UNIVERSITÀ DEGLI STUDI DI SALERNO

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Bioactives from agricultural food by-products: application in food and health

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To my sister

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ABSTRACT

Agriculture and agri-food industry produce a large amount of residues in non-edible portions from cultivation and processing of crops. These wastes, considered by-products, give serious environment damage, if not properly disposed, also because of their seasonal production. Until a few years ago the only goal was the disposal of these by-products. Recently the researchers discovered the characteristics of these wastes and their possible exploitation as sources of interesting compounds and energy for pharmaceutical, cosmetics and nutraceutical sectors. Fruit and vegetable by-products, in fact, are rich in bioactive compounds, such as carotenoids, dietary fibers, minerals and phenolic compounds, with correlated beneficial health effects.

In this context the activity of the PhD project evaluated agricultural by-products as sources of bioactive compounds.

The aims of the present PhD project were the development of **innovative and selective analytical methodologies,** for determination of bioactive compounds, using liquid chromatography methods (UHPLC) coupled to UV-Vis spectroscopy and mass spectrometry (high resolution, HRMS and tandem mass, MS/MS), the improvement of **new extraction techniques,** which can optimize extraction yields, minimize costs and environmental impact, such as pressurized hot water extraction (PHWE), ultrasound assisted extraction (UAE) and supercritical antisolvent fractionation (SAF), and finally **the study of antioxidant activity** of obtained extracts, using chemical and biological assays (DPPH, ABTS, ORAC, CAA).

The analysis of bioactive compounds in plant extracts through advanced sample preparation techniques and modern separation tools allows a comprehensive study of the matrix. The project, very relevant, was to provide innovative methodologies, procedures and final products for different companies of healthcare area, in terms of innovation, costs and profits.

The project was divided into three major areas, corresponding to the three selected by-products. The determination of chemical profile and the development and optimization of innovative and green extraction procedure for recovery of bioactive compounds have been carried out for each matrix. Selected by-products were: the main **artichoke by-products**, bracts and leaves, derived from cultivation and industrial processing, **hazelnut by-products**, roasted skins, derived from kernel industrial processing, and **aromatic plants by-products**, distillation waste waters, derived from essential oils production.

All by-products studied have proved to be low cost sources of bioactive compounds with antioxidant activity. Furthermore, among innovative extraction techniques, PHWE procedures have allowed obtaining final extracts selectively rich in compounds of interest without formation of artefacts, and compared to existing extraction methods were simple, fast, environmentally friendly and fully automated. Moreover my PhD project provided new analytical methods to standardize vegetable extracts. The most relevant results showed that discarded raw materials are suitable ingredients for the production of formulated health products and nutraceuticals.

INTRODUCTION

The following section is a summary of the PhD thesis. It includes:

1) a brief introduction about agricultural by-products,
2) aim,
3) selected matrices,
4) general conclusions.

1) Introduction

Nowadays there is a modern vision of agriculture as an activity that can not only satisfy nutritional needs, but also supports other sectors such as nutraceutical, cosmetic and pharmaceutical. This perspective is based on the large amount of nutrients resulting from the primary metabolism of plants, as well as the variable presence of secondary metabolites. Secondary metabolites are bioactive compounds that through certain mechanisms are able to provide benefits to human organism, for therapeutic purposes. However pharmaceutical industry rarely makes use of the whole plant, but through special extraction techniques extracts required components, which are used as isolated ingredients in drugs. In addition, food industry selects only the edible portion of plant, promoting the formation of a large amount of wastes and organic by-products. Agriculture follows the market needs and produces only what can be sold. In fact, residues left in the fields and derived from industrial processing must be necessarily disposed. By-products are currently used as potential raw materials for re-use. In this perspective "waste" of a process becomes "resource" for another.

A large amount of by-products are generated by food industry at every stage of the production processes. Generally, agricultural by-products are valorised as animal feedstuff, in fuel and fiber production. In the last years, some vegetables by-products have been valorised as a source of bioactive

phytochemicals and several studies have focused on the recovery of bioactives from agricultural and food waste and their application in food, functional foods, dietary supplements and cosmetics.

2) Aim

The activity of the PhD project focused on the exploitation of agricultural by-products as sources of naturally bioactive compounds with health promoting, disease preventing, or medicinal properties. The major aims are the development and the evaluation of the applicability of agricultural waste derived products as components of foods, functional foods, dietary supplements and herbal products. The general aims of the present work were:

a) **To develop innovative and selective analytical methodologies** for the quali-quantitative characterization of bioactive compounds from selected by-products using liquid chromatography methods (UHPLC) coupled to UV-Vis spectroscopy and mass spectrometry (high resolution, HRMS and tandem mass, MS/MS). The identification of bioactive compounds is preliminary action to be carried out before extraction and purification processes, to be able to understand the nature and the complexity of the matrix and its constituents. The knowledge of the quantitative profile is however necessary to check the real added value of by-products and to understand their potential applicability. Moreover, chemical, enzymatic and spectrophotometric methods were applied in order to obtain a complete and comprehensive characterization of by-products.

b) **To develop new extraction procedures** in order to optimize extraction yields, to minimize costs and environmental impact and to obtain enriched extracts that add value to by-products. This goal was achieved using non-conventional extraction techniques as pressurized hot water extraction

(PHWE), ultrasound assisted extraction (UAE) and supercritical antisolvent fractionation (SAF). The recovery of bioactive compounds was designed with the aim to enrich the fraction of interest and to eliminate or to reduce inert component (lipids, polysaccharides, pigments, proteins) that could make problematic the subsequent handling of the obtained derivative products and reduce the enrichment factor.

c) **Assessment of the potential usefulness** of the products obtained, after appropriate modifications (enriched extracts, partially purified fractions and isolated components) to supply methodologies and procedures for industrial applications.
d) Finally **to study the antioxidant activity** of obtained extracts, using chemical and biological assays (DPPH, ABTS, ORAC, CAA).

3) Selected matrices

The following matrices, supplied by local companies, have been selected and studied: the main **artichoke by-products**, bracts and leaves, derived from cultivation and industrial processing, **hazelnut by-products**, roasted skins, derived from kernel industrial processing, and **aromatic plants by-products**, distillation waste waters, derived from essential oils production, taking into account the percentage of waste, the needs and problems of the territory and the richness in bioactive compounds level after a literature search. Results, discussion, materials and methods are reported in experimental part, divided in three main chapters: chapter III, artichoke by-products divided in Section A and Section B, chapter IV, hazelnut by-products divided in Section A and Section B, chapter V, aromatic plant by-products divided in Section A and Section B.

4) General conclusions

All by-products studied have proved to be low cost sources of bioactive compounds, mainly belonging to polyphenols natural products class. Among the sample preparation techniques, PHWE employed has allowed obtaining final extracts selectively rich in compounds of interest in a faster and economically friendly manner, without formation of artefacts, respecting the originality of vegetable matrix, while using SAF enriched and purified fractions were obtained. The antioxidant activity of exhaustive extracts and PHWE extracts were evaluated to assess future applications by chemical and biological assays (DPPH, ABTS, ORAC and CAA). In conclusion the project results provided three new characterized, optimized and standardized extracts. The above raw materials are suitable ingredients for the production of formulated health products and nutraceuticals.

CHAPTER I

AGRICULTURAL RESIDUES AS A SOURCE OF BIOACTIVE NATURAL PRODUCTS

1.1. Introduction

Agriculture and agri-food industry produce a large amount of residues in non-edible portions from cultivation and processing of crops and by-products; these wastes give serious environment damage, if not properly disposed, also because of their seasonal production. There are a lot of methods to correctly dispose or exploit different type of residues. Until a few years ago the only goal was the disposal of these by-products, but now the researchers found the characteristics of these wastes and their possible exploitation as sources of interesting compounds and energy for pharmaceutical, cosmetics and food industries (Peralbo-Molina and Luque de Castro, 2013). Food waste is generated in big quantities worldwide. It is estimated that are produced billions of metric tons of biomass each year in liquid, solid and gaseous form and they constitute perhaps the most renewable and cheap source in the world. This context has attracted the interest of researchers in order to develop sustainable processes. Agricultural by-products can be used as raw materials, directly or converting them using physical, chemical and biochemical processes or fractionating them in order to produce energy, fuels, materials and high-value products, to exert in most cases their biological activities (antioxidant and pharmacological properties). According to Waste Framework Directive (European Union 2006): *waste* is a material which the holder discards or indented or is required to discard; *production residue* is a material that is not deliberately produced in a production process that may or not may a waste; *by-product* is a production residue that can be used directly without any further process other than normal industrial practice, whereas unprocessed and unadulterated material, which has been separated from the crop could be considered a *co-products.*

Food industry processing is associated with transformation of raw material into a food product. Now there is the intention to develop processes with food

production, minimization of water and energy consumption and valorisation of by-product segments.

1.2. Agricultural residues

There are two important groups of agricultural residues: crop residues, derived from farm practices and agro-industrial residues (post-harvest process). Non-edible plant parts are left in the field after harvest of main crop, as branches, roots, haulms, leaves, stalks, stubble, straw, twigs and sticks (primary biomass). Agro-industrial residues derived from food processing industries, take the form of hulls, husks, peels, pomace (secondary biomass) (Mande, 2005). There is not statistical information about quantities of residues; it's only possible to estimate their amount from the product yield and residue to crop ratios (Chen et al., 2009).

1.3. Valorisation processes

Agricultural residues represent a huge amount of biomass inducing strong impact on environment and greenhouse effect (Galanakis, 2012). Combustion is usually the only practice in the farms but removing all crop residues could provoke soil deterioration and declining yields. So a little amount of these residues must return in farm in order to preserve soil quality avoiding erodible lands (Lal, 2005). Nowadays, physical, chemical, biological and enzymatic pre-treatments are performed. In fact, large quantities of residues, in particular lignocellulosic materials, are used for bio-energy production. At the same time, agricultural wastes are employed for animal feed production, compost and wood-based panels, bio-fertilizers, bio-fibers. Furthermore, bioactive natural products can be obtained by biological and chemical processes. Flow diagrams (Figure 1) reports agricultural biomass conversions.

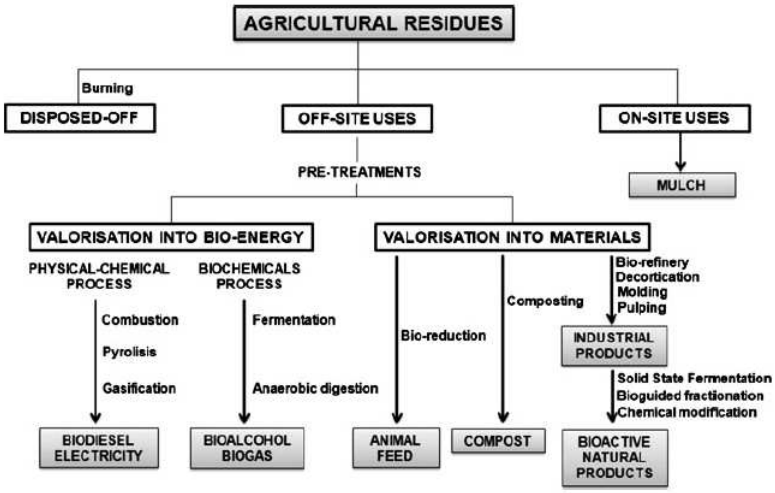

Figure 1 *Flow diagram of agricultural biomass conversions (Santana-Méridas et al., 2012).*

Agricultural and food processing segments produced wastes in a more concentrated way and with some related problems: serious pollution problem for high value of COD (Chemical Oxygen Demand) and BOD (Biological Oxygen Demand) due to mostly organic nature; varying pH conditions and chemical composition because of seasonal character; easily bacterial contamination (vegetable and fruit by-products); high water content; high accumulation (disposal problem). Current practices for food waste disposal are: animal feed, composting, incineration and landfill (Lin et al., 2013). High COD, high BOD and also high concentrations of substances with antimicrobial and antioxidant properties preclude disposal via the ordinary wastewater treatment (mainly sewage system) and irrigation and/or fertilization of cultivated fields (Ledesma-Escobar and de Castro, 2015). Pressing extraction, the oldest method to obtain olive oil, produces a liquid waste, known as OMWW (olive mill wastewaters), which is residual water after oil decantation. This waste has very high COD (up to 200 g/L) and BOD (up to 100 g/L) values, with several environmental problems. Its organic fraction

consists of pectins, lipids, sugars and aromatic compounds, which are responsible for antimicrobial activity and phytotoxicity (Ledesma-Escobar and de Castro, 2015).

