**	**MeOH**	**MeOH 3%HCOOH**	**Ethyl acetate/MeOH 3%HCOOH (50:50 v/v)**	**MeOH/H_2O (90:10 v/v)**
1	3,4-dyhydroxybenzoic acid	0,13 ± 0.21	< LOQ	< LOQ	0.56 ± 0.21	1.19 ± 1.09
2	chlorogenic acid	0.07 ± 0.22	4.26 ± 2.29	5.78 ± 0.09	2.32 ± 0.22	4.09 ± 2.43
3	caffeic acid	< LOQ	3.65 ± 0.29	10.36 ± 0.86	4.25 ± 0.31	9.45 ± 0.91
4	*p*-coumaric acid	0.13 ± 0.16	1.69 ± 0.30	1.70 ± 0.22	1.18 ± 0.16	2.06 ± 0.11
5	quercetin-*O*-rutinoside (Rutin)	< LOQ	0.27 ± 0.02	047 ± 0.01	0.29 ± 0.11	0.30 ± 0.01
6	quercetin	0.45 ± 0.11	ND	< LOQ	< LOQ	ND
7	kaempferol	0.35 ± 0.11	ND	< LOQ	< LOQ	ND
Total Phenolic Compounds		**1.13**	**9.87**	**18.32**	**8.61**	**17.10**

Dried weight (DW); ND= not detected

Quantitative results demonstrated that the different solvent extraction influenced the amount of the seven phenolic compounds in each extract. As can be seen in table 3.1.1.3., methanol/3% formic acid was the solvent presenting the highest concentration of detected phenolic compounds, while ethyl acetate the lowest one. Methanol, methanol 3% formic acid, ethyl acetate/methanol 3% formic acid (50:50 v/v) and water/methanol (90:10 v/v) were able to extract 3,4-dyhydroxybenzoic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid and rutin, even if in different concentration. Ethyl acetate was the only one of the tested solvents able to extract quercetin and kaempferol at concentration higher than the LOQ of the method. However, it was able to extract 3,4-dyhydroxybenzoic acid, chlorogenic acid, *p*-coumaric acid and rutin with low concentration. This fact could be attributed to ethyl acetate, which has a suitable polarity to extract phenolic compounds belonging to several classes, such as phenolic acids and flavonoids (Ignat et al., 2011). In general, the content of phenolic compounds detected in Goji berry

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studied in the present work showed a different profile respect to other studies reported in literature (Magiera & Zareba, 2015; Bondia-Pons et al., 2014; Mikulic-Petkovsek et al., 2012). The difference could be due to fruit chemical composition that is clearly influenced by the different geographic origin, climate, soil and cultivations method.

Antioxidant activity was tested with TEAC assay by using the free ABTS radical scavenging method. Trolox was used as reference standard and the results were reported as μmol Trolox equivalent per g of dried fruit weight. Figure 3.1.4. shows the results. Methanol/3% formic acid extract was the solvent presenting the highest TEAC value (56,71 μmol TE/g DW), while ethyl acetate extract was the lowest one (0,56 μmol TE/g DW). A positive correlation between concentration of detected phenolic compounds and sample antioxidant activity was observed.

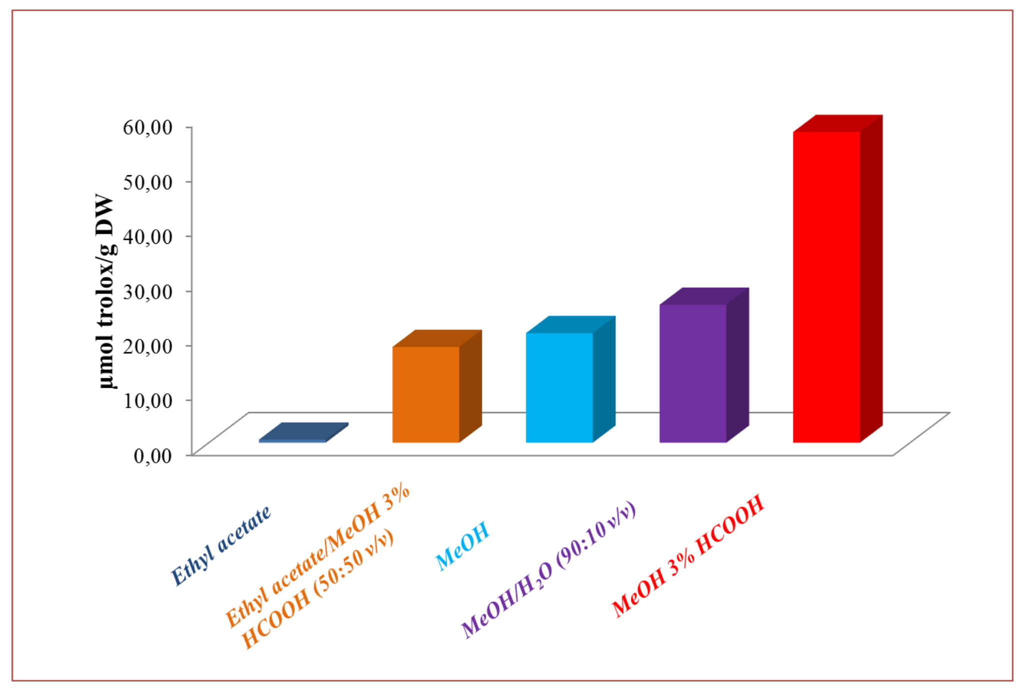

Figure 3.1.4. Antioxidant activity of goji phenolic compounds extracts obtained by using different solvents.

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3.1.1.3. Conclusions

Results demonstrated a different profile and content of phenolic compounds according to the choice of solvent extraction. A method for the analysis of Goji phenolic compounds belonging to different classes has been developed and validated. The optimized HPLC-DAD-ESI-QMS method, for qualitative and quantitative analyses of phenolic acids and flavonoids in fruits of a particular variety of *L. Barbarum L.*, called *Polonia*, was successfully applied based on extraction solvents. The method allowed qualitative and quantitative analysis of phenolic acids and flavonoids in the different extracts of the same fruit sample. Furthermore, the evaluation of antioxidant capacity by TEAC assay allowed obtaining a good correlation between the phenolic compounds concentration and antioxidant activity.

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3.1.2. **Pressurized liquid extraction of phenolic bioactive compounds in Goji berry (Lycium barbarum L.): Optimization by response surface methodology**

PLE extraction is an innovative extraction method, also known as accelerated solvent extraction (ASE), which employs organic solvents at high pressure and temperature above their boiling point. This extraction technique allows obtaining higher yields than those achieved by conventional extraction techniques, in a shorter time and with less solvent consumption. Furthermore, the use of food-grade solvents such as ethanol and water can be proposed as a green approach for the extraction of bioactive compounds.

As reviewed recently by Ameer et al. (2017), PLE is one of the techniques that have been used for the green extraction of polyphenols from many plant materials and fruits, however, to the best of my knowledge it has never been used before to investigate Goji berries.

This study aimed to apply for the first time the use of PLE for the extraction of phenolic compounds from Goji berry fruits. To do this, optimization of PLE conditions was carried out using mixtures water-ethanol as green solvent, by a design of experiments (DOE) based on response surface methodology (RSM). The impact of green solvent composition and temperature on total yield, total phenolic content (TPC) and total flavonoid (TF) concentration, as well antioxidant activity of the obtained extracts was evaluated. The extraction efficiency of PLE treatment in comparison with conventional solid-liquid extraction method was also studied. Finally, the extracts were analyzed by HPLC-DAD-MS/MS for the characterization of the polyphenols present in the samples.

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3.1.2.1. Materials and methods

Chemicals

Absolute ethanol for extractions was purchased from VWR International (Leuven, Belgium). ACN with HPLC–MS quality, was purchased from Fisher (Thermo Fisher Scientific, Leicestershire, UK). Ultrapure water with a resistivity value of 18.2 MΩ was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Folin–Ciocalteu reagent, ABTS (2,2-azinobis (3-ethylbenzothiazoline- 6-sulfonate)), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate, aluminum chloride and the standard compounds: rutin, p-coumaric acid, kaempferol, 3,4-dyhydroxybenzoic acid, quercetin, chlorogenic acid and caffeic acid were from Sigma–Aldrich (Steinheim, Germany). Sea sand was from Panreac (Barcelona, Spain).

Goji berry fruit samples and sample preparation

Commercial samples of Goji berry fruits, produced in Tibet (China), were purchased in herbalist's shop in Spain. Fresh fruits of varieties Polonia, Bigol and Selvatico mongolo were obtained from an Italian local producer (Lazio). Before extraction, all fruit samples were freeze-dried, ground in a mortar and stored at -20°C in darkness until phenolic compounds extraction.

Pressurized liquid extraction (PLE) method

PLE extraction of Goji berry fruits was performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, USA), equipped with a solvent controller.

Extractions were performed at different extraction temperatures and green solvent compositions (namely, ethanol/water), according to the experimental design described in the next section. Dried fruit sample (1 g) was mixed with 3 g

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of sea sand and placed into an 11 mL volume extraction cell. The extraction process was carried out under the following conditions: time, 20 min; pressure, 10 MPa (1500 psi), heat-up time, 5 min; static extraction time, 5 min; flush volume, 60%; purge, N_2 for 60 s; number of cycles, 1. The purged sample extract was collected into a collection vial by compressed gas.

The extract was protected from light and stored at -20°C. Samples extracted with 100% ethanol were dried under N_2 stream, samples extracted with ethanol and water mixtures were first dried under N_2 and then freeze-dried in a freeze-dryer (Lyobeta, Telstar, Terrassa, Spain), while those extracted with water were directly freeze-dried.

Experimental design and statistical analysis

A factorial experimental design 3^2 was employed for PLE optimization, considering extraction yield, total phenolic content (TPC), total flavonoid (TF) and antioxidant capacity (TEAC) as response variables to study the effects of temperature (50, 115, 180°C) and percentage of ethanol (0, 50, 100% in water) as independent variables. A total of 12 experiments were conducted in a randomized order for commercial Goji berry samples: nine points of the factorial design and three additional center points to consider the experimental errors. The experimental design and data analysis were carried out using RSM with Statgraphics Centurion XVI® software (Statpoint Technologies, Warrenton, Virginia, USA). The effects of the independent factors on the response variables in the separation process were evaluated at 95% confidence level ($p \leq 0.05$) for all the variables. The significance of the mathematical model was evaluated using ANOVA. A Pareto diagram was used to represent the effect of factors where bar color shadings indicate a positive or negative effect caused in the response variable. Moreover, a response surface plot was built to predict the most favorable PLE conditions to extract phenolic compounds from Goji berry. Optimum PLE extraction conditions were achieved by a multiple response optimization by the combination of experimental factors,

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aiming to maximize the desirability function for the responses in the extracts. To corroborate the suitability of predicted optimal conditions by the mathematical model, fruits were extracted under optimal conditions, in triplicate. Afterwards, the optimum extraction conditions obtained for commercial fruit were used for the extraction of Goji berry from *Polonia*, *Selvatico mongolo* and *Bigol* varieties.

