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# DEVELOPMENT, OPTIMIZATION AND VALIDATION OF CONVENTIONAL AND NON-CONVENTIONAL ANALYTICAL METHODS FOR PHENOLIC COMPOUNDS IN FOODS AND FOOD BY-PRODUCTS

Coordinator:
Prof. Giulio Iannello

Tutor:
Prof. Chiara Fanali

**Susanna Della Posta**

# Abstract

Phenolic compounds represent a group of highly heterogeneous secondary metabolites which in the last 15 years have been arousing strong interest especially for their beneficial effects for humans.

Traditionally, these molecules are extracted by using techniques such as solid-liquid extraction, liquid-liquid extraction, solid-phase extraction, using organic solvents, such as methanol, ethanol, methanol and water mixtures as extractive solvents.

Phenolic compounds extracts are typically analyzed by high performance liquid chromatography coupled with UV-Vis spectrophotometry and mass spectrometry (HPLC/PDA-ESI/MS).

With the advent of green chemistry, more and more attention has been paid to the search for sustainable extractive solvents, among these deep eutectic solvents (DESs) are having great success.

The present thesis study was based in the development, optimization and validation of conventional and non-conventional, extraction with deep eutectic solvent, analytical methods for the analysis of phenolic compounds in foods and food wastes using HPLC/PDA-ESI/MS system.

Specifically, two food matrices were considered being extra virgin olive oil (EVOO) and hazelnuts and two waste products from the food supply chain being spent coffee ground and hazelnut skin.

EVOO and hazelnuts are famous for their high content in phenolic compounds.

A very large quantity of spent coffee ground and hazelnut skin are produced annually following the production of the coffee beverage and the consumption of hazelnuts respectively.

These by-products are characterized by a high content of phenolic compounds, the same present in the food matrices from which they derive.

In the first project phenolic compounds in EVOO were analyzed using an HPLC/PDA-ESI/MS method. Thirty samples from four different Italian regions were analyzed. 15 phenolic compounds belonging to the following classes have been identified: secoiridoids, phenolic alcohols, flavonoids, and phenolic acid classes. Quantitative analysis was performed by UV detection considering eight standard phenolic compounds. In all samples secoiridoids were the main compounds ranging from 85 to more than 99% (w/w) of the total concentration of detected phenolic compounds while phenolic acids accounted for the lowest percentage (0.1-0.6%, w/w). Intra-day and inter-day retention time precision, limit of detection, limit of quantification, and linearity were considered for the validation of the optimized analytical method. Finally, total concentration of phenolic compounds and antioxidant activity were determined with different chemical assays. A good correlation was found between antioxidant activity and total phenolic content. It was not possible to cluster the samples according to the regions but statistically significant differences were found between the Puglia and Sicilia samples.

The second project involved the optimization and validation of an HPLC/DAD-ESI/MS method for the analysis of the most representative phenolic compounds in EVOO samples but using a green extraction approach based on deep eutectic solvents (DESs) at room temperature. Ten DESs based on choline chloride and betaine in combination with different hydrogen bond donors comprising six alcohols, two organic acids, and one urea were considered for the study. To evaluate the extraction capacity of each DES five phenolic compounds, secoridoids and phenolic alcohols were considered.

The optimization of the extraction method involved the study of the quantity of water to be added to the DES and evaluation of the sample-to-solvent ratio optimal condition. Betaine-based DES with glycerol (molar ratio 1:2), added of thirty percent of water considering for

the extraction a sample to solvent ratio 1:1 (g/mL) are resulted the best conditions for the extraction. The chromatographic method was validated by studying LOD, LOQ, intraday and interday retention time precision, and linearity range using two standard molecules. Good results about recovery (5 and 100 μg/g concentration) were obtained being in the range of 75.2% and 98.7%.

Other two work involved the development, optimization and validation of a conventional HPLC-DAD/ESI-MS method, studying best conditions of the extraction, for the analysis of phenolic compounds in hazelnuts, in the first case and in the second case the development of a green approach for the extraction of phenolic compounds in hazelnut skin, using DESs. For the first one, 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. 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. Six standard molecules were selected for the method validation and 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 R2 for quantitative analysis. Finally, the method was applied to the analysis of phenolic compounds in three different hazelnut kernel varieties.

The second project involved the study of fifteen DESs as extractive solvents for phenolic compounds in hazelnut skin. Eighteen phenolic compounds were identified in the extracts, using HPLC/DAD-ESI/MS optimized method. Total phenolic content in the extracts obtained using tested DESs was evaluated through FOLIN ASSAY with the aim to understand which DES guaranteed the best extraction. DES ChCl/lactic acid (1:2) resulted

better than the other DESs tested and also compared to an organic solvent. Extraction technique was then optimized considering the quantity of water to add to the DES to reduce its viscosity, time of extraction and sample-to-solvent ratio. The optimal conditions for obtain the best extraction of phenolic compounds from hazelnut skin are resulted: use of DES ChCl/lactic acid (1:2) as extraction solvent, without addition of water, extraction time of 30 min and quantity of matrix/ volume of solvent ratio of 0.2 g matrix/5mL of solvent.

Application of DESs was also tested for the extraction of chlorogenic acids, phenolic compounds, from another food by-product, spent coffee ground (SCG).

HPLC/PDA-ESI/MS method has been developed for the analysis of the most representative CGAs in SCG samples. Fifteen DESs based on choline chloride and betaine as hydrogen bond acceptor in combination with different hydrogen bond donors were considered to evaluate their extraction efficiency. Fifteen CGAs were detected, identified and quantified into the different extracts obtained using tested DESs with the aim to evaluate which of these was the best. A betaine-based DES with triethylene glycol (molar ratio 1:2) extracted the higher content in phenolic compounds also compared to the conventional extraction using organic solvents. Thirty percent of water added to DES and sample to solvent ratio 1:15 (g/mL) were selected as the best extraction conditions. The chromatographic method was validated studying LOD, LOQ, retention time and peak area precision, and linearity range. Good results of repeatability intra-day and inter-day were determined obtaining RSDs values below 0.63 % for retention time and 8.51 % for and peak area and below 0.58 % for retention time and 8.80 % for peak area, respectively.

**Sommario**

# CHAPTER 1: PHENOLIC COMPOUNDS

## 1.1 PHENOLIC COMPOUNDS: DEFINITION

Phenolic compounds or polyphenols are one of the largest and most widespread groups of secondary metabolites in the plant world. With more than 8000 phenolic structures identified, these compounds have aroused particular interest due to their massive presence and their properties. The main sources of phenolic compounds are fruits and vegetables, but they are also present in cereals, olives, legumes, chocolate and in some beverages such as tea, coffee and wine. Generally, they affect the quality, palatability and stability of the food by acting as flavoring, coloring and antioxidants (Manach C., et al., 2004). Plant foods are famous to be rich of phenolic compounds, which are molecules that can act as antioxidants to prevent heart disease, lower the incidence of cancers and diabetes, reduce inflammation. The protection afforded by the consumption of plant products is mostly associated with the presence of phenolic compounds. Their structure consists of an aromatic ring, containing one or more hydroxyl substituents (Tsao R., et al., 2010). Due to their structure diversity, there is a wide range of phenolic compounds that occur in nature. Phenolic compounds can range from simple phenolic molecules to highly polymerized compounds. The most phenolic compounds occur naturally as conjugates with mono- and polysaccharides, associated with one or more phenolic groups. They also can be linked to esters and methyl esters.

## 1.2 PHENOLIC COMPOUNDS: CLASSIFICATION

There are different classes of phenolic compounds in the world, ranging from simple molecules, such as phenolic acids, to highly polymerized compounds, such as tannins. The classification depends on the number of the carbon atoms as reported in Table 1 (Harborne J.B., et al., 1999). Following the description of these phenolic compounds.

Phenolic compounds that possesses only one functional carboxyl group are called ***phenolic acids***.

| Class | Structure |
|---|---|
| Simple phenolics, benxoquinones | C6 |
| Hydroxybenzoic acids | C6-C1 |
| Hydrozycinnamics acids, phenylpropanoids | C6-C3 |
| Naphtoquinones | C6-C4 |
| Xanthones | C6-C1-C6 |
| Stilbenes | C6-C2-C6 |
| Flavonoids, isoflavonoids | C6-C3-C6 |
| Lignans, neolignans | (C6-C3)2 |
| Bioflavonoids | (C6-C3-C6)2 |
| Lignins | (C6-C3)n |
| Condendes tannins | (C6-C3-C6)n |

Table 1: Classes of Phenolic compounds

This group of phenolic compounds 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 M.L., et al., 2013; Gallardo C., et al., 2006). The simplest phenolic acids found in nature take the name of benzoic acids. This class of polyphenols has characterized by the presence of seven carbons atoms ($C_6$-$C_1$) and, according to hydroxylation and methoxylation of the aromatic ring, can present variations in the basic structure. Hydroxybenzoic acids (HBAs) can be found as free or conjugates, with sugar, usually glucose, in fruits and vegetables. Gallic, 4-Hydroxybenzoic, protocatechuic, vanillic and syringic acids are part of phenolic acids can occur as esters of glucose to produce the so-called hydrolysable tannins. Hydroxybenzoic acid glycosides are also characteristic of some herbs and spices (Clifford M.N., et al., 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. Caffeic (3,4-dihydroxycinnamic acid), ferulic (3- methoxy-4-hydroxy), *p*-coumaric (4-hydroxy) and sinapic (3,5-dimethoxy-4- hydroxy) acid are the most common cinnamic acids. These

molecules are widely distributed as conjugates, mainly as esters of quinic acid. This group of phenolic compounds may be divided into following subgroups according to number and position of the acyl residues. The different classes of cinnamic acids are: 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 E., et al., 1994). It's possible to find cinnamic acids condense with molecules such as tartaric, rosmarinic and malic acid, aromatic amino acids, choline, mono- and polysaccharides, glycerol and different glycosides (Clifford M.N., et al., 2000). In nature cinnamic acids are present in fruits and vegetables such as blueberries, apples, green mate, broccoli, lettuce and spinach.

***Naphthoquinones*** are a class of quinone pigments that presents a basic structure similar to naftalene ($C_6$ - $C_4$). These molecules occur in plant tissues in the reduce form, colourless and conjugated to sugar moieties. *Avicenniaceae*, *Bignoniaceae*, *Boraginaceae*, *Droseraceae*, *Ebenaceae*, *Juglandaceae*, *Nepenthaceae*, and *Plumbaginaceae* are the principal plants in which we can find this type of phenolic compounds.

***Xanthones*** ($C_6$ - $C_1$- $C_6$) occur in the leaves, roots and wood of two families of higher plants, *Gentianaceae* and *Guttiferae*. This group of polyphenols can be classified into five major groups that are: simple oxygenated xanthones, subdivided into six groups according to the degree of oxygenation, xanthone glycosides, prenylated and related xanthones, xanthonolignoids, and miscellaneous.

Another class of phenolic compounds takes the name of ***Stilbenes*** that are characterized by the $C_6$ - $C_2$- $C_6$ structure. Stilbenes have two benzene rings divided by ethane or ethenic bridge. It's possible to find stilbenes in liverworts, in some ferns, in gymnosperms, and in

many dicotyledonous angiosperms. Resveratrol who is principally present in grape and wine is the most studied stilbene in the world.

The most common phenolic compounds present in nature are ***flavonoids*** that represent about half of the total phenolic compounds present in the world (Iwashina T., et al., 2000). Flavonoids are synthesized by the chalcone synthetase enzyme, starting from three molecules of Malonyl-CoA and one molecule of 4-Cumaroil-CoA. The biosynthesis of the various flavonoids, as well as the genetics of the plant and its biological cycle, is regulated by numerous external factors such as light, temperature, soil composition, mechanical damage, attack by parasites, etc. In recent years, various functions have been attributed to flavonoids such as capture of oxygen free radicals, anti-cancer, antidepressant, antibacterial, antibiotic and anti-allergic action (Kinoshita T., et al., 2005).

According to the degree of hydroxylation and the presence of the double bond between carbons 2 and 3, in the heterocyclic ring, flavonoids can be divided into 13 classes, the most important being flavonols, flavanols, isoflavones, anthocyanidins or anthocyanins and flavonones.

Within these categories there are many varieties at the level structural, depending on the degree of hydroxylation and hydroxylation of the three characteristic rings of such compounds. Flavonoids often occur as sulphate or methylated derivatives or combined with oligosaccharides, lipids, amines, carboxylic acids and organic acids to form complexes.

*Flavones* are characterized by a double bond between the C2 and C3 positions and are the least common flavonoids. The only food sources of flavones such as apigenin are parsley and celery. Large quantities of polymethoxylated flavones are found in the peel of fruits such as the peel of the mandarin where their content reaches up to 6.5g/L of essential oil of this fruit (D'Archivio M., et al., 2007; Manach C., et al., 2004).

*Flavonols* have a double bond between the C2 and C3 positions and a hydroxyl in C3. This class of flavonoids is the most abundant in foods and among these quercetin is the most representative compound. The richest food sources in flavonols are onions (up to 1.2 g / kg of fresh weight), kale, leeks, broccoli and blueberries. Beverages such as tea and red wine can contain up to 45 and 30 mg of flavonols/L, respectively (D'Archivio M., et al., 2007). Their biosynthesis is stimulated by light in fact they accumulate in the outer tissue of the fruit. Furthermore, different concentrations of flavonols may exist between fruits of the same tree and also in different parts of the same fruit, depending on the exposure to sunlight (Manach C., et al., 2004).

*Flavanones* are characterized by the presence of a saturated chain of three carbon atoms and one oxygen atom in C4. They are generally glycosylated from a disaccharide in C7. This class of compounds is present in high concentrations in citrus fruits, but also in tomatoes and some aromatic plants such as mint. The main ones are narigenin in grapefruit, hesperidin and narirutin in oranges and eriodictol in lemons. An orange juice contains about 470-761 mg/L of hesperidin and 20-86 mg/L of narirutin. Generally, a very high content of flavanones is found in the solid part of citrus fruits, in particular in the white spongy portion (the albedo) and in the membranes that separate the fruit into segments; this is the reason why the whole fruit can contain up to 5 times more flavanones than a glass of orange juice (D'Archivio M., et al., 2007; Manach C., et al., 2004).

*Flavan-3-oils* are characterized by the presence in the C ring of three saturated carbon atoms, a hydroxyl group in C3 and the absence of oxygen in C4. They can exist in the form of monomers (catechins) and polymers (proanthocyanidins). Unlike other classes of flavonoids, flavan-3-oils are not found in glycosylated form in foods, but are often found in acylated form. The catechins in turn can be classified into catechin, epicatechin, gallocatechin, epigallocatechin and epigallocatechin-gallate. Catechin and epicatechin are present in many fruits such as cherries (250 mg/kg of weight), apricots (250 mg/kg of

weight) and in red wine (up to 300 mg/L). Gallocatechin, epigallocatechin and epigallocatechin-gallate are found abundantly in tea (up to 80 mg/L) and in chocolate (up to 600 mg/L) (D'Archivio M., et al., 2007; Manach C., et al., 2004). Proanthocyanidins are also known as condensed tannins and are dimers, oligomers and polymers of catechins. Difficult to quantify in foods because they are very different in structure and molecular weight, they are responsible for the astringent character of fruit (grapes, apples, berries, etc.) and some beverages (wine, cider, tea, beer, etc.) and the bitterness of chocolate. This astringency changes as it ripens and often disappears when the fruit is fully ripe, which can be linked to a change in the levels of these molecules (Manach C., et al., 2004).

*Isoflavones* have the basic structure of 1-2 diphenyl-propane. They are characterized by the presence of hydroxyl groups in position C7 and C4, such as the estradiol molecule, in fact, precisely this characteristic unites them structurally to estrogen. They can bind to estrogen receptors, in fact they are classified as phytoestrogens. (D'Archivio M., et al., 2007; Manach C., et al., 2004)

They are contained exclusively in legumes. In fact, soy and its derivatives represent the main source of isoflavones, and contain the three main molecules such as genistein, daidzein and glycitein which occur as aglycones or more frequently in conjugated forms of glucose. The soybean sprouts come to contain isoflavone levels between 140 and 1530 mg/kg by weight and soy milk can contain between 12 and 130 mg/L (D'Archivio M., et al., 2007; Manach C., et al., 2004).

*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 $C_6\text{-}C_3$ 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 $C_6\text{-}C_3$ 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. 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* are phenolic compounds of intermediate to high molecular weight (500-3000 Da) and can be classified into two main groups: hydrolysable and non-hydrolysable tannins or condensed tannins. There is a third group of tannins, phlorotannins, which have been found solely within brown algae and are not commonly consumed by humans. Hydrolyzable tannins have at the center either a glucose molecule or a polyvalent alcohol, partially or completely esterified with gallic acid or hexahydroxydiphenic acid, forming gallotannins and ellagitannins, respectively. These metabolites are readily hydrolyzed with acids, bases or enzymes. However, they can also be oxidatively condensed to other galoyl and hexahydroxydiphenics and form high molecular weight polymers. The most studied hydrolyzable tannin is tannic acid. The condensed tannins are polymers of catechin and/or leukoanthocyanidin, not easily hydrolysed by acid treatment, and constitute the phenolic fraction mainly responsible for the astringency characteristics of vegetables. Although the term condensed tannins is still widely used, the chemically more descriptive term "proanthocyanidins" is gaining acceptance. These substances are flavonoid polymers that form anthocyanidin pigments. The most studied proanthocyanidins are based on flavan-3-oils, (-)-epicatechin and(+)-catechin. Although the antioxidant activity of tannins is much less marked than the activity of flavonoids, recent studies have shown that the degree of polymerization of these substances is linked to their antioxidant activity. In condensed and hydrolysable tannins (ellagitannins) with a high molecular weight, the activity can be up to fifteen to thirty times higher than those attributed to simple phenols (Sánchez-Moreno C., et al., 2002).

## 1.3 PHENOLIC COMPOUNDS: HEALTS BENEFITS

Polyphenols are the secondary metabolites that plants produce to protect themselves from organisms. It has been found that the polyphenol diet plays an important role in human health. The high intake of fruit, vegetables and some grains, rich in polyphenols have been correlated with the reduction of the risk of many chronic diseases including cancer, cardiovascular disease, chronic inflammation and many degenerative diseases. Recent studies have shown that many of these discomforts are linked to oxidative stress caused by reactive oxygen and nitrogen species. Photochemicals, especially polyphenols, are the main contributors to the total antioxidant activity of fruit, in addition to vitamin C. Phenolic compounds have been found to be strong antioxidants capable of neutralizing free radicals by donating an electron or a hydrogen atom. Highly conjugated systems and some hydroxylation patterns such as the 3-hydroxy group in flavonols are considered important for antioxidant activity (Stalikas C.D., et al., 2007). Polyphenols suppress the generation of free radicals, so as to reduce the oxidation rate, inhibiting the formation or deactivating the active species or the precursors of free radicals. More frequently they act as scavengers of the radicals of the chain reactions of lipid peroxidation. The chain switches donate an electron to the free radical, neutralizing the radical and itself, becoming a stable radical, thus blocking the reaction. In addition to their scavenger activity, polyphenols are known as metal chelators. The chelation of transition metals such as $Fe^{(2+)}$ can directly reduce the rate of the Fenton reaction, thus preventing oxidation caused by highly reactive hydroxyl radicals. Phenolic compounds can also act as coantioxidants, and are involved in the regeneration of essential vitamins. Various in vitro antioxidant model systems have been devised to assess total antioxidant activity. Although these methods are limited in terms of similarity to antioxidant mechanisms of action in biological systems, together they may well represent the functioning of polyphenols as antioxidants, hence their role in human health. Care should be taken when antioxidant activity is assessed by in vitro models. From the chemical point

of view, the molecules of polyphenols, after having donated an electron or a hydrogen atom, become free radicals, and, at fairly high concentrations, can potentially cause pro-oxidant activity. n addition to the antioxidant activity reported above, other activities, such as inhibition of xanthine oxidase and increase of endogenous antioxidants, are considered important. Polyphenols can induce antioxidant enzymes such as glutathione peroxidase, catalase and superoxide desmutase to degrade hydroperoxides, hydrogen peroxide and superoxide anion, respectively, and inhibit the expression of enzymes such as xanthine oxidase. While the direct and indirect antioxidant activity of polyphenols could play an important role in reducing oxidative stress, as described above, the cellular roles of these compounds could be more complicated. There is a new view regarding phytochemicals, mainly regarding polyphenols and their metabolites in vitro, according to which they do not act through the typical donation of an electron or a hydrogen atom but through a modulatory action in cells on the path of protein kinase and lipid kinase signaling. Although polyphenols such as flavonoids can be absorbed through the gastrointestinal tract, the plasma concentration is low, usually less than 1 μmol/L, in part due to the rapid metabolism of human tissues. Most polyphenols are found in a very low concentration, not sufficient to exert any significant and direct antioxidant activity, so many researchers believe it is unlikely that polyphenols exert antioxidant activity in vivo. Therefore the focus must be shifted to activities other than the usual one of antioxidants. Nutrigenomics is emerging as a new multidisciplinary area of research, not only for polyphenols, but for phytochemicals in general. Effects on biomarkers involved in various signaling pathways can lead to changes in cell functions with possible beneficial effects on health. Williams et al. states that: << A clear understanding of the mechanisms of action of flavonoids, both as antioxidants and as cell signaling modulators, and the influence of their metabolism on these properties, are the key to the evaluation of these powerful biomolecules as anti-cancer agents, cardiac

protectors, and neuro degeneration inhibitors >>. Future research on polyphenols will likely move in the same direction (Tsao R., et al., 2010).

# CHAPTER 2: TECHNIQUES FOR EXTRACTION OF FOOD PHENOLIC COMPOUNDS

Sample preparation is an important and essential step of the analytical process, each analytical method provides for a sample preparation procedure, before the actual analytical determination. The analytical process involves several steps, which usually include: sampling, extraction, purification of the extract, which in some cases may be conducted simultaneously with the extraction, possible concentration or dilution of the extract, analytical determination and results interpretation. Also for the analysis of phenolic compounds it's necessary to follow these step.

## 2.1 SAMPLE STORAGE AND PREPARATION

The storage and preparation of the sample must be carried out in such a way as to avoid physical, chemical-physical or biological phenomena capable of altering the representativeness of the sample itself. Volatilization of some component and the diffusion and absorption on the surfaces with which the sample comes into contact, represent the physical processes that can alter a sample.

The choice of the container in which to place the sample is therefore extremely important. Usually, taking into consideration the possible interactions between analytes and container, we choose between glass, metals and plastics. Among the physical changes that the sample can take into account is the variation of the water content, which can determine, in the case of an increase in the quantity, an increase in the value of Aw, water activity, with consequent possible action of enzymes or proliferation of microorganisms. Chemical changes include photochemical reactions, redoxes and precipitations. It is therefore essential to protect the sample from light or contact with air, if the parameters of interest are related to the level of oxidation or, for example, to the antioxidant capabilities of a particular matrix or food.

Samples are generally stored in the freezer (-18 ° C), or in the refrigerator, reducing storage time as much as possible. In some cases, treatment with low temperatures may not be adequate and cause irreversible precipitation of some components. Before the actual preparation of the sample, the homogenization and dissolution of the sample is usually carried out. Generally, homogenization mainly concerns solid samples, therefore a frequent operation is grinding. It is necessary to avoid unwanted and side effects, such as the loss of volatile components caused by heating and the absorption of moisture due to the increase in the surface area. To increase the extraction effectiveness, some preliminary operations can be carried out, such as dehydration or freeze-drying to remove the water, in order to ensure greater contact between the analyte and the extractive solvent (Koziel J.A., et al., 2002).

## 2.2 SAMPLE PRETREATMENT

The type of pre-treatment to choose is strictly dependent on the physical nature of the sample, the method of analysis that will be used and the concentration of the analyte itself in solution. The pre-treatment can simply consist in bringing the sample into the most appropriate form of the analysis rather than being characterized by many more steps.

During sample pre-treatment it is possible to isolate, so as to select only the analyte of interest; concentrate, compounds present in too low concentrations must first be concentrated in order to be analyzed and purify, to eliminate interferents and try to have the analyte of interest with as few molecules as possible in solution.

In the sample pretreatment, three types of analysis approach are possible:

- Direct analysis on the sample without pre-treatment, or with simple mechanical manipulations: Eg. a solid food that must be ground using a mortar rather than blades, followed by a method that allows you to analyze the solid sample.

- Wet analysis: requires the need to bring the sample or analyte into solution. In some cases it is not only sufficient to bring into solution but the analyte extraction procedure is also necessary.

- Non-homogeneous phase analysis (rare cases, as nephelometry in which it is necessary to have the coexistence of several phases in the same sample).

## 2.3 EXTRACTION TECHIQUE FOR PHENOLIC COMPOUNDS

In this phase, the most important aspect is given by the physical state of the sample as different approaches will have to be used depending on the liquid, solid or gaseous state of my sample.

There are various extraction techniques that can be applied to derive the analyte of interest from our sample. It is possible to distinguish the various extraction techniques depending on the characteristics of the analyte of interest. The protocol for the extraction of phenolic compounds from plant-based matrices must be set up taking into consideration numerous factors related to the complexity of the sample, such as the presence of interfering substances and the solubility of the analytes of interest, their degree of polymerization and the formation of complexes with carbohydrates and proteins. These evaluations are followed by the choice of solvents and the size of the particulate in which the sample is reduced in view of extraction. The large number of factors to consider means that there is no universal and completely satisfactory procedure for the extraction of phenolic compounds, in whole or in classes, from different matrices of plant origin and involves the study of a peculiar extraction procedure for each sample examined (Naczk M., et al., 2004).

In the literature, the most used method for this purpose is solid/liquid extraction. The most common solvents are methanol, ethanol, water, acetone and their solutions in different proportions. The interactions, in particular hydrogen bonds, close with the hydrophilic portion of the phenolic compounds may explain the effectiveness of these solvents

(Schuelter Boeing J., et al., 2009). Furthermore, solvents are often acidified as it is known that polyphenols are more stable at low pH. Usually the use of weak acids or low concentrations of strong acids is preferred to avoid the hydrolysis of glycosides; an exception is made for samples in which the molecules of interest are linked to structural materials or particularly complex glycosidic structures for which acid, alkaline or enzymatic hydrolysis is specifically sought in order to obtain a more efficient chromatographic separation and to simplify the operations of quantification and qualification (Tsao R., et al., 2010). The extraction times vary between one minute and 24 hours, with increasing time the risk of oxidation of phenolic compounds increases, unless antioxidants are added to the mixture of solvents (Naczk M. et al., 2004). In Table 2 are reported a series of optimized condition for extraction of phenolic compounds from plants using different extraction techniques subsequently explained.

Table 2: Optimized condition for extraction of phenolic compounds from plants

| Sample | Phenolic compound | Extraction technique | Solvent | Time (min) | Temperature (°C) | Ref. |
|---|---|---|---|---|---|---|
| Coffee beans | Chlorogenic acids | Solid-liquid extraction | acetonitrile:water solution (5:95; v/v) | 10 | 80 | Farah et al., 2019 |
| Coffee beans | Chlorogenic acids | Solid-liquid extraction | methanol:water solution (50% v/v) | 90 | Room temperature | Mussatto et al., 2011 |
| flowers of *Cassia angustifolia* | Flavonoids | Microwave-assisted extraction | 70% aqueous ethanol | 9 | 50 | Laghari et al., 2011 |
| Medicinal plants | Flavonoids Phenolic acids | Microwave-assisted extraction | 50% aqueous ethanol | 3–5 | 70 | Salinas-Moreno et al., 2015 |
| Green coffee beans | Chlorogenic acids | Microwave-assisted extraction | Water | 5 | 50 | Upadhyay et al., 2012 |
| Olive leaves | oleuropein, luteolin-40-O-glucoside | Ultrasound-assisted extraction | 80% aqueous ethanol | 1 | 60 | Giacometti et al., 2018 |
| Flax seeds | Lignan, flavonol, hydroxycinnamic acids (HA) | Ultrasound-assisted extraction | water with 0.2 N of sodium hydroxide | 60 | 25 | Corbin et al., 2015 |
| Brosimum alicastrum leaf | Total phenolic content, total monomeric anthocyanin | Ultrasound-assisted extraction | 80% aqueous methanol | 20–10 | 28 | Gullian Klanian et al., 2017 |
| Eugenia uniflora | Phenolic compounds | Supercritical fluid extraction | | 360 | 60 | Garmus et al., 2014 |
| Guaraná seeds | Phenolic compounds (pyrogallol) | Supercritical fluid extraction | | 40 | 40 | Marques et al., 2016 |

### 2.3.1 SOLVENT EXTRACTION

This is a very common pre-treatment technique. This technique allows the analytes of interest to be selectively brought into solution, leaving the matrix almost intact, i.e. depleted of the analytes of interest, but without destroying it. It is carried out in a closed container by placing the sample in contact with a solvent immiscible with it, in which however the analytes are soluble (Baiano A., et al., 2011). Exist two type of solvent extraction: liquid-liquid extraction, if both the sample and the extracting solvent are liquid and liquid-solid extration if carried out with a liquid solvent on a solid sample.