1.4. Recovery of bioactive natural products

Methodologies for the use of by-products can be grouped into three categories:

1. recovery of natural compounds using conventional and non-conventional extraction techniques;
2. utilization and optimization of lead compounds in order to develop analogues by synthetic strategies;
3. bioprocess development to produce bioactive compounds.

Table 1 summarizes the potential value of crop-based and processing-based agricultural residues as raw materials for some applications.

Table 1 *High added-value product from crop-based and processing-based residues (Santana-Meridas et al., 2012).*

Activity	Waste type	Bioactive compounds	Applications	Reference
Melon	Aerial biomass	Xanthan	Rheology modifier, food additive	Lopez et al., 2004
Carrot	Roots	Hydroxycinnamic acid, anthocyanins	Antioxidant	Guinot et al., 2007
Wheat	Straw	Xylose, polyphenols	Food ingredient	Akpinar et al., 2012
Olive	Leaves	Polyphenols	Antimicrobial, antioxidant	Lee and Lee, 2010
Mango	Peels, pits, seeds	Tannins, vanillin, mangiferin	antioxidant	Masibo and He, 2009
Orange	Peels	Citric acid	Additive, detergent, cosmetic	Torrado et al., 2011
Artichoke	Bracts, receptacles, stems, juice	Caffeoylquinic acids	Antioxidant	Llorach et al., 2002
Onion	Fresh peeling	Condensed tannins, quercetin	Antioxidant, textile, dyes	Giunot et al., 2007
Hazelnut	Skins, hard shells, leafy covers	Phenolic acids	Antioxidant	Shahidi et al., 2007
Rosemary	Solid residues	Phenolic acids	Antioxidant	Navarrete et al., 2011

Bioactive compounds are recovered using conventional and non-conventional extraction techniques. Conventional methods are solid-liquid and liquid-liquid

extractions that changing parameters such as type of solvent, time and temperature of extraction can influence recovery of valuable compounds. Recently, new extraction non-conventional techniques are developed in order to recover more efficiently bioactive compounds: supercritical carbon dioxide extraction, ultrasound assisted extraction, pressurized liquid extraction and others (Wijngaard et al., 2012). Moreover, in literature for example are reported enzymatic modifications of hydroxytirosol and chemo-enzymatic modifications of resveratrol respectively from olive and grape by-products in order to produce semi-synthetic analogues perhaps with incremented pharmacological activity (Spatafora and Tringali, 2012). Solid state fermentation (SSF) is employed like a bioprocess for growth of microorganism capable of producing bioactive compounds (Martins et al., 2011).

1.5. Bioactive compounds from vegetable and fruit by-products

Nowadays the eating habits of the population have changed and as a result there is a greater awareness of the effects of nutrition on health and quality of life. Consumers demand fresh or minimally processed products that can provide nutrients and at the same time positive biological effects and of course safe products from a microbiological point of view. Phytochemicals, in fruits and vegetables, are responsible for the beneficial effects on human health. Furthermore, consumers demand safer products without synthetic additives because of negative effects that these may exercise. In this scenario, there is demand for food quality, natural ingredients and safe additives with positive biological effect and nutritional values. Recently there is a trend for consumption of products rich in phytochemicals or food enriched in them, in order to assure the health effects, because of greater knowledge of beneficial activities obtained. Fruit and vegetable by-products are rich in phytochemicals,

such as carotenoids, dietary fibers, minerals and phenolic compounds. In literature there are many studies, *in vitro* and *in vivo*, that correlate a diet rich in fruits and vegetables with a low incidence of degenerative and cardiovascular diseases (De Ancos et al., 2015). It's possible to define phytochemicals as bioactive compounds due to their biological properties. They can exert beneficial effects with synergistic or additive interactions by acting on target pathway in human organism (Liu, 2013).

Each stage in agricultural and food industry – food production, processing, transportation, storage, distribution and marketing – provokes an impact on pollution with formation of by-products (derived from packaging, canning, freezing, drying, washing, peeling, blanching steps), waste and air emissions. Wastes are different in quality and quantity and there is the need to reduce the impact technologies and processing methods on the environment. In many countries there is the necessity to increase agricultural production due to increasing population but it's required to focus attention on environmental problems searching a balance between agricultural development and environment protection. There are several side effects of agricultural inputs (fertilizers, pesticides, feed additives) such as contamination of ground and water, ammonia emissions, acid rains, accumulation of heavy metals in soils.

Figure 2 represents the percentage of the initial weight of vegetables and fruits that are disposed at each step of food supply chain (FSC). It's possible to assess that in some countries over than 40% of initial weight is discarded (Europe) and this percentage becomes higher in less industrialized countries (North Africa & East Central Asia and Latin America). In Europe agriculture has the major impact on food waste (about 20%). Waste regarding consumption is also considerable (15-30%); postharvest step waste is high because of perishable and seasonal characters of crops and fruits.

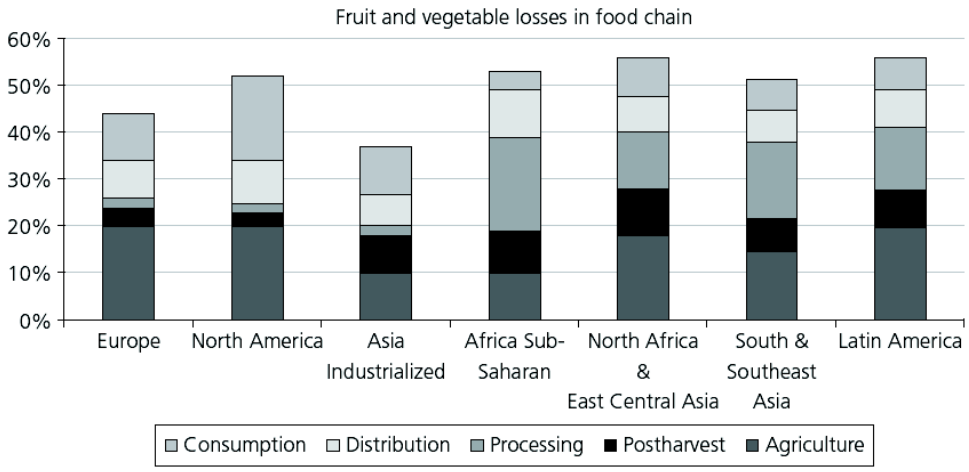

Figure 2 *Percentage of fruit and vegetable production discarded at different steps of FSC (De Ancos et al., 2015).*

Normally in vegetable processing step, canning and freezing operations produce large amounts of by-products, but also at level of selection and classification there is a big quantity discarded because of fungal attack or mechanical injury, or size and maturity not-compliant. The step of elimination of the non-edible portion involves that a further significant amount is discarded. At this level, for example, artichoke's waste is about 50-60% of the initial weight (Larrosa et al., 2002). The most important processed fruits produced worldwide are fruit juices and it's evaluated that 40% of citrus fruit production is involved in fruit juices and consequently are produced as wastes peels, pulps, stones, seeds, pomace. According to Directive 2006/12/EC, integrated in Directive 2008/98/EC, there are some laws and requirements that oblige to practice recycling, recovery and reuse of food waste as source of energy and valuable compounds with nutritional and functional characteristics. In Table 2 is reported approximately the percentage amount of some agricultural by-products generated by processing industry. It is evident that in

some cases the percentage discarded is more than 40%, as artichoke, mango, orange, asparagus (Goñi and Hervert-Hernández, 2011).

Table 2 *Amount of by-products generated from fruit and vegetable processing industry (Goñi and Hervert-Hernández, 2011).*

	By-products	Edible part	Reference
Agave	40% (rind and pith)	60%	Iniguez-Covarrubias et al.,2001
Apple	11% (pulp and seed core)	89%	Ayala-Zavala et al., 2010
Artichoke	Around 60% (outer bracts, receptacle and stems)	40%	Llorach et al., 2002
Asparagus	Up to 40-50%	50-60%	Rodrìguez et al.,2006
Banana	Up to 30% (buccia)	70%	Schieber et al., 2001
Cactus pear cladodes	20% (spines, glochids and peel)	80%	Bensadon et al., 2010
Cactus pear fruit	45% (spines, glochids,peel and unusable pulp)	65%	Bensadon et al., 2010
Carrot	30-40%	60-70%	Schieber et al.,2001
Cyfhoman-dra betacea	15-35% (skin, pulp, seeds)	65-85%	Ordonez et al., .2010
Guava	10-15% (peel and seeds)	85-90%	Schieber et al., 2001
Mandarin	16% (peels)	84%	Ayala-Zavala et al., 2010
Mango	13.5% (seeds),11% peels) e 17.9% (unusable pulp)	58%	Ayala-Zavala et al., 2010
Orange	66% (peel)	44%	Li et al., 2006
Papaia	6.5% (seeds), 8.5% (peels) and 32.1% (unusable pulp)	53%	Ayala-Zavala et al., 2010
Passion fruit	>75% (rind and seeds)	25%	Schieber et al., 2001
Potato	15-40% (peel)	60-85%	Schieber et al., 2001
Tiger nuts ("chufa")	Up to 60% (solid and liquid waste)	40%	Sanchez-Zapata et al., 2009
Tomato	3-7% (peels and seeds)	93-97%	Schieber et al., 2001

1.6. Remarks on the use of by-products and future prospects

Valorisation of fruit and vegetable by-products involves some considerations before their utilization. First of all, starting materials are not homogenous and

may come from different fields, cultivation procedures, conditions and processing and consequently standardization is necessary. Agricultural by-products are characterized by biological instability because of high microbial load – than can affects product safety and naturally to accelerate microbial degradation – and high water load – which entails higher costs for the transport because of the weight increased. For example drying costs and pressing for artichoke (90% water content) and tomato (71% water content) involve very high costs (Peschel et al., 2006). Obviously the water after pressing has a high organic load and must be properly recycled. Furthermore by-products with high fat content may undergo to oxidation of fatty acids and consequently formation of bad smells. A problem very important is the presence of active enzyme inside by-products that can be inactivate for example using blanching or thermal step, in order to avoid degradation of phytochemicals. Some pre-treatments are necessary before to use by-products: wet grinding (to reduce particle size), drying (lyophilisation, oven) (O'Shea et al., 2012). Drying should be done as close to the location production to reduce volume and cost of transport. Several and additionally studies are necessary to ensure safe bioactive compounds extracted from by-products, free of pesticides and toxic molecules (solanin, patulin, ochratoxin, dioxins). Bioavailability studies are very important to determine the effective activity of phytochemicals. So are necessary interdisciplinary studies and coordination among them (De Ancos et al., 2015).

The future prospect related to waste management is the utilization of by-products as raw materials to provide the generation of different products with various applications. Moreover by-products reuse together with minimization waste production will allow the development of zero-waste production processes.