Conventional solid-liquid extraction method

A conventional solid-liquid extraction using methanol was used as benchmark method, considering that methanol is the most commonly used solvent for the conventional solid-liquid extraction of phenolic compounds from fruits and vegetables (Ignat et al., 2011; Garcia-Salas et al., 2010), and that it has been used by several authors for the extraction of phenolic compounds from Goji berries (Protti et al., 2017; Carvalho et al., 2016; Magiera & Zareba, 2015) also carried out in triplicate for comparison with PLE. Briefly, 1 g of dried fruit sample was extracted with 20 mL of methanol under agitation in ultrasonic bath for 5 minutes and then centrifuged at 3500 × g for 10 minutes. The extraction was repeated three times and the obtained supernatants were collected together. The solvent was evaporated under vacuum at 40°C. The residue was dissolved in 1 mL of mixture methanol/water (50:50 v/v), centrifuged at 12100 × g for 5 minutes and filtered through a 0.45 μm pore size syringe filter. The extraction was carried out in triplicate for each sample.

Total yield

Glass vials (40-60 mL) were weighed before collecting the extracts and after drying the extracts, to calculate the extract mass. Then the global extraction yields obtained by PLE and conventional methods were calculated as the ratio between

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the extract mass in dry basis (x) and the mass of initial dry sample fed into the extraction cell (y). The total yield was calculated as following:

extraction yield % (w/dw) = x (extract mass)/y (initial mass) x 100

Total phenolic content (TPC)

The total phenolic content was determined according to Folin–Ciocalteu assay (Kosar et al., 2005) using gallic acid as standard. Briefly, 10 µL (concentration 10 mg/mL) of extract (adequately dissolved) were added to 50 µl of Folin reagent. After 1 min, 150 µL of a 20% (w/v) aqueous sodium carbonate solution was added and the volume was made up to 1 mL with water. After 2 h of incubation at room temperature in darkness, 300 µL of the mixture was transferred into a microwellplate. The absorbance of solutions was measured at 760 nm with a Synergy HT microplate reader, by Bio-Tek Instruments (Winooski, VT, USA). TPC was calculated from a calibration curve using gallic acid as standard (0.031–1.000 mg/L). The results were expressed as mg of gallic acid equivalents (GAE) per g of dry fruit. All the analyses were performed in triplicate.

Total flavonoids (TF)

The TF content was measured by the aluminum chloride colorimetric assay (Furnari et al., 2006). Briefly, 100 µL of extract (concentration 2.5 mg/mL) were added to 140 µL of methanol and 60 µL of an 8 mM aqueous solution of AlCl3. After 30 min of incubation in darkness, the absorbance was measured at 425 nm. TF was calculated from a calibration curve using quercetin as standard (from 1 to 14 mg/L). Results were expressed as mg of equivalent of quercetin per gram of dry fruit. All the analyses were performed in triplicate.

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Trolox equivalents antioxidant capacity (TEAC) assay

Antioxidant capacity was measured using the Trolox Equivalents Antioxidant capacity (TEAC) methodology (Re et al., 1999) with some modifications. $ABTS^{\bullet +}$ (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) radical cation was produced by reacting 7 mM ABTS solution and 2.45 mM potassium persulfate solution in the dark at room temperature for 16 h. The aqueous $ABTS^{\bullet +}$ solution was diluted with 5 mM sodium phosphate buffer at pH 7.4 till an absorbance of 0.7 (±0.02) at 734 nm. The extracts were prepared at five different concentrations and 10 µL of each was mixed with 1 mL of $ABTS^{\bullet +}$ solution and 300 µL of the mixture were transferred to a 96-multiwell microplate. After 45 min of incubation in darkness the absorbance was recorded at 734 nm in a Synergy HT Microplate reader, by Bio-Tek Instruments (Winooski, VT, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as a reference standard and TEAC values were calculated from the Trolox (from 0.25 to 2 mM) standard curve. The percentage of inhibition of ABTS was calculated for Trolox standard and samples using values of absorbance obtained (absorbance ABTS, absorbance standard (Trolox) and absorbance of sample):

%Inhibition = [(AABTScontrol -AABTSstandard)/AABTScontrol] *100,

%Inhibition = [(AABTScontrol -AABTSsample)/AABTScontrol] *100

These values were obtained from five different concentrations of each sample tested in the assay giving a linear response between 20 and 80% of the blank absorbance. All the analyses were performed in triplicate. Results were expressed as mmol of equivalent of trolox per gram of dry fruit.

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HPLC-DAD-ESI-IT-MS of Goji berry fruits

High pressure liquid chromatography- diode array detector- tandem mass spectrometry (HPLC-DAD-MS/MS) analysis of the extracts was carried out on an Agilent 1200 series liquid chromatograph (Agilent Technologies, Santa Clara, CA) consisting in a binary pump, an autosampler and a diode-array detector (DAD), directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) with an electrospray ionization (ESI) interface. HPLC-DAD-MS/MS method was based on previously studies conducted for phenolic compounds analysis in Goji berry (Carvalho et al., 2016; Magiera & Zareba, 2015) with minor modifications. HPLC separation was carried out using a C18 reversed-phase column (150 × 4.6 mm i.d., 3 µm particle size, from ACE Ltd, Aberdeen, Scotland) with a Security Guard Cartridge of the same material. Elution was conducted at 30 °C with water/0.1% formic acid (solvent A) and acetonitrile/0.1% formic acid (solvent B) at a constant flow rate of 0.6 mL min. The gradient program was as follows: 0 min, 0%B; 10 min, 5%B; 15 min, 10%B; 45 min, 60%B; 65 min, 70%B; 70 min, 100%B; 72 min 0%B. The injection volume was 10 µL. Data were acquired using photodiode array detector in the range 200-700 nm and the chromatograms were extracted at 280 and 370 nm. The instrument was controlled by LC ChemStation 3D Software Rev. B.04.03 (Agilent Technologies, Santa Clara, CA, USA).

Chromatograms were acquired in the MS instrument using negative ionization mode with the following parameters: capillary voltage, −3.5 kV; drying temperature, 350 °C; drying gas (N2) flow rate, 9 L min−1; nebulizer gas pressure, 40 psi. Full scan was acquired in the m/z range 50–2200. Automatic MS/MS analyses were also performed, fragmenting the two highest precursor ions (10,000 counts threshold; 1 V Fragmentor amplitude).

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3.1.2.2. Results and Discussion

PLE of Goji berry phenolic compounds by a multiresponse surfaces

An experimental design was applied to optimize the PLE extraction conditions. The optimization of the PLE procedure was performed using freeze-dried commercial Goji berry and the optimal conditions found were then applied for the other three varieties studied in the present work. Therefore, a factorial experimental design at three levels was set-up as follows: temperature (50, 115, 180 °C) and ethanol percentage in water (0%, 50%, 100%). Employing this design, the influence of extraction conditions on four different response variables was studied.

RSM was employed to maximize four different variables, extraction yield, TPC, TF and antioxidant activity (TEAC) through the optimization of two independent variables, extraction temperature (50-180 °C) and solvent composition (water/ethanol mixtures). Extraction time and pressure were kept constant at 20 min and 1500 psi, respectively, taking into account results obtained in previous published works (Rodriguez-Pérez et al., 2016; Herrero et al., 2011).

The results are reported in Table 3.1.2.1. As can be seen in the table, the extraction yields, TPC, TF and TEAC values obtained for different temperatures and solvent compositions were statistically analyzed. Regression analysis was performed on the experimental data and the coefficients of the model were evaluated for significance. The response variables showed a different behavior depending on the extraction conditions. Thus, the highest global yields of the PLE extracts were achieved with 50% ethanol in water as extraction solvent. Moreover, the experimental data showed that an increase in the temperature from 50 to 180 °C led to a substantial increase in the global extraction yield for all the different extraction solvents. The lowest total phenolic content (TPC of 5.72 mg GAE/g)

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was found in the extract obtained with pure water at 50 °C, and the highest TPC value (74.84 mg GAE/g) was obtained for 100% ethanolic extract at 180 °C. For all employed solvents, TPC increased with temperature. Summarizing, the solvents that extracted more phenolic compounds are, in decreasing order: pure ethanol, ethanol + water (50% v/v), pure water. In a similar manner as TPC, TF values showed a minimum of 0.16 mg QE/g extract when pure water at 50 °C was used and a maximum value of 3.20 mg QE/g extract with 100% ethanol at 180 °C. In addition, TF increased with temperature for all tested solvents. Respect to the antioxidant activity, the highest TEAC value of 1.21 was found in the extract obtained with 50% ethanol solvent at 180 °C, which are the same conditions that gave the highest yield. Also antioxidant activity increased with the temperature except for 100% ethanol as extraction solvent. Thus, based on TPC and TF results, it can be concluded that the higher yields obtained with 50% ethanol as extraction solvent are due to the extraction of other non-phenolic compounds, which have antioxidant activity.

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Table 3.1.2.1. PLE design of three-level two-factor experimental design (3^2) for commercial Goji berries extraction: results observed in the studied response variables (yield, TPC, TF and TEAC).

	Factors		**Response Variables**			
Run	**T (°C)**	**Solvent (%)[1]**	**Yield (%)**	**TPC (mg GAE/g)**	**TF mg (Eq quercetin/g)**	**TEAC (mmol Eq Trolox/g)**
1	50	0	12,74	5,72	0,16	0,22
2	115	0	28,81	8,31	0,25	0,30
3	180	0	62,70	28,80	1,68	1,05
4	50	50	41,74	8,35	0,37	0,27
5	115	50	56,11	10,15	0,58	0,36
6	115	50	51,26	11,74	0,51	0,40
7	115	50	53,46	12,54	0,73	0,37
8	115	50	56,58	12,14	0,55	0,38
9	180	50	74,84	35,60	2,86	1,21
10	50	100	6,43	6,97	0,38	0,15
11	115	100	46,74	44,83	1,82	0,49
12	180	100	59,22	75,84	3,20	0,47
Individual Optimum	180	52,6	**78,84**			
	180	100		**75,30**	**3,48**	
	180	77,2				**1,05**

% ethanol in water

Figure 3.1.2.1. shows the standardized Pareto charts for the four response variables studied and their corresponding response surface plot. Different bar color shadings indicate the positive (grey) and negative (black) effects whereas the vertical line tests the significance of the effects at the 95% confidence level (see Figure 1).