#### 2.3.1.1 Liquid-liquid extraction

Liquid-liquid extraction is a selective extraction and the need to choose the most suitable experimental conditions, such as to favor only the extraction of the analytes, allowing their concentration and eliminating the interferents. This technique is based on the principle of dividing an analyte between two liquid phases that are immiscible to each other, since each substance tends to distribute itself between two liquids in a ratio corresponding approximately to the solubility that the compound itself has in each solvent. Solution, in which a second solvent is added, will tend to be solubilized also in the second solvent in an amount which will depend on the solubility of the analyte in its own solvents.

The distribution of the analyte between the two liquids occurs according to a quantity that takes the name of the partition coefficient, which is given by the ratio between the concentration of the analyte in the solvent in which it is dissolved and the concentration of the same analyte in the extraction solvent.

$K = Cs/Ce$

K = Partition coefficient

Cs = Concentration of analyte in the solvent

Ce = Concentration of analyte in the extraction solvent

One of the crucial steps in liquid-liquid extraction is the choice of the extraction solvent. The selected extraction solvent must have the following characteristics:

- Be immiscible with the solution on which the extraction is applied, so that it can subsequently have a phase separation.
- Having a K that is high for compounds of interest, and low for impurities and compounds that I don't want to extract.
- Be easily eliminated. Once the extraction has taken place, the solvent must be easily removed (physical methods, evaporation).
- Characterized by a polarity that is suitable for the extraction of the analyte of interest.

This extraction is usually carried out in a separating funnel.

Initially, the solution containing the analytes of interest is added, then the second solvent, suitably chosen, is added and the funnel is stirred, favoring the contact of the analyte with the two solvents. The analytes present in the solution will also be distributed in the second solvent, based on the partition coefficient. In this phase often emulsions are formed at the contact interface between the two liquids, and this makes the solution cloudy. To avoid this problem and making separation more effective is possible add inorganic salts, such as sodium chloride or magnesium chloride, which vary the ionic strength and minimize the formation of emulsions. At the end of the procedure the solvent with higher density is will find in the lower portion while the one with lower density in the higher portion. The wide range of solvents allows for highly selective extractions.

This technique, widely used for the extraction of various analytes from food matrices, however, has several disadvantages:

- Use of large quantities of solvent with consequent disposal problems. It is not a green technique.

- Tendency to form emulsions when the two solvents are mixed. Difficulty in physical separation

- The extraction solvents are limited to those immiscible with water (for aqueous samples). Limited choice.

- The operations are usually manual and take a long time.

Once the extraction is complete, the collected solvent is evaporated, the solutes are taken up in a smaller volume in order to obtain the concentration of the extract (Baiano A. et al., 2011).

#### 2.3.1.2 Microwave-assisted solid-liquid extraction

The solid-liquid extraction can be carried out simply by stirring, ie by placing the solid in contact with the appropriate solvent and shaking in such a way as to allow the extraction of the analyte.

To facilitate the extraction of the analyte from the solid matrix, it is possible to use a solid-liquid extraction assisted by microwave.

This technique is carried out through the use of microwave baths. The solid matrix, once reduced into smaller particles, is placed in contact with a suitable solvent which is subsequently heated by microwaves. The microwaves heat and facilitate the extraction of the analyte as by increasing the energy of the particles, it increases their movement and therefore the contact surface with the solvent (Baiano A. et al., 2011).

### 2.3.2 ULTRASOUND ASSISTED EXTRACTION (UAE)

Ultrasound-assisted extraction (UAE) is an advanced extraction technique that dramatically reduces extraction time, increasing yields. UAE is based on the use of mechanical waves that need an elastic medium to spread. Ultrasound applications, characterized by sound power (W), sound intensity (W/m2) or sound energy density (W/m3), may be subdivided

into two groups: high intensity low frequency (20 kHz ≤ f ≤ 100 kHz), that do not alter the physical or chemical properties of the material through which the ultrasonic wave propagates and low intensity high frequency (f > 100 kHz), that generate intense pressures and temperature gradient due to the bubble cavitation producing a disruption effect within matrix.

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 P., et al., 2013).

### 2.3.3 SOLID PHASE EXTRACTION (SPE)

The principle of SPE is similar to that of liquid-solid extraction (LSE): in a suitable container, the liquid sample is placed in contact with the solid adsorbent, and then is stirred for a predetermined time (Huck C.W., et al., 2000). The analytes related to the solid adsorbent are adsorbed by it and can be subsequently eluted, using a solvent that has a greater affinity towards them.

The SPE is placed between the classic LSE and liquid chromatography on column, in fact, the adsorbent phase is placed in a column or cartridge in which the liquid sample is passed. Analytes are retained and concentrated on the surface of the adsorbent if they have a greater affinity for the adsorbent than for the solvent in which they are dissolved, while the interferents are removed by the use of an elution solvent or by subsequent washing with a specific solvent. On the contrary, if the adsorbent phase has a greater affinity towards the interferents, these will be retained by the solid phase, while the analytes will subsequently be eluted with a suitable solvent. The first approach allows to obtain a concentration of the analyte, since the elution volume is lower than the loading one and requires a lower quantity of adsorbent, for this reason it is the most used.

The SPE exploits the same analyte-adsorbent interactions, used in a very powerful separation technique, widely used in laboratories, that is, HPLC, of course, greatly varies the purpose of the two techniques. In the case of HPLC, the goal is to separate the analytes based on the different affinity they have for a stationary phase rather than for a mobile phase, in the SPE we want to strongly consider the analyte or a class of analytes in phase loading the sample, and then eluting them, using the least amount of solvent possible, completely. The SPE column has a number of theoretical plates (N) that is around twenty, much lower than the value of theoretical plates that characterizes the columns for HPLC.

The cartridges used in SPE have a cylindrical body made of polyethylene (PE), or polypropylene (PP), with a female luer connection at the upper end, to apply positive pressure, and a male luer connection at the lower end, to connect between of them multiple cartridges or apply vacuum. The packaging material is held in place by two frits (septa) made of PE or PP. In the past, the materials used for the frits released impurities, therefore low-release medical-grade materials were subsequently introduced (Fritz G. et al., 2002).

The SPE column, often referred to by the term cartridge, consists of a cylindrical body similar to that of a syringe without a piston. The adsorbent phase, with a particle diameter between 20 and 60 μm, is held in place by two frits (Poole et al., 2012). There are commercially available columns of different sizes and mass of adsorbent: the dimensions can vary from 1 to 60 mL, while the weight of the adsorbent varies from 50 mg to 10 g (Camel V. et al., 2003).

The characteristics of the phase mirror those used in chromatography.

- Reverse phase (modified non-polar solid phase, polar liquid phase): alkyl chains bind to silica, a material of this type has hydrophobic characteristics, so it will be a cartridge capable of retaining hydrophobic and non-hydrophilic compounds.

- Normal phase (polar modified solid phase, non-polar liquid phase): the interaction between the adsorbent material and the analytes is polar. It is therefore a solid phase with polar characteristics and requires the use of less polar solvents for elution.
- Ion exchange: It is based on the electrostatic attraction of groups with opposite charge. The solid material is derivatized with a charged group (amino group, deprotonated carboxylic acid group) and the analytes that bind to the adsorbent material are those that have the opposite charge to that of the adsorbent itself.

The columns can be processed in special devices connected to a vacuum pump and equipped with connections for the cartridges and a test tube holder to position the collection vials (Figure 8). In addition to facilitating the passage of samples or viscous solvents, the application of vacuum allows the adsorbent to be dried more easily when non-miscible solvents are used in succession (Moret S. et al., 2014).

There are various advantages due to the use of the SPE technique, compared to the use of conventional techniques. In fact, its use requires rather short extraction times and relatively low costs. In addition, the SPE is suitable for field sampling, simplifies the sample storage phase and is easily automatable and interfaceable with chromatographic and spectroscopic separation techniques (Camel V. et al., 2003).

### 2.3.4 MODERN EXTRACTION TECHINIQUES FOR PHENOLIC COMPOUNDS

In the last time the use of ultrasound-microwave-assisted extraction (UMAE), supercritical fluid extraction (SFE), sub-critical water extraction (SCWE) and high hydrostatic pressure processing (HHPP) is increasing. These methods are relatively simple to perform, are characterized by short extraction time and decrease the release of toxic pollutants through reducing organic solvent consumption.

#### 2.3.4.1 Ultrasound/Microwave Assisted Extraction (UMAE)

A new efficient approach to extract bioactive compounds is represents by the coupling of two powerful radiation techniques (ultrasonic and microwave). MAE (Microwave Assisted Extraction**),** as previously described, is a simple and rapid technique using dielectric mechanisms to heat samples and extract the plant bioactive compounds, whereas UAE (Ultrasonic Assisted Extraction) trough the formation of cavitations, increase mass transfer and improve penetration of the solvent into the sample. UMAE is a powerful technique characterizes by short extraction time, low consume of volumes of solvents and with an higher extraction yields than conventional extraction, MAE and UAE.

#### 2.3.4.2 Supercritical Fluid Extraction (SFE)

Another environmentally friendly extraction technique, which is a good alternative to conventional organic solvent extraction methods can be the Supercritical fluid extraction (SFE). It may lower the requirement for toxic organic solvents, increase safety and selectivity, lower extraction time and facilitate separation of the extract from the supercritical fluids (SCF). Degradation of extracted compounds can be avoided in the absence of air and light and the possibility of contaminating the sample with solvent impurities is much lower than in other conventional methods. The only big problem related to this new technique is the high capital investment.

#### 2.3.4.3 Subcritical Water Extraction (SCWE)

Subcritical water extraction (SCWE), also known as superheated water, pressurized water or hot liquid water extraction, is a modern technique efficiently used to isolate phenolic compounds. SCWE is very simple and has an higher extraction quality compare to that of the other extractions. It's characterized by low extraction time and environmental friendliness due to water being used as the solvent. This technique also allows polar compounds to be extracted and is less expensive than SFE.

#### 2.3.4.4 High Hydrostatic Pressure Extraction (HHPE)

HHPE utilizes non-thermal super-high hydraulic pressure (1.000–8.000 bar) and works on the basis of mass transport phenomena. The pressure applied increases plant cell permeability, leading to cell component diffusivity according to mass transfer and phase behavior theories. A main disadvantage of methods such as HHPE, SCWE and SFE is that expensive equipment is required; *i.e.*, a solvent transporting pump, a pressure vessel and system controller, and a collection device for the extract. In the case of antioxidant extraction, in which products are in great demand and high purity of extract and processing efficiency are expected, the price of equipment might not play a critical role in selection of these methods (Khoddami A., et al., 2013).

### 2.3.5 EXTRACTION WITH NON CONVENTIONAL SOLVENTS: DEEP EUTECTIC SOLVENTS

As previously reported several extraction techniques can be used for the extraction of phenolic compounds from food matrices. Most of the previous studies used organic solvents like methanol, ethanol, dichloromethane, ethyl acetate, isopropanol and hexane for the extraction. Moreover, green solvents like supercritical fluids, subcritical water and deep eutectic solvents (DESs) have also been tested. Recently, supramolecular solvents, nanostructured solvents made up of assembled amphiphile aggregates, have also been proposed (Fanali C., et al., 2020).

DESs with the objective to reduce toxic waste and to improve selectivity and extraction efficiency have been recently explored. These new type of solvents consist of a mixture of a halide salt, which acts as hydrogen-bond acceptor (HBA), and a hydrogen bond donor (HBD), which can be carbohydrates, amines, carboxylic acids or alcohol. Choline chloride (ChCl), a quaternary ammonium salt, has always been the most used HBA for the formation

of DESs. In recent times, betaine is widely used as an HBA. This molecules is cheaper and less toxic than ChCl (Chianioti S., et al., 2018). When each components thet composed the DES is natural we can defined it as Natural Deep Eutectic Solvents (NADES). On a chemical level, what happens between the components of the mixture is a charge delocalization that causes the lowering of the melting temperature of the mixture compared to the individual components, giving the DES particular chemical-physical characteristics. The mixture of HBA and HBD has a lower melting point than those of the individual components making possible its use as extractive solvent. A problem related to the application of DES as an extraction solvent is their high viscosity, which can result in low extraction repeatability. The viscosity of DES can be lowered by carrying out the extraction at high temperatures or by adding a certain amount of water to the DES (Musatto S.I., et al., 2011).

The preparation of a DES involves the mixing of easily available, cheap and stable compounds at temperatures between 60 and 80 ° C, without generating any waste. Precisely these aspects make their use on a large scale appear much more advantageous than polar organic solvents and ionic liquids. (Tommasi E., et al., 2017)

It has been seen that the application fields of DES can be numerous, from biocatalysis to organic synthesis, to electrochemistry and development of materials (nanoparticles, metal-phosphates, etc.); moreover, they can also be used for the pretreatment of biomass as, by causing a partial disintegration of the cell walls, they increase their permeability. Thanks to this property, they have recently been used to facilitate the extraction of lipids from algal biomass. Moreover, due to their strong ability to solubilize a certain type of compounds more than water, they have been studied as alternative means for the administration of active ingredients, for the separation of glycerol from biodisel and for the extraction of a large variety of bioactive molecules from plant matrices. Until now, DES have been used successfully for the extraction of various molecules of natural origin including phenolic acids, flavonoids, proteins and polysaccharides (Tommasi E., et al., 2017).

The chemical-physical properties of DES, such as melting point, density, conductivity and viscosity, vary according to the structure of the DES. DESs and NADESs can exhibit highly variable viscosity and density based on their composition and temperature. These properties are directly influenced by the molecular size, structure and intermolecular forces of the constituents of DESs and NADESs. For example, solvents containing lactic acid with one carboxylic group have a lower viscosity than those consisting of citric acid with three carboxylic groups, this is due to an increase in intermolecular forces. Likewise, solvents made up of polyalcohols have a lower viscosity compared to a solvent made up of acid or sugar, because small molecules like polyalcohols have weaker molecular interactions. (Benvenutti L., et al., 2019)

The type of hydrogen bond donor, the type of salt and their molar ratios are factors that influence the density, surface tension and viscosity of DESs which generally have a higher viscosity and lower conductivity than other solvents. molecular and ionic liquids. (Zainal-Abidin M. H., et al., 2017). The peculiar characteristics of the DESs (polarity, viscosity and solvent capacity) are easily modulated by varying the molar ratios of the components and the water content. This enhances its versatility. DES generally have a polarity between that of acetone and that of water. DES and NADES are generally defined hydrophilic due to their high electronegativity and ability to form hydrogen bonds through dipole-dipole interactions. Hence, they are usually comparable with polar solvents, although some mixtures, such as citric acid and methanol, show polar and non-polar properties to each other. (Benvenutti L., et al., 2019)

In addition, by adding water, up to about 40% by weight, it is possible to create a ternary mixture (aqueous DES, aDES) which greatly reduces its viscosity and preserves the intermolecular interaction of the components. (Tommasi E., et al., 2017)

The viscosity of DES or NADES can therefore be reduced by adding a certain amount of water. Bajkacz and Adamek (2017), evaluated the water content in NADES (10 to 75% w/w)

for the micro-extraction of flavonoids from plant matrices. According to the authors, the extraction yield increases with the increase in water content up to 30%. For water concentrations of 75% a dilution was obtained so high as to weaken the hydrogen bonds, reducing the solvation capacity of the DESs or NADESs. (Benvenutti L., et al., 2019) It is also appropriate to mention the importance of the pH of DESs/ NADESs solvents in the extraction process of the target compounds. The pH of the DESs/NADESs varies according to the constituents of the solvent in question. In general, ADES (acid-based DES), low pH solvents, have an excellent dissolving property due to their ability to donate protons and accept electrons, and are suitable for the recovery of low polarity compounds such as catechins and polar compounds. such as anthocyanins. While, DES/NADES consisting of polyalcohols such as 1,2-propylene glycol, with a neutral pH, have a large extraction capacity of compounds such as phenolic acids. DES/NADES consisting of polysaccharide components (sugar-based), also with neutral pH, have a high selectivity for the recovery of flavonoids (Benvenutti L., et al., 2019). Based on the aforementioned characteristics, it was seen how DES can be "custom built" to meet the needs and flexibility desired by the chemical industry. (Tommasi E., et al., 2017)

# CHAPTER 3: TECHNIQUES FOR DETERMINATION OF PHENOLIC COMPOUNDS

Using separative techniques it's possible to isolate the single phenolic compounds and to do an accurate quantitative and qualitative analyses of them. The instrumental analysis commonly used for this purpose is High Performance Liquid Chromatography (HPLC) conducted with a reversed phase column and coupled to a diode array spectrophotometric detector (DAD) and a mass spectrometer (LC-MS) (Tsao R. et al., 2010). The most recent studies report the use of UHPLC instrumentation, equipped with columns packed with particles with a diameter of less than 3 μm, which are therefore very efficient, and pumps capable of supporting the relative back pressure. The total antioxidant activity of the extract can be measured easily and quickly with spectrophotometric assays. The chromatographic technique was first used by Mikhail Tswett in the early 1900s. Between 1960 and early 1970 there were notable improvements, thanks to the development of algorithms, including Fourier's, and related innovations, such as for example, the packaging of high performance liquid chromatography (HPLC) columns (Thorngate J.H. et al., 2006). The term HPLC refers to a modern technique, with characteristics strongly different from the other techniques, which are however still used for preparatory purposes. The HPLC technique is efficient, highly selective, of wide applicability, requires only small quantities of sample, may not involve the destruction of the sample and is easily adaptable to quantitative analysis. The introduction of the diode array detector (Diode Array Detector or DAD) has improved the performance of HPLC in the analysis of phenolic compounds, for example by increasing the sensitivity of the detector as it allows reading at maximum absorbance and evaluation purity of chromatographic peaks (Santos G. et al., 2003). Another technique used in the determination of phenolic compounds is Mass Spectrometry. This analytical technique of structural elucidation is based on the ionization of a molecule brought into the gas phase and

on the subsequent fragmentation of ions of different mass/charge ratio (M /z). MS electronic ionization was initially used to study chalcone-reduced flavones (Brown B.R., et al., 1964). While electronic ionization techniques have been used to characterize monomeric phenolic compounds, the application to oligomers and polymers, due to the increase in volatility, was very limited. (Lazarus S.A., et al., 2003).

Among the spectrometry/spectroscopy techniques are to be mentioned:

- UV-Vis spectrometry (ultraviolet and visible): has limited utility for identifying the classes of phenolic compounds.

- IR spectrometry: powerful tool in the identification of structures, as the covalent bonds absorb electromagnetic radiation in the IR region. It can only be used in the sample preparation phase, since many phenolic compounds are insoluble in the solvents used for IR analysis (Thorngate J.H., et al., 2006).

- NMR spectrometry or nuclear magnetic resonance: examines the magnetic moments of atomic nuclei with an odd mass or atomic number. Initially, due to the complex structures of flavonoids, protons were difficult to stabilize and their application to phenolic compounds was limited. The technique was able to identify these protons displaced on defined regions of the spectrum. With the application of the Fourier derivative to NMR, the conditions have improved. (Ernst R.R., et al., 1966).

### 3.1 HPLC (High pressure liquid chromatography)

Liquid chromatography (LC) represents a technique used to obtain the separation of a mixture of several 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. 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, that is the widely used for analysis of phenolic compounds, has a non-polar stationary phase and an aqueous, moderately polar mobile phase.

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 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.

### 3.1.2 Components of the instrument

High performance liquid chromatography instrumentation tends to be much more elaborate, sophisticated and expensive than that encountered in other types of chromatography, having to ensure the achievement of much higher pressures (Holler J.F. et al., 2015).

#### 3.1.2.1 Containers for mobile phases and treatment systems of sample

A modern HPLC equipment is equipped with one or more glass containers, each of which can contain 500 mL or more of solvent. Systems are often provided to remove dissolved gases in powder form from solvents. The gases can produce bubbles in the column and cause bandwidths, in addition, both the gas bubbles and any dust could interfere with the detector's performance, reducing its reliability. The degassing system can consist of a vacuum pump, a distillation system, a system for heating and stirring the solvent, or a bubbling system in which the dissolved gases are removed from the solution by a stream of small bubbles of an inert gas insoluble in the mobile phase. If the elution is carried out using as mobile phase

only a solvent or a mixture of the solvent, with constant composition, it is called isocratic, in the case in which instead two solvent systems are used, whose polarity differs significantly, and whose ratio it is made to vary in a programmed way during the separation, the elution is called gradient (Holler J.F. et al., 2015).

### 3.1.2.2 Pumping systems

A liquid chromatography pump system needs some requirements such as ability to generate pressures up to 6000 psi (Ib/in$^2$), a pulse-free output signal, flow rates ranging from 0.1 to 10 mL/min, reproducibility of flow greater than or equal to 0.5%, resistance to corrosion by various solvents. The high pressures generated by liquid chromatography pumps are not at risk of explosion since liquids are not very compressible, for this reason the failure of a component will only cause the solvent to escape. However, in the specific case of some solvents, this loss can lead to fires or environmental damage. The types of pumps most used are:

-Syringe Pumps: These pumps produce pulse-free delivery, the flow of which is easily controlled; they have the disadvantage of insufficient capacity and are not convenient when must be used different solvents.

- Reciprocating pump: this pump consists of a small cylindrical chamber which is filled and emptied by the reciprocating movement of the piston. This pumping system produces a pulsed flow, which must subsequently be damped. The advantages of reciprocating pumps are small internal volume, high external pressures, easy adaptability to gradient elutions and constant flow rates, largely independent of column back pressure and solvent viscosity. Most of the chromatographs on the market use reciprocating pumps.

- Pneumatic pump: this pump consists of a container of compressible solvent, housed in a container that can be pressurized by a compressed gas. Such knobs are simple, inexpensive and do not give impulses; they have as a disadvantage limited capacity and output pressure

and the dependence of the pumping speed on the viscosity of the solvent, so they are not adaptable to gradient elution (Holler J.F. et al., 2015).

#### 3.1.2.3 Sample injection system

The most widely used method for introducing the sample in liquid chromatography is based on the use of a sampler (loop). Interchangeable samplers are often possible that allow sample sizes between 5 and 500 $\mu$L. The reproducibility of injections with a sampler of this type is within a few tenths percentages.

Many HPLC instruments contain an autosampler with an automatic injection system capable of continuously injecting variable volumes (Holler J.F. et al., 2015).

#### 3.1.2.4 Columns for high performance liquid chromatography

Although chromatography is a continuous process, it's necessary to consider that the column is divided into N sections, in each of which the equilibrium between stationary phase and mobile phase is reached. Each of these sections is defined as theoretical dish. If the total length of the column is L, the height equivalent to a theoretical plate (HETP) is: HETP = L/N.

The efficiency of a column is expressed in N theoretical plates. The higher the number of theoretical plates, the higher the column efficiency. Narrow peak corresponds to good chromatographic separation. In accordance with the above formula, the greater the number of theoretical plates, the lower the height of the theoretical plate and the greater the efficiency of the column.

Liquid chromatography columns are generally made up of stainless steel tubes, and in the case the applied pressures are less than 600 psi, glass or Tygon tubes are also used. Most of the columns are characterized by a length between 10 and 30 cm and internal diameters of 2-5 mm. The packages consist of 3 or 10 $\mu$m particles. Columns of this type typically provide

40.000 to 60.000 theoretical plates per meter. Micro-columns with internal diameters from 1 to 4.6 mm and lengths from 3 to 7.5 cm have recently been proposed: they are packed with 3 or 5 um particles, contain up to 100.000 theoretical plates per meter and have the advantage of analysis speed and minimum solvent consumption. Reduce the consume of solvents is very important because the high purity solvents required in liquid chromatography are very expensive both in terms of their purchase and in terms of their disposal. The most common packing for liquid chromatography is based on silica, prepared by agglomerating particles of silica at the size of submicron in conditions such as to give rise to larger particles of uniform diameter. The resulting particles are often coated with thin organic films, chemically or physically bonded to the surface. Particles of alumina, porous polymers or ion exchange resins are other typical packaging materials (Holler J.F. et al., 2015).

#### 3.1.2.5 Detectors

The most widely used detectors in liquid chromatography are based on absorbance measurements in the ultraviolet or visible region. There are commercially available photometers and spectrophotometers specifically built to be used as detectors in chromatography. The former usually make use of the spectral lines at 254 and 280 nm, originating from a mercury source, because many organic functional groups absorb in this region. Sources of deuterium or tungsten filaments, associated with interference filters, also provide a simple means of detecting the species they absorb. Spectrophotometric detectors are much more versatile than photometers and are also widely used in high performance instruments. Modern instruments use diode-array detectors, which can show the entire spectrum of the analyte, leaving the column. Lately the use of a combined HPLC / MS detector is becoming quite widespread (Holler J.F. et al., 2015).

## 3.2 MASS SPECTROMETRY

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, based on the ionization of a molecule and its subsequent fragmentation into ions of different mass/charge ratio (M/z). This is a destructive method of analysis, the molecule does not remain intact after analysis, and above all it is not based on the interaction between radiation and matter. 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 ions from the sample, under investigation, are separated according to their specific mass-to-charge ratio (m/z).

This technique allows to measure the molecular masses, both nominal and exact, and to obtain fragmentation profiles that are specific for each compound, of which they therefore constitute a fingerprint. It is thus possible to identify the structural formula of unknown compounds, even if small quantities are available.

The interpretation of the mass spectrum consists in the study of the signals due to the ions generated in the experiment, from which the original molecular structure can be reconstructed. The vacuum, which is around $10^{-6}$-$10^{-5}$ torr, is necessary to prevent a loss of ionization due to collision with atmospheric gases. The introduction of the sample into the ionization chamber can be carried out either by means of a probe, therefore in the solid state, or by using a system of valves that allow access to the ionization chamber without it coming into contact with the outside, therefore the liquid or gaseous state. The amount of product necessary to record a spectrum is in the order of micrograms/nanograms. If a molecule is invested in the vapor phase by an electron beam of considerable kinetic energy, it can be impacted by its ionization to positive or negative ion. Generally, the instruments are adjusted

to work only with positive ions, which can spontaneously or by collision decompose into a series of fragments of lower mass and these in turn in others. Each molecule will therefore have its own characteristic and specific fragmentation which will depend both on the nature of the molecules and on the operating conditions of ionization.

The ionization of the sample takes place in a special ionization chamber, in which the electron beam is produced by an ion source that varies depending on the technique used. Generally the electrons are emitted from a hot of tungsten or rhenium filament, and pass through a duct, which creates the beam, in the central part of the chamber that contains the gaseous sample. The fraction of electrons that does not collide with the molecules is collected by an electron trap, the molecules that are not ionized are removed from the high vacuum pump, while the ionized ones are accelerated and conveyed to the analyzer. The ionization system plays an essential role in mass spectrometry, because the number, nature and abundance of molecular fragments that appear in the mass spectrum also depend on it. Once ionized, the sample is conveyed to the analyzer. The analyzer has the function to differentiate the ions generated based on their mass/charge ratio. An electronic multiplier is commonly used as a collector and detector of the ions, consisting of a series of electrodes in cascade. When an ion arrives on the first electrode it emits a beam of electrons that hit the second electrode, which in turn emits a greater quantity of electrons, thus generating a cascade effect. The signal is then strongly amplified, then digitized and finally processed by the spectrometer computer for the realization of the mass spectrum. The mass spectrum is therefore presented as a set of peaks, represented by vertical lines, of different intensity, each corresponding to the mass value of a fragment ion. The molecular ion will correspond to the peak with the greatest intensity. From the mass spectrum it's therefore possible to trace the structure of an unknown compound, attributing an elementary composition to the individual ions and reconstructing the fragmentation mechanisms following typical schemes for the various classes of compounds.

A typical mass spectrometer consists of three main parts: the ion source, the mass analyser and the detector (De Hoffmann E., et al., 2007).

### 3.2.1 ION SOURCE

The ion source is crucial in the quality and type of the spectrum obtained at the end of the analyses. There are sources in the gaseous phase and sources for desorption. In the case of gas phase sources, the sample is first vaporized and then ionized. In desorption sources, the sample in the liquid or solid state is directly converted into ions in the gaseous phase. It is also possible to classify sources into HARD sources and SOFT sources. The HARD sources are such as to transfer such high energy to the molecule, as to allow its fragmentation directly in the source. The SOFT sources, on the other hand, transfer a quantity of energy that allows only ionization and possibly partial fragmentation. The gas phase sources most used are the electron impact (EI) and chemical ionization (CI) sources, while the sources for desorption are the electrospray ionization (ESI), chemical ionization at atmospheric pressure (APCI) sources, and matrix-assisted laser desorption ionization (MALDI). Among the sources listed above, the only HARD source is the electronic impact. The desorption sources allow the analysis of high molecular weight molecules. ESI and APCI have very similar ionization mechanisms and provide for the introduction of the sample in the liquid state, in fact they couple very easily to a chromatographic system. MALDI, allows the analysis of samples in the liquid state but the source sample must be in a solid state, so it is necessary to evaporate the solvent previously.