1.7. Food waste valorisation: winery waste and by-products

The following explains a study focusing on integrated valorisation of food supply chain waste by green chemical conversion routes and biotechnological applications. Worldwide wine production is about 280 million hectolitres. Wine residues are mainly composed of grape stalks, grape pomace, marc, waste waters and waste lees. The novel biorefinery concepts could provide a suitable solution. Grape stalk is lignocellulosic material, with great mineral content, and normally are burned (greenhouse effect and release of toxic compounds). It's possible to employ grape stalks for C5-C6 sugar production by fermentation processes. Hemicellulose hydrolyzates were fermented by *L. Pentosus* for lactic acid and biosurfactants production. Furthermore grape stalks are sources of antioxidant compounds, hydroxicinnamic and hydroxibenzoic acids. In this scenario, extraction of antioxidants could be done prior to fermentation processes. Similarly grape marc, generated after pressing, is employed for fermentative lactic acid production. Grape marc is also a source of tannins to produce wood adhesives and is used in solid state fermentation for bioethanol production.

Wine lees is the residue at the bottom of recipients containing wine, after fermentation and the residue obtained following filtration and centrifugation of wine: it represents about 2-6% of total volume of wine and is rich of ethanol, tartaric acid and yeast cells. This by-product for its high phenolic content is not suitable for animal feed production. In literature extraction of phenolic compounds is reported from wine lees by microwave-assisted extraction and supercritical fluids (Lin et al., 2014).

Furthermore grape seeds represent valuable by-products for their lipid profile mainly composed of linoleic acid and vitamin E. Recently these seeds are used as culinary oil. Grape seeds are important sources of proanthocyanidins too, with antioxidant properties: their extracts were employed as antimicrobial

additives in soy protein edible films, inhibiting the growth of *L. monocytogenes*, *E. coli* and *S. tyhimurium*. Grape seeds extracts could find applications in cosmetic and nutraceutical sectors as colorants, flavour modifiers and antioxidants (Ledesma-Escobar and Luque de Castro, 2015)

CHAPTER II

TECHNIQUES FOR ANALYSIS OF BIOACTIVE COMPOUNDS

For chemical determination of bioactive compounds in vegetable extracts is advisable the combination of advanced sample preparation techniques and separation tools. Sample preparation is the most important phase due to necessity of selective and efficient extraction of target compounds. It converts real matrix into a sample suitable for subsequent analytical techniques.
Recently the development and application of modern sample preparation techniques provide various advantages.

2.1. Extraction

The preparation and extraction of bioactive compounds depends on the nature of sample matrix and chemical properties of its compounds. For liquid sample, liquid-liquid extraction (LLE) is employed, while for solid sample, solid-liquid extraction (SLE) is used.
In the case of phenolic compounds, polarity, molecular structure and number of hydroxyl groups are important. Some of these compounds are complexed with proteins, carbohydrates and for sample preparation freeze-drying, air-drying and oven-drying are useful.
Afterwards dried matrices are milled in order to obtain fine particle size while liquid samples undergo centrifugation, filtration and purification. For samples with lipids are usually applied defatting processes. These steps are performed prior to extraction, because is necessary to prepare samples for subsequent analysis.
Organic and inorganic solvents are employed to extract bioactive compounds. Some parameters can influence extraction yields, such as temperature, time, solvent to sample ratio, solvent. The most used solvents are ethanol, acetone, ethyl acetate, water and methanol. Higher temperature and extraction time can allow increased analytes solubility, but at high temperatures degradation phenomena can occur. Solvent to sample ratio and number of extraction cycles

can enhance recovery. Bioactive compounds can be linked to other inert materials: addition of enzymes and acidic/alkaline hydrolysis can promote release of compounds and their stability, respectively.

Extraction is fundamental for separation and recovery of bioactive compounds, after sample preparation and removal of undesirable compounds. Soxhlet and maceration are conventional extraction techniques and are performed for several hours. Main disadvantages are: large volumes solvents, hazardous organic solvents and long extraction times. Recently there is an increased demand for alternative and non-conventional techniques to overcome all these disadvantages. New techniques allow short extraction times, reduced volume organic and hazardous solvents and are very simple to perform, achieving a decrease in the use of energy and water too (Khoddami et al., 2013).

Before the coming of Green Chemistry, green extract was obtained using lower energy and solvent consumption, but now it is the final result after a long process that is wholly green, starting from production and harvesting of plant to transformation and formulation. Pharmaceutical, cosmetic and food industries have recognized these concepts and reached a point of meeting increasing extraction efficiency, reducing or eliminating hazardous solvents, moderating energy consumption. Chemat gives a definition of green extraction: "Green extraction is based on the discovery and design of extraction processes which will reduce energy consumption, allows use of alternative solvents and renewable natural product, and ensure a safe and high quality extract/product" (Chemat et al., 2012).

The objectives are:

- use of alternative solvents;
- use of renewable raw material for botanical extracts;
- energy consumption by energy recovery.

First of all, in the industry solvent selection depends on nature of botanical raw material and its bioactive compounds, cost and availability. Some frameworks report which solvent can or cannot be used. Almost all solvents are derived from crude oil, only ethanol derived from fermentation. Water represents the obvious solution but only for polar compounds extractions.
Simultaneously, agricultural by-products are already used as raw materials for extraction purposes, allowing revenue for farmers and improving agricultural economy. By-products valorisation permits to increase value added of these wastes, initially used only as fertilizers or feedstocks and recently utilized as source of bioactive ingredients (Chemat et al., 2015). Despite this valorisation, the majority of by-products are not exploited as rich source due to lack of extraction techniques. Recently pressurized liquid extraction (PLE), supercritical fluid extraction (SFE) and ultrasound assisted extraction (UAE) are the most used techniques because of their high efficiency extraction and reduced consumption solvent.
Recovery of bioactive compounds from by-products follows basic principles:

- maximizing yield of extraction;
- adapting demands of industry;
- clarifying impurities and toxic compounds;
- preventing deterioration and loss of functionality during extraction;
- ensuring food grade of final products (Galanakis, 2012).

2.1.1. PLE

It's possible to determine qualitative and quantitative profile of an extract only after an exhaustive extraction using conventional solid-liquid techniques. Solvents at elevated pressures and temperatures possess different characteristics and improved speed extraction. Ideally, compounds should have higher solubility in solvent while interfering compounds should remain

insoluble in solid matrix (Pronyk and Mazza, 2009). In order to realize this effect, some parameters must be optimized, such as solvent to feed ratio, particle size, modifier concentration, time, temperature. Conventional techniques as Soxhlet and maceration are time and solvent consuming. Furthermore, parameters as diffusivity, viscosity and density can be controlled by varying pressure and temperature. During PLE extraction, a certain pressure is applied and consequently temperature is above the boiling point of solvents. High temperatures allow increased mass transfer and extraction rate: solvent is able to solubilise compounds, diffusion rate is increased, destruction of solute-matrix linkage is easier, solvent viscosity and surface tension were decreased. Application of pressure (range 580-2900 psi) assures that solvent is in liquid state. The pressure allows the solvent to penetrate into the pores of matrix and increases solute solubility. In PLE extraction is usually used pressure value of 1500 psi. PLE extraction permits low consumption of solvent and time and for this reason is the most utilized technique to recover valuable and bioactive compounds from by-products. Obviously food grade solvents were employed as water or ethanol or mixture of both. Ethanol is cheap and has GRAS status (generally-recognized as safe, according to American Food and Drug Administration, FDA). It's possible to change some parameters, as temperature, pressure, flow rate and extraction time. Type of compound influences efficiency extraction because polarity, size and medium differ so much. It's possible to change concentration solvent and temperature. Ethanol and water are the most utilized solvents in PLE experiments, but if ethanol is completely eliminated, regulations about organic solvent can be avoided and costs for evaporating solvent deleted. The pressure to maintain water at liquid state is 217 psi at 200 °C and 1232 psi at 300 °C. Its dielectric constant changes when temperature is increased and becomes similar to that of methanol if is employed temperature of 200 °C. Furthermore there are some advantages when mixture of ethanol-water was utilized: ethanol improves

solubility of solute while water helps desorption of solute from matrix. The addition of ethanol lowers boiling point and can improve efficiency yield. Elevated temperature also can affect yield for increased mass transfer and extraction rate. Range temperature is about 100-180 °C, because is not recommended to use temperature more high than 140-150 °C in order to avoid degradation of thermo-labile compounds or formation of undesirable products of the Maillard reaction (Wijngaard et al., 2012). PLE is a fully automated and environmentally friendly technique. For example this technique was employed for the extraction of bioactive compounds from marine sponges (Ibañez et al., 2012).

2.1.2. SFE

Supercritical fluid extraction was used at laboratory and commercial scale to extract exhaustively target compounds or to recovery bioactive compounds on a large scale, for example the well-know decaffeination of coffee beans. CO_2 has a low critical temperature (31 °C) and low critical pressure (7.4 MPa), is safe, cheap and possesses food-grade too. Supercritical region is located above its critical temperature and critical pressure and CO_2 is present in this case as one phase, with gas and liquid properties. Density is high, like a liquid (high solvent power and diffusion coefficients) but viscosity is low, like a gas. Sc-CO_2 is usually used to extract apolar compounds but if a co-solvent such as ethanol was added, also polar compounds can be extracted. If ethanol is added, it's necessary to increase temperature and pressure to remain in supercritical region, due to critical temperature increased. It's possible to change temperature, pressure, time, solvent-to-feed ratio, co-solvent concentration (Wijngaard et al., 2012). For example phenolic compounds are recovered from grape by-products at these operational conditions: 400 bar, 35 °C, 5% v/v ethanol (Casas et al., 2010).

2.1.3. UAE

Ultrasound is a sound wave that man is not able to perceive; ultrasound passes through a medium creating a compression and an expansion. This phenomenon enables to produce a phenomenon called cavitation, which provides production, growth and collapse of bubbles. Bubbles stay at 5000 K and 1000 atm. This phenomenon can only occur in liquid and in liquid containing solids. In plant matrix, cavitation permits to facilitate leaching from matrix by increased mass transfer and diffusivity solvent in plant parts, with diffusion across cell wall and capture of solutes after breaking cell wall. Frequency, temperature, pressure and time are the most important parameters that can affect UAE extraction. UAE is not a time and solvent consuming technique (Azmir et al., 2013). Li and co-workers reported UAE extraction for recovery of chlorogenic acid from leaves and barks of *Eucommia ulmodies* Oliv. at these operational conditions: 70% v/v, methanol, 30 min extraction time and 20:1 solvent to sample ratio (Li et al., 2005).

2.2. Analytical techniques

After sample preparation and extraction, analytical techniques are necessary in order to identify bioactive compounds, to determine chemical composition of crude extracts and to obtain chemical fingerprint. Several analytical techniques are employed, also coupled to mass spectrometry (MS): thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC).

HPLC allows separation, identification and quantification of bioactive compounds, varying sample purification, mobile phases, columns and detectors. The main advantages are high reproducibility, good linear range, automation. For phenolic compounds for example reverse phase columns,

photo diode array detectors (PDA) and acidic polar mobile phases were used. HPLC performance also depends on complexity of crude extracts after undergoing purification steps (open column chromatography or adsorption-desorption processes using SPE (solid phase extraction) cartridge, styrene-divinylbenzene or acrylic resins.

Methanol and acetonitrile mixed with water are employed as mobile phases. It's important to maintain constant pH. In fact for phenolic compounds, acidified phase are utilized (with formic acid or acetic acid) in order to avoid ionization phenomena. A gradient elution program is usually performed.

Columns are C18 normal phase or C18 reverse phase, with variable length (5-30 cm) and particle size (3-10 μm). Recently new columns are employed, monolithic and porous forms, with smaller particle size by UHPLC (ultra high performance liquid chromatography) and two-dimensional liquid chromatography (LC x LC).