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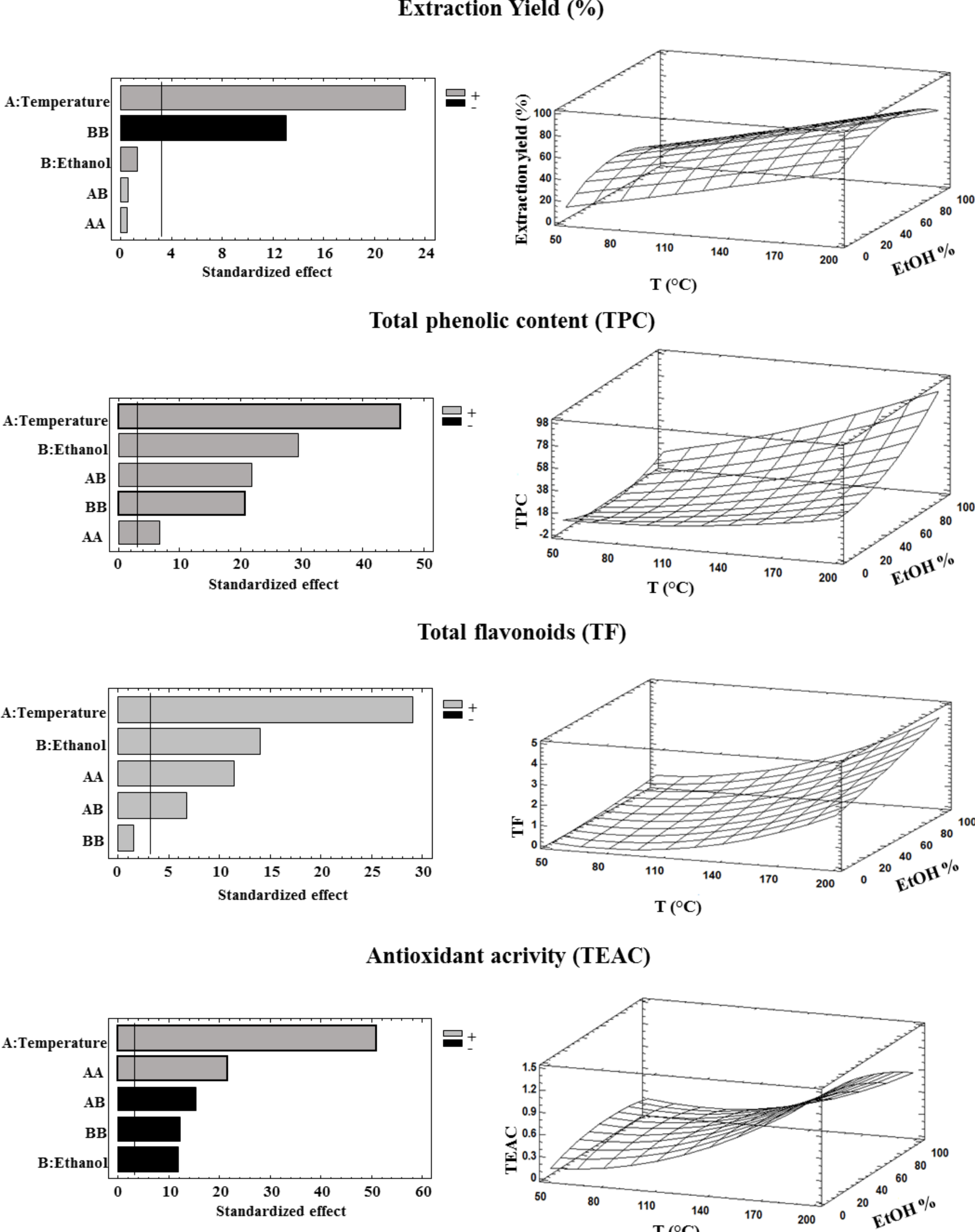

Figure 3.1.2.1. Standardized Pareto charts for: extraction yield, TPC, TF and TEAC according to the experimental factors temperature and ethanol percentage.

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Extraction yield was positively influenced by temperature. This behavior can be explained by (i) improvement of mass transfer from the sample to the extraction solvent, (ii) increased solubility of compounds and (iii) reduction in solvent viscosity giving more penetration of the solvent in the matrix (Syahariza et al., 2017). The composition of the solvent also showed a significant effect (see Figure 3.1.2.1.), obtaining the highest yield with the mixture of water:ethanol (50:50, v:v). According to the mathematical model, 180 °C and 52.6 % ethanol were the optimum conditions to maximize the extraction yield.

TPC and TF values showed the same behavior. These responses were positively influenced by temperature giving pure ethanol the highest values (see Figure 3.1.2.1.). In this case, according to the mathematical model 180 °C and 100% ethanol were the optimum conditions to maximize TPC and TF values. The TEAC values were also positively influenced by temperature, while the increasing amount of EtOH in the solvent showed a significant negative effect on TEAC values as shown in Figure 3.1.2.1. According to the mathematical model, the highest TEAC values can be achieved by using 180 °C and 77% ethanol as optimum conditions.

Since all response variables had equal importance, a multiple response optimization was carried out including extraction yield, TPC, TF and TEAC (see Figure 3.1.2.2.). The optimum PLE conditions predicted by the model were as follows: 180°C and 86% ethanol with a good desirability value of 0.815.

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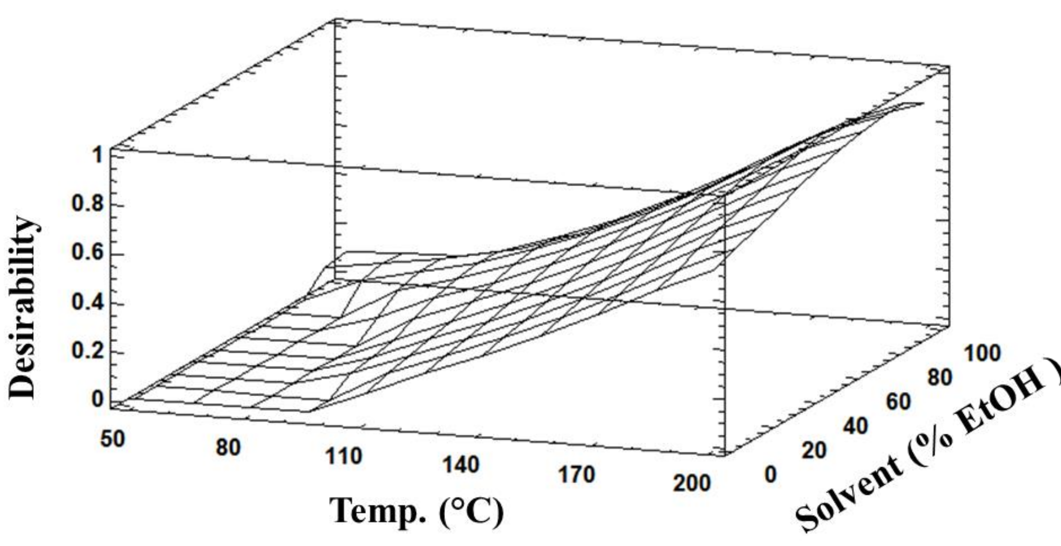

Figure 3.1.2.2. Response surface plot for the effects of solvent (percentage of ethanol in water) and temperature on the overall desirability in PLE extractions.

To corroborate the usefulness of our model, three replicate extractions for commercial Goji berry were carried out under the optimum PLE conditions predicted by the model. Table 3.1.2.2. shows the predicted and the experimental values. As can be seen, the values experimentally obtained for yield, TPC, TF and TEAC (77.64, 65.98, 3.02 and 0.80, respectively) were in good agreement with those theoretically predicted (69.95, 62.22, 3.20 and 0.78, respectively), besides, all the RSD values were lower than 8%.

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Table 3.1.2.2. Predicted and observed values of each individual response variable (i.e., yield, TPC, TF and TEAC) for commercial Goji berries PLE extraction.

Sample	Response	Predicted	Observed	SD	RSD
Commercial	Yield %	69,95	77,64	5,44	7,37
	TPC, mg GAE/g	62,22	65,98	2,66	4,15
	TF, mg QE/g	3,20	3,02	0,13	4,16
	TEAC, mmol TE/g	0,78	0,80	0,02	2,25

Once the experimental design was validated for the commercial Goji berries samples, these parameters were applied for the extraction of phenolic compounds from the other three varieties: *Selvatico mongolo*, *Bigol* and *Polonia*. The results are given in Table 3.1.2.3.

Among the total yield, the results obtained under the PLE optimum conditions for the four fruits samples analyzed in the present study indicate potential differences between commercial (origin: China) and fresh berry varieties (origin: Italy). The three fresh fruit samples (*Selvatico mongolo*, *Bigol* and *Polonia*) gave a lower yield values respect to commercial fruit sample (see Tables 3.1.2.2. and 3.1.2.3.). Various factors contribute to the phenolic profile of plants: genotype, site location, climatic conditions and year. It is probable that fresh berry fruits, growing in a different habitat with different treatment, have a different phenolic content. In addition, among TPC, TF and TEAC, results obtained were in good agreement with those theoretically predicted (low RSD values). However, interestingly, an appreciable relationship between total phenols, total flavonoids and antioxidant activity was observed.

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Table 3.1.2.3. Observed values of each individual response variable (i.e., yield, TPC, TF and TEAC) after PLE extraction of different Goji berries varieties (*Polonia*, *Selvatico mongolo* and *Bigol*).