Following the description of all the mass ion sources previously mentioned, but ESI 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.

#### 3.2.1.1 Electronic Ionization (EI)

Electron impact ionization is the most common technique.

An incandescent tungsten filament emits a beam of electrons which, accelerated towards an anode placed on the opposite side of the filament, acquire a high energy (70 eV). When these electrons come into contact with the electronic sphere of a molecule (electronic impact), energy is transferred to it and an electron is expelled with the formation of a radical cation (molecular ion) $M^{+\bullet}$. The energy needed to ionize an organic molecule is about 13-14 eV, so the radical cations are produced at a very high vibrational energy, which can cause their fragmentation with the formation of a radical and a cation. All positive ions, cations and radical cations, are then rejected by a plate, whose potential is kept positive, towards a series of perforated plates, held at an increasing positive potential, called accelerator plates. On their way, the ions undergo an acceleration proportional to the potential V of the accelerator plates and are expelled, through an exit slit, with a kinetic energy: $\frac{1}{2}\, m\, v^2 = z\, V$.

Where z is the charge of the ions, typically = 1, V is the potential of the grid, m is the mass of the ion and v is the velocity of the ion. By varying the intensity of the magnetic field B, or that of the potential of the grids V, it is possible to condition the trajectory of the ions in such a way that they reach the detector. Thus, for each B·r/V value, only the ions possessing the m/z value that satisfies the previous equation will reach the detector. This type of ionization is hard, this means that the ionization involves very high energies that often cause the fragmentation of the ion and for this reason it will be difficult to find the molecular ion in the spectrum.

Advantages: very reproducible and well representable spectra are obtained, therefore structural information of the molecule can be obtained from an electron impact spectrum. There are databases of electron impact spectra.

Disadvantages: for many compounds it's impossible to see the molecular ion, so structural information is obtained but no info on its PM, even if this disadvantage is compensated by

the databases because it's possible to identify the molecule comparing data with that reported on the datavase. Another disadvantage is given by the fact that this source can be apply only to volatile compounds.

### 3.2.1.2 Chemical Ionization (CI)

The chemical ionization technique is a more "mild" ionization technique, compared to the electronic impact technique. It is based on the interaction of the vaporized sample with an ionized reagent, which is usually a gaseous Bronsted acid. The most commonly used reagents are those that derive from the electron impact ionization of methane.

If the M molecule has a higher affinity for the proton than that of methane, then the $M\text{-}H^+$ ion will be formed.

M-H ions do not possess such high energy and therefore undergo less fragmentation. In general, electronic ionization gives more significant fragments than does chemical ionization. In fact, after C.I. C-C bonds tend to break only if the break product is particularly stable. Frequently the skeleton of the carbon atoms remains intact and the cleavage is limited to C-O, C-S, C-N bonds. CI is particularly suitable for molecules such as hydrocarbons, alcohols, esters, amines, amino acids, small peptides which in EI conditions would give an excessive fragmentation.

The method consists in introducing methane in large excess, together with the sample. Methane will be ionized by electronic impact, generating $CH4^+$; this, meeting another molecule of CH4, forms CH3. and $CH5^+$, which acts as an acid for an organic molecule M generating the conjugated acid $MH^+$. This species is not generated at an excited vibrational level, and it does not fragment. The peculiarity is that in the spectrum it's possible to see the molecular ion +1.

Advantages: CI is a soft ionization technique, it does not lead to analyte fragmentation, because the electrons do not directly transfer energy to the molecules. The advantage it's the possibility to observe the molecular ion in the spectrum.

Disadvantages: It is less used because it is not reproducible like the electronic impact, because it's strictly dependent on many conditions (vacuum used, the T, the type of reagent gas used) therefore it is not possible to obtain reproducible spectra as can be obtained with the electronic impact. Another important drawback is that there aren't CI-MS spectra libraries for the identification of compounds and this is a major.

#### 3.2.1.3 Electrospray ionization (ESI)

ESI is one of the most versatile ionization sources that provide a simple, realtimemeans of analysing a wide range of polar molecules (100-200,000 Daltonrange).

The liquid sample passes through a metal capillary which is inside an atmospheric pressure chamber. A potential difference is applied to the capillary and the chamber is kept at high temperatures. Inside the chamber where the sample will enter trough the capillary, there are nitrogen molecules. The sample exiting the capillary encounters high temperatures and nitrogen in countercurrent and a spray is formed. The sample molecules are in the liquid state but ionized. The electrospray source only has the function of bringing the molecules into the gaseous phase and letting them enter the analyzer, so it works at a controlled pH to keep the molecules ionized.

The collisions of the sample droplets with the nitrogen molecules initially cause the solvent molecules to passing into the gas phase, because its are more volatile than the analyte molecules, The diameter of the drops decreases until there are very small drops with a large positive charge, or negative, according to the method of analysis. This process continues until the electrostatic repulsion becomes so high as to cause the phenomenon called

Columbian explosion and the molecules pass into the gaseous state. ESI ionization theory is reported in figure 1.

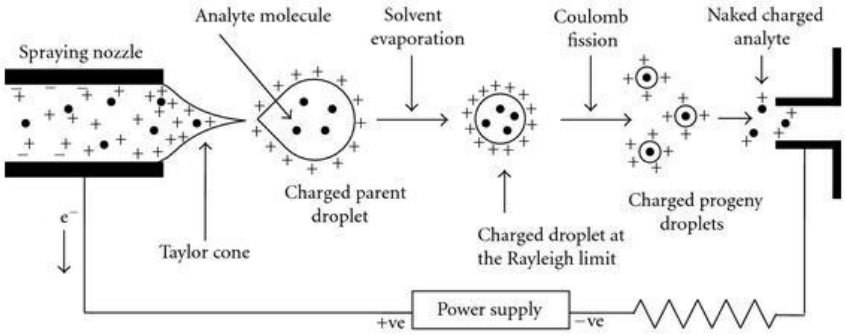



Figure 1: Electrospray ionization theory scheme. Banerjee S., et al., 2011.

Advantages: it's a soft ionization technique so it's possible to observe the molecular ion, in addition it allows the formation of multicharged ions, with consequent reduction of the m/z ratio, and therefore the possibility to analyze compounds with high PM even with detectors that have a low upper limit of detectable ions.

Disadvantages: it's a technique that involves the ionization of the sample by changing the pH, so it is not suitable for the analysis of apolar molecules.

### 3.2.1.4 Chemical ionization at atmospheric pressure (APCI)

SOFT ionization technique, which therefore does not involve the fragmentation of the molecule. The operation is similar to that of chemical ionization but, in this case, we don't work under vacuum but at atmospheric pressure and the ionization of the solvent takes place by means of a strong electric discharge which is called a corona discharge. Ionization occurs by reaction between the ions of the reagent gas and the analyte molecules.

Advantages: technique that has a good reproducibility as it involves a rather limited number of variables. It is suitable for the analysis of volatile and not very polar compounds.

Disadvantages: it's a SOFT technique but involves more drastic conditions than ESI, for this reason the thermolabile compounds are difficult to analyze. It doesn't form multi-charge ions and is not suitable for the analysis of high molecular weight compounds.

#### 3.2.1.5 Matrix-assisted laser desorption ionization (MALDI)

Maldi ionization involves the irradiation with a laser light (l337nm) of a small surface (100 mm in diameter) where the sample is placed, crystallized with a matrix, with consequent heating of the irradiation area and vaporization of the organic molecules.

The matrix absorbs energy from the irradiation of laser light, heats up and can consequently act as a proton donor forming $[M+H]^+$ pseudomolecular ions. For compounds with a high affinity for cations, adducts of type $[M+Na]^+$, $[M+K]^+$ can also be formed.

Advantages: SOFT ionization technique, so there is no fragmentation of the molecule. It allows the ionization of high molecular weight molecules and to analyze non-volatile molecules. It is a very sensitive technique, and with the application of this is possible to detect low concentrations of analyte, starting from small sample volumes. Finally, it has a wide choice of possibly usable matrices.

Disadvantages: the formation of clusters of matrix molecules doesn't allow to detect species with low m/z value (<600). In addition, the nature of the ion source is pulsed, it is not continuous, so it's necessary to deposit all the samples and then carry out the analysis individually, and coupling with a chromatographic system is more complex. Finally, not all analytes ionize easily, some of these absorb energy from the laser, degrade but aren't brought to the gaseous state.

### 3.2.2 MASS ANALYZERS

The first mass analyzers to be used were magnetic analyzers. This type of analyzer was expensive and bulky and is no longer used today. Quadrupole, ion trap and time-of-flight analyzers are the most widely used mass analyzers.

#### 3.2.2.1 Quadrupole

Quadrupole consists of four cylindrical metal bars, about 20 cm long, which delimit the "path" traveled by the ions coming from the ionization chamber and directed to the detector. The bars are kept at an oscillating electromagnetic potential, so that when the two vertical bars have positive potential, the horizontal ones have negative potential, and vice versa. Figure 2 shows the generic structure of a quadrupole. The electrons, accelerated by the accelerator plates, enter the tunnel delimited by the bars and are repelled by the positive poles and attracted by the negative ones. The ions, due to the quadrupole oscillation, take a zigzag trajectory and end up discharging on one of the bars, except those which, for a certain oscillation frequency, have a kinetic energy such that the trajectory becomes sinusoidal and ions manage to exit the

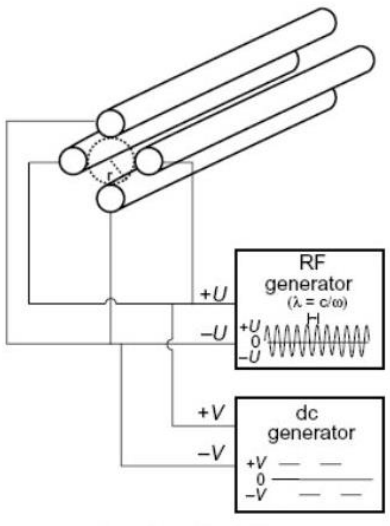



Figure 2: Quadrupole mass analyser scheme (Smith R.M., et al., 2004).

tunnel and enter the detection system (photomultiplier). Therefore, by scanning the oscillation frequency of the field, it is possible to release ions with increasing molecular mass.

#### 3.2.2.2 Ion Trap

The electronic trap analyzer can be considered a variant of the quadrupole analyzer; rather than allowing for the ions to cross the quadrupolar field, the ion trap holds all the ions inside it. This technique is based on the use of three electrodes. The structure provides an annular electrode placed between two hemispherical inlet and outlet electrodes. This structure has the aim of trapping and accumulating the ions in a cavity of restricted volume, called ion trap, in order to obtain a high sensitivity. The two side electrodes have a small hole in the center through which the ions pass. The mass spectrum is generated by varying the electric potential in order to expel the ions in sequence from the trap towards the detector according to an increasing m/z value.

#### 3.2.2.3 Time Of Flight

The basic principle of flight time analyzer is that ions with different mass/charge value have equal energy, but different speed after acceleration suffered in the room of ionization. It follows that the time each takes to go through the analyzer is different (Skoog D.A., et al., 2009).

### 3.3 SPECTROPHOTOMETRIC ASSAYS

Folin-Ciocalteu is an assay used to determine the phenolic compounds total content. Folin-Denis method was Initially used to determinate the total phenolic content. This method

involved redox reactions in which the phenolate ion was oxidized and the phosphotungstic or molybdic ions reduced (Graham H.N., et al., 1992). Folin-Ciocalteu consists of a mixture of phosphomolybdateand phosphotungstate which, in a basic environment, oxidizes the -OH group contained in the carbonyl group polyphenols, reducing to a mixture of oxides of molybdenum and tungsten having a blue color that has a maximum of absorption around 750 nm.

The calibration curve is built using a solution of gallic acid at different concentrations alkalized with carbonate to which the Folin-Ciocalteu reagent is added. After 30 minutes, the absorbance of the individual solutions is measured and the calibration line is constructed, reporting the concentration on the abscissa and the absorbance on the ordinate. The analysis of the total phenolic compounds is then carried out under the same conditions and the absorbance is measured. From the calibration line, once the absorbance is known, it is possible to trace the concentration of total polyphenols present in the extracts which is expressed in g/L of equivalents of gallic acid (Singleton V.L., et al., 1986).

### 3.4 METHODS FOR MEASURING ANTIOXIDANT POWER

The existence of different action mechanisms by which an antioxidant substance can act has led to the development of various tests for measuring the antioxidant capacity of single compounds and/or complex mixtures. TEAC (trolox equivalent antioxidant capacity ) method is one of the most widely used methods for the determination of the antioxidant activity fo an extract (Baiano A., et al., 2011).

TEAC is an analytical method that uses a spectrophotometric measurement to determine the antioxidant capacity of a sample. Using a UV-Vis spectrophotometer, this method is based on measuring the absorbance of a solution containing the radical $ABTS^{\bullet+}$, generated by oxidation of ABST (2,2'-azinobis (3-ethylbenzothiazolin-6-sulfonate), a colorless substance that in the form radical is colored by absorbing at characteristic wavelengths in the visible

range. The addition of antioxidant molecules to the $ABTS^{\bullet+}$ solution, which can act through the transfer of both hydrogen and an electron, determines the reduction of the radical to the colorless form, with consequent discoloration of the mixture, which will be proportional to the reduction in absorbance over a certain time at a specific wavelength (734 nm). The antioxidant power is expressed by comparison with the absorbance values measured for known quantities of a chosen antioxidant molecule as a reference standard, which is usually ascorbic acid or Trolox. Other assays reported in literature to define the antioxidant capacity of an extract are: ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), dyphenyl-1-picrylhydrazyl (DPPH) radical scavenging, following described.

# AIMS OF PhD RESEARCH PROJECT

This PhD research project is aimed at develop and optimize analytical methods for the analysis of phenolic compounds in food and their by-products. Conventional and non-conventional solvents have been selected and tested for the extraction of these molecules. Extracted compounds has been characterized through High performance liquid chromatography coupled to photodiode array and mass spectrometry detectors.

Specifically, my PhD period involved the study of five different project, about optimization of analytical method for phenolic compounds in hazelnuts, extra virgin olive oils and two by-products being spent coffee ground and hazelnut skin.

Conventional methods, based on the use of traditional organic solvents, for phenolic compounds from hazelnut and extra virgin olive oils were developed, optimized and validated.

The application of deep eutectic solvents was tested for the extraction of phenolic compounds from extra virgin olive oils, spent coffee ground and hazelnut skin.

# CHAPTER 4: EXPERIMENTAL SECTION

## 4.1 DEVELOPMENT, OPTIMIZATION AND VALIDATION OF ANALIYCAL MEHOTDS FOR EXTRA VIRGIN OLIVE OIL PHENOLIC COMPOUNDS

### 4.1.1 Introduction

The product obtained from the fruit of the olive tree (*Olea europea L.*) solely by mechanical or other physical means such as washing, decantation, centrifugation, or filtration takes the name of Virgin olive oil (VOO). Extra virgin olive oils (EVOOs) are classified as VOOs having a free acidity, in terms of oleic acid, lower than 0.8 g per 100 g (Council regulation (EC), 2001). EVOOs whose production steps are closely related to a specific production area can be marked as Protected Designation of Origin (PDO). EVOOs are characterized by two fractions, a saponificable fraction, consisting of triglycerides, and an unsaponifiable fraction, consisting of several metabolites.

Phenolic compounds represent one of the most important classes of molecules in the EVOOs unsaponifiable fraction. A wide range of polyphenols has been identified in VOO. Among them, phenolic alcohols (e.g., hydroxytyrosol, tyrosol) and their secoiridoid derivatives linked to the aldehydic and dialdehydic forms of elenolic acid (such as oleuropein, the dialdehydic form of decarboxymethylelenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), an isomer of oleuropein aglycone (3,4-DHPEA-EA), among others) are the main ones. Flavonoids (e.g., luteolin and apigenin), phenolic acids (e.g., vanillic and p-coumaric acid) and lignans (e.g., pinoresinol) are present as minor compounds (Servili M., et al., 2014; Mazzotti F., et al., 2012).

Thanks to their antioxidant power, phenolic compounds have aroused strong interest.

According to the literature the natural antioxidants of EVOO with the highest antioxidant efficacy are 3,4-DHPEA and secoiridoids such as 3,4-DHPEA-EDA and 3,4-DHPEA-EA, that in their molecular structure contain 3,4-DHPEA. It has been demonstrated that the antioxidant power is strictly related to the concentration of that phenolic compound in

EVOOs (Servili M., et al., 2014). Total Phenolic content is usually determinated applying a spectrophotometric method (Folin-Ciocalteau), while, for the determination of the antioxidant activity it's possible to use different assays such as TEAC assay, ORAC assay, FRAP assay (Leporini M., et al., 2018; Mulinacci N., et al., 2018).

According to previous studies the most efficient extraction techniques for the extraction of phenolic compounds from EVOOs are LLE and SPE, using traditionally methanol or methanol/water mixtures ($H_2O$ < 40%, *v*/*v*) as extraction solvents (Fu S., et al., 2009; Carrasco-Pancorbo A., et al., 2006; Carrasco-Pancorbo A., et al., 2005) .

With the objective to reduce toxic waste and to improve selectivity and extraction efficiency non-conventional solvents, such as DESs have been explored in the extraction of bioactive compounds, (Pena-Pereira F., et al., 2014).

Two studies evaluated the extractive efficiency of DESs for phenolic compounds in EVOO. Garcia et al, tested three alcohol-based, four sugar-based, two organic acid-based and two urea-based DESs for the extraction of phenolic compounds, while Paradiso et al., evaluated the UV/Vis spectra characteristics of the EVOO extracts obtained using two DESs based on ChCl and lactic acid or glucose . DESs tested in the works showed a good results regarding the extraction capacity both for the most water-soluble phenolic compounds, such as tyrosol and hydroxytyrosol, and for those insoluble in water but soluble in MeOH, such as flavonoids. DESs, according to obtained results could be considered as a green valid alternative for a rapid and sustainable method to screen phenolic compounds in EVOO (Garcia A., et al., 2016; Paradiso V.M., et al., 2016).

At our knowledge any EVOO phenolic compounds extraction procedure using DESs has never been proposed working at room temperature probably due to the high solvent viscosity and consequent low extraction repeatability and only ChCl-based DESs have been tested. DESs with betaine as HBA have been tested for the extraction of phenolic compounds from food matrices, but never for the EVOO'S phenolic compounds.

The most used techniques for the analysis of phenolic compounds in oil are high-performance liquid chromatography (HPLC) coupled with ultraviolet or diode array detection (PDA), electrochemical detection, mass spectrometry (MS), gas chromatography coupled to MS, capillary electrophoresis (CE) with UV or MS, nuclear magnetic resonance spectroscopy and infrared spectroscopy (Liu X., et al., 2018; Garcia A., et al., 2016).

In a first moment, the purpose of the PhD project envisaged the optimization and validation of an analytical method, for the determination of phenolic compounds in EVOOs, based on (HPLC/DAD-ESI/MS). The project involved the characterization of the phenolic profile of thirty samples of monovarietal and multivarietal EVOOs samples including PDO oils from different areas in Italy (Sicily, Lazio, Tuscany, and Puglia) and to investigate the difference in total phenolic content and antioxidant activity.

The total phenols content was assessed by the Folin-Ciocalteau method, while the antioxidant activity was evaluated by four different assays: ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), dyphenyl-1-picrylhydrazyl (DPPH) radical scavenging, and Trolox equivalent antioxidant capacity (TEAC).

At later time, we decided to optimize an extraction technique for phenolic compounds from EVOO through the use of different DESs, including ChCl and betaine-based DESs, working at room temperature using the best conditions and to compare extractive efficiency with that of a conventional method. Different parameters were optimized like the type of DES most suitable for extraction, the quantity of water to be added to DES and the ratio of solvent extractive volume/quantity of matrix, optimal to guarantee a good extractive efficiency. In order to evaluate the extractive capacity of each tested DES, phenolic compounds were separated and detected by HPLC/DAD-ESI/MS.

### 4.1.2 Optimization and validation of an analytical method for the analysis of phenolic compounds in different Italian extra-virgin olive oils and evaluation of their total phenolic content and antioxidant activity.

#### 4.1.2.1 Materials and Methods

4.1.2.1.1 Chemicals

Ethanol (EtOH) (99.8%) n-Hexane (purity 99.8%), methanol (MeOH) (purity 99.9%), water (HPLC-MS grade), acetonitrile (purity 99.9%), dimethyl sulfoxide (DMSO), and formic acid (purity 95–97%) and were purchased from Sigma-Aldrich (Milan, Italy) were the solvents used for the extraction process and for the HPLC-MS anlysis. The standard compounds namely 4-hydroxyphenylacetic acid (purity 98%), p-coumaric acid (purity 98%), vanillic acid (purity 97%), oleuropein (purity 98%), apigenin (purity 99%), pinoresinol (purity 95%), luteolin (purity 98%), and 2-(4-hydroxyphenyl)ethanol (purity 98%), gallic acid, and Trolox were purchased from Merck KGaA (Darmstadt, Germany).

The reagents as TPTZ (2,4,6-tripyridyl-s-triazine), $FeCl_3$ $6H_2O$, glacial acetic acid, fluorescein, 2,20-azobis(2-methylpropionamidine) dihydrochloride (AAPH), sodium carbonate, Folin-Ciocalteu reagent, potassium persulfate, ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic) diammonium salt), and DPPH (2,2-dyphenyl-1-picrylhydrazyl) were purchased from Merck KGaA (Darmstadt, Germany).

4.1.2.1.2 Samples

The analyzed samples consisted of thirty mono- and multivarietal and PDO extra-virgin olive oils collected from Italian producers located in different areas belonging to the harvest year 2017. The cultivars were as follows: Sicily: Nocellara del belice, Tonda iblea, Biancolilla, Cerasuola; Lazio: Frantoio, Leccino, Moraiolo, Rajo, Mariolo; Tuscany: Frantoio, Moraiolo, Maurino, Picholine, Pendolino, Correggiolo, Lecino, Olivastra saggese, Mariolo; Puglia: Coratina, Ogliarola, Cima di Bitonto, Cima di Mola. All extra virgin oil

samples, until the time of analysis, were kept at 4 °C, away from light. In table 3 are reported the geographical origins of the analyzed samples.

| Sample | Geographical origin | Variety |
|---|---|---|
| 1 | Sicily | Monocultivar |
| 2 | Tuscany | Monocultivar |
| 3 | Puglia | Monocultivar |
| 4 | Tuscany | Blend |
| 5 | Puglia | Blend |
| 6 | Puglia | Monocultivar |
| 7 | Sicily | Monocultivar |
| 8 | Tuscany | Blend |
| 9 | Lazio | Blend |
| 10 | Sicily | Monocultivar |
| 11 | Puglia | Monocultivar |
| 12 | Lazio | Blend |
| 13 | Tuscany | Blend |
| 14 | Tuscany | Monocultivar |
| 15 | Sicily | Blend |
| 16 | Puglia | Monocultivar |
| 17 | Tuscany | Monocultivar |
| 18 | Lazio | Monocultivar |
| 19 | Lazio | Monocultivar |
| 20 | Tuscany | Blend |
| 21 | Tuscany | Blend |
| 22 | Puglia | Monocultivar |
| 23 | Lazio | Blend |
| 24 | Lazio | Blend |
| 25 | Sicily | Monocultivar |
| 26 | Tuscany | Blend |
| 27 | Tuscany | Blend |
| 28 | Abruzzo | Blend |
| 29 | Abruzzo | Blend |
| 30 | Sicily | Blend |

Table 3: geographical origin and olive varieties of the thirty EVOO samples

4.1.2.1.3 Extraction of Phenolic Compounds

The technique selected for the extraction of phenolic compounds from EVOOs is the conventional LLE, according to the procedure reported by Ricciutelli and co-workers, with some modification (Ricciutelli M., et al., 2017). The first step of the procedure involved the use of one gram of EVOO, dissolved in 1 mL of hexane in a 15 mL centrifuge tube. The extraction was carried out using a mixture of MeOH/$H_2O$ (3:2, *v*/*v*), added to the tube that consequentially was vortexed and centrifuged for 3 min at 3000 rpm. To improve the extraction efficiency, the extraction was made in an ultrasound bath (Elmasonic S30H, Elma Schmidbauer GmbH, Singen, Germany) at room temperature, frequency of 37 kHz and heating power of 200 W for 20 min.The procedure was repeated four times. A volume of 2

mL of hexane was added to the extracted solution, vortexed and centrifuged for 5 min 3000 rpm, and then hexane was discarded. Solvent was evaporated in a rotary evaporator (Eyela, Tokyo, Japan) and the extract was then dissolved in 500 µL of a mixture of MeOH/$H_2O$ (1:1, *v*/*v*).

4.1.2.1.4 Folin-Ciocalteau method for the determination of total phenolic content

Folin-Ciocalteau method, according to the method described by Singleton and co-workers was applied for the determination of total phenolic content (Singleton V.L., et al., 1999 ). This method involved using 20 $\mu$L of sample extracts (diluted two times) added of 1580 $\mu$L of a MeOH/$H_2O$ (50:50, *v*/*v*) mixture. The blank consisted in 1600 $\mu$L of a MeOH/$H_2O$ (50:50, *v*/*v*). According to the protocol 100 $\mu$L of Folin-Ciocalteu reagent was added to the previously prepared mixture, that consequentially was stored at room temperature in the dark for 8 min. 300 $\mu$L of sodium carbonate solution (20% *w*/*v*) was added, mixed and incubated for 2 h in a dark environment at room temperature, and finally was centrifuged at 20,817× *g* for 5 min. The absorbance reading was 765 nm in a 96-well cell culture plate (Greiner Bio-one, Kremsmünster, Germany) using a multifunctional microplate reader (Infinite®, 200 PRO multimode reader, Tecan, Männedorf, Switzerland). The obtained results were reported as mg of gallic acid equivalents (GAE)$g^{-1}$ of sample. Total phenolic content was determined from the gallic acid calibration curve (0.05–1.6 mg $mL^{-1}$) (y = 0.8008x + 0.0065. $R^2$ = 0.9987).

4.1.2.1.5 Antioxidant Activity

For the evaluation of antioxidant activity were selected four different assays reported below. The FRAP (ferric reducing antioxidant power) assay was carried out according to the procedure described in literature by Fu et al. ( Fu L., et al., 2010). Before the analysis EVOO extracts were diluted 20 times. 10 µL of diluted sample solution was added to 190 µL of

FRAP reagent. A blank sample was also prepared as described above, 10 µL of ethanol was added instead of the sample. After 6 min at room temperature, the absorbance was measured at wavelength of 593 nm in a 96-well cell culture plate (Greiner Bio-one, Germany) using a multifunctional microplate reader (Infinite®, 200 PRO multimode reader, Tecan, Männedorf, Switzerland). The analysis was performed in triplicate. Using Trolox as reference compound, a calibration curve for the determination of the extracts antioxidant activity, was constructed (25–500 $\mu$M). Results were expressed in µmol of Trolox Equivalent (TE) $g^{-1}$ of oil.

The ORAC assay was performed as described by Trombetta et al. (Trombetta D., et al., 2017) with some modifications following reported. First step allowed the dilution of the samples of 500 times. A fluorescein stock solution was prepared in 75 mM phosphate buffer ($NaH_2PO_4$-$H_2O$ and $Na_2HPO_4$-$2H_2O$, pH 7.4) aqueous solution with a final concentration of 70 nM. 20 µL of diluted extract was added with 120 µL of Fluorescein and solutions were mixed and incubated for 15 min at 37 °C. Additing of 60 µL AAPH solution (12 mM, final concentration), the reaction was started. The fluorescence decay was measured every minute for 90 cycles in a 96-well cell culture plate (Greiner Bio-one, Germany) using a multifunctional microplate reader (Infinite®, 200 PRO multimode reader, Tecan, Männedorf, Switzerland). Fluorescence was read with an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

The area under the curve (AUC) of the blank and samples, due to the decay curves of fluorescein, were calculated by One-way analysis of variance (ANOVA) followed by area under curve using GraphPad Prism version 4 for Windows (GraphPad software, versio 4). The net AUC was calculated by subtracting the AUC of the blank to the sample. The activity of the sample was expressed as µmol TE $g^{-1}$ of oil. Also with this assay the antioxidant activity was evaluated using a calibration curve, made with Trolox as reference compound (0–100 $\mu$M) ($y = 14697x + 32669$; $R^2 = 0.997$).