Phenolic compounds are determined using UV-Vis and PDA (190-380 nm) and FLD (fluorimetric) detectors, but also spectrophotometric and colorimetric assay are performed.

The combination of integrated (hyphenated) techniques, i.e. LC-DAD (liquid chromatography-diode array detector), provides a simultaneous detection of analytes at different wavelengths and UV spectra, monitoring different classes of bioactive compounds.

But the best results are obtained with mass spectrometric detectors (MS) coupled to liquid chromatography systems, which allow obtaining detailed molecular structural information (selective techniques). Mass spectrometry provides structural molecular characterization (Khoddami et al., 2013).

New advanced mass spectrometric techniques are spreading with high sensitivity, selectivity and productivity. When MS is employed coupled to HPLC it is obligatory to use volatile buffer, with low ion strength. Strong acids are not recommended, due to ion suppressive effects. Low flows are

advised, in particular for ESI (electrospray source ionization) source. There are different mass spectrometers. The simple quadrupole is the best for quantification. If tandem mass spectrometry (MS/MS) is done, there is triple quadrupole equipment. The advantage of high mass resolution is reached with time of flight (TOF) instrument. TOF is usually coupled to quadrupole analyzer (qTOF) and allows exploiting high mass resolution and tandem mass capability. Another common instrument is ion trap (IT), very sensitive, inexpensive but without high mass resolution. If IT is combined with Orbitrap or ion cyclotronic resonance analyzers, it's possible to have high resolution and to characterize unknown compounds. ESI ionization is the most employed because it is suitable for HPLC-MS equipment, both in negative and positive mode for polar compounds (Di Stefano et al., 2012).

EXPERIMENTAL PART

CHAPTER III
SECTION A

CHEMICAL PROFILE AND CELLULAR ANTIOXIDANT ACTIVITY OF ARTICHOKE BY-PRODUCTS

Pagano, **I**., Piccinelli, A. L., Celano, R., Campone, L., Gazzerro, P., De Falco, E., & Rastrelli, L. (2016). Chemical profile and cellular antioxidant activity of artichoke by-products. *Food & Function*, *7*(12), 4841-4850.

3A 1. Introduction

The globe artichoke (*Cynara scolymus* L., *Asteraceae*) is a perennial herbaceous crop, originating from the Mediterranean area. The edible portions are large immature inflorescences (capitula or heads) consisting of fleshy leaves, named bracts, and receptacle. More than 60% of its production is done in Europe and Italy represents the first world producer. The heads are sold fresh, or they undergo industrial processes and marketed as frozen, cooked, canned or preserved in oil (de Falco et al., 2015; Lattanzio et al., 2009). Artichoke is considered a functional food due to its high content of phenolic compounds and inulin. The main compounds are caffeoylquinic acids, mono- and di-caffeoylquinic acids (CQAs and diCQAs) and the most abundant are chlorogenic acid (5-CQA) and 1,5-dicaffeoylquinic acid (1,5-diCQA). Moreover flavones glycosides represent another important class and the major compounds are luteolin-7-O-rutinoside (L-RUT) and apigenin-7-O-glucoside (A-GLU). Artichoke has been used in folk medicine against liver and gallbladder complaints and leaves extracts have been used as hepatoprotective, choleretic and lipid-lowering agents. The beneficial and therapeutic activity can be ascribed mainly to presence of caffeoylquinic acids and flavones. Furthermore edible portion of artichoke is a great resource of inulin (18-36% dry matter), a reserve carbohydrate with specific prebiotic properties. The cultivation and industrial processing of artichoke produces a large amount of waste material (80-85% of total plant biomass) (Lattanzio et al., 2009). This waste is constituted mainly of leaves and stems, removed during cultivation, and external bracts discarded during industrial processing. These by-products are used as compost or for animal feed production. In the last years, numerous studies have demonstrated that artichoke by-products are a rich source of phenolic compounds (Palermo et al., 2013; Pandino et al., 2011; Pandino et al., 2013a) and inulin (Lopez-Molina et al., 2005; Machado et al., 2015; Ruiz-

Cano et al., 2014). Then leaves and bracts begin suitable raw materials to recovery bioactive compounds for food additives and nutraceutical/pharmaceutical production. From the point of view of valorisation of food by-products and considering beneficial properties of artichoke, the aim of this study was to evaluate the added value of artichoke by-products, bracts and leaves, by chemical characterization of bioactive constituents (phenolic compounds and inulin content) and biological study of cellular antioxidant activity. Two artichoke varieties of Campania region (Italy) were utilized: bracts (ABs) and leaves (ALs) of Bianco di Pertosa and Tondo di Paestum. Chemical profiles were determined using high performance liquid chromatography-ultraviolet detection-high resolution mass spectrometry (HPLC-UV-HRMS) and inulin content was determined using AOAC official enzymatic-spectrophotometric method. Cellular antioxidant activity of AB and AL extracts and of main compounds is investigated by cellular antioxidant activity (CAA) assay in human hepatocellular carcinoma HepG2 cell line.

3A 2. Results and discussion

The artichoke by-products ABs and ALs of "Bianco di Pertosa" and "Tondo di Paestum" were characterized in terms of phenolic compounds and inulin contents, as well as their cellular antioxidant activity.

3A 2.1. Phenolic profile of artichoke by-products by UHPLC-DAD-HRMS^n

The artichoke by-product extracts were analyzed by UHPLC-DAD-(–)-HRMS^n to investigate their qualitative phenolic profile. Figure 3 shows representative UHPLC chromatogram at 325 nm of AB-Pa and AL-Pe extracts. A total of 16 peaks (1-16) were detected. Metabolite assignments

were made by comparing retention time, UV/Vis spectra and MS data (accurate mass and MS^n fragment ions) of the compounds detected with standard compounds, whenever available, and artichoke compounds reported in the literature and databases. The identities, retention times, UV and MS data for individual components are listed in Table 3.

Identified metabolites belonged to caffeoylquinic acid and flavone classes (Figure 3). Peaks 1-3, 5, 8, 10 and 14 showed characteristic UV spectra of caffeoylquinic acids (λ_{max} 325-330 nm, cinnamate chromophore), whereas peaks 6, 7, 9, 11-13 and 15-16 exhibited typical UV absorption of flavones (λ_{max}340-350 nm, B ring cinnamoyl system, and 267 nm, A ring benzoyl system). Molecular formulas, established by HRMS, allowed identifying three CQAs (1-3, $C_{16}H_{18}O_9$), four diCQAs (5, 8, 10 and 14, $C_{25}H_{24}O_{12}$), the flavones luteolin (L 15, $C_{15}H_{10}O_6$) and apigenin (A 16, $C_{15}H_{10}O_5$) and their rutinoside (L-RUT 6, $C_{27}H_{30}O_{15}$ and A-RUT 11, $C_{27}H_{30}O_{14}$), glucoside (L-GLU 7, $C_{21}H_{20}O_{11}$and A-GLU 12, $C_{21}H_{20}O_{10}$) and glucuronide (L-GLA 9, $C_{21}H_{18}O_{12}$ and A-GLA 13, $C_{21}H_{18}O_{11}$) derivatives. MS^n experiments revealed further structural information on the nature of CQAs and aglycone of flavones glycosides. Particularly, Clifford's hierarchical schemes were employed to characterize CQA and diCQA isomers. In fact, the CQA and diCQA fragmentation patterns are dependent upon the particular stereochemical relationships between the individual substituents on the quinic acid moiety (Clifford et al., 2003; Clifford et al., 2005; Kuhnert et al., 2012). Therefore, based on characteristic products ion at m/z 191.0566 ($[QA-H]^-$) and 179.0343 ($[CAA-H]^-$), compounds 1-3 were identified as 1-, 3- and 5-CQA, respectively. Identity of 5-CQA was confirmed by comparison with reference standard. 3-CQA (2) was distinguished from 5-CQA (3) by a comparatively intense $[CAA-H]^-$ ion (38% in 2 compared with 5% in 3). Additionally, 1-CQA isomer was identified by retention time in comparison with available literature data (Clifford et al., 2005). Likewise, the diCQA positional isomers

(5, 8, 10 and 14) were identified by their product ions in MS^2 ($[diCQA-H_2O-H]^-$ at *m/z* 335.0764) and MS^3 ($[QA-H]^-$, $[CAA-H]^-$ and $[QA-H_2O-H]^-$ at m/z 173.0454) spectra. Only 3,4- (8) and 4,5-diCQA (14) gave the diagnostic ion for substitution at position 4 ($[QA-H_2O-H]^-$) as MS^3 base peak (Clifford et al., 2003). These two isomers were discriminated on the basis of ($[diCQA-H_2O-H]^-$ and $[QA-H_2O-H]^-$ ion intensities (Table 3) (Clifford et al., 2003). Peak 5 was identified as 1,3-diCQA by comparison with reference standard, and the structure of 10 was established as 1,5-diCQA, on the basis of MS and chromatographic data (Table 3) and literature data of artichoke compounds (Clifford et al., 2005).

Regarding, the flavone glycosides (6, 7, 9 and 11-13), the nature of sugar moieties was established by molecular formulas of MS^2 product ions (neutral losses of $C_6H_{10}O_5$, $C_6H_{10}O_4$ and $C_6H_8O_6$ residues) and their aglycone skeleton was confirmed by comparison of MS^3 spectra with the reference standards L-GLU and A-GLU. Therefore, flavones 6, 7, 9 and 11-13 were identified as rutinoside, glucoside and glucuronide derivatives of luteolin (6, 7 and 9) and apigenin (11-13), according to artichoke compounds reported in the literature (de Falco et al., 2015; Lattanzio et al., 2009).

Table 3 *UV and high-resolution mass spectrometry identification of phenolic compounds in artichoke by-products (bracts and leaves) by UHPLC-DAD-(–)-HRMS^n.*

N	t_R min	λ_{MAX} nm	$[M-H]^-$ *m/z*	ppm	Formula	MS^n product ion *m/z* (relative abundance)	Compound[a]
1	2.5	325	353.0879	3.4	$C_{16}H_{18}O_9$	179 (4.2) 191 (100)	1-CQA
2	3.3	325	353.0870	0.8	$C_{16}H_{18}O_9$	135 (7) 179 (38) 191 (100)	3-CQA (neochlorogenic acid)
3	5.9	325	353.0870	0.8	$C_{16}H_{18}O_9$	135 (1) 179 (5) 191 (100)	5-CQA (chlorogenic acid) [b]
4	6.4		179.0344	3.0	$C_9H_8O_4$	135 (100)	CAA (caffeic acid) [b]
5	8.8	325	515.1201	3.2	$C_{25}H_{24}O_{12}$	179 (13) 335 (28) 353 (100); MS^3 (353): 135 (4) 179 (26) 191 (100)	1,3-diCQA (cynarin) [b]
6	13.1	267, 350	593.1515	2.3	$C_{27}H_{30}O_{15}$	285 ($C_{15}H_9O_6$, 3.0 ppm); MS^3 (285): 133 ($B^{1,3}$) 151 ($A^{1,3}$) 241 (-CO_2)	L-RUT (scolymoside)
7	13.4	267, 350	447.0924	0.4	$C_{21}H_{20}O_{11}$	285 ($C_{15}H_9O_6$, 3.0 ppm); MS^3 (285): 133 ($B^{1,3}$) 151 ($A^{1,3}$) 241 (-CO_2)	L-GLU (cynaroside) [b]
8	13.8	330	515.1195	2.0	$C_{25}H_{24}O_{12}$	173 (2) 179 (1) 191 (3) 203 (18) 255 (8) 299 (19) 317 (17) 335 (17) 353 (100); MS^3 (353): 173 (100) 179 (19) 191 (13)	3,4-diCQA
9	14.1	267, 340	461.0723	1.9	$C_{21}H_{18}O_{12}$	285 ($C_{15}H_9O_6$, 3.0 ppm); MS^3 (285): 133 ($B^{1,3}$) 151 ($A^{1,3}$) 241 (-CO_2)	L-GLA
10	14.5	330	515.1196	2.3	$C_{25}H_{24}O_{12}$	191 (8) 335 (6) 353 (100); MS^3 (353): 179 (5) 191 (100)	1,5-diCQA