Sample	Response	Observed	SD	RSD
Polonia	**Yield %**	32,85	0,85	2,58
	TPC mg GAE/g	66,02	3,07	4,64
	TF mg QE/g	2,73	0,25	9,33
	TEAC mmol TE/g	0,83	0,02	2,60
Selvatico mongolo	**Yield %**	26,92	3,31	12,29
	TPC mg GAE/g	75,15	3,03	4,04
	TF mg QE/g	3,15	0,20	6,34
	TEAC mmol TE/g	1,06	0,07	6,48
Bigol	**Yield %**	26,57	1,75	6,60
	TPC mg GAE/g	59,18	1,13	1,91
	TF mg QE/g	1,66	0,07	4,48
	TEAC mmol TE/g	0,86	0,05	5,33

Effects of PLE vs. SLE for phenolic compounds extraction from Goji berry

Solid-liquid extraction (SLE) is commonly used for extraction of phenolic compounds using solvents as methanol, ethanol and ethyl acetate (Herrero et al., 2011). This traditional extraction method consists of a direct extraction with an appropriate solvent using an extractor, homogenizer or ultrasonic bath for a given time. Often, traditional methods have some disadvantages like long extraction times and the massive use of solvents. In the present work, PLE is proposed as an alternative method to reduce common disadvantages like extraction time and solvents usage. In this work, a conventional solid-liquid extraction using methanol

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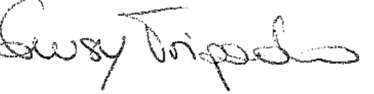
was used as benchmark method, since methanol is the most widely used solvent for the extraction of phenolic compounds (Ignat et al., 2011; Garcia-Salas et al., 2010) and has been used previously for the extraction of phenolic compounds from Goji berries (Protti et al., 2017; Carvalho et al., 2016; Magiera & Zareba, 2015). Table 3.1.2.4. shows the results obtained for phenolic compounds extraction from commercial Goji berry samples using PLE and SLE. The results presented in Table 3.1.2.4. show that PLE is quite more effective than the conventional SLE method for phenolic and flavonoid extraction. The PLE extract also presented higher antioxidant activity than SLE while PLE gave a slightly lower extraction yield than SLE. These results confirm that the combined application of high pressure and temperature in PLE is effective in the recovery of phenolic compounds from Goji berries when compared to a traditional method. By considering economic and practical aspects, PLE can be considered clearly advantageous respect to SLE for Goji berry phenolic compounds extraction. Furthermore, the use of environmentally friendly solvents as ethanol and water can be also proposed as a green approach compared to the use of methanol in conventional SLE method.

Table 3.1.2.4. Comparison of the four response variables (yield, TPC, TF and TEAC) obtained after using PLE or conventional solid-liquid extraction (SLE) of Goji berries.

Extraction method	**Time**	**Solvent**	**Yield %**	**TPC mg GAE/g**	**TF mg QE/g**	**TEAC mmol TE/g**
SLE	45	Methanol	81,63	40,82	0,61	0,69
PLE	20	Ethanol/Water	77,64	65,98	3,02	0,80

Determination of major phenolic compounds by HPLC-DAD-MS/MS

HPLC-DAD-MS/MS was used to analyze the PLE Goji berry extracts obtained at the aforementioned optimum PLE conditions in order to separate and identify the phenolic compounds in the extracts. Considering previously studies (Carvalho



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et al., 2016; Magiera & Zareba, 2015), the optimization of analytical method was performed. Taking into account the chemical structure of phenolic compounds, the separation was optimized using a C18 stationary phase. Due to the wide range of polarity of phenolic compounds, a gradient elution was developed.

Different mobile phases were tested to optimize the analytical method and acetonitrile (instead methanol) was found to improve the chromatographic peak resolution of phenolic compounds. In addition, the use of formic acid as modifier was proved in different amounts to improve the peak shape. The best separation was achieved by using water/0.1% formic acid as solvent A and acetonitrile/0.1% formic acid as solvent B. Data on the phenolic compounds found in the different samples are summarized in Table 3.1.2.5. Figure 3.1.2.3. shows representative chromatograms obtained for the four studied Goji berry samples at 280 and 370 nm. As it can be seen, the qualitative profile varied depending on the sample studied. In order to improve compound identification, the MS/MS spectrum was recorded in negative ionization mode because of the best performance of for phenolic compounds in negative ionization mode than in positive one. Compounds identification was based on DAD and fragmentation mass spectra (MS/MS), comparison of retention time of commercial standards when available and data reported in the literature.

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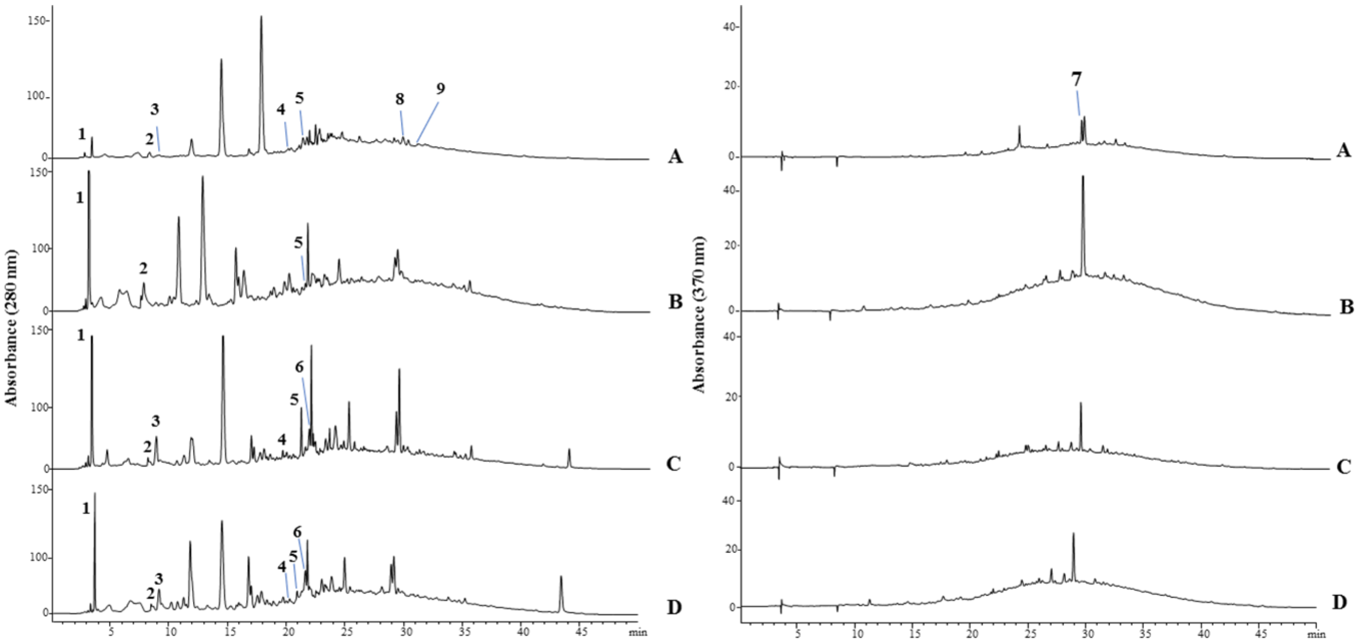

Figure 3.1.2.3. HPLC-DAD chromatograms (λ=280 and 370 nm) obtained under the optimal PLE conditions (180°C and 86% ethanol in water) corresponding to: A) Commercial fruit, B) *Selvatico mongolo*, C) *Bigol* and D) *Polonia* Goji berry extracts.

A total of nine phenolic compounds were tentatively identified in the four extracts including hydroxycinnamic acids and their derivatives and flavonols, namely:

Hydroxycinnamic acids derivatives. Peak 1 (Rt: 3.7 min, λ max: 290 nm) was identified as caffeoylhexose on the basis of the fragmentation of the molecular ion ([M-H]-) at m/z 341 showing a loss of 162 amu, [M-H-hex]- at m/z 179 (Maatta et al., 2003). Peak 2 (Rt: 8.0 min, λ max: 250 nm) was identified as p-coumaroylquinic acid having a molecular ion ([M-H]-) at m/z 337 and MS/MS fragmentation at m/z 179 as major fragment (Gouveia & Castilho, 2011). Peaks 9 (Rt: 21.0 min, λmax: 280 nm) and 10 were identified as two isomers of the dicaffeoylquinic acid having a molecular ion ([M-H]-) at m/z 515 and 514 respectively. Mass spectrum of these compounds showed fragmentation ions at m/z 353, m/z 191, m/z, m/z 179 and m/z 173 that is the characteristic mass spectrum of this chlorogenic acid (Gouveia & Castilho, 2011).

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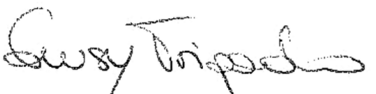
Flavonols. Three flavonols were tentatively identified, quercetin-O-rutinoside (rutin), quercetin-3-O-glucoside and isorhamnetin 3-O-rutinoside. The identity of Peak 18 (Rt: 28.8 min, λmax: 280,360 nm) as rutin was corroborated comparing UV-visible spectrum, retention time and MS/MS data after injecting the standard (Maatta et al., 2003). Quercetin-3-O-glucoside was assigned at peak 20 (Rt: 29.8 min, λmax: 300 nm) (Kajdžanoska et al., 2010). The MS spectrum showed a molecular ion ([M-H]-) at m/z 462.2 and MS2 fragmentation ion at m/z 301 as quercetin ion. Peak 22 (Rt: 30.3 min, λmax: 240, 290 and 340 nm) was identified as isorhamnetin 3-O-rutinoside. This identification was confirmed by the mass spectral data with molecular ion ([M-H]-) at m/z 623 and MS2 fragment at m/z 315 (Mikulic-Petkovsek et al., 2012).

Pyroglutamic acid hexose was assigned at peak 3 (Rt: 9.4 min, λmax: 280 nm) and exhibited a deprotonated ion ([M-H]-) at m/z 290. In the MS-MS spectra was observed an ion at m/z 128, after elimination of hexose moiety at m/z 128. Identification of this compound is consistent with a previous study (Bondia-Pons et al., 2014).

According to my results, Lycium barbarum fruits are mainly rich in flavonoids and phenolic acids derivatives, in good agreement with previously published works (Forino et al., 2016; Bondia-Pons et al., 2014; Inbaraj et al., 2010).