The DPPH assay was carried out following the procedure wrote by Padmanabhan and Jangle (Padmanabhan P., et al., 2012), with some modifications. First of all EVOO extracts were diluted 20 times. In the well of the microplate 20 $\mu$L of diluted sample were added with 180 $\mu$L of ethanolic solution of DPPH (0.1 mM). The blank was prepared in ethanol 100%, while the control sample was prepared with 20 $\mu$L of ethanol and 180 $\mu$L of DPPH (0.1 mM). The absorbance was measured at 518 nm in a 96-well cell culture plate (Greiner Bio-one, Germany) using a multifunctional microplate reader (Infinite®, 200 PRO multimode reader, Tecan, Männedorf, Switzerland) after 20 min of incubation in the dark at room temperature. Trolox was used for create the following calibration curve (20–200 $\mu$M) ($y = 0.0015x - 0.0246$, $R^2 = 0.995$).

Free radical scavenging activity of EVOO extract was evaluated according to a previously described colorimetric method, with few modification (Re R., et al., 1999). 10 $\mu$L of each diluted sample was mixed with 190 $\mu$L of $ABTS^{\bullet+}$ working solution in a 96-multiwell plate (Greiner Bio-one, Kremsmünster, Germany). After 20 min the absorbance was measured at 734 nm using a multifunctional microplate reader (Infinite®, 200 PRO multimode reader, Tecan, Männedorf, Switzerland). Also for this last assay the evaluation of antioxidant activity was calculated using a trolox calibration curve at the concentration range of 50–700 $\mu$M. Results were expressed as $\mu$mol $TEg^{-1}$ of oil ($y = 0.0009x + 0.0158$; $R^2 = 0.998$).

4.1.2.1.6 HPLC/DAD-ESI/MS Analysis of Phenolic Compounds

Shimadzu Prominence LC-20A instrument (Shimadzu, Milan, Italy) equipped with two LC-20 AD XR pumps, SIL-10ADvp, CTO-20 AC column oven and DGU-20 A3 degasser coupled to a SPD-M10Avp PDA detector and a mass spectrometer detector (LCMS-2010, Shimadzu, Tokyo, Janpan) equipped with ESI interface was selected to carry out the analysis of EVOOs phenolic compounds. MS data were acquired by Shimadzu LCsolution Ver. 3.7 software (Shimadzu, Version 3.7). PFPColumn (150 × 2.1 mm I.D., 2.7 µm d.p.) (Merck

KGaA, Darmstadt, Germany) was selected to realize the separation. $H_2O$/0.1% HCOOH was chosen as solvent A, and methanol/acetonitrile at 1:1 (*v*/*v*)/0.1% HCOOH as solvent B to constitute the mobile phase with a constant flow–rate of 0.2 mL/min. the analysis were carried out at 40 °C. The gradient elution profile was as follows: 0–5 min 5% B, 5–15 min 5–30% B, 15–40 min 30–50% B, 40–50 min 50–100% B. The injection volume was 2 µL. PDA was used to register data in a range of 210-400 nm and the chromatograms were axtracted at 280 nm, wavelength of maximum absorbance of polyphenols. Chromatograms were acquired in the MS instrument with ESI interface in negative ionization mode using the following parameters: nebulizing gas flow ($N_2$): 1.5 mL $min^{-1}$; Event time: 1 s; 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.

4.1.2.1.7 HPLC/DAD Quantitative Analysis Method Validation

A mixture of eight phenolic standard compounds (4-hydroxyphenylacetic acid, p-coumaric acid, vanillic acid, oleuropein, apigenin, pinoresinol, luteolin, and 2-(4-hydroxyphenyl)ethanol), characterizing EVOO, was selected for quantitative analysis and method validation. The analytical method was validated considering linearity, repeatability (intra-day and inter-day precision), limit of detection (LOD), and limit of quantification (LOQ).

Stock standard solutions of each compound were prepared at a concentration of 10,000 $mg{\cdot}L^{-1}$ in MeOH for 4-hydroxyphenylacetic acid, p-coumaric acid, vanillic acid, 2-(4 hydroxyphenyl)ethanol) and oleuropein; at a concentration of 10,000 mg $L^{-1}$ in DMSO for pinoresinol; at a concentration of 2500 $mgL^{-1}$ in DMSO for luteolin, and at a concentration of 2000 mg $L^{-1}$ in EtOH for apigenin and were used to obtaine the calibration curve, through HPLC, for the evaluation of method linearity.

#### 4.1.2.1.8 Statistical Analysis

All statistical analysis were made using Stata 14 statistical software (StataCorp LLC, College Station Texas, USA). All samples analyses were performed in triplicate. After verifying the normality of the distribution of the dependent variable, differences between samples were evaluated by one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test and correlations were evaluated using Pearson correlation analysis. Probability values of $p \leq 0.05$ were considered statistically significant.

### 4.1.2.2 Results and Discussion

#### 4.1.2.2.1 HPLC Method Optimization and Validation

As previously reported the validation of the analytical method was carried out using several standard molecules of phenolic compounds characterizing EVOO, namely 4-hydroxyphenylacetic acid, p-coumaric acid, vanillic acid, oleuropein, apigenin, pinoresinol, luteolin, and 2-(4-hydroxyphenyl)ethanol. A column containing pentafluorophenyl (PFP) stationary phase (SP) was selected for the study considering the physical-chemical properties of the known phenolic compounds.

In a first moment we decided to test as solvent B, in the formulation of the mobile phase, acetonitrile or methanol alone, but we obtained the best results in terms of peak separation using methanol and acetonitrile together slightly acidified. PDA detector was used to record the several peaks correspond to EVOO phenolic compounds, detected at 280 nm.

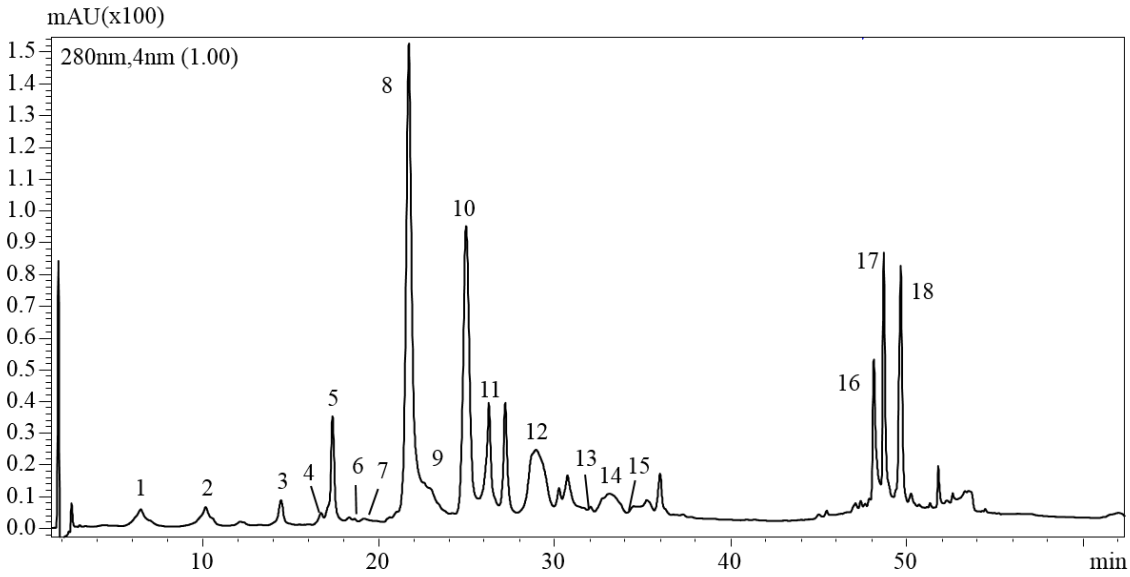
Repeatability of retention time, limit of detection (LOD), limit of quantitation (LOQ), linearity range, and recovery are considered to validate the analytical method. Validation was carried out using the eight standard molecules previously described.

Linearity was studied analyzing a standard mixture containing 4-hydroxyphenylacetic acid, p-coumaric acid, vanillic acid, oleuropein, apigenin, pinoresinol, luteolin, and 2-(4-hydroxyphenyl)ethanol at concentration range for each analyte of LOQ-140 mg $L^{-1}$ without an internal standard. A good correlation coefficient was obtained ($R^2$ between 0.989 and 0.998). Recovery was verified considering two points of concentraztion 10 and 50 $\mu$g $g^{-1}$ of each standard compound recording good results of 73.9–92.1% and 79.1–102.8%, respectively. LOD and LOQ values were below 1 mg $L^{-1}$ and 2 mg $L^{-1}$, respectively. Good results were obtained regarding intra-day repeatability and intra-day repeatability, for retention time ($t_R$) (five runs) and peak area, being 0.4–0.8% and 0.3-0.8% respectively for retention time and 5.9–10.2% and 5.8–10.5% for peak area. RSD% values mirrored those reported in the literature about EVOO phenolic compounds analysis (Bonoli M., et al., 2003; Luque-Muñoz A., et al., 2018).

4.1.2.2.2 Identification and quantification of phenolic compounds in EVOOs

The identification of phenolic compounds in the extracts was done considering: $t_R$ and UV spectra data, MS spectra, use of standard compounds and data available in literature. Eighteen peaks was detected and correlated to phenolic componds. Only fiftheen of these phenolic compounds were identified and as can be observed in figure 3 the analyzed EVOO extract contained secoiridoids (compounds 4, 6–11, and 14 of the chromatogram), phenolic alcohols (1 and 2), flavonoids (13 and 15) and phenolic acids (3 and 5). Nine different secoiridoid derivatives were detected and tentatively identified as being the dialdehydic form of oleuropeine aglycon, seven aglycon isomers of oleuropein and ligstroside, elenolic acid, and a derivative of elenolic acid. In total we applied the method to the analysis of thirty oil samples and cultivar.

Qualitative profile of the different EVOO extracts was very similar with small differences. In all samples of different geographical origin the same compounds have been found.




Quantification of single phenolic compounds in the extract was done through HPLC analysis. Summing the single concentrations of detected phenolic compounds we obtained a total concentration of polyphenols in the several extracts ranging from 814 to 5920 mg $kg^{-1}$ a mean value of 3049 mg $kg^{-1}$. It was possible to observe differences in the total concentration oh phenolic compounds between samples from different region but also between sample with the same origin. It was in accordance with literature (Ouni Y., et al., 2011). Most abundant class of phenolic compounds was that of secoridoids with a total concentration, calculated as the sum of concentrations of individual secoiridoids, ranged between 2671 and 5034 mg $kg^{-1}$, 1088 and 5077 mg $kg^{-1}$, 1276 and 3294 mg $kg^{-1}$, 471 and 1577 mg $kg^{-1}$, for Puglia, Tuscany, Lazio, and Sicily, respectively. Puglia and Tuscany were the regions with the higher content in secoridoids while Lazio and Sicily the two with the lowest content. Olmo-Garcia et al. reported that secoiridoids concentration ranged from 127.5 to 2327.7 mg $kg^{-1}$, these results are in agreement with our data also considering that we included in the secoridoids group, as well as elenoic acid and their derivatives, while they considered these last compounds separately (Olmo-García L., et al., 2018). The dialdehydic form of oleuropein aglycon (3,4-DHPEA-EDA), oleuropein, and ligstroside and

aglycone isomers showed the highest concentrations ranging from 88 to 1547 mg $kg^{-1}$, from 22 to 1683 mg $kg^{-1}$, from 81 to 900 mg $kg^{-1}$, respectively, for all analyzed samples. Luteolin and apigenin concentrations did not show a considerable variability respect to the other classes ranging between 12 and 17 mg $kg^{-1}$, and 1 and 3 mg $kg^{-1}$, respectively. Tyrosol and hydroxytyrosol concentrations ranged between 0.03 and 62 mg $kg^{-1}$, and 0.7 and 63 mg $kg^{-1}$, respectively. Concentrations of vanillic and p-coumaric acids were below 4 mg $kg^{-1}$ for all analyzed samples with low variability.

In literature it's possible to find work in wich the total concentration of EVOO phenolic compounds calculated through HPLC method is very variable, data are following reported: 260–613 mg $kg^{-1}$ (Antonini E., et al., 2015), between 172 and 326 mg $kg^{-1}$ (Trombetta D., et al., 2017) and between 336 and 978 mg $kg^{-1}$ (Veneziani G., et al., 2018).

These strong variations in the total concentration value of phenolic compounds in the oil may be due to various factors, olive cultivar, the maturation stage, geographical region, and extraction conditions, as well as the agricultural practices furthermore in literature there isn't an official method, which would be practical and would guarantee the comparison of results exists.

Puglia showed the highest concentration of total phenolic compounds and an ANOVA test showed a significant statistical difference between it and Sicily total concentration of phenolic compounds determined by HPLC method. This result was in agreement with that reported by Antonini et al. (Antonini E., et al., 2015).

Despite this, it wasn't possible to cluster the samples according to the regions, probably for the above-mentioned reasons that cause the high variability in the content of phenolic compounds in the various samples (Sicari V., et al., 2017).

To better describe the differences in phenolic compounds between the various samples in Figure 4 are reported the percentage results of the phenolic compounds according to the different classes present in the oil varieties studied.

Results obtained was in agreement with literature, infact in the analyzed extracts the class of phenolic compounds most abundant was that of secoirideds, whose percentage of the total ranged in all samples from 85 to more than 99% (w/w) (Bakhouche A., et al., 2013; Karkoula E., et al., 2012; Sánchez de Medina V., et al., 2015. The two most representative flavonoids in EVOO, namely luteolin and apigenin, were detected in analyzed extracts and their percentage ranged from 0.3 to 1.5% (w/w) of the total. Hydroxytyrosol and tyrosol, simple alcohols conjugated to form oleuropein derivatives, were identified and quantified and their

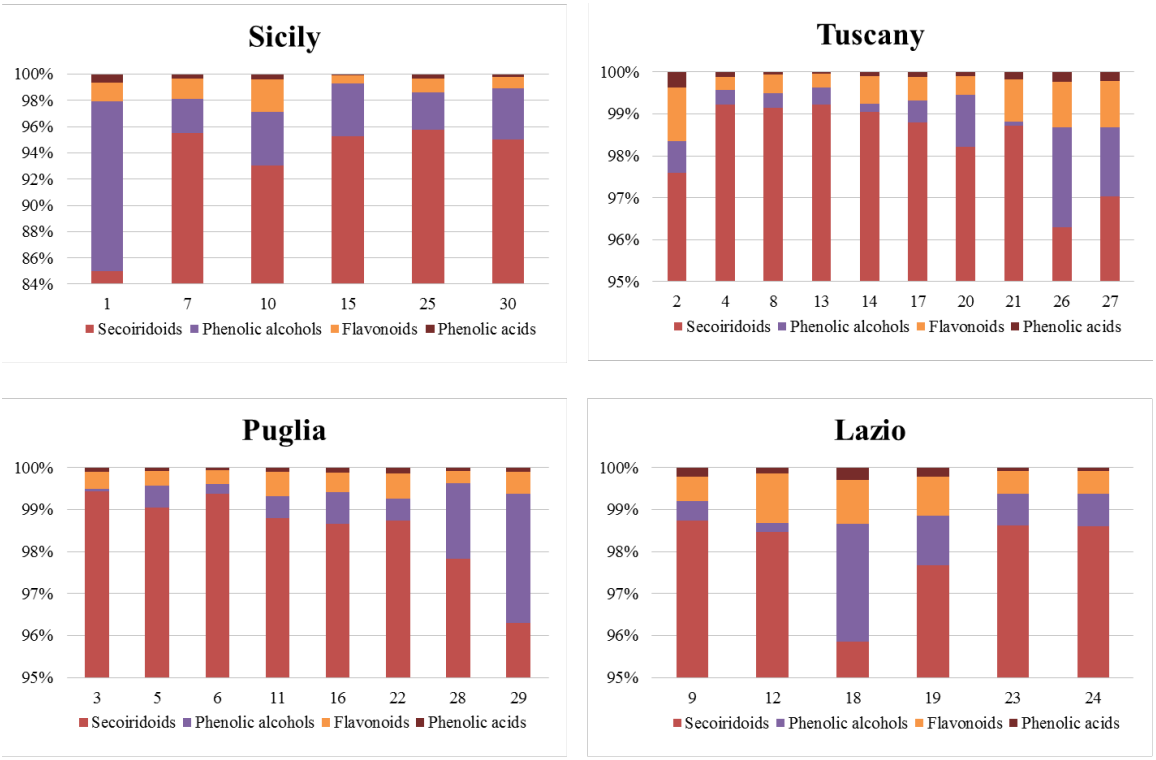



Figure 4:Percentage distribution of phenolic compounds classes (phenolic acids, phenolic alcohols, secoiridoids and flavonoids) for each analyzed sample in the four different Italian areas (Sicily, Tuscany, Lazio and Puglia). Sample number refers to table 4.

percentage was from 0.1 to 12% (w/w) of the total concentration of phenolic compounds. Also molecules belonging to the class of phenolic acids, were detected, specifically Vanillic and p-coumaric acids whose percentage of the total concentration of phenolic compounds found, was in the range of 0.1–0.6% (w/w). All the results reported were in accordance with data presents in literature (Bakhouche, A et al., 2013)

4.1.2.2.3 Evaluation of antioxidant activity of EVOO extracts

Table 4 shows ranges values of TPC, TEAC, DPPH, FRAP and HPLC grouped by geographical areas. Results show that TPC values of all tested samples were between 98 and 573 mg GAE $kg^{-1}$ of oil. The obtained results was in agreement with wich reported in literature that stated TPC values ranging between 121.3 and 388.0 mg GAE $kg^{-1}$ (Tuberoso C.I.G, et al., 2016), between 14.8 and 121.2 mg GAE100 $g^{-1}$ (Galvano F., et al., 2007), between 133.7 and 322.2 µg GAE $g^{-1}$ (Baiano A., et al., 2009).

TEAC and DPPH values ranged between 2.11 and 8.94 $\mu$mol $TEg^{-1}$, and 0.42 and 2.41 $\mu$mol $TEg^{-1}$, respectively. FRAP and ORAC values ranged between 0.59 and 3.19 $\mu$mol $TEg^{-1}$, and 1.67 and 17.99 $\mu$mol $TEg^{-1}$, respectively. Also these results was in accordance with literature (Tuberoso C.I.G., et al., 2016, Trombetta D., et al., 2017).

Both for TPC and antioxidant activities was noted differences between samples of different region but also between samples of the same region, according with results regarding total phenolic concentration obtained with HPLC analysis. However, a significant difference between antioxidant activities measured by TEAC and ORAC assays between samples from Puglia (highest values) and Sicily (lowest values), applying ANOVA test was observed as previously seen for total phenolic compounds concentration measured by HPLC.

| | Sample Number | TPC (mg GAE $kg^{-1}$ ) | TEAC (µmol TE $g^{-1}$) | DPPH (µmol TE $g^{-1}$) | ORAC (µmol TE $g^{-1}$) | FRAP (µmol TE $g^{-1}$) | HPLC (mg $Kg^{-1}$ ) |
|---|---|---|---|---|---|---|---|
| SICILY | **1** | 159.70 ± 16.11 | 3.13 ± 0.56 | 0.59 ± 0.08 | 3.03 ± 0.43 | 1.00 ± 0.16 | 1435.20 ± 2.00 |
| | **7** | 138.67 ± 9.89 | 3.45 ± 0.38 | 0.67 ± 0.10 | 3.28 ± 0.59 | 0.90 ± 0.13 | 4821.47 ± 1.23 |
| | **10** | 97.63 ± 7.10 | 2.11 ± 0.27 | 0.46 ± 0.05 | 1.67 ± 0.19 | 0.59 ± 0.03 | 3093.91 ± 1.53 |
| | **15** | 236.41± 18.10 | 3.86 ± 0.32 | 1.03 ± 0.08 | 10.65 ± 0.58 | 1.03 ± 0.15 | 2707.48 ± 0.65 |
| | **25** | 165.46 ± 24.97 | 4.59 ± 0.74 | 0.76 ± 0.10 | 5.00 ± 0.74 | 0.93 ± 0.09 | 3424.21 ± 0.06 |
| | **30** | 156.50 ± 16.84 | 3.32 ± 0.23 | 0.53 ± 0.06 | 3.34 ± 0.35 | 1.18 ± 0.07 | 2075.92 ± 0.31 |
| PUGLIA | **3** | 419.53 ± 14.44 | 7.18 ± 0.81 | 1.32 ± 0.12 | 9.35 ± 1.18 | 1.82 ± 0.30 | 1552.15 ± 1.67 |
| | **5** | 509.00 ± 37.87 | 8.02 ± 0.61 | 1.55 ± 0.11 | 11.76 ± 0.91 | 2.77 ± 0.09 | 5920.32 ± 0.71 |
| | **6** | 409.75 ± 16.29 | 7.86 ± 1.26 | 1.23 ± 0.07 | 14.69 ± 1.44 | 2.04 ± 0.06 | 4785.86 ± 0.44 |
| | **11** | 334.90 ± 19.01 | 5.82 ± 040 | 0.99 ± 0.06 | 6.70 ± 0.83 | 1.27 ± 0.18 | 813.96 ± 1.40 |
| | **16** | 301.77 ± 14.20 | 5.38 ± 0.30 | 0.96 ± 0.08 | 11.01 ± 0.95 | 0.80 ± 0.12 | 2625.00 ± 0.63 |
| | **22** | 268.63 ± 19.59 | 4.83 ± 0.65 | 0.89 ± 0.10 | 8.30 ± 0.66 | 1.41 ± 0.04 | 2089.20 ± 0.73 |
| | **28** | 136.21 ± 13.47 | 2.50 ± 0.19 | 0.42 ± 0.03 | 6.05 ± 0.54 | 0.94 ± 0.05 | 5613.64 ± 0.28 |
| | **29** | 301.46± 26.97 | 5.24 ± 0.54 | 1.00 ± 0.15 | 11.92 ± 0.60 | 1.35 ± 0.10 | 3511.29 ± 0.15 |
| TUSCANY | **2** | 213.14 ± 15.15 | 4.28 ± 0.23 | 0.75 ± 0.07 | 7.58 ± 0.86 | 0.93 ± 0.08 | 2929.45 ± 4.05 |
| | **4** | 573.20 ± 33.66 | 8.94 ± 1.22 | 2.41 ± 0.15 | 15.17 ± 1.66 | 3.19 ± 0.17 | 4317.37 ± 5.08 |
| | **8** | 488.59 ± 33.67 | 7.48 ± 0.14 | 1.71 ± 0.04 | 8.61 ± 0.53 | 2.43 ± 0.30 | 1180.14 ± 0.57 |
| | **13** | 513.03 ± 40.79 | 6.69 ± 0.82 | 1.78 ± 0.11 | 17.99 ± 1.01 | 2.42 ± 0.14 | 2221.55 ± 0.66 |
| | **14** | 246.80 ± 19.05 | 4.34 ± 0.61 | 0.83 ± 0.07 | 9.46 ± 0.75 | 0.90 ± 0.17 | 5429.22 ± 1.49 |
| | **17** | 338.95 ± 31.47 | 4.40 ± 0.54 | 1.30 ± 0.08 | 10.65 ± 0.58 | 1.57 ± 0.13 | 3652.16 ± 0.67 |
| | **20** | 374.12 ± 36.61 | 5.00 ± 0.45 | 1.25 ± 0.11 | 7.29 ± 0.63 | 2.06 ± 0.12 | 2185.76 ± 0.50 |
| | **21** | 171.16 ± 6.24 | 3.27 ± 0.38 | 0.60 ± 0.04 | 5.61 ± 0.91 | 1.00 ± 0.10 | 4197.36 ± 0.32 |
| | **26** | 407.49 ± 34.86 | 7.61 ± 0.48 | 1.05 ± 0.03 | 13.72 ± 1.51 | 2.51 ± 0.26 | 1676.81 ± 0.54 |
| | **27** | 156.00 ± 10.82 | 3.99 ± 0.39 | 0.57 ± 0.05 | 6.17 ± 1.64 | 0.83 ± 0.07 | 1778.42 ± 0.10 |
| LAZIO | **9** | 287.47 ± 9.35 | 4.79 ± 0.31 | 1.07 ± 0.04 | 7.21 ± 0.30 | 1.71 ± 0.10 | 3592.33 ± 2.11 |
| | **12** | 164.64 ± 11.11 | 3.47 ± 0.34 | 0.75 ± 0.04 | 4.68 ± 0.87 | 0.96 ± 0.05 | 3635.78 ± 1.02 |
| | **18** | 161.82 ± 15.60 | 2.91 ± 0.32 | 0.62 ± 0.05 | 2.99 ± 0.43 | 1.04 ± 0.11 | 3490.66 ± 0.30 |
| | **19** | 202.88 ± 20.62 | 3.25 ± 0.28 | 0.82 ± 0.07 | 4.55 ± 0.74 | 1.33 ± 0.10 | 1592.28 ± 0.63 |
| | **23** | 316.04 ± 28.41 | 6.21 ± 0.38 | 0.82 ± 0.05 | 8.26 ± 1.52 | 1.57 ± 0.19 | 2425.75 ± 0.07 |
| | **24** | 298.23 ± 28.96 | 4.93 ± 0.49 | 1.01 ± 0.12 | 8.37 ± 1.37 | 2.07 ± 0.20 | 3260.89 ± 0.30 |

Table 4:TPC, TEAC, DPPH, FRAP values of analyzed EVOO samples grouped by geographical areas. Values are expressed as mean ± SD. Sample number refers to table x

The pearson correlation was evaluated between the total concentration values of phenolic compounds obtained with the two methods (Folin-Ciocalteau and HPLC) and between these and the antioxidant activity values obtained with the four methods reported above (Table 5).

Table 5: Pearson correlation coefficient among total phenolic compounds concentration determined by HPLC method, TPC, TEAC, DPPH, ORAC, and FRAP values.

| | **Total Phenolic Compounds Concentration HPLC** | **TPC** | **TEAC** | **DPPH** | **ORAC** | **FRAP** |
|---|---|---|---|---|---|---|
| Total phenolic compounds concentration HPLC | 1 | | | | | |
| TPC | 0.893 *** | 1 | | | | |
| TEAC | 0.812 *** | 0.817 *** | 1 | | | |
| DPPH | 0.8659 *** | 0.790 *** | 0.658 *** | 1 | | |
| ORAC | 0.798 *** | 0.706 *** | 0.789 *** | 0.607 *** | 1 | |
| FRAP | 0.873 *** | 0.836 *** | 0.801 *** | 0.863 *** | 0.649 *** | 1 |

Pearson R2 values ranged between 0.607 and 0.893. A very good positive correlation was found between the values obtained with the four assays for the determination of the antioxidant activity, demonstrating that all methods are valid for the evaluation of the antioxidant activity, but also between the values of antioxidant activity and the concentration of total polyphenols calculated with the two methods. This last result shows that is the concentration of phenolic compounds in the samples increases, their antioxidant capacity also increases. The correlation between the concentrations of the individual phenolic compounds and the antioxidant activity of the extract was also evaluated. Results showed a very good positive and significant correlation between concentration of oleuropein aglycon and ligstroside aglycon and antioxidant activity determined by the four assays, $R^2$ values higher than 0.6 and a good positive significant correlation even if with lower $R^2$ values was determined also between the concentration of dialdeydic form of oleuropein aglycon and oleuropein aglycon isomer and antioxidant activity. These molecules, ad previously reported belong to the class of secoridoids, whose powerful antioxidant activity has already been demonstrated in literature (Servili M., et al., 2014).

### 4.1.2.3 Conclusion

A HPLC-PDA/ESI-MS method has been developed and validated for the analysis of the most representative phenolic compound in EVOO samples. The method was optimized and validated and then applied to the analysis of thirty EVOO samples from four Italian regions.

Quali-quantitative analysis of phenolic compounds was done and quantitative results showed differences with data reported in the literature. This results has been discussed considering the limits existing for the determination of real total concentration of phenolic compounds in EVOO. Subsequently antioxidant activity of the EVOO samples was evaluated and the correlation between results obtained with used method studied. Also the correlation between antioxidant activity of the extract and the concentration of phenolic compounds was evaluated. Obtained results show that antioxidant activity increases in accordance with the increasing of phenolic concentration. Although it was possible to observe the characteristics of the oils related to the region of origin, it is impossible to clasterize the oils by region due to the many factors that affect the chemical characteristics of the oil. The described results were published in the journal "molecules" (Fanali C., et al., 2018).