N	t_R min	λ_{MAX} nm	$[M-H]^-$ *m/z*	ppm	Formula	MS^n product ion *m/z* (relative abundance)	Compound[a]
11	15.1	267, 340	577.1563	2.0	$C_{27}H_{30}O_{14}$	269 ($C_{15}H_9O_5$, 2.2 ppm); MS^3 (269): 117 ($B^{1,3}$) 151 ($A^{1,3}$) 225 (-CO_2)	A-RUT
12	15.4	267, 340	431.0973	0.1	$C_{21}H_{20}O_{10}$	269 ($C_{15}H_9O_5$, 2.2 ppm); MS^3 (269): 117 ($B^{1,3}$) 151 ($A^{1,3}$) 225 (-CO_2)	A-GLU [b]
13	15.8	267, 340	445.0777	2.6	$C_{21}H_{18}O_{11}$	269 ($C_{15}H_9O_5$, 2.2 ppm); MS^3 (269): 117 ($B^{1,3}$) 151 ($A^{1,3}$) 225 (-CO_2)	A-GLA
14	16.4	330	515.1197	2.6	$C_{25}H_{24}O_{12}$	173 (3) 179 (2) 191 (3) 203 (6) 255 (3) 299 (7) 317 (3) 335 (2) 353 (100); MS^3 (353): 173 (100) 179 (66) 191 (35)	4,5-diCQA
15	20.5	267, 350	285.0405	4.1	$C_{15}H_{10}O_6$		L (luteolin) [b]
16	21.8		269.0454	3.5	$C_{15}H_{10}O_5$		A (apigenin) [b]

[a] IUPAC nomenclature is used for caffeoyl quinic acid and the number denominates the position of acyl substitution. CQA, caffeoylquinic acid; diCQA, dicaffeoylquinic acid; CAA, caffeic acid; RUT, rutinoside; GLU, glucoside; GLA, glucuronide. [b] Comparison with a reference standard.

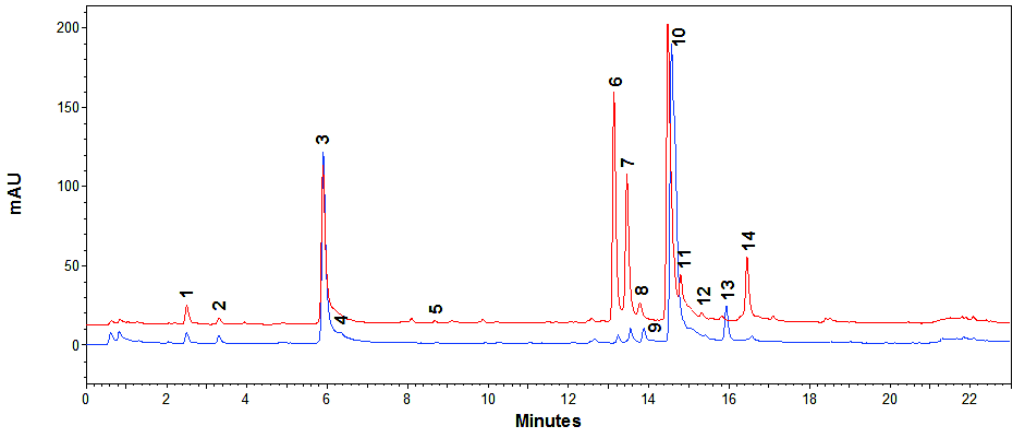

Figure 3 *UHPLC chromatograms (325 nm) of AB-Pa (blue line) and AL-Pe (red line) extracts.*
1, 1-CQA; **2**, 3-CQA; **3**, 5-CQA; **4**, CAA; **5**, 1,3-diCQA; **6**, L-RUT; **7**, L-GLU; **8**, 3,4-diCQA; **9**, L-GLA; **10**, 1,5-diCQA; **11**, A-RUT; **12**, A-GLU; **13**, A-GLA; **14**, 4,5-diCQA.

3A 2.2. Quantitative profile of artichoke by-products

The contents of the phenolic compounds (1−16) of ABs and ALs of two artichoke varieties, “Bianco di Pertosa” and “Tondo di Paestum”, were estimated by UHPLC-UV analysis of their exhaustive extracts. Quantitative data are listed in Table 4, while the Figure 4 displays the phenolic profile of the main compounds, expressed as percent of the total phenolic content.

The studied artichoke by-products showed an overall phenolic content, calculated as the sum of individual phenolic compounds, between 0.5 and 1.7 g/100 g DM, in agreement with previous quantitative data of these artichoke by-products (Palermo et al., 2013, Pandino et al., 2013a; Ruiz-Cano et al. 2014). The highest value was recorded in AL-Pe, but the content of bioactive compounds in plant tissue is highly dependent on cultivar, climatic and agricultural conditions, and also on the physiological stage of the tissues. For example in artichoke, the total caffeoylquinic acid content ranges from about 8% of DM in young tissues to less of 1% in senescent tissues (Lattanzio et al., 2009). Although the phenolic content in artichoke by-products is lower than

the edible part, the levels of total phenolic compounds found in ABs and ALs reveal in the present study their potential as a promising and cheap source of bioactive compounds.

CQAs were the most abundant bioactive compounds of both by-products (63.9-87.7 and 78.2-92.9% of the total phenolic content in AB and AL samples, respectively), and the major constituents were 5-CQA (19.5-52.0 and 28.1-59.2% in AB and AL samples, respectively) and 1,5-diCQA (35.9-52.9 and 23.0-31.4% in AB and AL samples, respectively) (Figure 4). Also in artichoke heads of marketable quality these two CQAs are the predominant compounds of this functional food (Lattanzio et al., 2009). Cynarin (1,3-diCQA) content in ABs and ALs, instead, was very low (0.2-0.4% of the total phenolic content), as well as the levels of 4,5-diCQA (0.1-0.3%), 1- (1.9-2.6%) and 3-CQA (0.6-1.7%). Many studies reported that in the raw artichoke materials only 5-CQA and 1,5-diCQA were found at high amounts, whereas, the other CQA isomers have very low concentrations. The enhancement of 1,3-diCQA content, and of the other CQA isomers, was caused by intramolecular transesterification of 5-CQA and 1,5-diCQA promoted by hot water extraction (Adzet and Puigmacia, 1985) or cooking processes (Ferracane et al., 2008).

In the case of flavones, the more prevalent compounds were the flavones glycosides L-RUT (0.9-1.0 and 7.9-20.3% in AB and AL samples, respectively), L-GLU (0.7-1.1 and 4.4-12.4% in AB and AL samples, respectively) and A-GLA (3.2-16.3 and 0.1-0.3% in AB and AL samples, respectively). These compounds, compared to CQAs, revealed a greater quantitative variation (Figure 4). Flavone aglycones were detected at trace levels in all samples.

The quantitative profile of two agricultural artichoke by-products, AB and AL, displayed relevant differences (Figure 4). ABs of both artichoke cultivars contained mostly CQAs (78.2-92.9%). Previous quantitative data of external

bracts reported a CQA content of about 99% (Palermo et al., 2013; Ruiz-Cano et al., 2014). The flavones glycosides, instead, occurred in ABs at very low amounts, with the exception of A-GLA, which was the third most abundant component of AB in both cultivars (Figure 4). In contrast, A-GLA content in ALs (< 0.3%) was at least 10 times lower than to ABs (3.2 -16.3%). A-GLA was the major flavone also in the outer bracts of the artichoke cultivar 'Violetto di Sicilia', whereas it doesn't detect in its leaves and floral stems (Pandino et al. 2013b). Similar A-GLA amounts observed in AB-Pe and AB-Pa (Table 4) were previously reported for the external bracts of the Italian cultivars Romanesco clone C3, Tondo di Paestum and Violetto di Sicilia (Pandino et al., 2011; Lombardo et al., 2010).

Regarding ALs, luteolin glycosides were well represented, especially in AL-Pe, with L-RUT and L-GLU present as the main compounds after CQAs (Figure 4). L-RUT and L-GLU incidence on the total phenolic content varied significantly among AB and AL, with higher amounts for ALs (7.9-20.3% and 4.4-12.4%, respectively) than ABs (0.9-1.0% and 0.7-1.1%, respectively).The same AB and AL phenolic profiles were previously described for Romanesco clone C3 (Palermo et al., 2013; Pandino et al., 2013 b), Tondo di Paestum (Lombardo et al., 2010) and Violetto di Sicilia (Pandino et al., 2013 a; Lombardo et al., 2010).

Quantitative differences were observed between two studied varieties of artichoke. The absolute values are strongly influenced by cultivar, climatic and agricultural conditions, (Lattanzio et al., 2009) anyway "Bianco di Pertosa" artichoke showed higher amounts of phenolic compounds in the leaves, and "Tondo di Paestum" in the bracts (Table 4). The most significant difference was the highest 5-CQA content in by-products of "Bianco di Pertosa" (52-59%) than to "Tondo di Paestum" (20-28%) (Figure 4).

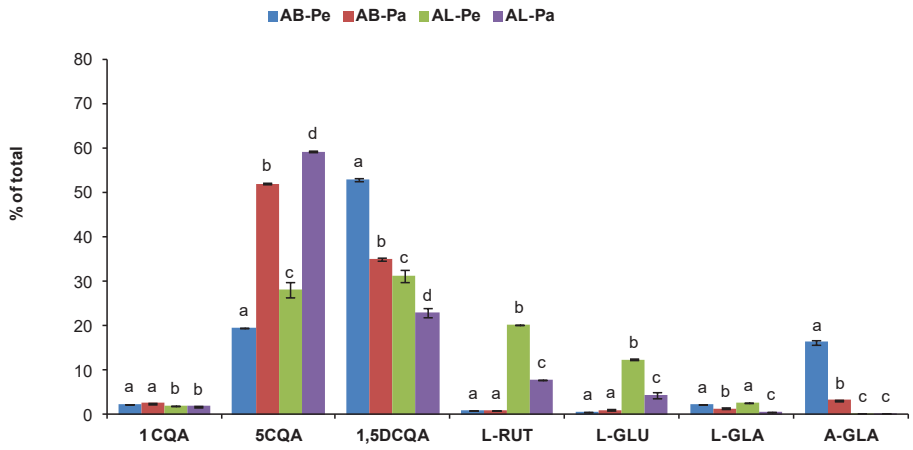

Figure 4 *Phenolic profile of the main compounds (each component expressed as percent of total) of "Bianco di Pertosa" (Pe) and "Tondo di Paestum" (Pa) artichoke by-products (AB and AL).*
Reported only compounds >2%. Different superscript letters within each compound indicate significant differences amongst extracts ($p < 0.05$).