In this regard, it has already been shown that the phenolic profile of Goji berries from Mongolia, China and Tibet depends on the cultivation area (Bondia-Pons et al., 2014). These berries showed differences in terms of quercetin and isorhamnetin derivatives, rutin, narcissin and kaempferol derivatives, among flavonoids. Furthermore, there was also a significant difference in terms of phenolic acids derivatives. These results are in good agreement with my study, in which the chemical composition in flavonoids and phenolic acid derivatives is clearly influenced by the different geographic origin, climate, soil and cultivations method. Besides, the four samples studied in the present study showed a different qualitative profile, which it is certainly due to the different cultivars and origins.



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Among flavonoids, rutin is the most frequent compound (Protti et al., 2017; Carvalho et al., 2016; Forino et al., 2016; Mikulic-Petkovsek et al., 2012).

Rutin was detected only in the commercial Goji berry sample. Also the other two flavonoids, quercetin 3-o-glu and isorhamnetin 3-o-rut, were detected only in the commercial Goji berry sample and not in the *Polonia*, *Selvatico mongolo* and *Bigol* varieties. Among phenolic acid derivatives, some compounds as caffeoylhexose, p-coumaroylquinic acid and dycaffeoylquinic acid were identified in all varieties of Goji berry fruits. Caffeic acid derivative was identified in all samples except in variety *Selvatico mongolo*. Dycaffeoylquinic acid was identified in varieties *Polonia* and *Bigol*.

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Table 3.1.2.5. Retention time (R_t), wavelengths of maximum absorbance in the UV-Vis region, molecular ions ($[M-H]^-$), major fragment ions, and tentative identification of phenolic compounds in four different varieties of Goji berries.

Peak number	**R_t (min)**	**$[M-H]^-$**	**Main fragments**	**UV-Vis (nm)**	**Tentative identification**	**Goji berry variety**			
						Commercial (A)	*Selvatico mongolo* (B)	*Bigol* (C)	*Polonia* (D)
1	3.7	341	191,179	290	caffeoylhexose	x	x	x	x
2	8.0	337	191,179,173	250	p-coumaroylquinic acid	x	x	x	x
3	9.4	290	200,128	280	pyroglutamic acid hexose	x		x	x
4	12.2	439	393,351,321,115	280	n.i.		x	x	
5	13.4	283	211,151	280	n.i.		x		
6	18.6	453	291	280	n.i.		x		x
7	19.6	629	347,275	280	n.i.		x		
8	20.3	283	179,151	280	caffeic acid derivate	x		x	x
9	21.0	515	353,191,173	280	dycaffeoylquinic acid	x	x	x	x
10	21.4	514	353,179	280	dycaffeoylquinic acid			x	x
11	21.9	796	634,472,308	280	n.i.	x	x	x	x
12	22.8	634	472,308	280,320	n.i.	x	x	x	x
13	23.6	632	470,334,217	320,370	n.i.	x		x	

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14	24.0	355	309,211,151	280	n.i.	x	x	x	x
15	24.7	497	335,305,292	280	n.i.			x	
16	25.6	543	381,179,135	280	n.i.			x	x
17	27.0	625	300,271,179	280	n.i.				x
18	28.8	609	301	280,360	rutin*	x			
19	29	717	681,519,357	260,300	n.i.			x	
20	29.8	462.2	418, 301, 151	300	quercetin 3-o-glu	x			
21	30.0	517	471,399,345,247,157	280	n.i.	x			
22	30.3	623	315, 299, 271	240,290,340	isorhamnetin 3-o-rut	x			
23	33.6	916			n.i.		x	x	x

* identification corroborated by co-injection of the standard compound

n.i.: not identified

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3.1.2.3. *Conclusions*

A green extraction method based on pressurized liquid extraction has been developed for the first time to obtain phenolic bioactive compounds from Goji berries.

Optimum extraction conditions were optimized using RSM. An experimental design was applied to optimize the extraction conditions in order to maximize the selected response variables (extraction yield, total phenols, total flavonoid and antioxidant activity of the extracts). The optimal PLE conditions were achieved at 180°C and 86% ethanol in water as solvent, showing better figures of merit than the conventional SLE method using methanol. At optimal conditions, experimental values obtained coincided with the predicted theoretical values by RSM. The chemical characterization of those extracts, carried out by HPLC-DAD-MS/MS, allowed the tentative identification of nine phenolic compounds in four different Goji berry varieties. Major fragments of the non-elucidated compounds included in the present work may help future comparisons about phenolic composition of Goji berry fruits. The qualitative differences found among the Italian varieties and the commercial sample native to China indicate that an in-depth characterization of Goji berry phenolic compounds from different varieties may be of potential interest for future works addressing biomarkers for geographical and authentication studies.

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3.2. *Hazelnut kernel (Coryllus avellana L.) phenolic compounds analysis and extraction*

Hazelnut derives from the common name "Hazel" used for the flowering plant genus *Corylus*, belonging the *Betulacee* family, although some botanists consider it a separate family named *Corylaceae*. *Corylus* comprises about 15 species, including the European *Corylus avellana L.* The fruit of core is a walnut. Internally, the edible portion of the hazelnut is the roughly spherical to oval, about 15-25 mm long and 10-15 mm in diameter (Contini et al, 2011).

The most popular commercial hazelnut varieties are from Turkey, Italy, Spain, Portugal and USA. About 90% of global production of shelled hazelnut is absorbed by food industry such as confectionery industry. Hazelnut oil is used not only as food, but also widely used by cosmetic industry, for its astringent and emollient properties.

Hazelnuts are well known for their mild, sweet, exotic flavour; moreover, they are very nutritious and healthful for their chemical composition. This fruit is a rich source of dietary fibers (~ 10%) and beneficial nutrients such as lipids (~ 60%) and proteins, but also micronutrients such as essential minerals, vitamin E, B complex and phenolic compounds which contribute to its organoleptic properties such as astringent and bitter taste. Particularly, phenolic compounds play an important role on human health for their beneficial effects, such as antioxidant activity (Tas & Gokmen, 2017).

According to several studies reporting the chemical composition of phenolic compounds in hazelnut kernels, the most common classes are flavan-3-ols, phenolic acids, flavonols and anthocyanins. Flavan-3-ols is the most abundant phenolic class in hazelnut. Catechin and epicatechin are the most represented ones, especially in polymerized form, condensed tannins monomers and dimers, mainly consisting of B-type proanthocyanidins. Several phenolic acids have been detected

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in hazelnut kernels such as benzoic acids like protocatechuic and gallic acids, and cinnamic acids like caffeic, *p*-coumaric and ferulic acids.

Different studies are present in literature regarding the phenolic compounds extraction from hazelnut kernels (*Coryllus avellana L.*) by-products like skin and shell (Contini et al., 2008; Locatelli et al., 2010; Odabas H. et al., 2006; Alasalvar et al., 2009; Tas & Gokmen, 2017). Some of these papers were focused on the analysis of individual and total phenolic compounds and antioxidant capacity in the edible hazelnut kernels (Yurttas et al., 2000; Schmitzer et al., 2011; Gültekin-Ozgüven et al., 2015; Altun et al., 2013; Ciarmello et al., 2014; Ghirardello et al., 2010; Pelvan et al., 2012; Delgado et al., 2010).

As pre-treatment, hazelnuts are generally milled and treated with a non-polar solvent as hexane for removing of fat portion (defatting) due the high percentage of lipids (~ 60%). Defatting has been carried out by solid-liquid extraction (Ciarmiello et al., 2014; Alasalvar et al., 2006), or by using a soxhlet system (Locatelli et al., 2010). The only extraction technique exploited for phenolic compounds was the conventional solid-liquid extraction, generally, supported by an ultrasound bath improving the mass transfer. The choice of extraction solvent was one of important parameters and it was based on the chemical nature of target analytes. The most common solvent used were methanol, ethanol, acetone, water and mixture of them with or without some percentage of acid as hydrochloric or formic acid (Ghirardello et al., 2010; Yurttas et al., 2000; Amarowicz et al., 2008).

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3.2.1. **Effect of solvent on the extraction of phenolic compounds and antioxidant capacity of hazelnut kernel**

The aim of this study was to develop a high performance liquid chromatography with UV/Visible and mass spectrometry detection (HPLC-PDA/ESI-MS) method for qualitative and quantitative analyses of phenolic compounds in hazelnut kernels evaluating different extraction methods. The isolation and enrichment of phenolic compounds from hazelnut kernels were carried out by ultrasound assisted solid-liquid extraction and solid phase extraction, with a C18 cartridge, evaluating the effects of different extraction conditions on the total phenolic compounds concentration determined by Folin-Ciocalteau assay and on antioxidant activity. Identification and quantification of phenolic compounds was performed by RP-HPLC coupled with UV and MS detectors. The optimized method was validated and then applied to the analysis of phenolic compounds in three varieties of hazelnut kernels, *Akcakoca*, *Ordu* and *Romane*.

3.2.1.1. Materials and methods

Chemicals

Solvents employed for the extraction procedure and for HPLC-MS analyses were, methanol, ethanol, acetone, ethyl acetate, water, acetonitrile, hydrochloric acid and formic acid and were purchased from Sigma-Aldrich (Milan, Italy). The standard compounds and reagents namely gallic acid, protocatechuic acid, catechin, caffeic acid, (-) epicatechin, *p*-coumaric acid, epicatechin gallate, quercetin, kaempferol, potassium persulfate, sodium carbonate, Folin reagent, ABTS (2,2-Azinobis-(3-ethylbenzothiazoline-6-sulfonic) diammoniumsalt), Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2 carboxylic acid) and were

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purchased from Sigma-Aldrich (Milan, Italy). Procyanidin A2 and Procyanidin B2 were purchased from Extrasynthese (Genay Cedex, France).

Samples

Hazelnut were purchased in a local market and used for the optimization of extraction conditions. Three cultivars of hazelnut (*Corylus avellana L.*), namely *Akcakoca*, *Ordu* and *Romane*, were also included in the study. Roasted, shelled, and calibrated (12-13 mm diameter) kernels were obtained in dark vacuum plastic bags and stored at 4 °C until analysis.

HPLC-PDA-ESI-QMS

The analyses were carried out on Shimadzu Prominence LC-20A instrument (Shimadzu, Milan, Italy) equipped with two LC-20 AD XR pumps, SPD-M10Avp PDA detector, SIL-10ADvp, CTO-20 AC column oven and DGU-20 A_3 degasser. For MS analyses was used a mass spectrometer detector (LCMS-2010, Shimadzu) equipped with ESI interface in negative ionization mode. MS data were acquired by Shimadzu Lcsolution Ver. 3.7 software (Shimadzu).