### 4.1.3 Optimization of a green analytical method, extraction with Deep Eutetic Solvents, for the analysis of EVOOs phenolic compounds

#### 4.1.3.1 Materials and methods

##### 4.1.3.1.1 Chemicals

Solvents employed for the traditional extraction procedure and for HPLC-MS analyses were n-hexane (purity 99.8% v/v), MeOH (purity 99.9% v/v), H2O (HPLC-MS grade), ACN (purity 99.9% v/v), formic acid (purity 95–97% v/v) and were purchased from Sigma-Aldrich (Milan, Italy). The components used to prepare DESs, such as ChCl, betaine, triethylene glycol, ethylene glycol, glycerol and urea, were obtained from Sigma-Aldrich (Milan, Italy). The standard compounds namely oleuropein (purity 98%) and 2-(4-hydroxyphenyl)ethanol (purity 98%) were purchased from Merck KGaA (Darmstadt, Germany).

##### 4.1.3.1.2 Preparation of the deep eutectic solvents

Table 6: Composition of DESs tested

| Abbreviation | Components | | Molar ratio |
|---|---|---|---|
| | Component 1 | Component 2 | |
| DES1 | Choline chloride | Glycerol | 1:2 |
| DES2 | Choline chloride | Lactic acid | 1:2 |
| DES3 | Choline chloride | Triethylene glycol | 1:2 |
| DES4 | Choline chloride | Urea | 1:2 |
| DES5 | Choline chloride | Ethylene glycol | 1:2 |
| DES6 | Betaine | Glycerol | 1:2 |
| DES7 | Betaine | Lactic acid | 1:2 |
| DES8 | Betaine | Triethylene glycol | 1:2 |
| DES9 | Betaine | Urea | 1:2 |
| DES10 | Betaine | Ethylene glycol | 1:2 |
| Control | MeOH/$H_2O$ (3:2 v/v) | | |

Ten DESs shown in the Table 6, were tested and compare to a conventional extraction for the analysis of EVOOs phenolic compounds. Betaine and ChCl were selected as HBA. About the preparation of ChCl DESs, ChCl was dried before use in a heater for 12 h at 100°C. With the aim to produce DESs it's necessary that the components of each DES were

weighed in a tube, respecting the molar ratio established, and kept in a thermoblock for 30 min at a temperature of 80°C until a homogeneous colorless liquid was formed.

4.1.3.1.3 Optimization of DES extraction method

For the optimization of the extraction method applying DESs we decided to allow the study of several parameters. First of the extraction efficiency of selected DESs was evaluated and subsequantialy water content in DES (10, 20, 30, 40, 50 and 70 %) and ratio of sample amount (mg) to DES volume (mL) (1:1, 2:1, 3:1, w/v), considering DES that guaranteed the best extraction, were studied.

4.1.3.1.4 Conventional and Green extraction protocols for EVOOs phenolic compounds

The conventional extraction technique for EVOOs phenolic compounds chose as reference to evaluate the extraction efficiency of DESs is an organic solvent extraction according to the protocol reported in literature by Ricciutelli et al., with few modifications (Ricciutelli, M., et al., 2017). An amount of 0.5 g of oil was dissolved in 0.5 mL of hexane in a centrifuge tube. The phenolic extraction was carried out using 0.5 mL of a mixture of MeOH/H2O (3:2, v/v). Centrifuge tube was kept in a ultrasound bath at room temperature, frequency of 37 kHz and heating power of 200 W for 20 min and consequentially the was vortexed and centrifuged for 3 min at 9.5xg. The solution to be analyzed was obtained following three repetitions of this procedure

For the extraction with DESs 0.5 g of EVOO were dissolved in 0.5 mL of hexane and added with 0.5 mL of DES, vortexed and centrifuged for 3 min at 9.5xg, in a centrifuge tube. The extraction was carried out in the same condition of the traditional extraction so also in this case using an ultrasound bath (Elmasonic S30H, Elma Schmidbauer GmbH, Singen, Germany) at room temperature, frequency of 37 kHz and heating power of 200 W for 20 min. The procedure was repeated three times and the formed solution analyzed.

#### 4.1.3.1.5 HPLC-DAD-ESI-MS analysis of phenolic compounds

HPLC-DAD-ESI-MS method used for the analysis of the extracts obtained with DES and convention extractions was set in the same conditions as the one used in the above research project (Fanali C., et al., 2020).

#### 4.1.3.1.6 HPLC-DAD quantitative analysis method validation

Oleuropein and 2-(4-hydroxyphenyl) ethanol, two of the most characteristics phenolic compounds in EVOO were selected for the method validation which included the study of the linearity, retention time and peak area repeatability (intra-day and inter-day precision), LOD and LOQ.

Linearity was determined by the calibration curves obtained from the HPLC analysis of the two standard solutions, whose stock solution was prepared at a concentration of 10.000 mg $L^{-1}$ in MeOH for each one.

#### 4.1.3.1.7 Statistical analysis

All the analysis were performed in triplicate. Statistical analysis where conducted using Stata 14 statistical software (StataCorp LLC, College Station, Tx). One-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test, if applicable, after verifying the normality of the distribution of the dependent variable was considered for the evaluation of results differences. Probability values of $p \leq 0.05$ were considered statistically significant.

### 4.1.3.2 Results and Discussion

#### 4.1.3.2.1 Optimization of the DES extraction for EVOOs phenolic compounds

The tested DESs previously descibed, was chosen on the bases of which reported in literature (Zainal-Abidin M.H., et al., 2017). The first step of the optimization involved the evaluation

of DES that guaranteed the best extraction. With this aim the evaluation of the DESs extraction efficiency was carried out considering for all DESs the same extraction conditions being: 30 % of water, added to each DES in order to reduce their viscosity and a sample to solvent ratio of (w/v). All the extracts obtained were analyzed through HPLC/DAD-MS method, previously described and optimize (Fanali C., et al., 2020).

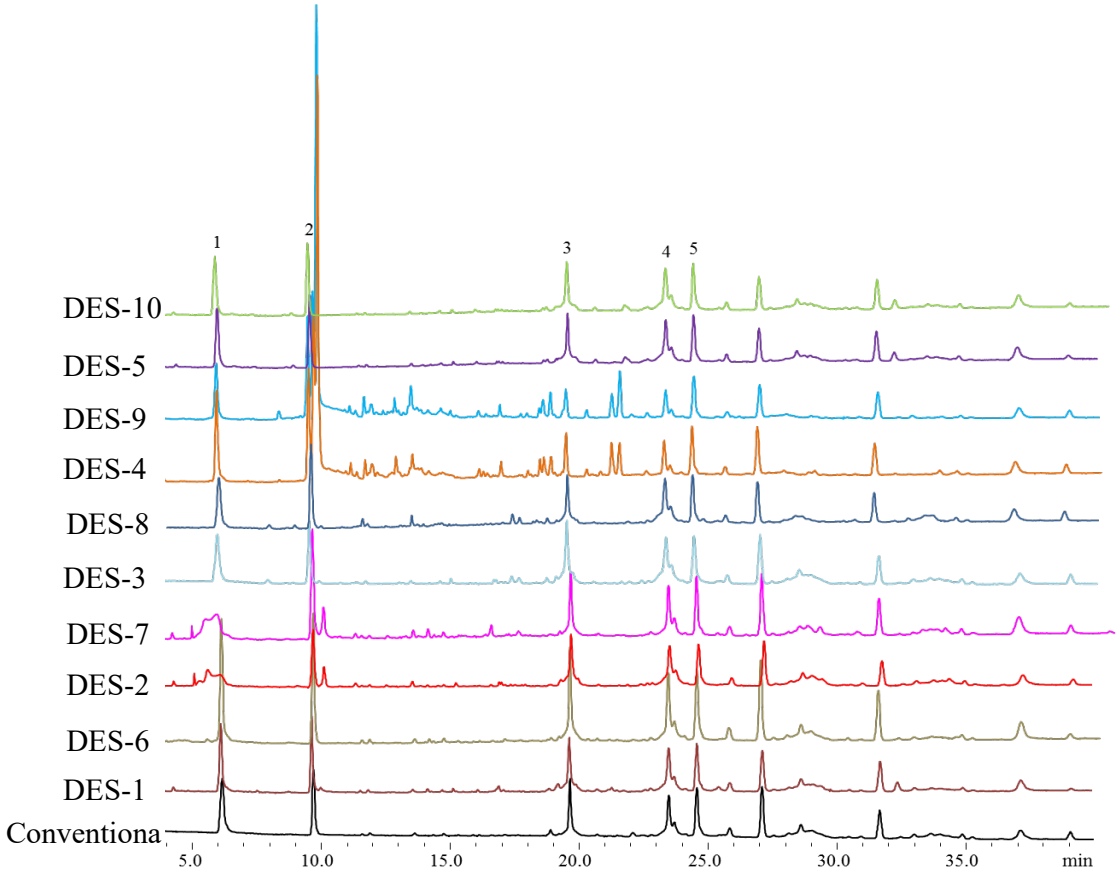



Figure 5: Chormatograms obtained used differnt tested DESs (Fanali C., et al., 2020).

Phenolic compounds in the extracts were identified considering the retention time and UV spectra data, MS spectra, use of standard compounds and data available in literature. The dialdehydic form of oleuropeine aglycon, aglycon isomer of oleuropein and ligstroside aglycon (compounds 3, 4 and 5 of the chromatogram), secoridoid dervates and tyrosol and hydroxytyrosol (compounds 1 and 2 of the chromatogram), phenolic alcohols were selected for the evaluation of the extraction efficiency of each DES.

It's possible to see in figure 5 that the qualitative profile of phenolic compounds in the different extracts was very similar. Only DES 4 and DES 9 reported some differences and according to the literature, this could be due to the formation of adducts due to the interaction between MeOH and urea. Quantitative differences between extracts were evaluated considering Standard of 2-(4-hydroxyphenyl)ethanol for the quantitation of the two phenolic alcohols while oleuropein standard was considered for the quantitation of secoiridoid derivatives. Results are reported in table 7, DES 4 and DES 9 were immediately considered unsuitable for the purpose as they had a too high RSD% probably due to their high viscosity at room temperature.

Table 7: Concentration and SD of each phenolic compounds extracted with tested DESs

| Phenolic compounds µg/g ± SD | | | | | | |
|---|---|---|---|---|---|---|
| Solvent | Hydroxytyrosol | Tyrosol | Dialdehydic form of oleuropein aglycon | Oleuropein aglycon isomer | Lygstroside aglycon | Total |
| DES1 | 62.84 ± 2.07 | 55.18 ± 2.71 | 131.78 ± 10.31 | 134.99 ± 11.98 | 113.07 ± 4.19 | 497.86 ± 16.70 |
| DES2 | 37.69 ± 6.29 | 81.13 ± 4.11 | 129.41 ± 5.61 | 138.34 ± 18.70 | 122.26 ± 12.74 | 509.10 ± 24.49 |
| DES3 | 70.60 ± 3.38 | 58.94 ± 8.06 | 196.63 ± 8.85 | 158.90 ± 28.77 | 137.48 ± 13.14 | 622.55 ± 33.99 |
| DES4 | 53.20 ± 2.36 | 224.56 ± 64.42 | 74.95 ± 9.01 | 83.21 ±18.04 | 94.93 ± 6.13 | 530.84 ± 67.82 |
| DES5 | 56.04 ± 0.45 | 50.54 ± 1.13 | 133.17 ±6.84 | 138.69 ± 8.13 | 144.56 ± 0.63 | 523.00 ± 10.71 |
| DES6 | 116.20 ± 6.17 | 89.55 ± 5.42 | 206.67 ± 6.48 | 178.88 ± 9.20 | 181.74 ± 5.86 | 773.03 ± 15.11 |
| DES7 | 53.21 ± 14.52 | 98.30 ± 0.67 | 146.14 ± 21.23 | 156.20 ± 2.71 | 158.51 ± 3.23 | 612.35 ± 26.07 |
| DES8 | 46.35 ± 3.92 | 44.05 ± 5.54 | 116.16 ± 3.68 | 129.43 ± 12.82 | 118.65 ± 1.69 | 454.64 ± 15.06 |
| DES9 | 54.32 ± 30.17 | 291.31 ± 73.95 | 71.03 ± 25.92 | 90.48 ± 28.77 | 135.60 ± 13.45 | 642.74 ± 89.77 |
| DES10 | 65.75 ± 1.73 | 66.18 ± 0.45 | 94.69 ± 5.25 | 130.28 ± 7.86 | 133.17 ± 2.42 | 490.08 ± 9.92 |
| Conventional solvent | 97.54 ± 6 .05 | 67.83 ± 1.18 | 170.06 ± 4.47 | 27.52 ± 5.48 | 134.52 ± 0.83 | 597.47 ± 9.42 |

In all extracts secoridoids were resulted the most abundant class of phenolic compounds, in accordance with our other project and literature data and the oleuropein aglycon isomer was

the phenolic compound with the highest concentration in each extracts. (Karkoula E., et al., 2012; Sánchez de Medina V., et al., 2015; Fanali C., et al, 2020).

Good results in terms of extraction efficiency were obtained with all DESs, also compare with conventional extraction but only DES 6 extracted higher total concentration of phenolic compounds (sum of concentrations of single phenolic compounds) than that extracted with organic solvents and differences were statistically significant.

2-(4-hydroxyphenyl)ethanol concentration was very similar in all extracts analyzed except for DES characterized by the presence of lactic acid as HBD, that have higher concentration than the other DESs.

This result could be explain considering that the low pH of lactic acid DESs can cause the degradation of secoridoids containing tyrosol and hydroxytyrosol in their structure (García A., et al., 2016).

Considering data described, DES 6 was selected for the subsequent phases of method optimization.

The selected alcohol-based DES (DES6) was prepared adding 10, 20, 30, 40, 50 and 70% (v/v) of water. Adding a certain amount of water to the DES is very important in order to reduce its high viscosity and guarantee extraction repeatability. However the optimal water percentage is strictly dependent on the DES composition and the polarity of extracted molecules and in some cases adding an high quantity of water, >50% can cause the reduction of extraction efficiency due to the the loss of the structure of the DESs or the lowering of the interactions between analyte and DES itself (Bosiljkova T., et al., 2017).

EVOO phenolic compounds extraction was then performed at sample to solvent ratio of 1:1 (w/v) for the DESs prepared with different water quantity.

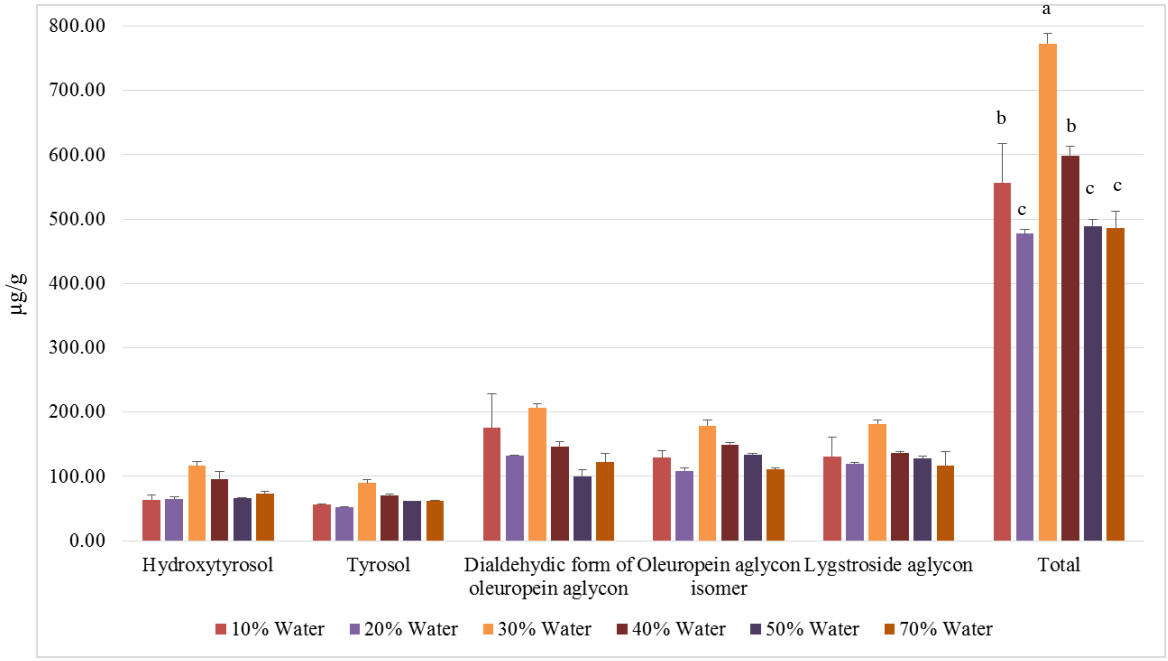



Figure 6: concentrations of single phenolic compounds obtained through application that DES6 added of different percentages of water

In figure 6 it's possible to observe results obtained in this phase of optimization, in particular in figure are reported concentrations of single phenolic compounds extracted using DES 6, added of previously reported percentage of water. Extraction repeatability was good for all extractions except for that allowed using DES added with 10% of water, probably due to its high viscosity. The best extraction in terms of total concentration of phenolic compounds was with DES added of 30% of water. From 30 % to 70 % inceasing the quantity of water, deacreased the extraction efficiency and this phenomena was the same for the concentration of secoridoids. At the contrary extracted phenolic alcohols concertation increased, incrising the quantity of water. Different effects of water on the extraction of different compounds, could be due to the different polarity of the same. The concentration of polyphenols extracted drastically drops by adding 50% of water to the DES, confirming the data in the literature (Bosiljkova T., et al., 2017). Based on obtained results we selected DES 6, added with 30% of water for the further method optimization, that involved the evaluation of the sample to solvent ratio optimal condition considering 1:1, 1:2 and 1:3 (w/v) ratio. The best

results, shown in table 8, in term of extracted phenolic content were obtained using 1:1 sample to solvent ratio with statistically significant differences among samples. Betaine-glycerol 1:2 (molar ratio) with 30% water using a sample to solvent ratio of 1:1 (w/v) and room temperature was selected as optimal condition for the method validation.

Table 8: Concentration of phenolic compounds extracted from EVOO sample using DES betaine-glycerol 1:2 (molar ratio) with 30% H2O using three matrix/extractive solvent ratios.

| Sample to solvent ratio µg/g ± SD | | | |
|---|---|---|---|
| Compound | 1:1 | 1:2 | 1:3 |
| Hydroxytyrosol | 116.20 ± 6.17 | 74.80 ± 8.96 | 91.03 ± 6.31 |
| Tyrosol | 89.55 ± 5.42 | 54.42 ± 2.65 | 55.79 ± 5.06 |
| Dialdehydic form of oleuropein aglycon | 206.67 ± 6.48 | 159.05 ± 17.20 | 172.35 ± 13.37 |
| Oleuropein aglycon isomer | 178.88 ± 9.20 | 185.53 ± 23.75 | 145.99 ± 5.65 |
| Lygstroside aglycon | 181.74 ± 5.86 | 190.02 ± 43.68 | 174.25 ± 16.97 |
| Total | 773.03 ± 15.11 | 663.82 ± 53.44 | 639.41 ± 23.75 |

4.1.3.2.2 High-performance liquid chromatography method optimization and validation

Oleuropein and 2-(4-hydroxyphenyl)ethanol were selected to complete the optimization of the analytical method. HPLC-DAD-MS-ESI conditions are the same described in the previously published project (Fanali et al., 2020).

Repeatability of retention time and peak area, LOD, LOQ, linearity concentration range and recovery were studied for the validation of the method.

Calibration curves with external standard were constructed for each analytical standards 2-(4-hydroxyphenyl)ethanol and oleuropein obtaining good determination coefficients (R2) being 0.995 and 0.997 rispectively. RSDs values below 0.12 % and 9.91% for retention time and peak area repeatability were obtained and 0.37% and 10.94% for inter-day precision, respectively. LOD and LOQ values were 0.1 and 0.8 µg mL-1for 2-(4-hydroxyphenyl)ethanol and 1.0 and 2.0 µg mL-1, respectively.

Recovery was calculated using seed oil sample aliquots spiked with 5 and 100 µg $g^{-1}$ of each standard compound. Good results were obtained both for 2-(4-hydroxyphenyl)ethanol being 98.7(RSD%= 2.4) and 89.1% (RSD%= 0.37) at 5 and 100 g g-1, respectively and for

oleuropein being 88.6 (RSD%= 3.3) and 75.2 (RSD%= 1.95) at 5 and 100 g g-1, respectively.

#### 4.1.3.3 Conclusion

A HPLC-DAD/ESI-MS method was developed and validated for the analysis of the most representative phenolic compounds in EVOO samples using a green extraction approach. Betaine-glycerol 1:2 (molar ratio) resulted to be a good green alternative to the conventional organic solvents for the extraction of phenolic compounds from EVOOs. The addition of a certain quantity of water to the DES results to be very important to reduce its viscosity and improve its extraction capacity. This validated method represents an eco-friendly alternative to the conventional method traditionally used and could be applied also for the extraction of phenolic compounds in EVOO food waste products.

Described data are published on "Electrophoresis" journal (Fanali C., et al., 2020).

## 4.2 DEVELOPMENT, OPTIMIZATION AND VALIDATION OF ANALIYCAL MEHOTDS FOR THE ANALYSIS OF PHENOLIC COMPOUNDS IN HAZELNUTS AND HAZELNUT SKIN USING HPLC/DAD-ESI/MS

### 4.2.1 Introduction

Hazelnut (Corylus avellana L.) is a type of dried fruit most consumed in many countries, including: Turkey, Italy, Spain and the United States. Hazelnut is a rich source of dietary fiber and beneficial nutrients such as lipids, proteins, but also micronutrients such as essential minerals, vitamin E, vitamin B complexes and phenolic compounds, which contribute to its organoleptic properties such as astringency and bitter taste.

In particular, phenolic compounds play an important role on human health due to their beneficial effects such as antioxidant activity (Schmitzer V., et al., 2011). The content in phenolic compounds could be a significant parameter for evaluating the quality of hazelnuts. In fact, this depends on various factors such as the cultivar, the geographic origin, the process conditions such as for example roasting (Contini M., et al., 2011). Published papers reported the chemical composition of phenolic compounds in hazelnut, the most common classes are flavan-3-ols, phenolic acids, flavonols and anthocyanins. During processing of hazelnut, a large number of waste products such as hazelnut skin and other by-products including hard shell, green leafy cover and tree leaf are produced. The skin represents about 2.5% of the total hazelnut kernel weight, and is usually removed by blanching or roasting in order to improve the kernel flavor, color, and crunch and for the use of the kernel in the confectionery industry and bakery (Piccinelli A.L., et al., 2015).

According to the literature, the main polyphenolic subclass is by far the flavan-3-ols in both their monomeric and polymeric forms accounting for more than 95% of the total hazelnuts skin polyphenols. Flavonols and dihydrochalcones represent an additional 3.5% while phenolic acids are responsible for less than 1% of the total identified phenolicsproanthocyanidins (PAs).

The solid-liquid technique is the most widely used method for the extraction of phenolic compounds from hazelnuts. Table 9, shown below, lists some solid-liquid methods, found in the literature, which involve the use of different solvents.

Table 9: Optimized extraction tecnhiques for phenolic compounds in hazelnuts

| Extraction solvents | |
|---|---|
| MeOH/H2O (2:1) | Yurttas H.C., et al., 2000 |
| Acetone/ H2O/acetic acid (70:29.5:0.5) | Gu L., et al., 2003 |
| Acetone/ H2O/acetic acid (70:29.5:0.5) | Gu L., et al., 2004 |
| EtOH/ H2O (80:20) or Acetone/ H2O(80:20) | Alasalvar C., et al., 2006 |
| EtOH/ H2O (80:20) | Shahidi F., et al., 2007 |
| Acetone/ H2O (80:20) | Amarowicz R., et al., 2008 |
| EtOH/ H2O (80:20) or Acetone/ H2O (80:20) | Prosperini S., et al., 2009 |

Also phenolic compounds from hazelnut skin are typically extracted using a solid-liquid extraction technique, using organic solvents such as methanol, ethanol and, methanol water mixtures (Del Rio D., et al., 2011; Piccinelli A.L., et al., 2015). The most commonly used instrumental analysis for the analysis of the phenolic compounds present in the extracts, obtained from hazelnuts and hazelnut skin, is the high-performance liquid chromatography HPLC, conducted with a reversed phase column and coupled to a series spectrophotometric detector. of diodes (DAD) and a mass spectrometer (LC-MS) (Tsao R., et al., 2010, Del Rio D., et al., 2011). From what has been found in the bibliography, the most suitable source for the analysis of phenolic compounds, by means of mass spectrometry, is the electrospray ionization source, ESI, coupled to single quadrupole analyzers or time-of-flight analyzers, which, characterized by a higher resolution, allow to obtain more reliable results, guaranteeing a better identification efficacy (Riethmuller E., et al., 2014; Del Rio D., et al., 2011).

Considering this food matrices in a first moment we dicided to develop, optimize and validate an extraction method for phenolic compounds in hazelnuts of different cultivars and analyze them by HPLC/DAD-ESI/MS. The procedure chosen and optimized for the extraction of phenolic compounds from hazelnut kernels was the solid-liquid, using a mix of solvents. The samples were analyzed for total phenolic content and antioxidant capacity by the Folin-Ciocalteu and TEAC (Trolox equivalent antioxidant capability) assays, respectively. Subsequently a green extraction technique was optimized for the extraction of phenolic compounds from the hazelnut skin.

### 4.2.2 Optimization of an analytical methods for the analysis of phenolic compounds in hazelnuts using HPLC/PDA-ESI/MS

#### 4.2.2.1 Materials and methods

##### 4.2.2.1.1 Chemicals

The analytical method was developed and validated using a mix of different standards of phenolic acids and flavonoids: Gallic Acid, Protocatechuic Acid, Catechin, Caffeic Acid, Epicatechin, p-Coumaric Acid, Epicatechin Gallate, Quercitin and Kampferol, supplied by Sigma-Aldrich , Procyanidin A2, Procyanidin B2 provided by extrasynthese.

The reagents used for the development and optimization of the analytical method are: water, methanol, ethanol, acetonitrile, acetone, ethyl acetate, hydrochloric acid and formic acid, supplied by Sigma-Aldrich.

##### 4.2.2.1.2 Samples

Three different cultivars of hazelnuts were analyzed, being Roman, of Italian origin, Ordu, of Turkish origin and Akacoca, of Turkish origin, supplied by a food company that uses them as raw material for the production of the finished product.

##### 4.2.2.1.3 Development and optimization of the extraction method

To carry out the extraction procedure and consequently the validation of the analytical method, it was necessary to carry out a delipidation procedure on the three varieties of hazelnuts to be analyzed. The delipidation was carried out by repeating an extraction three times that involved the addition of 10 ml of hexane to 1 g of each sample. This extraction was coupled with the use of an ultrasonic bath which increased its effectiveness, at a temperature of 25 ° C, for a time of 18 minutes.

Two techniques were used to optimize the extraction method: the conventional solid-liquid (SLE), and the SPE technique. Seven methods were developed with the solid-liquid

technique, varying the solvents used and the quantity of the starting sample, other three methods, with the SPE technique. The choice of the most suitable method was carried out by subjecting the various extracts obtained to the Folin Ciocalteu and TEAC assays, and evaluating the results. Table 10 summarizes all the extraction methods used. The first six solid-liquid extractions tested, involved two extraction cycles on 1.5g of delipidated sample, varying the extraction solvents:

A. 50 mL methanol 0.1% HCl

B. 50 mL ethanol 0.1% HCl

C. 50 mL acetone 0.1% HCl

D. 50 mL methanol / water (8: 2 v/v)

E. 50 mL ethanol / water (8: 2 v/v)

F. 50 mL ethyl acetate, methanol and methanol water (1: 1 v/v)

The seventh solid-liquid extraction (G), involved two extraction cycles, using 0.1 g of delipidated sample and 15 mL of the following solvents: methanol/water (1:1 v/v), 2 ml; methanol 0.1% HCOOH, 8 ml; acetonitrile, 5 ml.

The SPE technique involved the use of a C18 column and 2 ml of water and 2 ml of methanol for the conditioning step. Three procedures were tested starting from a 0.1 g delipidated sample quantity:

H. 6 mL methanol 0.1% HCl

I. 15 mL methanol/water (1: 1 v/v); methanol 0.1% HCOOH; acetonitrile

L. 20 mL methanol/water (1: 1 v/v); methanol 0.1% HCOOH; acetonitrile; n-propanol

Table 10: extraction procedures tested for the method optimization

| | Delipidated sample | Solvents (2x) | |
|---|---|---|---|
| SLE | 1.5 g | 50 mL methanol 0.1% HCl | A |
| | | 50 mL ethanol 0.1% HCl | B |
| | | 50 mL acetone 0.1% HCl | C |
| | | 50 mL methanol/water (8:2 v/v) | D |
| | | 50 mL ethanol/water (8:2 v/v) | E |
| | | 50 mL ethyl acetate, methanol and methanol water (1: 1 v/v) | F |
| | 0.1 g | 15 mL methanol / water (1:1 v/v); methanol 0.1% HCOOH; acetonitrile | G |
| SPE | 0.1 g | 6 mL methanol 0.1% HCl | H |
| | | 15 mL methanol/water (1:1 v/v); methanol 0.1% HCOOH; acetonitrile | I |
| | | 20 mL methanol/water (1:1 v/v); methanol 0.1% HCOOH; acetonitrile; n-propanol | L |

4.2.2.1.4 HPLC/PDA-ESI/MS analysis

The analyzes of the extracts of the hazelnut samples were carried out using an LC-20A system (Shimadzu Prominence LC-20AD XR) consisting of: two LC-20 AD XR pumps, a CBM-20A controller, a DGU-20A3 degasser, a SIL-10 AD vp autosampler, a CTO-20 AC oven, an SPD-M10A UV photodiode detector and a single quadrupole mass spectrometer (Shimadzu 20-10 EV).