Table 4 *Phenolic and inulin composition of artichoke bracts (AB) and leaves (AL) of "Bianco di Pertosa" (Pe) and "Tondo di Paestum" (Pa) cultivars.*

	AB-Pe	**AB-Pa**	**AL-Pe**	**AL-Pa**
	mg/100 g DM	**mg/100 g DM**	**mg/100 g DM**	**mg/100 g DM**
1-CQA	12.5 ± 0.7^{a}	21.9 ± 1.9^{b}	35.6 ± 0.5^{c}	8.8 ± 0.5^{d}
3-CQA	6.5 ± 0.4^{a}	13.6 ± 1.1^{b}	11.2 ± 0.4^{c}	8.1 ± 0.6^{a}
5-CQA	100.5 ± 3.5^{a}	443.5 ± 23.4^{b}	491.4 ± 42.4^{b}	277.4 ± 27.1^{c}
CAA	nd	nd	nd	nd
1,3-diCQA	2.2 ± 0.1^{a}	1.9 ± 0.3^{a}	2.7 ± 0.0^{b}	1.2 ± 0.1^{c}
3,4-diCQA	7.3 ± 0.6^{a}	10.4 ± 1.2^{a}	22.4 ± 3.1^{b}	2.3 ± 0.1^{c}
1,5-diCQA	272.9 ± 12.1^{a}	299.2 ± 14.5^{a}	547.0 ± 12.1^{b}	108.2 ± 16.3^{c}
4,5-diCQA	1.3 ± 0.1^{a}	1.0 ± 0.1^{b}	5.3 ± 0.0^{c}	0.8 ± 0.0^{d}
CQA total	**403.1** ± 17.2^{a}	**791.5** ± 42.5^{b}	**1115.6** ± 26.4^{c}	**406.7** ± 44.6^{a}
L-RUT	5.0 ± 0.1^{a}	7.6 ± 0.8^{a}	353.9 ± 8.6^{b}	37.1 ± 3.9^{c}
L-GLU	3.5 ± 0.0^{a}	9.1 ± 1.1^{b}	216.0 ± 2.7^{c}	20.5 ± 1.7^{d}
L-GLA	12.1 ± 1.2^{a}	12.3 ± 2.6^{a}	46.7 ± 3.3^{b}	3.2 ± 0.3^{c}
A-RUT	4.5 ± 0.9^{a}	2.9 ± 0.2^{b}	nd	nd
A-GLU	1.8 ± 0.2^{a}	1.6 ± 0.2^{a}	8.0 ± 0.3^{b}	0.8 ± 0.1^{c}
A-GLA	83.8 ± 0.9^{a}	27.3 ± 2.9^{b}	5.36 ± 0.7^{c}	0.7 ± 0.2^{d}
L	nd	nd	nd	nd
A	1.8 ± 0.0	nd	nd	nd
FL total	**112.4** ± 1.6^{a}	**60.9** ± 7.4^{b}	**630.0** ± 14.1^{c}	**62.2** ± 2.5^{b}
Phenolic total	**515.5** ± 18.8^{a}	**852.4** ± 49.8^{b}	**1745.6** ± 40.5^{c}	**468.9** ± 47.1^{a}
Inulin (g/100 g DM)	3.8 ± 0.1^{a}	8.2 ± 0.6^{b}	0.2 ± 0.02^{c}	0.5 ± 0.02^{c}

CQA, caffeoylquinic acid; diCQA, dicaffeoylquinic acid; CAA, caffeic acid; A, apigenin; L, luteolin; RUT, rutinoside; GLU, glucoside; GLA, glucuronide; FL, flavone.Values are means of three replicates ± standard deviation (SD). nd = not detected.Different superscript letters within each row indicate significant differences amongst extracts according to ANOVA, Multiple Range Tests ($p < 0.05$).

3A 2.3. Inulin content of artichoke by-products

Inulin is a component of the soluble dietary fiber with recognized prebiotic properties and used as a technological ingredient (Lattanzio et al., 2009). Artichoke is considered one of the vegetables with the highest quantity of inulin (Muir et al., 2007): a content of 7-30 g/100 g, based on dry weight has been reported in the edible part of artichoke, mainly inner bracts and receptacles (Lattanzio et al., 2009; Ruiz-Cano et al., 2014; Muir et al., 2007; Leroy et al., 2010).

Inulin contents of AB and AL samples were estimated by the enzymatic/spectrophotometric method (AOAC 999.03, 2002) and are presented in Table 4. Inulin amounts differed widely among AB and AL with levels in bracts (3.8-8.2 g/100 g DM) much higher than in leaves (0.2-0.5 g/100 g DM). As for phenolic compounds, the highest inulin content was revealed in "Tondo di Paestum" cultivar (Table 4).

The results of ABs are consistent with those found in raw outer bracts by industrial canning (Ruiz-Cano et al., 2014) and obtained by microwave assisted extraction from external bracts (Ruiz-Aceituno et al., 2016). The recommended formulation dose, based on tests with volunteers and experience in the food industry, is 4-10 g inulin (Coussement, 1999; Costabile et al., 2010). These data support the observation that ABs could be a valuable source of inulin for the ingredient in functional foods and dietary supplements.

3A 2.4. Cellular antioxidant activity (CAA) of artichoke by-products

Several studies suggest that the hepatoprotective effect of artichoke extracts are due to their antioxidant activity (Gebhardt, 1997; Gebhardt and Fausel, 1997; Miccadei et al., 2008). Artichoke leaf extracts showed antioxidant action against hydroperoxide-induced oxidative stress in cultured rat hepatocytes,

(Gebhardt, 1997; Gebhardt and Fausel, 1997) human hepatoma HepG2 cells (Miccadei et al., 2008) and human leukocytes, (Perez-Garcia et al., 2010) and chlorogenic acid (5-CQA) and cynarin (1,3-diCQA) accounted for part of this activity (Gebhardt, 1997; Gebhardt and Fausel, 1997; Miccadei et al., 2008; Perez-Garcia et al., 2010). Thus, in this study the potential antioxidant activity of artichoke by-product extracts and their major phenolic compounds (5-CQA, 1,3-diCQA, L-GLU and L) was investigated by cellular antioxidant activity (CAA) assay in human hepatocarcinoma HepG2 cells.

To exhibit their antioxidant effects in CAA model the compounds can break the peroxyl radical chain reactions at the cell membrane surface, or they can be taken up by the cell and react with ROS intracellular. The comparisons in antioxidant activities using the CAA protocols with and without a PBS wash may provide information on the degree of uptake and membrane association of the compounds. The PBS wash protocol evaluates only the antioxidant effects of compounds taken up by the cells or closely associated with the cell membrane. Conversely, the protocols without PBS wash allows estimating also the activity of more polar compounds that interact with membrane surfaces via hydrogen bonding, where they are able to protect membranes from external and internal oxidative stresses (Wolf and Liu, 2007; Wolf and Liu, 2008).

Preliminary experiments (data not shown) indicated that AB and AL extracts, as well as the pure compounds, exhibited the highest cellular antioxidant activity in no PBS wash protocol, which was then adopted to evaluate their activities. The EC_{50} values of CAA for extracts and pure compounds are listed in Table 5. The range of concentrations tested was non-cytotoxic against HepG2 cells.

AB-Pa and AL-Pe were the most efficient at inhibiting peroxyl radical-induced DCFH oxidation, followed by AB-Pe and AL-Pa. By-product extracts, except AL-Pa, showed a higher CAA compared to a commercial

product (artichoke leaf extract), indicating that artichoke by-products could provide suitable extracts for the traditional application of artichoke. The greatest activity was observed for by-product extracts with the highest content of phenolic compounds, indicating that they are responsible for the antioxidant activity. The relationship between EC_{50} values and the extract quantitative data was examined by Pearson's correlation coefficients (Table 5). EC_{50} values were well correlated with the total phenolic compounds and CQAs, whereas a poor correlation was observed between CAA and flavones content. Particularly, a significant correlation was obtained with diCQA levels ($p = 0.005$). As for individual components, 1,5-diCQA contents in the extracts showed the best correlation with EC_{50} values of extracts ($p = 0.024$), whereas no significant correlation was found between activity and 5-CQA and flavones glycosides (Table 5). These correlations were in agreement with the EC_{50} of 1,3-diCQA, 5-CQA, L-GLU and their levels in the extracts (Table 5). In addition, the significant correlations with diCQAs and 1,5-diCQA levels and the high activity of 1,3-diCQA suggested that CAA is due primarily to diCQA isomers. Therefore, 1,5-diCQA can be considered the main contributor to the CAA of the artichoke by-product extracts.

Table 5 *Cellular antioxidant activity (CAA) of artichoke by-product extracts and main phenolic compounds, and the correlations between CAA and phenolic content.*

		mg/g of extract								
Extract	**EC_{50} (μg/mL)**	**P-T**	**5-CQA**	**1,5-diCQA**	**diCQAs**	**CQA-T**	**L-RUT**	**L-GLU**	**A-GLA**	**FL-T**
AB-Pe	44.6 ± 5.5^{a}	26.8^{a}	5.2^{a}	14.2^{a}	14.8^{a}	21.0^{a}	0.3^{a}	0.2^{a}	4.4^{a}	5.9^{a}
AB-Pa	29.5 ± 3.3^{a}	55.7^{b}	29.0^{b}	19.6^{b}	20.4^{b}	51.7^{b}	0.5^{b}	0.6^{a}	1.8^{b}	4.0^{b}
AL-Pe	26.6 ± 3.5^{a}	66.9^{c}	18.8^{c}	21.0^{b}	22.1^{b}	42.7^{c}	13.6^{c}	8.3^{b}	0.2^{c}	24.1^{c}
AL- Pa	124.1 ± 9.4^{b}	17.1^{d}	10.1^{d}	3.9^{c}	4.1^{c}	14.8^{d}	1.3^{d}	0.7^{a}	0.0^{c}	2.3^{d}
Com. product	89.4 ± 8.4^{c}	34.5^{e}	7.9^{d}	2.1^{d}	$7.8^{d,\#}$	28.6^{e}	0.1^{a}	3.6^{c}	0.3^{c}	5.9^{a}
Pearson correlation coefficient		-0.80	-0.55	-0.93^{*}	-0.98^{**}	-0.77	-0.44	-0.32	-0.44	-0.54
Compound	**EC_{50} (μM)**									
Quercetin	7.5 ± 0.5									
5-CQA	3.8 ± 0.5									
1,3-diCQA	2.2 ± 0.3									
L-GLU	10.0 ± 1.6									
L	10.9 ± 0.8									

PT, phenolic total; CQA, caffeoylquinic acid; diCQA, dicaffeoylquinic acid; CQA-T, total of CQA; A, apigenin; L, luteolin; RUT, rutinoside; GLU, glucoside; GLA, glucuronide; FL-T, total of flavones;$^{\#}$1,3-, and 3,4-diCQA levels of 4.5 and 1.2 mg/g, respectively; $^{*}p$ value < 0.05; $^{**}p$ value < 0.01. Different superscript letters within each column indicate significant differences amongst extracts according to ANOVA, Multiple Range Tests ($p < 0.05$).

3A 3. Conclusions

Chemical profile and cellular antioxidant activity of artichoke by-products of two artichoke varieties of Campania region (Italy) prove that they are a suitable and cheap source of bioactive compounds in the production of food additives and nutraceuticals.

Phenolic profiles indicate that the flavones glycosides distribution is able to discriminate between AB and AL, and the quantitative results reveal high 5-CQA and 1,5-diCQA amounts in both artichoke by-products, and remarkable inulin level in ABs.

The cellular antioxidant activities of AB and AL extracts are better or similar of a commercial leaf extract, and appear to be related to the diCQA levels.

These results confirm the use of artichoke as functional food and the popular use of leaves extracts, revealing that this therapeutical activity is primarily due to the phenolic content, and highlight the potential utilization of the bracts as raw material rich in CQAs and inulin.