Hazelnut extracts were analysed at the following analytical conditions. HPLC separations was carried out using a column (Poroshell C18 150 x 2.1 mm I.D., 2.7 μm d.p., Supelco). Elution was conducted at 40 °C with H_2O/0.1 % HCOOH (solvent A) and acetonitrile/0.1 % HCOOH (solvent B) at a constant flow of 0.2 mL/min. The gradient mode was applied: 0-5 min 0-0% B, 5-45 min 0-25% B, 45-60 min 25-100%B, 53. The injection volume was 2 μL. Data were acquired using a PDA in the range 210-400 nm and the chromatograms were extracted at 280 and 325 nm.

Chromatograms were acquired in the MS instrument using ESI as interface in negative ionization mode at the following parameters: nebulizing gas flow (N_2):

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1.5 mL/min; Event time: 1 sec; mass spectral range: 100-800 m/z; scan speed: 1000 amu/sec; detector voltage: 1.5 kV; Interface temperature: 250 °C; CDL temperature: 300 °C; Heat Block: 300 °C; Interface Voltage: - 3.50 kV; Q-array: 0.0 V; Q-array RF: 150.0 V.

Analytical method validation

Standard compounds were chosen, according to the phenolic composition of hazelnut kernel, as representative of each class of phenolics. A mixture of seven phenolic standard compounds (gallic acid, catechin, (-) epicatechin, procyanidin A2, epicatechin gallate, procyanidin B2, quercetin) was employed for quantitative analysis and method validation.

The analytical method was validated considering linearity, repeatability (intra-day precision) and reproducibility (inter-day precision), limit of detection (LOD) and limit of quantification (LOQ).

Linearity was determined by the calibration curves obtained from the HPLC analysis of the standard solutions. Stock standard solutions of each compound were prepared at concentration 10000 mg/L in methanol for gallic acid; 5000 mg/L for catechin; 2000 mg/L for procyanidin A2, procyanidin B2, (-) epicatechin and epicatechin gallate; 1000 mg/L for quercetin. External standard calibration curves were constructed by using five dilutions. Evaluation of each points was conducted in five replicates and the calibration curves were fitted by linear regression. LOD and LOQ were calculated based on the signal (S)-to-noise (N) ratio by the following equations (3.2.1.1. and 3.2.1.2.):

$$LOD = 3 \times S/N \qquad (3.2.1.1.)$$

$$LOQ = 10 \times S/N \qquad (3.2.1.2.)$$

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In order to study the method's precision, six injections of standards were done in the same day for intra-day repeatability, and six injections for three consecutive days for inter-day reproducibility. Then, the %RSD of retention times and peak area were calculated. The accuracy of the method was calculated by means of the recovery (%). A known concentration of standard compounds (100 mg/Kg) was added on a sample that did not contain the studied standard molecules. Every extract was analysed in triplicate. Recovery was calculated according to the follow equation 3.2.1.3.:

Recovery % = [(Conc. Sample Fortified – Conc. Sample Unfortified)/Fortification] *100 (3.2.1.3.)

Sample preparation and phenolic compounds extraction

<u>Preparation of defatted sample</u>: Hazelnut kernels were finely ground in a domestic electric grinder and defatted by mixing with n-hexane (1:10, w/v) with the help of ultrasonic bath at room temperature for 15 min. The procedure was repeated for three times. Defatted sample was subsequently dried under nitrogen and then stored in vacuum bags at -20 °C until phenolic compounds extraction.

<u>Extraction of phenolic compounds</u>

The extraction of phenolic compounds from roasted hazelnut kernels was performed using two different methods: i) solid-liquid extraction (SLE) and ii) solid phase extraction (SPE). For both methods, different solvents were employed. Samples of hazelnuts (a quantitative mixture of the three *C. avellana* cultivars referred to above) were used for these studies.

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Solid-liquid extraction: Phenolic compounds were extracted from defatted sample using different solvents at two different solid to solvent ratios. Table 3.2.1.1. reports the different extraction conditions. Extraction was carried out with ultrasound bath at room temperature for 18 min. The extraction was repeated twice, supernatants were combined and the solvent was evaporated under vacuum at 40 °C in a rotary evaporator (Eyela, Tokyo, Japan). The extract was then dissolved in 400 µL of a mixture of MeOH/H_2O (8:2, v/v).

Solid-phase extraction: Phenolic compounds were extracted from defatted sample employing a SPE cartridge packing with 1 g of C18 sorbent (Supelco, Milan, Italy). The cartridge was conditioned with 2 mL of MeOH, and subsequently equilibrated with 2 mL of water. After cartridge activation, 0,1 g of defatted sample was loaded directly on the top of the cartridge and phenolic compounds were extracted and eluted with a total of 15 mL of solvents. Different solvents were used for phenolic compounds elution (Table 3.2.1.1.). The eluted solution was evaporated to dryness by using a rotary evaporator and then dissolved in 400 µL of a mixture of MeOH/H_2O (8:2, v/v).

Total phenolic compounds

Total phenolic content (TPC) of hazelnut kernels extract was determined by Folin-Ciocalteu phenol reagent method, according to the procedure reported by Singleton and co-workers (Singleton et al., 1999). A volume of 1580 µL of distilled water and 100 µL of Folin-Ciocalteu were added to 20 µL of sample extract (or standard solution). The mixture was incubated for 8 minutes at room temperature. Then 300 µL of sodium carbonate solution (20% w/v) were added. The solution was mixed and allowed to stand for two hours at room temperature and in the dark before measuring the absorbance at 765 nm in a 96-well cell culture plate (Greiner Bio-one, Germany) using a multifunctional microplate reader

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(Infinite®, 200 PRO multimode reader, Tecan, IT). The results were expressed as mg of gallic acid equivalents (GAE) per g of hazelnut fresh weight.

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Table 3.2.1.1. Extraction protocols conditions of SLE and SPE techniques.

SLE			
Extraction protocol	**Type of solvent**	**Defatted sample (g)**	**Solvent volume (mL)**
A	methanol 0.1 % HCl	1.5	50
B	ethanol 0.1 % HCl	1.5	50
C	acetone 0.1 % HCl	1.5	50
D	methanol/water (8:2, v/v)	1.5	50
E	ethanol/water (8:2, v/v)	1.5	50
F	acetone/water (8:2, v/v)	1.5	50
G	ethyl acetate/methanol/water (1:1:1, v/v/v)	1.5	50
H	methanol/water/methanol 0.1 % HCOOH/acetonitrile (1:1:8:5, v/v/v/v)	0.1	15
SPE			
Extraction protocol	**Type of solvent**	**Defatted sample (g)**	**Solvent volume (mL)**
I	methanol 0.1% HCl	0.1	15
L	methanol/water/methanol 0.1 % HCOOH/acetonitrile (1:1:8:5, v/v/v/v)	0.1	15
M	methanol/water/methanol 0.1 % HCOOH/acetonitrile/n-propanol (1:1:8:5:5, v/v/v/v/v)	0.1	20

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Trolox equivalent antioxidant capacity assay (TEAC)

Free radical scavenging activity of hazelnut kernel extract was evaluated by colorimetric assay according to a previously described method with some modification (Re et al., 1999). The radical cation ABTS•+ was produced by reacting 7mM ABTS aqueous phosphate buffer (5mM NaH2PO4-H2O and 5mM Na2HPO4-2H2O, pH 7.4) solution with 2.5 mM potassium persulfate (final concentration). This solution was allowed to react for 12 hours at room temperature and in the dark. ABTS•+ working solution was obtained diluting in ethanol stock solution to an absorbance of 0.70 ± 0.05 at 734 nm. A volume of 10 µL of sample (or standard) was mixed with 200 µL of ABTS•+ working solution in a 96-multiwell plate. The absorbance was recorded at 734 nm after 10 minutes. A calibration curve was prepared with Trolox as a standard (final concentration 0-300 µM). Results were expressed as mmol Trolox equivalent (TE) per g of hazelnut fresh weight.

Determination of total flavonoid content

Total flavonoid (TF) content was determined according to the method described by Taş & Gökmen (2015). A volume of 100 µL sample extract was mixed with 50 µL of a 5% $NaNO_2$ solution. The mixture was incubated for 6 minutes at room temperature. Then 500 µL of 10% $AlCl_3$ solution were added to form aluminium-flavonoid complex. After 7 min, 250 µL of 1 N NaOH solution were added and the mixture was kept in the dark for 10 min. Then, the mixture was centrifuged at 6000 x g for 5 min and the clear supernatant was measured against methanol at 510 nm. A calibration curve was built with different concentrations of catechin dissolved in methanol and results were given as mg catechin equivalent (CE) per g of hazelnut fresh weight.

Statistical analysis

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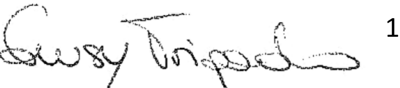
Analyses were performed in triplicate. Means and standard deviations were calculated and statistical differences were analyzed analysed by One-way analysis of variance (ANOVA) followed by Tukey's test using GraphPad Prism version 4 for Windows (GraphPad software). Differences were considered to be significant at p-Values lower than 0.05. To calculate correlations, the Pearson correlation analysis was performed (IBM SPSS statistics 19; SPSS Inc., Chicago, IL). A two-tailed Spearman's correlation was conducted to determine the correlations among mean values. Probability values of $p \leq 0.05$ were chosen as the criteria for statistically significant difference.