Considering the nature of the compounds, the analytes were separated with a C18 column, using a binary mobile phase which, following various tests, was found to be more effective

if composed of: an aqueous solution, acidified at 0.1% of formic acid and acetonitrile with 0.1% formic acid, in gradient elution:

0-5 min 0-0% B

5-45 min 0-25% B

45-60 min 25-100% B.

The column temperature was set at 40 ° C and the flow rate at 0.2 mL/min. The wavelength range of the PDA set was: 180-400 nm.

Mass Spectrometry Settings:

Nebulizing gas (N2): 1.5 mL/min; Time scan: 1 sec; m/z range: 100 800; scan speed: 1000 amu/sec; Detector voltage: 1.5 kV; Interface temperature: 250 C; CDL temperatures: 300 ° C; Heat Block: 300 ° C; Interface Voltage: 3.50 kV; Q array: 0.0V; Q array RF: 150.0V

4.2.2.1.5 Validation of the analytical method

A mixture of 11 standards was used for the validation of the analytical method: gallic acid, protocatechuic acid, catechin, caffeic acid, procyanidin B2, epicatechin, p-coumaric acid, epicatechin gallate, procyanidin A2, quercetin, kampferol. For these compounds, the Limit of Detection (LOD) and Limit of Quantitation (LOQ) values were identified. LOD and LOQ, were calculated considering the ratio between signal (S) and noise (N), corresponding to a standard concentration that was three times and ten times higher than the background noise, respectively:

LOD = 3x (Y/N); LOQ = 10x (S/N).

To carry out the quantitative analysis on hazelnut samples, calibration lines, external standard, were constructed for each of the standards used.

For each standard, the construction of the calibration line required the use of different known concentrations, obtained by dilution starting from a stock solution, and the absorbance reading of the same at a wavelength of 280 nm, for gallic acid, protocatechuic acid, catechin,

procyanidin B2, epicatechin, p-coumaric acid, epicatechin gallate, procyanidin A2, of 325 nm for caffeic acid and of 375 nm for quercitin and kampferol.

The accuracy of the analytical method was assessed through the use of recovery tests. The hazelnut samples were first delipidated, then deprived of their phenolic fraction. The delipidated samples were subjected to two solid-liquid extraction cycles with 15 mL of a mixture of solvents (methanol/water (1:1 v/v), methanol 0.1% HCOOH, acetonitrile). The exhausted samples, matrix deprived of the lipid fraction and the phenolic component, was then added with a mixture of standards of known concentration and by means of HPLC/PDA-ESI/MS analysis, the real extracted concentration of each standard was obtained.

$$R\% = \left(\frac{X}{M}\right) * 100$$

X = Average of the results

M = Concentration of standard added

The accuracy of the chromatographic system was evaluated by performing intra-day and inter-day analyzes of the mix of standards and obtaining% RSD of the retention times and peak areas.

4.2.2.1.6 Trolox equivalent antioxidant capacity assay

The antioxidant activity of the extracts obtained was evaluated by TEAC assay.

The ABTS radical cation was generated by reaction between a 7 mM ABTS solution and a 2.45 mM potassium persulfate solution, in the dark and at room temperature for 16 h. The radical ABTS was then diluted in a ratio of 1:50 in phosphate buffer (5mM pH 7.4), so as to obtain an absorbance of 0.70 ± 0.05 at λ = 734 nm.

The reaction mixture was prepared by mixing 10 µL of each diluted extract with 190 µL of the radical ABTS solution.

After 10 minutes of incubation in the dark, the absorbance of each extract was revealed, in triplicate, at the wavelength λ=734.

4.2.2.1.7 Total phenolic content

The total phenolic content of each extract was measured using the Folin Ciocalteu assay, using gallic acid as a standard.

The reaction mixture was prepared, in triplicate for each extract, by mixing 1550 µl of MetOH:H2O 50:50 with 50 µl of extract and 100 µl of Folin-Ciocalteu reagent. Following incubation of 8 minutes, in the dark and at room temperature, 300 µl of an aqueous solution of sodium carbonate Na2CO3 [0.2 g/ml] was added. The reaction mixture was then stirred and incubated for 2 h in the dark and at room temperature. At the end of the incubation, all the eppendorf containing the mixture were centrifuged and the reading was carried out with TECAN, loading 200 µl of supernatant per well on a transparent Greiner plate, measuring the absorbance at a wavelength λ=765 nm.

4.2.2.1.8 Statistical analysis

The statistical analysis of the values obtained by the Folin-Ciocalteu assay, relating to the total phenolic content, and the TEAC assay, as regards the antioxidant activity of the analyzed extracts, was carried out by means of analysis of variance, ANOVA test.

The results obtained through the recovery tests, for the validation of the analytical method and the results obtained from the quantitative analysis of the three varieties of hazelnuts analyzed, were expressed as the mean ± the standard deviation.

#### 4.2.2.2 Results

4.2.2.2.1 Selection of the extraction technique

The extracts obtained by solid-liquid extractions A, B, C, D, E, F, previously described and extracts obtained with SPE, H, I, L techniques were subjected to the Folin Ciocalteau test, for the evaluation of the total phenolic content, and to the TEAC test, for the evaluation of the antioxidant activity.

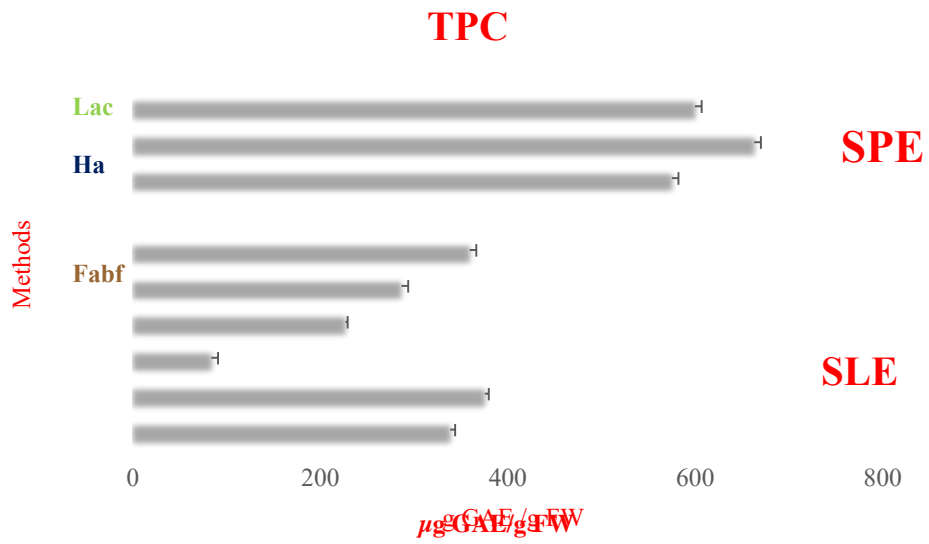



Figure 7: TPC of extractes obtained with technique extractions tested

Figure 7 shows the results relating to the total phenolic content, detected by the Foli-Ciocalteu assay, of the extracts obtained through the different SLE and SPE methods performed. It is possible to observe how the extracts obtained by the SPE technique had higher values in terms of total phenolic content.

In terms of total phenolic content, the most effective extraction technique, whose extract gave a higher value, was technique I, SPE, the one that used 15 mL of the methanol/water mixture (1: 1 v/v), methanol 0.1% HCOOH and acetonitrile, starting from 0.1 g of sample, also giving a good correlation with the values obtained by the TEAC assay (Figure 8). The

difference between the total phenolic content value of the extract obtained by technique I and those of the extracts obtained with techniques L and H, was statistically significant.

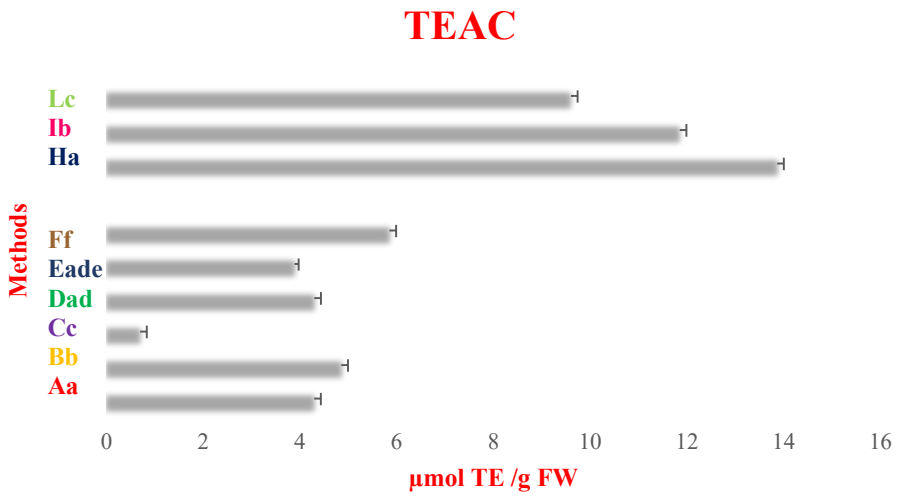



Figure 8: antioxidant capacity of extracts obtained with tested technique extration

The extracts with the greatest antioxidant activity were found to be those obtained using the SPE technique, in particular the most abundant content was found in the extract obtained with the H technique, which included 6 mL methanol 0.1% HCl as solvents, and 0.1 g of sample. The differences in the antioxidant activity value of the extracts, obtained by means of the three SPE techniques, were statistically significant.

It is not possible to confirm with certainty that the antioxidant activity characterizing the various extracts is solely related to the phenolic compounds present within them, consequently the total phenolic content was considered as the main discriminant of the extractive choice.

Observing the results obtained by these extractions, it was decided to carry out a tenth extraction (Method G), using the solid-liquid extraction technique, with the same quantity of starting delipidate sample (0.1g) and volume and mixture of solvents used for the extraction I.

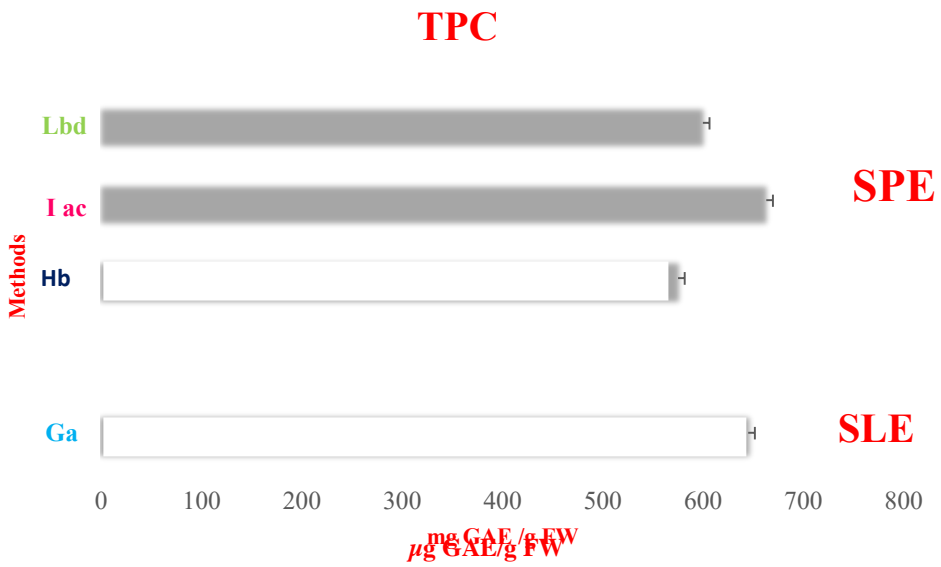



Figura 9: Total phenolic content of extracts obtained with selected extraction

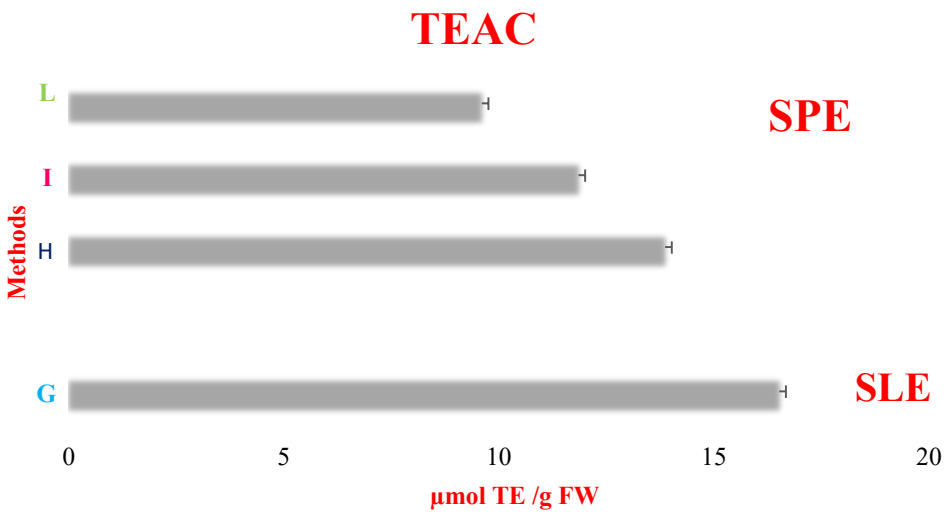



Figura 10: Antioxidant capacity of extracts obtained with selected extractions

The last extraction tested, extraction G, proved to be comparable, in terms of results and effectiveness, with the technique I, SPE, as regards the total content in polyphenols. As for the antioxidant activity, the G technique was better than the H, SPE technique (Figure 9, Figure 10).

By subjecting the values for the total phenolic content, obtained by the Folin-Ciocalteu assay, to statistical analysis, it was found that the difference in the total phenolic content of

the extracts obtained by techniques G and I was not statistically significant. The antioxidant activity found in the extract obtained using the G technique was significantly greater than the antioxidant activity of the extracts obtained with the other techniques.

For the extraction of phenolic compounds, in hazelnut samples, the choice fell on the G technique. This technique, using the same quantity of initial sample and the same quantity of solvents, used for the I technique, SPE, guaranteed same effectiveness but with reduced times and costs.

4.2.2.2.2 Development and optimization of the analytical method

Once the method of extraction of the phenolic compounds from the hazelnut samples was chosen, their analysis was carried out by HPLC/PDA-ESI/MS.

The separation of the compounds was carried out using reverse phase chromatography with C18 stationary phase. The optimization of the separation of the compounds involved an accurate study through which it was possible to make the choice of the most suitable mobile phases for the analysis. The method therefore envisaged a binary mobile phase, composed of an aqueous solution, acidified at 0.1% of formic acid and acetonitrile at 0.1% of formic acid, in gradient elution. The addition of 0.1% acid to water, rather than acetonitrile, has ensured an increased separative efficacy. The elution gradient of the compounds was then optimized in order to obtain the best possible separation of all the phenolic compounds detected. The best conditions in terms of analyte resolution and analysis times were obtained by applying a gradient elution mode: 0-5 min 0-0% B, 5-45 min 0-25% B, 45-60 min 25-100% B.

The quantitative analysis and validation of the method was carried out using a mixture of 11 standard compounds belonging to the phenolic classes of polyphenols present in hazelnuts: gallic acid, protocatechuic acid, catechin, caffeic acid, epicatechin, p-coumaric acid, epicatechin gallate, quercetin, kampferol, procyanidin A2, procyanidin B2.

To validate the HPLC method, intra-day and inter-day precision were evaluated, expressed as RSD% of retention times and peak areas, LOD, LOQ linearity range and recovery tests. The method accuracy of the retention times and areas was evaluated by injecting the standard mixture of phenolic compounds 5 times on the same day and on 3 different days. The RSD% for the retention times and for the peak areas of the intra-day tests was found to be included in a range of 0.05-2.01% and 0.38- 2.65%, respectively. The RSD% for retention times and peak areas of the inter-day tests, on the other hand, fell within a range of 0.04-0.91% and 2.31-7.57%, respectively.

The quantitative analysis of the compounds involved the construction of calibration curves for each standard available, in order to obtain the concentration of each compound present within the hazelnut extracts, by interpolating the areas of the peaks, obtained through validated chromatographic method, with the calibration curves of each standard.

| | LOD (mg/L) | LOQ (mg/L) |
|---|---|---|
| Gallic acid | 0.03 | 0.05 |
| Protocatecuic acid | 0.04 | 0.07 |
| Catechin | 0.04 | 0.08 |
| Caffeic Acid | 0.01 | 0.02 |
| Procyanidin B2 | 0.09 | 0.17 |
| Epocatechin | 0.09 | 0.17 |
| P-coumaric acid | 0.01 | 0.02 |
| Epicatechin Gallate | 0.04 | 0.08 |
| Procyanidin A2 | 0.08 | 0.14 |
| Quercetin | 0.02 | 0.03 |
| Kampferol | 0.01 | 0.03 |

Table 11: LOD and LOQ values for each standard molecule

The calibration curves for the standards were constructed using the same chromatographic conditions optimized for the analysis of the samples. The areas of the peaks were related to the known concentrations of the various standards used, expressed in mg/L, obtaining good correlation coefficient values, R2, between 0.997 and 0.999.

The linearity range was considered in the concentration range between LOQ values and a concentration of 100 mg/L. The limit of detection (LOD) and limit of quantification (LOQ) values, characteristic of the method, are shown in Table 11.

The accuracy of the extraction method was evaluated by carrying out recovery tests of the mixture of polyphenol standards, adding 100 μg/g of each standard to the exhausted starting sample, i.e. the sample deprived of its lipid and phenolic component, and analyzing it by chromatography, in triplicate. Some values were satisfactory, as in the case of Kampferol (77 ± 0.67)%, quercetin (73 ± 0.29)%, p-coumaric acid (102 ± 0.71)% and caffeic acid (95 ± 0.66)% ; for the remaining compounds the values were lower. This result could be related to the strong heterogeneity of the phenolic compounds and to the complexity of the starting matrix. In the literature there are no values obtained from recovery tests considering these molecules, so it was not possible to make comparisons.

4.2.2.2.3 Identification and quantification of phenolic compounds in hazelnut samples

The characterization of the phenolic compounds present within the hazelnut extract was carried out by HPLC/PDA-ESI/MS. The use of the mass spectrometer, with a single quadrupole mass analyzer, made it possible to determine the molecular weight of the molecules, based on the comparison of the m/z ratios of the standards available, or present in the literature. Figures 11 shows the PDA chromatograms, acquired at the wavelength λ = 280 nm, of a phenolic compounds hazelnut extract. Within the chromatograms, the peaks of the 18 compounds detected are numbered as shown in Table 12.

The qualitative analysis was conducted by considering various parameters such as the elution order, absorption spectrum, molecular weight, literature data and the comparison of retention times with standard molecules, if commercially available.

Table 12: Retention times and molecular ions of detected phenolic compounds

| | Compounds | Phenolic classes | [M-H]- (m/z) /[M-H]+(m/z) |
|---|---|---|---|
| 1 | Syringic acid | Phenolic acids | 197/195 |
| 2 | Ellagic acid | Phenolic acids | 300/301 |
| 3 | Unknow | - | 206 |
| 4+5 | Catechin / Vanillic acid | Flavan-3-ols / Phenolic acid | 289/167 |
| 6 | Unknow | - | 444 |
| 7 | Unknow | - | 243/323 |
| 8 | Unknow | - | 449 |
| 9 | procyanidin B2 | Proanthocyanidins | 577/575 |
| 10 | epicatechin | Flavan-3-ols | 289 |
| 11 | Unknow | - | 461 |
| 12 | 3-caffeoyl-5-feruilquinic acid | Phenolic acids | |
| 13 | Unknow | - | 499 |
| 14 | epicatechin gallate | Flavan-3-ols | 441 |
| 15 | procyanidin A2 | Proanthocyanidins | |
| 16 | Unknow | - | 541 |
| 17 | quercitrin | Flavonols | 301 |

Seventeen compounds were detected, 10 of which were identified, while for the remaining 7 it was not possible to define an identity.

The revealed and identified compounds were found to belong to 5 different phenolic classes: phenolic acids, proanthocyanidins, flavan-3-ols and flavonols.

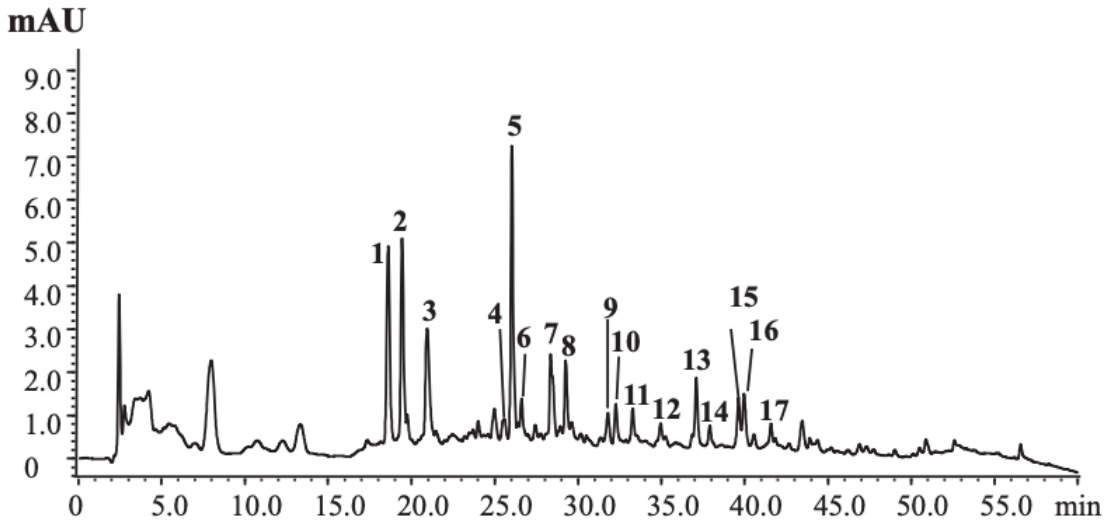



Figure 11: PDA chromatograms, acquired at the wavelength λ = 280 nm, of a phenolic compounds hazelnut extract

Table 12 shows the phenolic compounds identified, with respective phenolic classes, the corresponding retention times and the m/z ions obtained in positive rather than negative mode. Information on molecular ions and retention times can also be observed for phenolic compounds that have not been identified.

The 17 compounds were revealed in all three hazelnut varieties, showing a similar quality profile.

The quantitative analysis was carried out by interpolating the area of the peaks, obtained by applying the validated chromatographic method, relating to the various identified and non-identified compounds, with the previously obtained calibration curves (Table 13).

The variety of hazelnuts most rich in phenolic compounds was the Ordu variety, 128.9 μg/g, followed by Akcakoca, with an average quantity of 119.9 μg/g, and Romane 92.9 μg/g. The difference in the total concentration of polyphenols within the three varieties of hazelnuts is statistically significant, albeit small.

| | Compounds | Akakoca | Ordu | Romane |
|---|---|---|---|---|
| 1 | Syringic acid | 15.4 ± 0.38 | 16.8 ± 0.55 | 13.5 ± 0.58 |
| 2 | Ellagic acid | 20.8 ± 0.52 | 29.4 ± 0.33 | 13.4 ± 0.93 |
| 3 | Unknow | 13.7 ± 0.25 | 9.6 ± 1.75 | 6.7 ± 0.87 |
| 4+5 | Catechin / Vanillic acid | 2.2 ± 0.05 | 1.9 ± 0.07 | 1.7 ± 0.19 |
| 6 | Unknow | 20.4 ± 1.38 | 18.4 ± 0.89 | 19.4 ± 0.49 |
| 7 | Unknow | 1.8 ± 0.09 | 2.3 ± 0.15 | 2.4 ± 0.13 |
| 8 | Unknow | 5.2 ± 0.27 | 4.3 ± 0.18 | 4.4 ± 0.15 |
| 9 | procyanidin B2 | 5.1 ± 0.13 | 5.0 ± 0.15 | 4.6 ± 0.10 |
| 10 | epicatechin | 3.7 ± 1.85 | 2.5 ± 0.57 | 2.1 ± 1.11 |
| 11 | Unknow | 2.7 ± 0.06 | 2.8 ± 0.16 | 2.4 ± 0.15 |
| 12 | 3-caffeoyl-5-feruilquinic acid | 3.7 ± 0.04 | 4.5 ± 0.13 | 2.6 ± 0.20 |
| 13 | Unknow | 2.1 ± 0.09 | 1.8 ± 0.18 | 3.4 ± 0.40 |
| 14 | epicatechin gallate | 5.8 ± 0.22 | 4.4 ± 0.28 | 5.5 ± 0.14 |
| 15 | procyanidin A2 | 2.0 ± 0.04 | 2.3 ± 0.08 | 1.8 ± 0.04 |
| 16 | Unknow | 6.4 ± 0.19 | 10.5 ± 0.38 | 3.5 ± 0.08 |
| 17 | quercitrin | 6.2 ± 0.15 | 10.1 ± 0.21 | 3.4 ± 0.04 |
| **Tot.** | | **119.9±6.01** | **128.9± 5.01** | **92.9± 3.78** |

Table 13: total concentration and concentration of each phenolic compound detected in the extracts

In the Akcakoca and Ordu varieties, the phenolic compound most present is ellagic acid, with a concentration of 20.8 µg/g and 29.4 µg/g, respectively. In the Ordu variety, the phenolic compounds most present were found to be syringic acid and ellagic acid, with a concentration of 13.5 µg/g and 13.4 µg/g, respectively. In all three hazelnut varieties, the phenolic compounds present in smaller quantities were found to be catechin and vanillic acid, which coeluting to a tr = 19,455, were present in a total concentration of 2.2 µg/g in the Akcakoca variety, 1.9 µg/g in the Ordu variety and 1.7 µg/ g in the Romane variety. The concentration of quercetin within the three extracts varied significantly. In the Akcakoca

variety the quantity was 6.2 μg/g, in the Ordu variety the quantity was 10.1 μg/g and finally, in the Romane variety the quantity was 3.4 μg/g.

The composition in phenolic compounds found was found to be sufficiently in agreement with the data reported in the literature concerning the composition in phenolic compounds of other hazelnut varieties. In fact, the presence of the phenolic classes of flavan-3-oils, phenolic acids, proanthocyanidins, and flavonols within the hazelnut is confirmed (Schmitzer V., et al., 2011).

In particular, there are data in the literature that confirm the presence of Flavan-3-oils and flavonols (quercetin) and phenolic acids within hazelnuts of the Akcakoca and Ordu varieties (Del Rio D., et al., 2011).

Considering quantitative analysis some differences were found between the data obtained through this study and those present in the literature. An analysis conducted on extracts of 80% ethanol, hazelnuts, Corylus jacquemontii, by ultra-performance liquid chromatography coupled with tandem mass spectrometry, UPLC-MS/MS, revealed a quantity of catechin inside the extract. of 15.9 μg/g of catechin, higher than the quantity we found within the three varieties. The content of epicatechin 1.16 μg/g and quercetin 0.54 μg/g, was instead lower than what we found (Kumar A., et al., 2016).

Gallic acid, the presence of which within the hazelnut extracts is confirmed in the literature, has not been identified by us (Shahidi F., et al., 2007).

Differences between the qualitative-quantitative profile of the hazelnut varieties, Ordu, Akcakoca and Romane, analyzed in this study, and the qualitative-quantitative profiles of other hazelnut varieties reported in the literature could be related to various factors, such as the degree of ripeness of the hazelnuts analyzed and the origin of the hazelnuts analyzed, rather than the extraction technique used in the different studies (Slatnar A., et al., 2014).

#### 4.2.2.3 Conclusions

The purpose of the following project was to optimize an extraction method and consequently an analysis method, for the determination of phenolic compounds present within the hazelnuts. The optimized and validated method was applied to the analysis of polyphenols in three varieties of hazelnuts: Akcakoca, Ordu and Romane.

Two extraction methods were used, the conventional solid-liquid (SLE) and the SPE extraction technique. The optimization of the extraction method was carried out considering the total amount of phenols and the antioxidant activity obtained for the different extracts.

The solid-liquid technique and the SPE technique were both valid but, with the same efficiency, the choice fell on the solid-liquid technique, which represented a faster and less expensive alternative.