3A 4. Materials and Methods

3A 4.1. Materials

Analytical-grade methanol, MS-grade ammonium formate and formic acid, p-hydroxybenzoic acid hydrazide (PAHBAH) and sodium borohydride ($NaBH_4$) were obtained from Sigma-Aldrich (Milan, Italy). Ultrapure water (18 MΩ) was prepared by a Milli-Q purification system (Millipore, Bedford, USA). MS-grade acetonitrile (MeCN) and water were supplied by Romil (Cambridge, UK). Reference standards (≥98% HPLC grade) of caffeic acid (CA), chlorogenic acid (5-caffeoylquinc acid, 5-CQA), cynarin (1,3-dicaffeoylquinic acid, 1,3-diCQA), luteolin (L), apigenin (A), luteolin-7-O-

glucoside (L-GLU) and apigenin-7-O-glucoside (A-GLU) were purchased from Extrasynthase (Lyon, France). Standard stock solutions (1 mg/mL) of each compound were prepared in methanol and stored at 4 ºC. Diluted solutions and standard mixtures were prepared in MeOH/H_2O 1:9, v/v.

The k-FRUC enzymatic assay kit for the determination of fructo-oligosaccharides and fructan polysaccharides was provided by Megazyme (Bray, Ireland).

All the cell culture reagents were from Sigma–Aldrich, Inc. OxiSelect™ Cellular Antioxidant Activity Assay Kit (Cell Biolabs, San Diego, USA) was used to evaluate the cellular antioxidant activity.

3A 4.2. Artichoke by-product samples

The bracts and leaves of "Bianco di Pertosa" (AB-Pe and AL-Pe) and "Tondo di Paestum" (AB-Pa and AL-Pa) samples were kindly supplied by Cafaro Carmine (Caggiano, Vallo di Diano, Salerno, Italy) and Consorzio Meristema (Capaccio, Piana del Sele, Salerno, Italy), respectively.

The farms were located in the respective artichoke typical area of production and the cultivation was carried out according to the ordinary cultivation technique for the variety. The heads were harvested at commercial maturity. The bracts were representative samples of the residues that were discarded during the process of manufacturing of heads artichoke in oil. The leaves were representative samples collected in the field during the senescence phenological stage, before cutting the plants. ABs-Pe were collected during the first ten days of May 2014 and ALs-Pe at the end of June 2014. In fact this variety is the latest in the group of "late artichokes" (Ciancolini et al., 2012). ABs-Pa were recovered during the second ten days of March 2014 and ALs-Pa at the end of May 2014.

The raw materials were dried in an oven at 60 °C until constant dry weight. The dry samples were finely blended using a knife mill Grindomix GM 200 (Retsch, Haan, Germany), operating at intervals of brief cycles, to avoid any heating of the matrix and consequently increase in its adhesiveness. The resulting ground samples were sieved through a test sieve with a range of 300-600 μm to obtain powders with a homogeneous particle size distribution.

3A 4.3. Phenolic compounds extraction

Exhaustive extraction of phenolic fraction was performed by ultrasound-assisted extraction (UAE). The optimization of extraction parameters with respect to extraction time and number of extraction required for an exhaustive extraction was monitored by chromatographic analysis (area of main peaks < 2%).

AB-Pe, AL-Pe, AB-Pa and AL-Pa ground samples were extracted in triplicate, for 60 minutes (x 4) at 25 °C in a thermostat-controlled ultrasound bath (Labsonic LBS2, Treviglio, Italy) at the frequency of 20.0 kHz, using aqueous methanol (80% v/v) and a matrix/solvent ratio of 1:10. The extracts were pooled, filtered (Whatman No. 1 filter) and freeze-dried (freeze dryer Alpha 1-2 LD, Christ, Germany), after removal of organic solvent under vacuum at 40 °C in a rotary evaporator (Rotavapor R-200, Buchi Italia s.r.l, Cornaredo, Italy). Extraction yields obtained for AB-Pe, AL-Pe, AB-Pa and AL-Pa were 19.2 ± 0.1, 26.1 ± 0.4, 15.3 ± 0.1 and 27.5 ± 2.6 g extract/100 g dry matrix (DM), respectively.

3A 4.4. UHPLC-DAD-HRMS^n analysis

Chromatographic analyses were performed using a LTQ OrbiTrap XL mass spectrometer (ThermoFisher Scientific, Milan, Italy) connected to a Platin

Blue UHPLC system (Knauer, Labservice Analytica, Bologna, Italy), consisting of two Ultra High-Pressure Pumps, an autosampler, a diode array detector and a column temperature manager. A Kinetex PFP column (2.1x 100 mm, 2.6mm; Phenomenex, Bologna, Italy) was used at a flow rate of 400 µL/min. The column temperature was 25 °C, and the mobile phase was a gradient of water (A) and MeCN (B), both containing 0.1% formic acid. The gradient elution program is as follows: 5–60% B in 0–22 min and 60–98% B in 22–29 min. After each injection (5µL), cleaning and re-equilibration column were performed. UV spectra were acquired in the range of 200-600 nm, and the wavelengths 280, 325 and 350 nm were employed for the detection. The mass spectrometer, equipped with ESI source, was operated in negative mode. High purity nitrogen (N_2) was used as sheath gas (30 arbitrary units) and auxiliary gas (10 arbitrary units). High purity helium (He) was used as collision gas. Mass spectrometer parameters were as follows: source voltage 3.0 kV, capillary voltage –33 V, tube lens voltage –41.5 V, capillary temperature 300ºC. MS spectra were acquired by full range acquisition covering 140–1000 m/z. For fragmentation study, a data dependent scan was performed by deploying the collision-induced dissociation (CID). The normalized collision energy of the collision-induced dissociation (CID) cell was set at 30 eV and the isolation width of precursor ions was set at m/z 2.0. Phenolic compounds were characterized according to the corresponding spectral characteristics: UV and mass spectra, accurate mass, characteristic fragmentation, and retention time. Xcalibur software (version 2.2) was used for instrument control, data acquisition and data analysis.

3A 4.5. Quantitative analysis by UHPLC-UV

The analyses were performed using a Dionex Ultimate 3000 UHPLC system (ThermoFisher Scientific, Milan, Italy) constituted of an Ultimate 3000 RS

Pump, an Ultimate 3000 RS autosampler, an Ultimate 3000 RS column compartment and Ultimate 3000 RS variable wavelength detector. The chromatographic conditions were the same as those used for UHPLC–DAD-HRMS^n analysis. The UV chromatograms were recorded at 325 and 350 nm for quantification of caffeoylquinic acids and flavones, respectively. Calibration external standard method was used to quantify the phenolic compounds in artichoke by-product extracts (0.1, 0.5 and 3 mg/mL in MeOH/H_2O 1:9, v/v). Mixtures of 7 reference standards (CA, 5-CQA, 1,3-diCQA, L-GLU, A-GLU, L and A), at different concentrations, were used to prepare calibration curves. In the cases of the rutinoside and glucuronide derivatives of luteolin (L-RUT and L-GLA) and apigenin (A-RUT and A-GLA), L-GLU and A-GLU were used as reference standards for their quantification. CQAs and diCQAs were quantified using the calibration curves of 5-CQA and 1,3-diCQA, respectively. The amount of the compounds was finally expressed as mg per 100 g DM ± deviation standard (n = 3).

UHPLC quantitative method was validated, following the EURACHEM guidelines, in term of linearity, selectivity and precision. Linearity of calibration curves were evaluated in the concentration range of 0.1-10 µg/mL (0.5-10 µg/mL for 5-CQA and L) with 6 concentration levels, and triplicate injections for each level. UV peak areas of the external standards (at each concentration) were plotted against the corresponding standard concentrations (µg/mL). The regression curves were tested with the analysis of variance (ANOVA) and linear model was found appropriate over the tested concentration range (R^2 values > 0.99). Selectivity of the method was assessed by UHPLC-HRMS. Concerning the precision, intraday repeatability was estimated on all concentration levels (coefficient of variation <5%).

3A 4.6. Determination of inulin

The inulin content of AB and AL samples was estimated according to the AOAC enzymatic/spectrophotometric method (AOAC 999.03, 2002) and using the procedure described by the enzymatic k-FRUC kit. Briefly, 80 mL of hot distilled water (80 °C) were added to 1.0 g of dry sample and the solution was stirred at 80 °C for 15 min. Then, the solution was cooled to room temperature and diluted with distilled water at 100 mL in a volumetric flask and filtered (Whatman No. 1 filter). In order to remove sucrose, starch and reducing sugars, 0.2 mL of solution were treated with 0.2 mL of diluted sucrase/β-amylase/pullulanase/maltase solution for 40 °C for 30 min, and then with 0.2 mL of alkaline borohydride (10 mg/mL $NaBH_4$ in 50 mM sodium hydroxide) (40 °C for 30 min). To eliminate excess borohydride and to adjust the pH to 4.5, 0.5 mL of 200 mM acetic acid were added to the hydrolyzed solution. Successively, 0.1 mL of fructanase solution was added at two 0.2 mL aliquots of hydrolyzed solution (40 °C for 30 min) to affect the complete hydrolysis of fructans. A blank sample was obtained replacing fructanase with 0.1 mL of 0.1 M sodium acetate. Finally, 5.0 mL of PAHBAH working reagent were added to sample duplicates, sample blank, reagent sample (0.3 mL of 100 mM sodium acetate buffer), fructan/cellulose control sample and D-fructose standard (quadruplicate), and the mixtures were incubated in a boiling water bath for exactly 6 min. The test tubes were immediately placed in cold water for 5 min, and the absorbance of all solutions was measured at 410 nm against the reagent blank. The content of fructan (expressed as g/100 g DM) was calculated using Megazyme Mega-Calc^{TM} (www.megazyme.com).

3A 4.7. Cell cultures, treatments and viability assay

Human hepatocellular carcinoma Hep-G2 cell line was obtained from the Interlab Cell Line Collection (IST, Genoa, Italy). Hep-G2 cells were routinely grown in MEM (EBSS) medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% antibiotic mixture, 1% Non-Essential amino acids. All cell cultures were maintained at 37 °C in humidified 5% CO_2 atmosphere. To evaluate cell viability, the colorimetric MTT metabolic activity assay was used as described by Gazzerro et al., 2010. Briefly, Hep-G2 ($3x10^4$ cells/well) were cultured in a 96-well plate at 37 °C and exposed to various concentrations of extract for 24 or 48 hours. All experiments were performed in triplicate, and the relative cell viability was expressed as a percentage comparison with the untreated control cells.

3A 4.8. Cellular antioxidant activity (CAA) assay

CAA assay was performed according to the manufacturer's instructions (OxiSelect™ Cellular Antioxidant Activity Assay Kit, Cell Biolabs, San Diego, USA) and Wolfe's method (Wolfe and Liu, 2007) Experiments were performed using passages 4-8 of Hep-G2 cells. 2',7'-Dichlorodihydrofluorescin diacetate (DCFH-DA) probe, pure compound and extract solutions were prepared from stock solution in cell culture medium (MEM without FBS). Free radical initiator (ROS) was made in sterile PBS and diluted before use following the manufacturer's instructions. Briefly, 6×10^4/well were plated in a 96-well microplate in 100 µL complete medium, excluding the outside wells, and incubated for 24 h. After removal of medium, each well was treated with 50 µL of DCFH-DA probe solution. Then 50 µL of a range of concentrations of pure compounds and samples were added to the wells (at least in triplicate for each experimental point) and incubated at 37 °C

for 1 h in humidified 5% CO_2 atmosphere. Subsequently, the solution was gently removed and the cells were treated with 100 µL of free radical initiator (ROS). Emission fluorescence intensity at 530 nm with excitation at 485 nm was recorded every 5 min for 1 h at 37 °C using a multimode plate reader EnSpire 2300 (Perkin Elmer, Waltham , Massachusetts). A negative control without ROS (blank) and a positive control (PC) without quercetin or samples were tested in triplicate wells. The concentration range tested were: artichoke by-product extracts, 5-40 µg/mL; quercetin, 2-25 µM; 1,3-diCQA, 1-12 µM; 5-CQA, 2-20 µM; L-GLU and L, 7-30 µM.