3.2.1.2. Results and Discussion

Influence of the experimental conditions on the extraction of total phenols and antioxidant activity

It is well known that extraction of phenolic compounds is strongly affected by their chemical nature, the sample particles size, the extraction method employed, and the presence of interfering substances. Moreover, the solubility of phenolic compounds is governed by the polarity of the solvent used, as well as their degree of polymerization, interaction with other food constituents and formation of insoluble complexes (Naczk & Shahidi, 2004). In this work, the extraction of phenolic fraction from hazelnut kernels was performed by two different methods, ultrasound assisted solid-liquid extraction (UA-SLE) and solid phase extraction (SPE), using different solvents. The different extraction conditions are reported in table 3.2.1.1. together to sample quantity and solvents volume employed. Milled hazelnut kernels were defatted with hexane before phenolic compounds extraction in order to avoid the interference of lipid. In order to investigate the effect of different solvent systems on phenolic compounds extraction from hazelnut kernels, UA-SLE was performed employing the most common solvents reported in the literature like methanol, ethanol, acetone, ethyl acetate and water alone or in



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mixture with and without acid (Dai et al., 2010). Therefore, total phenolic and flavonoid contents and the consequent effect on antioxidant activities were investigated. The final objective of this work was to identify suitable conditions for extracting phenolic compounds from hazelnut kernels, as a base for future research. Table 3.2.1.2. shows total phenolic compounds and flavonoids concentration and antioxidant activities value obtained by spectrophotometric assays together to the extraction yields obtained for each extraction protocol. The first UA-SLE experiments were performed using 5 g of hazelnut kernels that was firstly defatted and then extracted with a volume of 50 mL of solvent, solvent to sample ratio 1:10 (w/v) (corresponding to extraction protocols from A to G). The extraction yield for these first extraction protocols ranged from 2.45 and 6.21%. The extraction obtained with methanol 0.1 % HCl (protocol A) gave the highest amounts of crude extract while acetone 0.1 % HCl (protocol C) gave the lowest value. As expected, also total phenolic compounds and total flavonoids concentration showed significant ($p<0.05$) differences among extraction protocols. TPC values ranged from 83.7 to 375.5 μg GAE/g FW. Some significant differences ($p< 0.05$) exist among extraction protocols. The highest TPC value was obtained with ethanol 0.1 % HCl (protocol B) while the lowest one with acetone 0.1 % HCl (protocol C). However a high TPC values were obtained also for acetone/water (8:2, v/v, protocol F) and ethyl acetate/methanol/water (1:1:1, v/v/v, protocol G) not statistically different from protocol B. TF values ranged from 127.8 μg CE/g FW for acetone 0.1 % HCl to 440.9 μg CE/g FW for ethyl acetate/methanol/water (1:1:1, v/v/v). Some significant differences ($p< 0.05$) exist among extraction protocols. TEAC values ranged from 0.70 μmol TE/g FW for acetone 0.1 % HCl to 6.01 μmol TE/g FW for acetone/water (8:2, v/v). Correlation between TPC, TF, yield and antioxidant activity is shown in Table 3.2.1.3. Results indicate a significant correlation between TPC and antioxidant activity (TPC vs. TEAC $r = 0.923$). A good but not significant correlation between TF and antioxidant activity (TPC vs. TF $r = 0.674$) was also observed.

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Table 3.2.1.2. Total phenolic content (TPC), antioxidant capacity (TEAC), total flavonoid (TF) and yield (%) of different extraction protocols of UA-SLE and SPE techniques.

		SLE		
Extraction protocol	**TPC (µg GAE/g FW)**	**TEAC (µmol TE/g FW)**	**TF (µg CE/g FW)**	**Yield (%)**
A	338.6 ± 5.13 d	4.30 ± 0.21 g, h, e	249.0 ± 51.1 c, d	6.21 ± 0.55 c
B	375.5 ± 4.16 d	4.86 ± 0.19 f, g, h,	301.7 ± 61.3 c, d	4.96 ± 1.32 c, d
C	83.7 ± 15.67 f	0.70 ± 0.19 i	127.8 ± 42.8 d	2.45 ± 0.35 d
D	226.9 ± 2.23 e	4.30 ± 0.19 g, h, e	263.6 ± 61.4 c, d	4.06 ± 0.40 c, d
E	286.7 ± 7.16 d, e	3.89 ± 0.08 h	339.1 ± 39.1 c, d	3.65 ± 0.42 c, d
F	340.6 ± 0.07 d,	6.01 ± 0.98 e, f	347.2 ± 43.18 c, d	5.28 ± 0.39 c, d
G	359.3 ± 31.17 d, e	5.85 ± 0.44 f, g	440.9 ± 32.6 b, c	5.11 ± 0.20 c, d
H	643.1 ± 3.90a	16.52 ± 0.39 a	658.9 ± 130.14 b	2.89 ± 0.07 c, d
		SPE		
Extraction protocol	**TPC (µg GAE/g FW)**	**TEAC (µmol TE/g FW)**	**TF (µg CE/g FW)**	**Yield (%)**
I	575.0 ± 43.62 c	13.87 ± 0.36 b	1383.7 ± 122.86 a	2.70 ± 1.34 d
L	663.0 ± 33.00a	11.85 ± 0.84 c	279.1 ± 62.01 c, d	13.79 ± 1.34 a
M	599.8 ± 36.00 b, c	9.60 ± 0.63 d	345.9 ± 242.91 c, d	11.19 ± 1.34 a

TPC expressed as mg of gallic acid per g of fresh weight (FW). TEAC expressed as µmol of trolox per g of fresh weight (FW). TF expressed as µg of catechin per g of fresh weight (FW). Yield expressed (extract mass per initial mass) * 100.
All values are expressed as mean ± SD (n = 3).
Different letters (a to i) in columns present statistically significant differences ($P < 0.05$) among samples.

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SPE was then applied for the extraction of phenolic compounds not in the classical way; in this work, we loaded a small quantity of defatted sample (0.1 g) directly on the top of the SPE cartridge after its conditioning (figure 3.2.1.1.). Phenolic compounds were then extracted by direct solvent elution from the cartridge.

Table 3.2.1.3. Correlation between total phenolic content (TPC), antioxidant activity (TEAC), total flavonoid (TF) and Yield by Pearson's correlation.

	TPC	TEAC	TF	Yield
TPC	1	0.923***	0.498	0.534
TEAC	0.923***	1	0.674	0.221
TF	0.498	0.674	1	-0.299
Yield	0.534	0.221	-0.299	1

Correlation expressed Pearson (*r*) as (*r*)*** as $p < 0,0001$.

Three different extraction and elution protocols were performed (table 3.2.1.1.). Yield, TPC, TF and antioxidant activity values obtained for SPE extraction for the three different protocols (I, L and M protocols of table 3.2.1.2.) were all higher and statistically different from the UA-SLE protocols discussed above (from A to G). In particular the solvent mixture of methanol/water/methanol 0.1 % HCOOH/acetonitrile (1:1:8:5, v/v/v/v, protocol L) gave the highest values. Based on these results we decided to perform another UA-SLE procedure using the same quantity of defatted sample (0.1 g) and solvent mixture and volume that gave the highest values for SPE extraction. The aim was to test the possibility to employ UA-SLE protocol using a small quantity of sample and solvent volume with the advantage of the lower cost respect to SPE. This extraction protocols is reported with letter H and the obtained results are reported in table 3.2.1.2.

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A high value of TPC was obtained, not statistically different from extraction protocol L (the highest TPC value among SPE protocols). For this reason and with the advantage of lower extraction cost per sample we selected this last extraction protocol for method validation and application to the analysis of phenolic compounds in different hazelnut kernel.

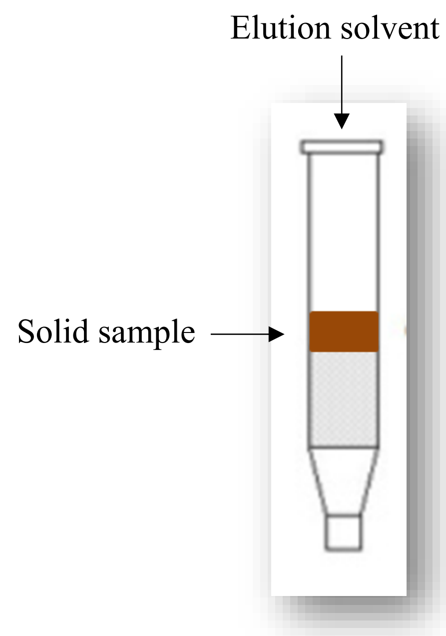

Figure 3.2.1.1. Scheme of defatted hazelnut kernel phenolic compounds SPE extraction.

Identification and quantification of phenolic compounds in hazelnut kernel (Coryllus avellana)

In this study, a fused core C18 stationary phase column (150 x 2.1 mm I.D. with particle size of 2.7 μm) was employed for the analysis of extracted phenolic compounds. Optimization of separation conditions was assessed on hazelnut kernel extract in order to have the best separation of all detected phenolic compounds. To

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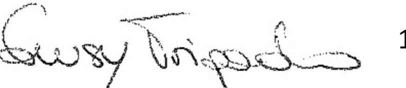
optimize the separation, in terms of resolution of analytes and analysis time, the mobile phase composition, including the formic acid concentration and the step gradient program were studied. The effect of ACN and MeOH as organic modifiers was evaluated. The use of an ACN/water mixture allowed the best separation of sample's compounds in the shortest time. Best conditions, in terms of resolution of analytes and analysis time, were obtained applying a step gradient elution mode (see materials and methods section). The use of a column with partially porous particle stationary phase allowed obtaining good chromatographic performance due to the increased column efficiency respect to a classical fully porous particles stationary phase. A mixture of 7 standard compounds belonging to phenolic compounds classes' representative of hazelnut was used for quantitative analysis. In order to validate the HPLC method, retention time precision, expressed as RSD% of retention time (tR), limit of detection (LOD), limit of quantitation (LOQ), linearity range and recovery, were considered (Table 3.2.1.4.).

Retention time precision of the method was studied by analyzing the standard mixture of phenolic compounds six times on the same day and in two different days (n=9). The calculated RSDs% values of retention times were in the range 0.05 % and 0.45 %.

Calibration curves of gallic acid, catechin, (-) epicatechin, procyanidin A2, epicatechin gallate, procyanidin B2, quercetin were constructed under the same chromatographic conditions optimized for samples analysis. Peak areas were plotted as a function of concentration expressed as mg/L, obtaining good values of correlation coefficients, R^2, between 0.997 to 0.999, without the employment of an internal standard. The linearity of the optimized method was calculated in the concentration range between LOQ value and 100 mg/L.

The recovery of extraction procedure was estimated by spiking an exhausted defatted sample, which do not contain the studied standard molecules with standard solutions of gallic acid, catechin, (-) epicatechin, procyanidin A2, epicatechin gallate, procyanidin B2 at concentration levels in the range of



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calibration curve (100 mg/Kg, final added concentration). Recovery values ranged from 30 to 73%, the low obtained valued for some of them could be due to the complexity of studied food matrix (Table 3.2.1.4.).

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Table 3.2.1.4. Calibration equations, coefficient of linearity (R^2), linearity range (mg/L), LOD (mg/L), LOQ (mg/L) and recovery values (%).