After selecting and validating the extraction method, the qualitative-quantitative analysis of the three varieties of hazelnuts was carried out by HPLC/PDA-ESI/MS, which was found to be appropriate for the identification and quantification of the phenolic compounds present in the hazelnuts. By applying the validated analysis method to the three hazelnut varieties: Akakoca, Ordu and Romane, it was possible to define the qualitative-quantitative profile.

The qualitative-quantitative profile of the three varieties of hazelnuts was similar. 18 compounds were detected, ten belonging to the classes were identified: Phenolic acids, Proanthocyanidins, Flavan-3-ols and Flavonols.

Ordu was the variety with the highest quantity of total phenolic compounds and the difference with the phenolic quantity of the other two varieties was statistically significant, albeit small.

The data obtained from the study were sufficiently in agreement with what is reported in the literature and were subsequently published on “Electrophoresis” journal (Fanali C., et al., 2018).

### 4.2.3 Development of an eco-friendly technique for the extraction of phenolic compounds from hazelnut skin.

#### 4.2.3.1 Materials and methods

4.2.3.1.1 Chemical Reagents

The solvents used for the traditional extraction procedure and for the analyzes with HPLC-MS were ethanol, water, Acetonitrile, formic acid all purchased from Sigma-Aldrich (Milan, Italy) with 99.9% v/v purity. The components used to prepare the DESs, choline chloride (ChCl), betaine, urea, citric acid, lactic acid, glucose, sorbitol, xylitol, glycerol, 1,6-hexanediol, triethylene glycol, ethylene glycol, propylene glycol, glycerol, malic acid and sucrose, were obtained from Sigma-Aldrich. The standard compounds used, such as gallic acid (98%), epicatechin (98%), quesrcitin (98%), campferol (98%), type B2 and type A2 procyanidin (98%), were purchased from Merck KGaA (Darmstadt, Germany).

2.2.3.1.2 Samples

Hazelnut skin were obtained from a company that uses hazelnuts to make food products. Before the experiments an aliquot of sample were ground in a coffee grinder and stored in a falcon at room temperature, until the use.

2.2.3.1.3 Preparation of DESs

DESs were prepared using ChCl and betaine as hydrogen bond acceptors (HBAs). Choline chloride was dried before use in an oven at about 100 ° C for 12h, as a highly hygroscopic substance, while betaine was used anhydrous. The components of each DES were weighed inside a test tube, respecting the established molar proportions and placed inside a thermo block at 80 ° C for 30 minutes until a homogeneous and colorless liquid has formed.

2.2.3.1.4 Extraction of phenolic compounds with DES and conventional solvent

The extraction of the analytes was performed using 0.2g of finely chopped hazelnut skin in a 15 mL test tube, added with 5 mL of extracting solvent and placed in an ultrasonic bath (Elmasonic S30H, Elma Schmidbauer GmbH, Singen, Germany) for 30 min, at 80°C. In order to compare the results obtained through the use of DES, with those obtained through a conventional extraction, a classical extraction method with organic solvent was used according to previously procedure reported (Del Rio D., et al., 2011), applying slight modifications. 0.5 g of sample was extracted using 5mL of a mixture of EtOH/H2O (75:25, v/v) in a centrifuge tube, vortexed for 15 min. and then put in an ultrasound bath (Elmasonic S30H, Elma Schmidbauer GmbH, Singen, Germany) at 50°C, frequency of 37 kHz and heating power of 200 W for 15 min.

2.2.3.1.5 HPLC/PDA-ESI/MS analysis of polyophenols

The analysis was performed using Shimadzu Prominence LC-20A instrument (Shimadzu, Milan, Italy) equipped with two LC-20 AD XR pumps, SIL-10ADvp, CTO-20 AC column oven and DGU-20 A3 degasser coupled to a SPD-M10Avp PDA detector and a mass spectrometer detector (LCMS-2010, Shimadzu, Tokyo, Japan) equipped with electrospray (ESI) interface. Shimadzu LCsolution Ver. 3.7 software (Shimadzu,Version 3.7) was used to acquire MS data. The separation of the analytes was carried out using a Core Shell column (150 × 4.6 mm I.D., 2.7 µm d.p.) (Merck KGaA, Darmstadt, Germany). Elution was performed at a constant flowrate of 1 mL/min and at temperature of 40°C. The mobile phase was (A) H2O/0.1% HCOOH and (B) acetonitrile/0.1% HCOOH. Polyphenols were separated using the following gradient : 0–40 min 0-30% B, 40–41 min 100% B. The injection volume was 2 µL. Data were acquired using a PDA in the range 200–400 nm and the chromatograms were extracted at 280 and 360 nm. MS-chromatograms were acquired in

negative ionization mode, using the following parameters: nebulizing gas flow rate (N2): 1.5 mL min−1; event time: 1 s; mass spectral range: m/z100–800; scan speed: 1000 amu/s; detector voltage: 1.5 kV; interface temperature: 250 °C; CDL temperature: 300 °C; heat block temperature: 300 °C; interface voltage: –3.50 kV; Q-array voltage: 0.0 V; Q-array RF: 150.0 V.

2.2.3.1.6 Quantification of total phenolic compounds (TPC) with the Folin-Ciocalteu assay

The quantification of total phenolic compounds (TPCs) was carried out with the Folin-Ciocalteu method. The TPC values was used to evaluate the extraction efficiency of the DES system. Folin's assay was performed according to a well-defined protocol, already validated previously, which initially involves the preparation of a sodium carbonate (Na2CO3) solution (0.2 g / mL). The solution is then filtered on a 0.22 µm filter and stored at room temperature until use. The sample is prepared by placing in a 2 mL eppendorf, 20 µL of sample or standard of gallic acid (for the calibration line) and 1,580 mL of extracting solvent (EtOH 50:50 v/v), for a total of 1.6 mL; then 100 µL of Folin's reagent were added. The solution is stirred and left to stand for 8 min, at room temperature in the dark. Then 300 µL of Na2CO3 solution are added. The solution is then stirred in the dark for 2h at room temperature. At the end of 2h the eppendorphols are all centrifuged at 25 ° C at 11000 rpm for 2 min. finally, 200 µL of solution are loaded into a transparent GREINER plate and the TECAN reading is started at λ 765 nm.

2.2.3.1.7 DES characterization

The DES selected as optimal (ChCl: lactic acid 1: 2 (mol/mol) was characterized by IR spectroscopy. In order to prove the effective formation of the eutectic mixture, the IR spectra of the individual HBAs and HBDs were acquired, and DES system format. All analyzes

were conducted at room temperature with FTIR Nicolet 8700. The spectrum was acquired in the range from 400 to 4000 cm-1 (64 scans per acquisition, resolution 4 cm-1).

4.2.3.1.8 Statistical analysis

All statistical analysis where conducted using Stata 14 statistical software (StataCorp LLC, College Station, Tx). One-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test, was selected to evaluate differences between the samples. Probability values of $p \leq 0.05$ were considered statistically significant. All the analysis were performed in triplicate.

## 2.2.3.2 Results and discussion

2.2.3.2.1 Qualitative analysis of the phenolic compounds contained in the hazelnut skin by HPLC/DAD-ESI/MS

The chemical characterization of the phenolic compounds extracted from the hazelnut skin was carried out using the HPLC/DAD-ESI/MS system considering the experimental conditions reported in the materials and methods section. In order to attempt to identify the analytes, the retention time and the UV and MS spectrum were taken into account, through the use of standard compounds and data available in the literature. The chromatograms were

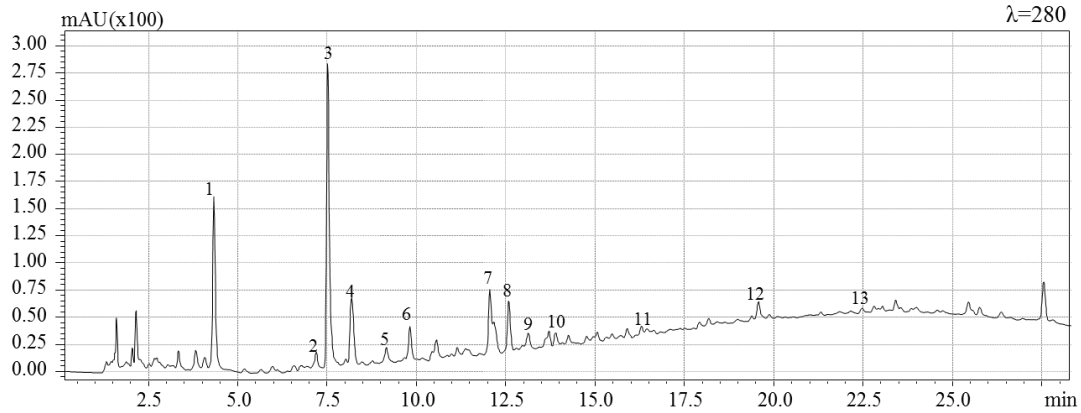



Figure 12: Chromatogram of phenolic compounds in hazelnut skin acquired at 280 nm.

acquired at the wavelengths of maximum absorption of the principal compounds present in the hazelnut skin, according to the literature, being λ=280 and λ=360.

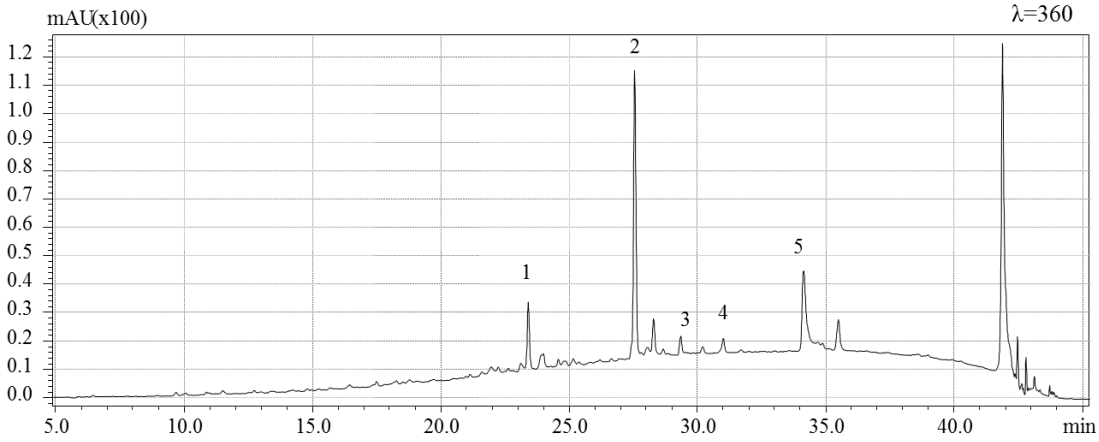



Figure 13: Chromatogram of phenolic compounds in hazelnut skin aquired at 360 nm.

Chromatograms are reported in figure 12 and figure 13.

Considering phenolic compounds detected at λ=280, a total of 13 phenolic compounds, reported in table 14, being eleven flavan-3-ols and two phenolic acids were identified basing on the mass-to-charge ratio (m/z) of the molecular ion and data available in literature.

Among flavan-3-oli (+)-catechin and (-)- epicatechin, having the same mass-to-charge ratio (m/z), were identified using the standard epicatechin molecule available and comparing the retention times. These compounds were previously identified in hazelnut skin (Del Rio D., et al., 2011).

Epicatechin 3-O-gallate, has been detected with [M-H]- at m/z 441, confirming the results obtained by Del Rio et al. (Del Rio D., et al., 2011). Among proanthocyanidins, one B-type dimer of procyanidins (PCs) was identified, presenting a [M-H]- at m/z 577. Three procyanidins trimers were detected and one was tentatively identified as procyanidin C2 on the basis of its elution time preceding procyanidin B1. One procyanidin gallate trimer with $[M-H]^-$ at m/z 729 and also three isomers of B-type PCs dimers were identified on the basis of their $[M-H]^-$ at m/z 593. Protocatechuic acid ([M-H]- =153) and gallic acid ([M-H]- =169)

were also identified. These two hydroxybenzoic acids were previously identified hazelnut skins (Del Rio D., et al., 2011).

| N | Identified compounds | Retention time (min) | m/z $(M-H)^-$ |
|---|---|---|---|
| 1 | Gallic acid | 4.43 | 169 |
| 2 | Protocatechuic acid | 7.32 | 153 |
| 3 | Procyanidin C2 trimer | 7.55 | 865 |
| 4 | Prodelphinidin beta type dimer | 8.19 | 593 |
| 5 | Prodelphinidin beta type dimer | 9.05 | 593 |
| 6 | Prodelphinidin beta type dimer | 9.59 | 593 |
| 7 | Procyanidin beta 1 dimer | 11.96 | 577 |
| 8 | (+) Catechin | 12.41 | 289 |
| 9 | Procyanidin beta type trimer | 13.03 | 865 |
| 10 | Procyanidin beta type trimer | 13.83 | 865 |
| 11 | (-) epicatechin | 16.20 | 289 |
| 12 | Procyanidin Beta type dimer gallate | 19.55 | 729 |
| 13 | Epicatechin 3-o-gallate | 22.15 | 441 |

Table 14: Abbreviation, components and molar ratio of components for each tested DESs (µg=280)

Considering phenolic compounds detected at λ=360 (Table 15), three flavonol-rhamnoside compounds were identified being myricetin rhamnoside ([M-H]- at 463 m/z), quercetin-3-o-rhamnoside ([M-H]- at 447 m/z) and kaempferol rhamnoside ([M-H]- at 431 m/z). The same compounds were previously detected in hazelnut skin (Del Rio D., et al., 2011). Quercetin [M-H]- at 301 m/z), was also detected by comparison with analytical standard. This aglycon has previously been identified in hazelnut skin (Del Rio D., et al., 2011). Finally, phloretin 2-O-glucoside, belonging to the dihydrochalcone subclass, was also identified.

| N | Identified compounds | Retention time (min) | m/z $(M-H)^-$ |
|---|---|---|---|
| 1 | myricetin rhamnoside | 23.40 | 463 |
| 2 | Quercitin 3-0-rhamnoside | 27.52 | 447 |
| 3 | Phloretin 2-o-glucoside | 29.30 | 435 |
| 4 | kaempferol rhamnoside | 30.96 | 431 |
| 5 | Quercetin | 34.16 | 301 |

Table 15: Abbreviation, components and molar ratio of components for each tested DESs (µg=360)

### 2.2.3.2.2 Screenin of DESs

Table 16: Abbreviation, components and molar ratio of comonents of each tested DES

| Abbreviation | Components | | Molar ratio |
|---|---|---|---|
| | Component 1 | Component 2 | |
| DES1 | Choline chloride | Urea | 1:2 |
| DES2 | Choline chloride | Lactic acid | 1:2 |
| DES3 | Choline chloride | Malic acid | 1:2 |
| DES4 | Choline chloride | Glucose | 2:1 |
| DES5 | Choline chloride | Xylitol | 1:7 |
| DES6 | Choline chloride | Glycerol | 1:2 |
| DES7 | Choline chloride | 1,6-Hexanediol | 1:7 |
| DES8 | Choline chloride | Triethylene glycol | 1:2 |
| DES9 | Choline chloride | Ethylene glycol | 1:2 |
| DES10 | Choline chloride | Propylene Glycol | 1:2 |
| DES11 | Betaine | Lactic acid | 1:2 |
| DES12 | Betaine | Malic acid | 1:2 |
| DES13 | Betaine | Glycerol | 1:2 |
| DES14 | Betaine | Ethylene glycol | 1:2 |
| DES15 | Betaine | Triethylene glycol | 1:2 |
| Control | EtOH (50:50 v/v) | | 1:2 |

The method developed in this study, which involves the use of deep eutectic solvents for the extraction of bioactive compounds from hazelnut skin, has been optimized for some parameters that determine its extraction efficiency, such as the choice of the ideal DES between a large number of deep eutectic solvents tested; water content (%) to add to the selected DES; the extraction time; the extraction temperature and the matrix/solvent ratio (w/v). The different components of the DESs have a significant influence on the physicochemical properties of the solvent itself, such as polarity, viscosity and solubilization capacity which influence the extraction efficiency of the target compounds.

With the aim of selecting the most suitable DES for the extraction of phenolic compounds from the hazelnut skin, seventeen DESs were tested and evaluated in this study. Ten of tested DESs had choline chloride as HBA, while seven had betaine, combined with different hydrogen bond donor compounds (urea, sugars, organic acids, polyalcohols) (Table 16).

Differences in the content of extracted phenolic compounds were not statistically significant but small differences in quantity were observed.

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The lowest amount of g of GAE is obtained using DES5 (7.144 % (g GAE/g)) and DES13 (7.854 % (g GAE/g)), while the highest one is reached by using DES1, DES2, DES8 which resulted in 13.983 %.These three DESs extracted a larger amount even than the Control ie the conventional extraction technique. Results are reported in figure 14.

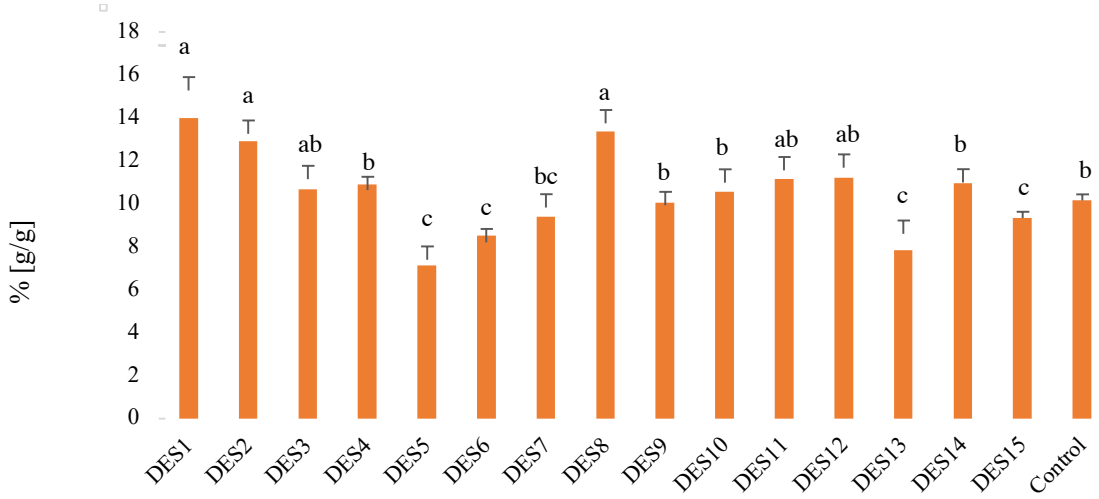



Figure 14: percentage concentration g/g of phenolic compounds extracted with tested DESs from hazelnut skin

DESs with a carboxyl groups extract a large amount of polyphenols compared to sugars based ones , this is probably due to the different strength of the interaction between the carboxyl group of the acid-based DESs with respect the carboxylic functionalities of the sugar-based DESs. The extraction efficiency of the alcohol-based DESs decreases with the length of the aliphatic-chain and probably for this way DES9 extracts better than the others. Considering the three DESs that guaranteed the best extraction in terms of phenolic compounds extracted, DES 1 was discarded as it gave repeatable results. DES2 was selected for the later steps of the method as its components were both natural and could be termed NADES, compared to DES8.

2.2.3.2.3 Characterization of the selected DES

To confirm the complete formation of DES-3, the variations in frequency, bandwidth and absorbance values of the same chemical bond in the FT-IR spectrum of DES and the corresponding individual components were evaluated (Figure 15). Briefly, the differences and the bandwidth between the spectra of the DES and the spectra of the individual corresponding components HBA and HBD were observed, indicating the formation of the DES itself.

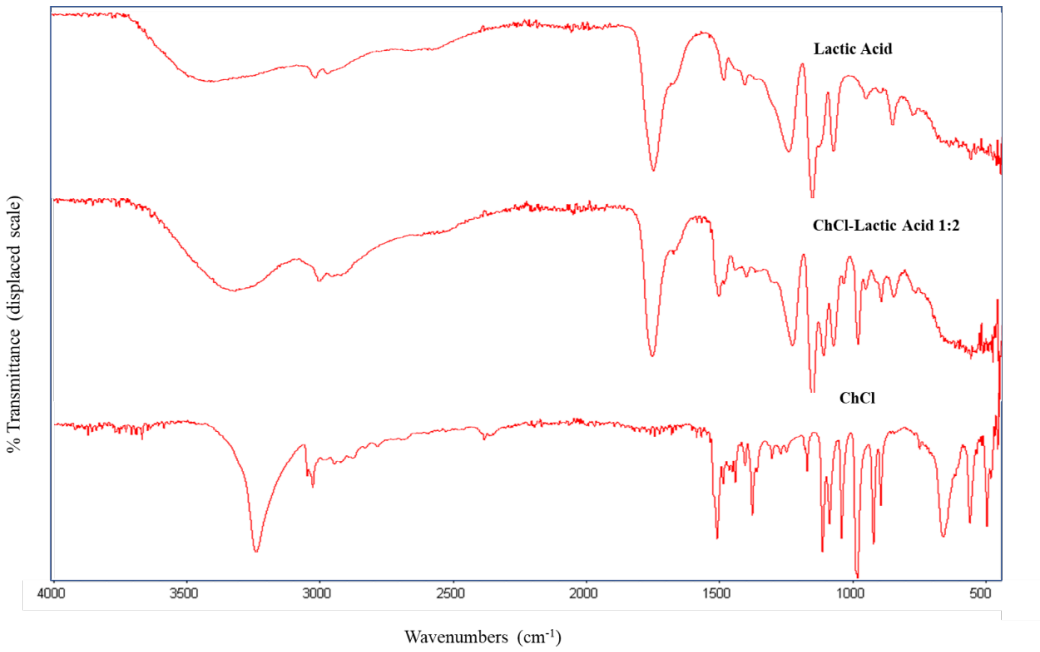



Figure 15: IR spectrum of the single components and of the DES as a system, obtained with FTIR

2.3.3.2.4 Evaluation of the quantity of water to add to selected DES

DES2 was prepared by adding 0%, 10%, 20%, and 30% water. It is well known that the addition of water to the DES allows a reduction in viscosity and an increase in the extraction capacity of the solvent. However, an excessive percentage of added water can cause a decrease in the solvent extraction efficiency, due to a weakening of the interaction between the components of the DES itself.

DES without addition of water, DES with addition of 10% of water and DES with addition of 20% of water extracted a quantity of comparable phenolic compounds. The extraction capacity of DES added with 30% of water was lower (Figure 16).

Since it is not necessary to add water to DES, it was decided to proceed for the next phases of optimization using DES without adding water.

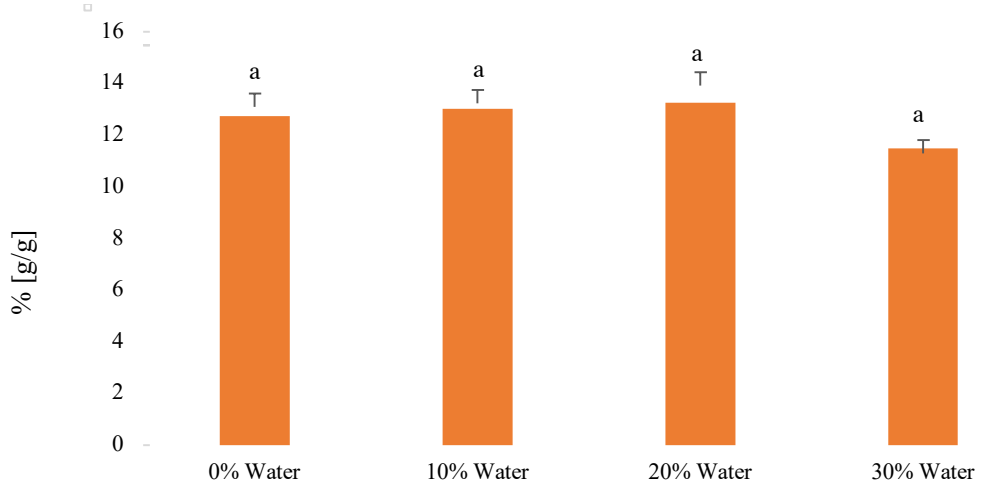
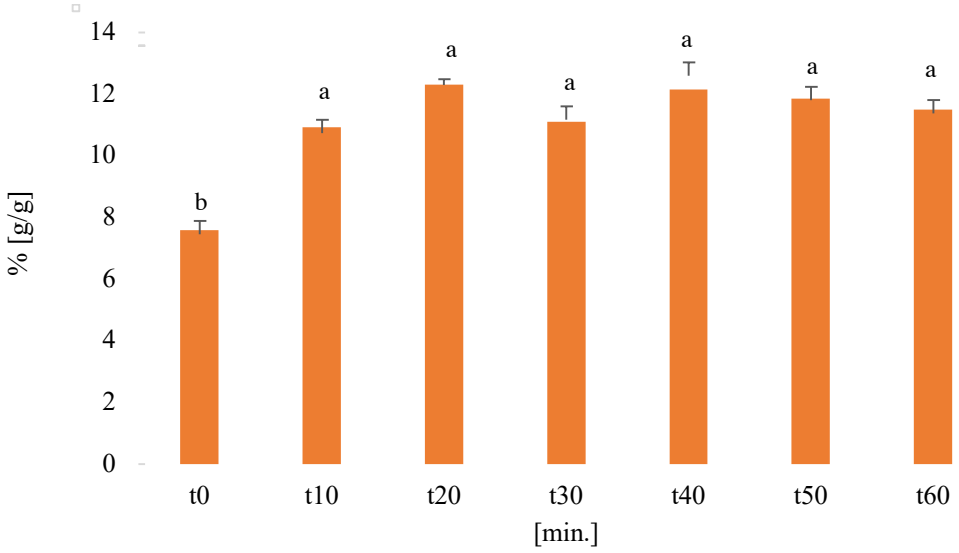



Figure 16: Phenolic content extracted using DES2, added of (0,10,20,30)% of water

2.3.3.2.5 Evaluation of time of the extraction to obtaine best extraction efficiency

To optimize the method, the ideal extraction time for conducting subsequent experiments was also investigated. The extraction of polyphenols from the hazelnut skin was carried out with DES2 at different times, such as 0, 10, 20, 30, 40, 50, and 60 min the other parameters being set at their values optimal. Results are shown in figure 17. It's possible to see that after 30 minutes there is no longer a significant increase in the extraction yield, but a sort of plateau is reached. In this regard, it was appropriate to consider 30 minutes as the extraction time.

Figure 17: total phenolic content extracted at different points of time




2.3.3.2.4 Optimization of the quantity of matrix/volume of solvent ratio

The extraction of phenolic compounds was then carried out with different matrix/solvent ratios to evaluate which could be the best for an optimal yield with DES2. The optimal condition of the matrix /solvent ratio was evaluated considering 0.2; 0.5; 0.8; 1.0; 1.5 g of matrix on 5 mL of solvent. The highest concentration of phenolic compounds was obtained with 0.2 g of matrix/5 mL of solvent.

This result is probably due to the fact that low matrix/solvent ratios favor a more efficient extraction because they allow a better and continuous surface contact. On the other hand, when larger quantities of film are used, an excessively dense system is obtained in which the ultrasounds lose efficiency.

On the basis of the results obtained in the experimental phase, the optimal conditions for obtain the best extraction of phenolic compounds from hazelnut skin are: use of DES ChCl/lactic acid (1:2) as extraction solvent, without addition of water, extraction time of 30 min and quantiy of matrix/ volume of solvent ratio of 0.2 g matrix/5mL of solvent.

#### 2.3.3.3 Conclusion

Results presented in this work show the development of a green analytical method for the extraction and analysis of phenolic compounds from hazelnut skin, an abundant by-product of industrial hazelnut processing. The extraction was performed through the use of new generation green solvents known as deep eutectic solvents, and this methodology represented a sustainable and economically advantageous approach compared to the use of traditional organic solvents. In conclusion, what emerged from this study is that, the extraction of phenolic compounds with DES from the hazelnut skin, has optimal extractive yields when compared to that with conventional solvents, representing an eco-friendly alternative to the traditional using of organic solvents.

## 4.3 Choline-chloride and betaine-based deep eutectic solvents for green extraction of nutraceutical compounds from spent coffee ground

### 4.3.1 Introduction

Coffee is the second most consumed beverage in the world. Its large consumption has led to a great interest in it and it has been the subject of many studies. Coffee is characterized by the presence of phenolic compounds, specifically chlorogenic acids. caffeoylquinic acids (CQAs), dicaffeoylquinic acids (DCQAs), feruloylquinic acids (FQAs) and p-coumaroylquinic acids (couQAs), typical of green coffee and quinolactones, generated due to high roasting temperatures, are mainly found in coffee beans and consequentially in coffee beverage (Farah C.M., et al., 2006). Several beneficial properties to human health have been demonstrated for CGAs like hepatoprotective, antioxidant (Sato Y., et al., 2011), antiplatelet, anticancer and neuronal cell death protection (Janissen T., et al., 2018). Considering the large consumption of coffee it is easy to imagine the large quantity of by-products that derive from its production. Among them spent coffee grounds (SCG) are generated during the production of coffee drink, specifically with SCG refers to the residue remaining after the water extraction of coffee powder. SCG quantity produced every year is about 6 million tons (Janissen T., et al., 2018). Some studies have concerned the possible use of this waste product and it has proved useful, for example, for bioethanol production and and for biodiesel production (Campos-Vega G., et al., 2015).

Various extraction techniques are tested for the extraction of chlorogenic acids from SCG, but only a few were found to be suitable as solid-liquid extraction (SLE) (Panusa A., et al., 2013) SFE, with and without co-solvent, soxhlet extraction and ultrasound (UAE) (Andrade K.S., et al., 2012) or microwave (MAE) assisted extraction (Getachew A.T., et al., 2017). The solvents mostly used for extraction are aqueous mixtures of organic solvents, such as ethanol, methanol and isopropanol (Panusa A., et al., 2013).

With the objective to reduce toxic waste green solvents have also been tested for the extraction of chlorogenic acids from SCG and when we started testing DESs only two works had applied them for that purpose. Yoo et al. (Yoo D.E., et al., 2018) tested thirteen ChCl-based DESs combined with amines, polyalcohols, organic acids. Total phenolic content (TPC), total flavonoid content, total chlorogenic acids, and/or antioxidant activity were measured to evaluate the DESs extraction capacity. Best extraction was obtained using the DES consisting of 1,6-hexanediol:ChCl at a molar ratio of 7:1, added of 32.5% w/w of water, with an extraction temperature of 65°C. Also Krisanti, E.A. et al. tried to use DESs for the extraction of CGAs from SCG and specifically they selected a series of betaine-based DESs, prepared using betaine as HBA and diols and organic acids as HBD, and evaluated the efficiency of DES extraction basing on TPC and antioxidant activity using UV-VIS Spectrophotometry. The best extraction efficiency was obtained with betaine-1,2-butanediol (Krisanti E.A., et al., 2019).

The aim of this project was to test a serveral ChCl and Betaine-based DESs for the extraction of bioactive compounds from SCG. Also for this work the optimization of the method involved the study of the type of DES most suitable for extraction, the quantity of water to be added to DES and the ratio of solvent extractive volume/quantity of matrix. Chlorogenic acids in the extract was identified and quantificated using an HPLC-PDA-MS-ESI method.

### 4.3.2 Materials and methods

#### 4.3.2.1 Chemicals

Solvents employed for the traditional extraction procedure and for HPLC-MS analyses were methanol (MeOH) (purity 99.9%), water (HPLC-MS grade), acetonitrile (purity 99.9%), formic acid (purity 95–97%); all of them were purchased from Sigma-Aldrich (Milan, Italy). ChCl, triethylene glycol for synthesis, glycerol and urea for synthesis were obtained from Sigma-Aldrich (Milan, Italy); sorbitol and glucose were from Fisher Scientific Italia (Milan,

Italy) and lactic acid, xylitol, citric acid, ethylene glycol, propylene glycol, 1,6-hexanediol and betaine were from Carlo Erba (Milan, Italy). 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3-O-(E)-feruloylquinic acid, 4-O-(E)-feruloylquinic acid standard molecules were purchased from Sigma-Aldrich (Milan, Italy).

4.3.2.2 Samples

Coffee samples were purchased from local markets. SCG was obtained after espresso extraction. Samples were weighted in triplicate and dried to constant weight in an oven set at 110 °C.

4.3.2.3 Preparation of DESs

| Abbreviation | Components | | Molar ratio |
|---|---|---|---|
| | Component 1 | Component 2 | |
| DES1 | Choline chloride | Xylitol | 1:2 |
| DES2 | Choline chloride | Glucose | 1:2 |
| DES3 | Choline chloride | Sorbitol | 1:2 |
| DES4 | Choline chloride | Citric acid | 1:2 |
| DES5 | Choline chloride | Lactic acid | 1:2 |
| DES6 | Choline chloride | Urea | 1:2 |
| DES7 | Choline chloride | 1,6-Hexanediol | 1:7 |
| DES8 | Choline chloride | 1,6-Hexanediol | 1:2 |
| DES9 | Choline chloride | Triethylene glycol | 1:2 |
| DES10 | Choline chloride | Ethylene glycol | 1:2 |
| DES11 | Choline chloride | Propylene Glycol | 1:2 |
| DES12 | Choline chloride | Glicerol | 1:2 |
| DES13 | Betaine | Lactic acid | 1:2 |
| DES14 | Betaine | Glicerol | 1:2 |
| DES15 | Betaine | Ethylene glycol | 1:2 |
| DES16 | Betaine | Triethylene glycol | 1:2 |
| Control | $MeOH/H_2O$ (70:30 v/v) | | |

Table 15: Abbreviation, components and molar ratio of each DESs tested in the study

Betaine and ChCl based DESs were prepared weighing the established quantity of each components, considering molar ratio selected, in a tube, kept in a thermo block for 30 minutes at a temperature of 80°C. All the mixtures were vortexed and subjected to ultrasound baths (Elm sonic S30H, Elma Schmidbauer GmbH, Singen, Germany) to guarantee the DES

solubilization. For the ChCl DESs, ChCl was dried before use in a heater for 12 h at 100°C. In table 15 are reported tested DESs.

#### 4.3.2.4 FTIR DESs characterization

The IR spectra of the individual HBAs and HBDs were acquired, and DES system format. All analyzes were conducted at room temperature with FTIR Nicolet 8700. The spectrum was acquired in the range from 400 to 4000 cm-1 (64 scans per acquisition, resolution 4 cm-1).

#### 4.3.2.5 Extraction of CGAs by DESs and conventional solvent

Conventional extraction used to evaluate the extraction capacity of DESs was carried out considering an organic solvent-based extraction reported by De Luca et al. (De luca S., et al., 2018) with some modification. A quantity of 0.2 g was extracted using 3 mL of a mixture of MeOH/$H_2O$ (70:30, v/v), vortexed and centrifuged for 5 min at 9.5 x g. Extraction was carried out in a ultrasound bath (Elmasonic S30H, Elma Schmidbauer GmbH, Singen, Germany) at 65°C, frequency of 37 kHz and heating power of 200 W for 20 min.

The extraction with DESs involved the addition of 3 mL of DES to 0.2 g of SCG in a centrifuge tube that then was vortexed and centrifuged for 5 min at 9.5 x g. Extraction was carried out in the same condition of conventional extraction.

#### 4.3.2.6 Optimization of DES extraction method

First step of optimization involved the study of which of selected DESs guaranteed the best extraction. Once selected the best DES, the optimization of the extraction was carried out studying water content in DES (10, 20, 30 and 40%), added to reduce viscosity, and optimal condition of the ratio of sample amount (mg) to DES volume (mL) (1:1, 2:1, 3:1, w/v).

4.3.2.7 HPLC/DAD-ESI/MS analysis of CGAs

Shimadzu Prominence LC-20A instrument (Shimadzu, Milan, Italy) equipped with two LC-20 AD XR pumps, SIL-10ADvp, CTO-20 AC column oven and DGU-20 A3 degasser coupled to a SPD-M10Avp PDA detector and a mass spectrometer detector (LCMS-2010, Shimadzu, Tokyo, Japan) equipped with electrospray (ESI) interface was selected for the analysis. Shimadzu LCsolution Ver. 3.7 software (Shimadzu,Version 3.7) was used to acquire MS data. The separation of CGAs was carried out using a Core Shell column (150 × 4.6 mm I.D., 2.7 μm d.p.) (Merck KGaA, Darmstadt, Germany). CGAs were separated using a mobile phase composed by $H_2O$/0.1% HCOOH, as solvent A and acetonitrile as solvent B and the following gradient: 0–5 min 5% B, 5–20 min 5–15% B, 20–42 min 15–35% B, 42–47 min 35–100% B. The oven temperature was set at 40°C and the flow rate was 1 mL/min. The injection volume was 2 μL. PDA was used to register in the range 210–400 nm and the chromatograms were extracted at 325 nm for CGAs. MS-chromatograms were acquired in negative ionization mode, using the following parameters: nebulizing gas flow rate ($N_2$): 1.5 mL $min^{-1}$; event time: 1 s; mass spectral range: *m*/*z*100–800; scan speed: 1000 amu/s; detector voltage: 1.5 kV; interface temperature: 250 °C; CDL temperature: 300 °C; heat block temperature: 300 °C; interface voltage: –3.50 kV; Q-array voltage: 0.0 V; Q-array RF: 150.0 V.

4.3.2.8 HPLC-PDA quantitative analysis method validation

The analytical method was validated considering seven standard molecules. Linearity range, repeatability (intra-day and inter-day precision), limit of detection (LOD), and limit of quantification (LOQ) were evaluated.

Stock standard solutions were prepared at a concentration of 10,000 mg $L^{-1}$ in $H_2O$ for all compounds. Linear range was determined by using the determination coefficients ($R^2$) obtained. Intra-day precision was calculated by injecting five standard solutions (N = 5) on

the same day. Inter-day precision was obtained by injecting five standard solutions for successive days (N = 10).

#### 4.3.2.9 Statistical analysis

All statistical analysis where conducted using Stata 14 statistical software (StataCorp LLC, College Station, Tx). One-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test, was selected to evaluate differences between the samples. Probability values of $p \leq 0.05$ were considered statistically significant. All the analysis were performed in triplicate.

### 4.3.3 **Results and Discussion**

#### 4.3.3.1 HPLC-PDA/MS qualitative and quantitative analysis of CGAs in SCG

Quali-quantitative analysis of CGAs in SCG were carried out by HPLC-PDA/ESI-MS considering experimental conditions reported in materials and methods section. The identification of chlorogenic acids was done, taken in account the retention time and UV and MS spectra, use of standard compounds and data available in literature. Fifteen CGAs were detected and tentatively identified. CQAs (3-CQA, 4-CQA, 5-CQA) and DCQAs (3,4-DCQA, 3,5-DCQA, 4,5-DCQA), two isomers of caffeoylepi-quinic acid, 5 couCQA, two isomers of caffeoylferuloylquinic acid and quinolactone were identified and quantified. Figure 18 shows a HPLC-PDA chromatogram ($\lambda$=325 nm) of the SCG extract obtained with organic solvent.

CGAs were quantified using HPLC-PDA data through calibration curve with external standard of seven CGAs being 3-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 3-O-(E)-feruloylquinic acid and 4-O-(E)-feruloylquinic acid.

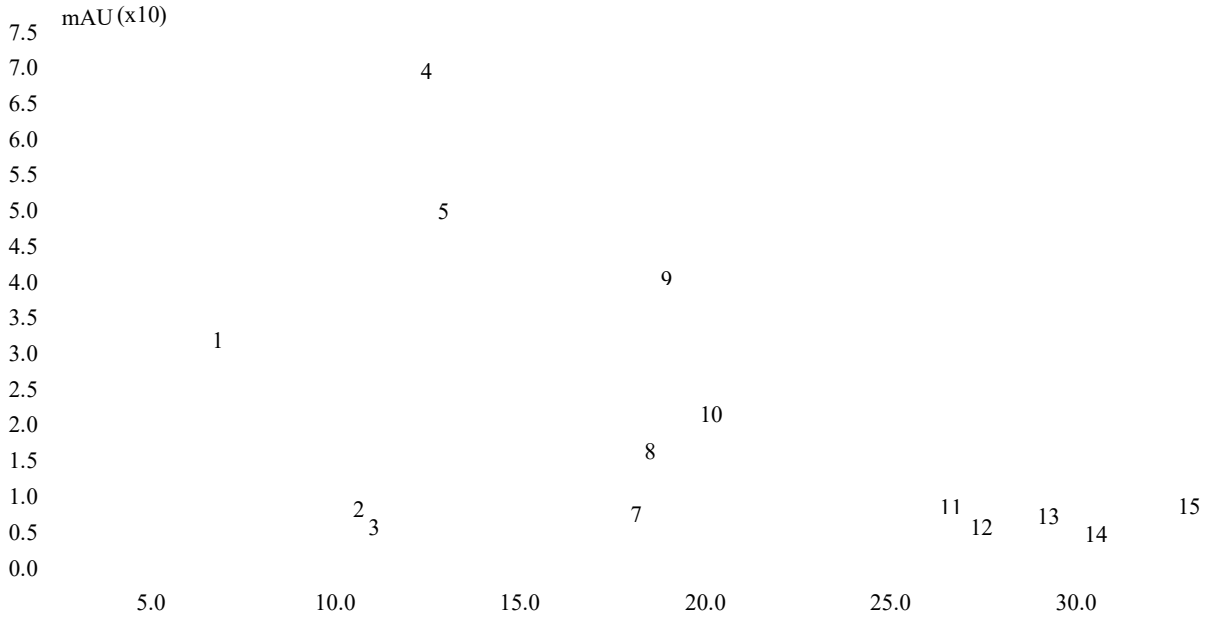



Figure 18: HPLC-UV/Vis chromatogram of CGAs in SCG extract with a mixture of MeOH/H2O (70:30, v/v) at 325 nm. Peaks correspond to (1) 3-O-caffeoylquinic acid, (2) caffeoylepi-quinic acid, (3) caffeoylepi-quinic acid, (4) 5-O-caffeoylquinic acid, (5) 4-O-caffeoylquinic acid, (6) 5-p-coumaroylquinic acid, (7) quinolactone, (8) 4-feruloylquinic acid, (9) 3-feruloylquinic acid, (10) quinolactone, (11) 3,4-dicaffeoylquinic acid, (12) 3,5-dicaffeoylquinic acid, (13) 4,5-dicaffeoylquinic acid, (14) caffeoylferuloylquinic acid, (15) caffeoylferuloylquinic acid.

Each compound was quantified using the calibration curve obtained with the corresponding standard molecule, if available. The compounds for which we didn't have the corresponding standard molecule were quantified using a standard molecule with similar structure, i.e. 5-p-coumaroylquinic acid, quinolactone were quantified with the calibration curve of 4-O-caffeoylquinic acid standard molecule. Caffeoylferuloylquinic acid were measured with the calibration curve of 3-feruloylquinic acid standard molecule. 4,5-dicaffeoylquinic acid were quantified with the calibration curve of 3,4-dicaffeoylquinic acid standard molecule.

Among detected CGAs in extracts of SCGs, CQAs (3-CQA, 4-CQA, 5-CQA) were the most abundant CGAs in accordance to literature data (Gabriele F., et al., 2019).

| Compounds | Linearity | LOD (ng/mL) | LOQ (ng/mL) | Run-to-Run Precision (RSD% n=5) | | Day-to-Day Precision (RSD% n=10) | |
|---|---|---|---|---|---|---|---|
| | $R^2$ | | | tr | Peak Area | tr | Peak Area |
| 3-O-caffeoylquinic acid | 0.997 | 0. 1 | 0.1 | 0.63 | 7.47 | 0.58 | 6.56 |
| 5-O-caffeoylquinic acid | 0.995 | 0.08 | 0.1 | 0.27 | 4.44 | 0.36 | 7.07 |
| 4-O-caffeoylquinic acid | 0.994 | 0.06 | 0.1 | 0.24 | 7.47 | 0.32 | 8.80 |
| 4-feruloylquinic acid | 0.994 | 0.04 | 0.1 | 0.17 | 7.55 | 0.28 | 8.36 |
| 3-feruloylquinic acid | 0.993 | 0.04 | 0.1 | 0.14 | 6.22 | 0.25 | 6.00 |
| 1,4-dicaffeoylquinic acid | 0.995 | 0.08 | 0.1 | 0.09 | 7.14 | 0.18 | 6.65 |
| 3,5-dicaffeoylquinic acid | 0.994 | 0.08 | 0.1 | 0.08 | 8.51 | 0.17 | 8.42 |

Table 16: results of validation method

Method was validated as previously reported and good results, reported in table 16, were obtained.

4.3.3.2 Optimization of the extraction with DESs of CGAs form SCG

Eleven ChCl-based DESs and four betaine-DESs were tested to evaluate the extraction capacity for CGAs from SCG. First of all we decide to confirm the formation of DESs evaluating frequency shifts, band widths and absorbance values of the same chemical bond in FT-IR spectra of DES and of the corresponding single components. Differences and band broadening among the spectra of the DES and the spectra of their single correspondent HBA and HBD were identified to prove the formation of DES. In compliance with other literature data ChCl spectrum shown OH stretching at 3210 $cm^{-1}$. The absorption bands between 2800−3000 $cm^{-1}$ are related to the $CH_3$ and $CH_2$ stretching, while the C–H scissor and bending are at 1450−1290 $cm^{-1}$. In the fingerprint region are visible the stretching of alcoholic C O at 1270 $cm^{-1}$, COC and COH bending, and the ammonium CN stretching in

the range 1100−950 $cm^{-1}$(Gabriele et al., 2019). The characteristic peaks for betaine are observed at around 1400 cm–1(CN stretching) and 1323 cm–1(C OO stretching), while the peak at 1625cm−1 corresponds to the asymmetric stretching of the carboxylate group. It was noticed in each betaine based DESs, the same shift of the carboxylate group towards greater wave number due to the formation of a H-bond between betaine and the HBD (Li T., et al., 2015; Zhao Z., et al., 2018).

As previously reported, the high viscosity of DESs can cause problem during the extraction, so to evaluate the extraction capacity of all DESs a percentage of 30% (w/w) of water was added to all DESs. The extraction was carried out for 20 min with a temperature of 65°C, which further reduced viscosity. Total CGAs concentration extracted ranged from 0.67 to 4.64 mg $g^{-1}$ of dry weight SCGs. Our results were in accordance with that reported in literature for which total CGAs concentration ranged from 1.39 to 19.8 mg $g^{-1}$ (Pena-Pereira F., et al., 2014; Li T., et al., 2015; Panusa A., et al., 2013).

In figure 19 are reported CGAs total concentration extracted using selected DESs.

Among all the DESs tested the one that gave the worst extraction was ChCl-xylitol, while the one that guaranteed the best extraction was Betaine-triethylene glycol.

Considering only ChCl-based DESs the organic acid-based DESs, two different acids (lactic and citric) were used showing a higher extraction capacity of lactic acid with a significant difference, polyalcohol-based DESs showed some significant differences for extraction of CGAs and specifically the lowest extraction yields was obtained for sugar alcohol-based DESs (xylitol and sorbitol), while a good even if not the highest extraction efficiency was observed for the others. ChCl-glucose showed a low extraction efficiency while ChCl-urea efficiency was not statistically different from that one of the alcohols-based DESs.

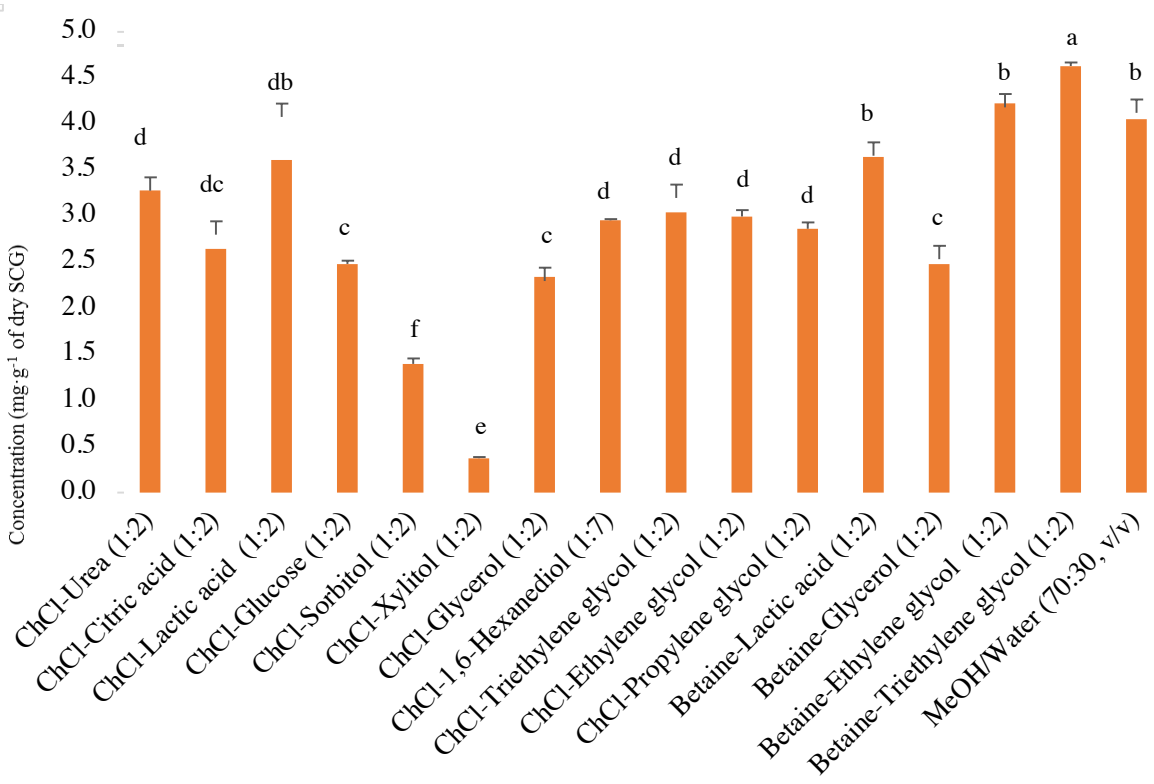



Figura 19: total concentracions of CGAs extracted from SCG with selected DESs

A similar result has been reported by Yoo et al. who, showed that the sugar alcohol and sugar HBDs yielded significantly less than did the amines, organic acids and polyalcohols (Yoo D.E., et al., 2018).

Betaine-based DESs showed higher extraction efficiency than ChCl-based DESs excepted for betaine-glycerol solvent. It has been reported that betaine, a trimethyl glycine, respect to ChCl is a zwitterionic amphiphilic surfactant being pH-sensitive with a pKa around 4.5. Its functionality can be reversibly protonated upon reducing the pH. This chemical characteristic allows it to form micelle-like assemblies through a self-assemble; act as hydrophilic and hydrophobic nanocontainers; be subjected to surface charge and size variations in dependence of pH (Zhao Z., et al., 2018) and these characteristics can be responsible for the observed higher extraction yields of betaine-based DESs respect to Ch-Cl-based ones.

Betaine-triethylene glycol DES guaranteed the best extraction in terms of total chlorogenic acids extracted, even greater than the conventional technique and the differences were statistically significant, so it was selected for the next step optimization.

For the evaluation of the best quantity to add to the selected DES four percentages of water were test being 10, 20, 30, 40 % (v/v). Results showed an increasing of extraction capacity of DES with the increase of water percentage and a decreasing when 40% of water was added probably due to the fact that higher water percentages can break the hydrogen bond framework of the DES, as previously reported in literature and through our project about the extraction of phenolic acids from EVOOS

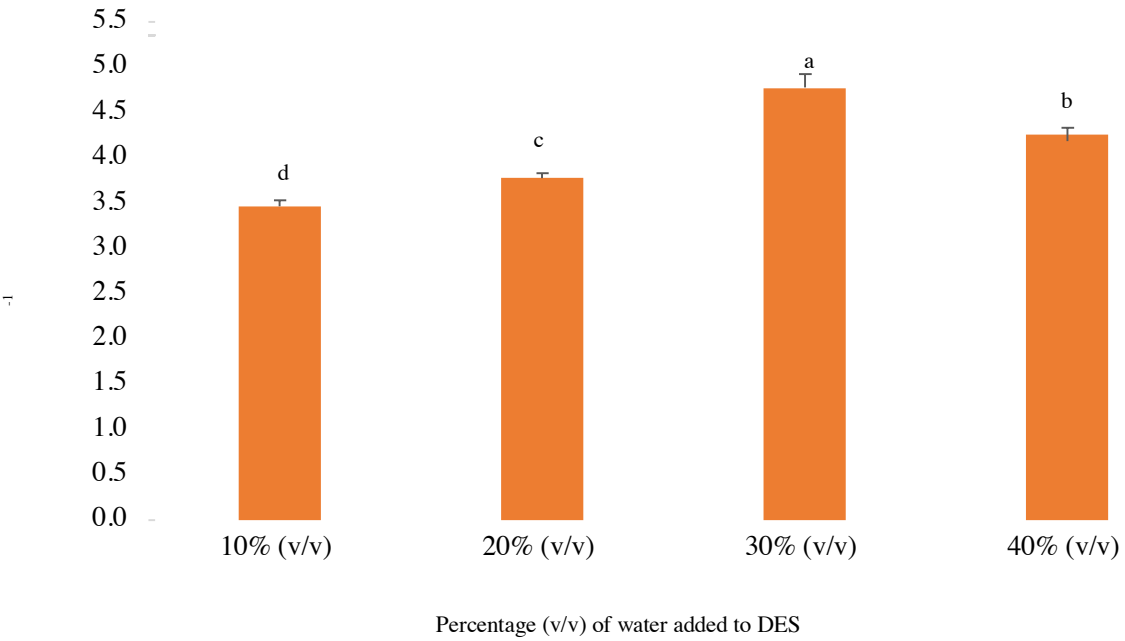



Figure 10: total concentration of phenolic compounds extracted using DES 6 added of 10, 20, 30, 40 % (v/v)

(Ala˜nón et al., 2020; Fanali et al., 2020). The highest and repeatable extraction efficiency was obtained with DES with 30% water (Figure 20).

Final step of the optimization involved the study of solvent ratio optimal condition considering (a) 1:1, (b) 1:15 and (c) 1:30 (w/v) ratio. 1:45 sample to solvent ratio conditions

gave the best results in term of extracted chlorogenic acids and differences with the other two ones were statistically significant (Figure 21).

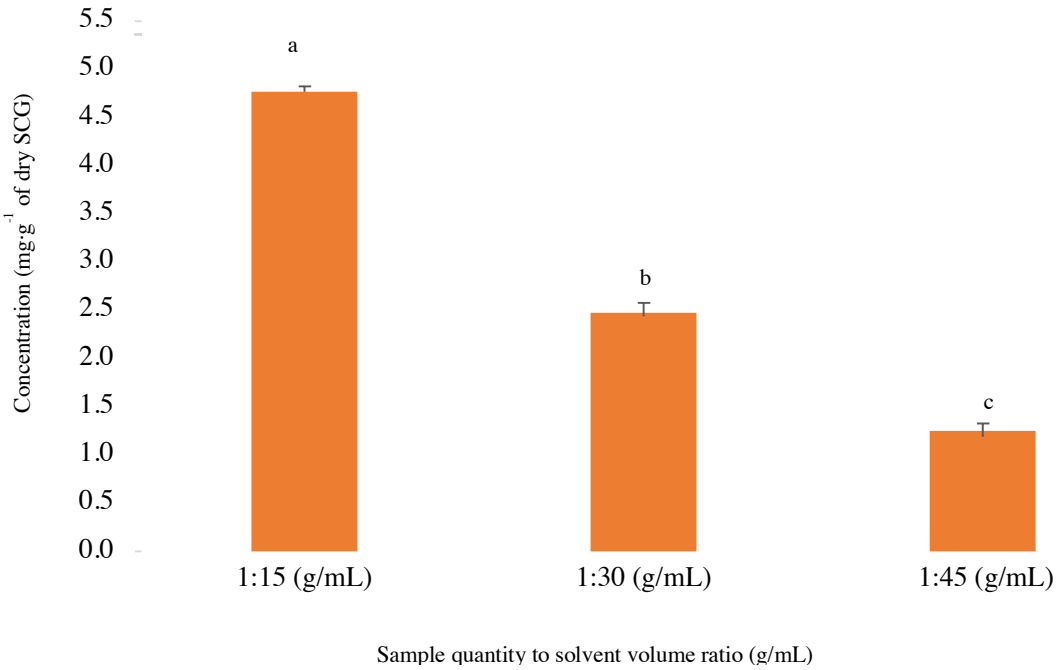



Figure 21: Results about optimization of solvent ratio optimal condition considering (a) 1:1, (b) 1:15 and (c) 1:30 (w/v) ratio

### 4.3.4 Conclusions

A green technique for the extraction of phenolic compounds from a waste product of the coffee beverages has been developed and proved to be valid and better than the conventional extraction with organic solvents. A HPLC-PDA/ESI-MS method was used for the analysis of the CGAs in the extracts allowing their identification through MS and their quantification. Betaine-triethylene glycol (1:2 molar ratio) guaranteed the best results in terms of chlorogenic acids extracted from the matrices and was selected for the further optimization steps. Extraction with betaine-triethylene glycol (1:2 molar ratio), added of 30% of water and considering sample quantity to solvent volume ratio of 1:15 (w/v), represented the best conditions. Results obtained in this work have been published on "Journal of Pharmaceutical and Biomedical Analysis" (Fanali C., et al., 2020).

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