Compound	**Phenolic class**	**Regression line**	**R^2**	**Linearity Range (mg/L)**	**LOD (mg/L)**	**LOQ (mg/L)**	**Recovery (mean%±SD)**
Gallic acid	Hydroxybenzoic acid	y = 24259x - 26798	0.999	0.05-100	0.03	0.05	40 ± 4.67
Catechin	Flavan-3-ol	y = 13889x – 2958.8	0.999	0.08-100	0.04	0.08	44 ± 3.35
Procyanidin B2	Proanthocyanidin	y = 6662.4x – 2792.8	0.998	0.17-100	0.09	0.17	30 ± 0.83
Epicatechin	Flavan-3-ol	y = 6441.8x – 2073.1	0.998	0.17-100	0.09	0.17	42 ± 0.06
Epicatechin gallate	Flavan-3-ol	y = 14316x - 18195	0.997	0.08-100	0.04	0.08	52 ± 0.28
Procyanidin A2	Proanthocyanidin	y = 7893.6x – 7516.1	0.998	0.14-100	0.08	0.14	55 ± 1.40
Quercetin	Flavonol	y = 38429x - 58467	0.999	0.03-100	0.02	0.03	73 ± 0.29

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Analytes were tentatively identified combining the information obtained from PDA and MS detectors and by comparison with literature data. Identification of phenolic compounds was achieved on the base of pseudo molecular ions $[M-H]^-$ and on characteristic fragment ions. MS and MS/MS spectra were acquired in negative ionization mode for phenolic compounds. When possible, the identification of compounds was confirmed by comparison with standards commercially available. Figure 3.2.1.2. shows the UV/Vis chromatogram of a hazelnut kernel extract at wavelengths of 280 nm obtained using the optimized separation conditions.

In general, a total of 17 phenolic compounds were detected and only 5 of them were tentatively identified. All detected compounds, their m/z, MS/MS fragments and compound's names are summarized in Table 3.2.1.5. Tentatively identified compounds have been reported in previous studies on phenolic composition of hazelnut (Ciemniewska-Zytkiewicz et al., 2015; Gultekin-Ozguven et al., 2015; Slatnar et al., 2014). Among them, monomeric and polymeric flavan-3-ols were identified. In particular catechin, epicatechin and epicatechin 3-gallate and two procyanidins were tentatively identified, moreover, an unknown compounds with a molecular ion m/z 368 have been detected and reported also in another work (Slatnar et al., 2014).

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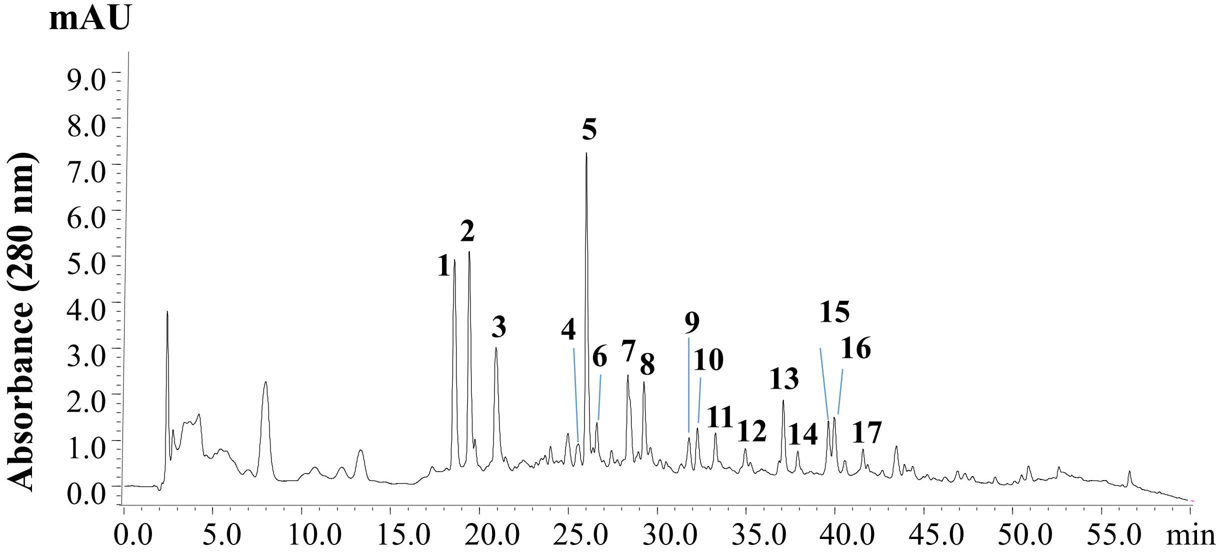

Figure 3.2.1.2. RP-HPLC/PDA chromatogram of the phenolic compounds content in hazelnut kernel. For peak identification, see Table 3.2.1.

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Table 3.2.1.5. Phenolic compounds identified in hazelnut kernel extract. Peak numbers are referred to the chromatogram shown in figure 3.2.1.2.

Peak No	$[M-H]^-$ (m/z)	MS/MS ions (m/z)	Tentative identification
1	485	341, 179, 161	Unknow
2	368	144, 89, 59	Unknow
3	341	low abundance ions	Unknow
4	289	203, 109, 123	Catechin
5	443	101, 119, 89	Unknow
6	456	190, 172, 146	Unknow
7	514	341, 190, 172	Unknow
8	577	low abundance ions	Procyanidin B2
9	289	203, 123, 109	Epicatechin
10	461	167, 341, 191	Unknow
11	529	223, 385, 305	Unknow
12	499	397, 355, 193	Unknow
13	441	330, 161, 119	Epicatechin gallate
14	575	529, 472, 430	Procyanidin A2
15	541	291, 261, 190	Unknow
16	605	low abundance ions	Unknow
17	429	low abundance ions	Unknow

The optimized and validated HPLC-PDA method was then applied to the analysis of extracted phenolic compounds from three different roasted hazelnut kernel cultivars called *Akcakoca*, *Ordu* and *Romane*. Table 3.2.1.6. reports quantitative results of phenolic compounds concentration expressed as μg/g of hazelnut fresh weight. Total concentration of detected phenolic compounds varied from 92.9 to 119.9 mg/Kg with significant statistical differences. Different studies have reported the concentration of individual phenolic compounds and their sum

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however different extraction methods have been employed making not possible a comparison among obtained results.

Table 3.2.1.6. Phenolic compounds concentration of hazelnut kernel extract of three varieties called. Peak numbers are referred to the chromatogram (Figure 3.2.1.2.). Data are expressed as mean ± SD (N=3).

	Roasted Hazelnut kernels (µg/g FW)		
Peak	***Akakoca***	***Ordu***	***Romane***
1	15.4 ± 0.38 b	16.8 ± 0.55	13.5 ± 0.58 c
2	20.8 ± 0.52 b	29.4 ± 0.33 a	13.4 ± 0.93 c
3	13.7 ± 0.25 a	9.6 ± 1.75 b	6.7 ± 0.87 c
4	2.2 ± 0.05 a	1.9 ± 0.07 a,	1.7 ± 0.19 b
5	20.4 ± 1.38 a	18.4 ± 0.89 a	19.4 ± 0.49 a
6	1.8 ± 0.09 b	2.3 ± 0.15 a	2.4 ± 0.13 a
7	5.2 ± 0.27 a	4.3 ± 0.18 b	4.4 ± 0.15 b
8	5.1 ± 0.13 a	5.0 ± 0.15 a	4.6 ± 0.10 b
9	3.7 ± 1.85 a	2.5 ± 0.57 a	2.1 ± 1.11 a
10	2.7 ± 0.06 a	2.8 ± 0.16 a	2.4 ± 0.15 b
11	3.7 ± 0.04 b	4.5 ± 0.13 a	2.6 ± 0.20 c
12	2.1± 0.09 b	1.8 ± 0.18 b	3.4 ± 0.40 a
13	5.8 ± 0.22 a	4.4 ± 0.28 b	5.5 ± 0.14 a
14	2.0 ± 0.04 b	2.3 ± 0.08 a	1.8 ± 0.04 c
15	6.4 ± 0.19 b	10.5 ± 0.38 a	3.5 ± 0.08 c
16	6.2 ± 0.15 b	10.1 ± 0.21 a	3.4 ± 0.04 c
17	2.7 ± 0.08 a	2.3 ± 0.10 b	2.0 ± 0.12 c
Total	119.9 ± 6.0 b	128.9 ± 5.0 a	92.9 ± 3.8 c

Different letters (a to c) in columns present statistically significant differences ($P < 0.05$) among samples.

Moreover, great differences in detected phenolic compounds and in their concentration among whole hazelnut, kernels without skin and roasted hazelnut. In particular the total phenolic concentration together to the individual phenolic

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compounds content decrease with roasting due to both the removal of the skin, which is very rich especially in condensed tannins, and to their chemical degradation (Schmitzer et al., 2011). Higher concentration values of catechin were determined respect to epicatechin ranging from 5.4 to 8.1 μg/g and from 2.6 and 5.0 μg/g, respectively. Obtained results agree (the same order of magnitude) with results reported in other published papers, taking in mind the differences due to the extraction methods and the different hazelnut parts (Schmitzer et al., 2011; Jakopic et al., 2011; Solar et al., 2011).

3.2.1.3. ***Conclusions***

A method for the analysis of hazelnut kernel phenolic compounds have been developed and validated with the aim to test different techniques and solvents for their extraction. Phenolic compounds were extracted employing two different methods: UA-SLE and SPE. Different extraction solvents were tested evaluation total phenolic and total flavonoids contents together to antioxidant activity. The best extraction conditions in terms of the highest value of total phenolic compounds extracted together to other parameters like simplicity and cost were selected for method validation and individual phenolic compounds analysis. The UA-SLE protocol performed using 0.1 g of defatted sample and 15 mL of extraction solvent (methanol/water/methanol 0.1 % HCOOH/acetonitrile (1:1:8:5, v/v/v/v)) was selected.

The analyses of individual phenolic compounds were performed by HPLC-PDA/ESI-MS. The method was fully validated and applied to the analysis of phenolic compounds in three different hazelnut kernel varieties. As expected differences in phenolic compounds concentration can be evidenced.

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Effect of hydroxytyrosol methyl carbonate on the thermal, migration and antioxidant properties of PVA based films for active food packaging.
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4) Tripodo G., Ibáñez E., Cifuentes A., Gilbert-Lopez B., Fanali C. (2017).
Optimization of pressurized liquid extraction by response surface methodology of Goji berry (Lycium barbarum L.) phenolic bioactive compounds.
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