CAA values were calculated by the area under-the curve (AUC) for the plot of fluorescence intensity against time, according to the equation:

$$CAA = 100 \times [1 - (AUC - AUC_{blank})/(AUC_{PC} - AUC_{blank})]$$

Subsequently, Fa/Fu (Fa = CAA and Fu = 100 – CAA) was plotted against the concentration of compounds on double logarithmic scale, and EC_{50} values were determined mathematically by linear regression of the data on the plot as the concentration where Fa/Fu = 1. EC_{50} values of quercetin and pure compounds were expressed in µM instead for extracts in µg/mL.

3A 4.9. Statistical analysis

Data were expressed as mean ± standard deviation of triplicates. The data were statistically analyzed using statistical software, Statgraphic Centurion XVI Version 16.1 from Statistical Graphics (Rockville, MD, USA). Statistically significant differences in the phenolic and inulin contents of artichoke by-product samples were evaluated by a multiple sample comparison procedure (ANOVA, Multiple Range Tests) that compare two or more independent samples of variable data. The method used to discriminate among the means is Fisher's least significant difference (LSD) procedure. Differences between means were considered statistically significant at the 95% confidence level

($p<0.05$). Pearson correlations between pair of variables (CAA and phenolic content) were evaluated by a Multiple-Variable Analysis procedure. P-value was used to estimate the statistical significance of the Pearson correlations and $p < 0.05$ indicate statistically significant non-zero correlations at the 95.0% confidence level.

CHAPTER III
SECTION B

RECOVERY OF BIOACTIVE COMPOUNDS FROM ARTICHOKE BY-PRODUCTS BY PRESSURIZED HOT WATER EXTRACTION (PHWE)

3B 1. Introduction

Over the past twenty years, numerous studies report that food industry by-products are a valuable source of bioactive compounds. Most of these products are not used for the lack of suitable extraction techniques (Herrero et al., 2006; Schieber et al., 2001). In solid-liquid extractions, the compounds of interest must be dissolved in the extraction solvent while interfering compounds must remain in the matrix (Pronyk and Mazza, 2009). These ideal conditions rarely is reached, therefore to improve recovery of bioactive compounds from plants is required a continuous research for the best extraction conditions in terms of solvent, particle size, temperature, pressure, time and amount of matrix (Kaur et al., 2008; Ku and Mun, 2008; Spigno et al., 2007; Wijngaard and Brunton, 2010). Furthermore, conventional extraction methods used have various drawbacks: long time, are laborious, have low selectivity and low yield of extraction and use large volumes of toxic solvents. Currently is seeking to develop and apply extraction methods that can overcome the above mentioned drawbacks (King, 2000). Primarily qualitative and quantitative studies of bioactive compounds in plants are based on the selection of a correct method of extraction (Smith, 2003; Sasidharan et al., 2011). The extraction is the first step in every study and plays a key role in the final result (Hennion et al., 1998). The development of modern techniques such as mass spectrometry makes the analysis of bioactive compounds simpler compared to before, even if the success mainly depends of extraction method used (Poole et al., 1990). Non-conventional extraction methods are environmentally friendly and are characterized by reduced use of organic and synthetic solvents, shorter cycle times and improved extraction yield, compared to conventional methods (maceration, Soxhlet). The Green Chemistry is a conception of chemistry that aims to protect the environment by developing procedures that restrict the use and generation of hazardous substances (de la Guardia and Armenta, 2011)

Another disadvantage of the traditional methods is that often are required concentration and washing steps after extraction. The non-conventional extraction methods have been developed over the last fifty years, and are based on the respect of the principles of Green Chemistry: the most used are ultrasound-assisted extraction (UAE), pulsed-extraction electric field (PEF), enzyme-assisted extraction (EAE), microwave assisted extraction (MAE), pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE) (Azmir et al., 2013).

Subsequently to qualitative and quantitative analysis of artichoke by-products and their evaluation as potential raw materials for nutraceutical, cosmetic and pharmaceutical sectors, a new non-conventional extraction method rapid, economic and environmentally friendly was developed and optimized using pressurized hot water extraction (PHWE) and response surface methodology (RSM) to recover main bioactive compounds (caffeoylquinic acids, CQAs and flavone glycosides, FLs) from bracts (ABs) and leaves (ALs).

In literature is reported evaluation of sequential process based on the use of ultrasound extraction and membrane technology for phenolic recovery from artichoke waste (Rabelo et al., 2016). Furthermore artichoke waste is reported as a potential source of phenolic compounds and bioenergy by environmentally friendly solvent extraction procedure using 50% v/v aqueous ethanol as solvent and response surface methodology (Zuorro et al., 2016; Zuorro et al., 2014). Tena and co-authors developed a method to determine caffeoylquinic acids in herbal extracts, feed additives and finished feed using for the first time focused ultrasound solid liquid extraction (FUSLE) with good recovery rates (Tena et al., 2015).

PHWE is a green extraction technique, fully automated, that employs water at elevated temperatures and pressures in liquid state. In these conditions water possesses polarity similar to alcohols, beginning a good solvent for a wide range of analytes, because of its dielectric constant can be reduced with

increasing temperature. PHWE allows reduction organic solvent consumption, short time, low costs and is environmentally-friendly due to water nature (non-toxic, recyclable, readily available) and also a suitable technique for industrial applications (Plaza and Turner, 2015).

RSM was employed to find the best extraction conditions, studying interaction among different variables, with a minimum number of experiments, through Experimental of Design (DoE). In particular Box-Behnken Design (BBD) utilized is an independent quadratic design (3-level design) that involves only three levels to run an experiment, because it doesn't contain any points at the vertices of the experiment region, avoiding all the corner points and the star points. Experiments are runs at different factor values, called levels. Each run of an experiment involves a combination of the levels of the factors that need to be investigated. The chemometric analysis applied to the optimization of extraction procedure consists in a first phase in which all parameters that can have an influence on the system are studied and significant parameters and the ranges for each of them are defined (named experimental domains). Then a second stage of practical experimentation is necessary to identify the most significant parameters and to obtain a model for system optimization (Ferreira et al., 2007).

The main aim of this work is the development of PHWE procedure allowing high bioactive compounds content, minimizing cynarin (1,3-diCQA) formation and low extraction yield in order to obtain final extracts in a more concentrated manner, through the construction of a response surface.

3B 2. Results and discussion

3B 2.1. Preliminary experiments

Preliminary experiments were first performed to set some specific technical parameters (particle size, matrix/dispersant ratio, maximum operating temperature), to reduce the number of independent variables and width of the ranges in the chemometric analysis. For each parameter the optimal values were selected taking into account extraction efficiency and operation of the employed equipment. Various dimensional ranges have been tested, and the selected size fraction was 300-600 μm, because it was observed greater extraction efficiency than the particles with larger particle size. In the case of particle size smaller, clogging of the extraction system occurred. Particle size must increase the contact surface between the solvent and the matrix avoiding formation of agglomerates. Moreover dispersant was employed to permit uniform particle distribution and the optimum matrix/dispersant ratio (diatomaceous earth) was 1:2 (no clogging of the system was observed). Previous experiments have demonstrated the absence of interaction between dispersant and analytes. As regards the size of the steel extraction cell, different volumes were evaluated (5, 11 and 33 mL). Comparable results were obtained with all kind of cells, and for all the subsequent experiments cells of 11 mL were utilized. Finally, the maximum operating temperature was established in order to avoid or reduce the typical isomerisation phenomena of caffeoyl quinic acids. For this purpose, extractions were carried out at different temperatures (100, 110, 115, 120 and 130 °C) to determine the limit temperature for the formation of cynarin (1,3-dicaffeoyl quinic acid, 1,3-diCQA), product of isomerisation of 1,5-dicaffeoyl quinic acid, 1,5-diCQA (Slanina et al., 2001), the most abundant compound found in artichoke by-

products. For temperatures higher than 115 °C was observed, by UHPLC-UV analysis of PHWE extracts, a significant conversion (>10%) of 1,5-diCQA in 1,3-diCQA (data not shown). Therefore, a temperature of 110 °C was chosen as the maximum operating temperature. The parameters flush volume % and pressure have been set to 150% and 1500 psi, respectively, based on previous experience. Ideal pressure recommended for extractions with pressurized water is 1500 psi (Teo et al., 2010).

3B 2.2. Optimization design

Subsequently, the effects of experimental factors, temperature, modifier %, number of cycles of extraction and static extraction time have been carefully studied by chemometric analysis. In fact, it is widely reported in literature (Teo et al., 2010) that they can exert a significant effect on extraction efficiency. These parameters can influence the process directly or indirectly, by interacting among them. Therefore it is crucial to simultaneously evaluate their effects on response factors. Regarding modifier, ethanol was utilized, in small percentages, to exert a positive effect on the solubility of the analytes maintaining the extraction conditions green (Mustafa and Turner, 2011). Ethanol is generally recognized as safe (GRAS). Low and high values of experimental parameters have been selected on the basis of preliminary experiments as: A. temperature: 60-110 °C; B. number of cycles: 2-4; C. modifier %: 0-10%, v/v; D. static extraction time: 4-10 minutes. Response factors were: area of the main chromatographic peaks (5-CQA, 1,5-diCQA, sum of luteolin glycosides, L-glycosides, sum of apigenin glycosides, A-glycosides, apigenin, A) to evaluate the efficiency extraction and recovery of main compounds and area of chromatographic peak of cynarin (1,3-diCQA) in order to control isomerisation phenomena. For each response factor has been set the desired effect (maximize area of main analytes and minimize extraction yields and area of cynarin).

Table 6 *Optimization design of experiments (Box–Behnken design).*

Experimental factors					Response factors				
Run	**Temp.**	**Cycles**	**Modifier**	**Static time**	**5-CQA**	**1,3-diCQA**	**A-GLA**	**1,5-diCQA**	**Yield**
n°	**°C**	**n°**	**%**	**min**	**area mAU*min**	**area mAU*min**	**area mAU*min**	**area mAU*min**	**%**
1	110	2	5	7	11.04	0.96	16.78	44.16	22.1
2	110	3	5	4	13.83	0.82	17.64	60.05	25.6
3	85	3	10	10	11.28	0.13	17.60	51.48	22.6
4	110	3	0	7	12.32	1.92	17.58	47.95	23.7
5	60	3	5	10	8.19	0.07	14.82	36.02	20.2
6	60	3	5	4	6.77	0.06	13.11	28.53	19.8
7	85	3	5	7	12.09	0.20	17.41	58.64	23.1
8	85	2	5	10	9.04	0.13	17.03	38.83	21.4
9	85	3	5	7	10.80	0.19	17.43	51.38	22.8
10	85	4	5	4	8.85	0.14	16.34	38.68	19.0
11	85	2	5	4	10.05	0.13	15.37	47.60	20.3
12	85	3	5	7	11.30	0.20	17.33	52.76	20.7
13	60	4	5	7	7.99	0.05	13.77	35.44	19.2
14	110	3	5	10	13.06	1.94	17.18	49.88	24.2
15	85	3	0	4	9.37	0.14	14.42	41.85	17.7
16	85	3	5	7	10.58	0.17	17.85	49.65	19.5
17	110	3	10	7	13.51	0.95	17.15	54.14	24.3
18	85	4	0	7	10.16	0.07	15.35	43.05	21.3
19	85	3	10	4	10.63	0.06	17.58	52.19	21.0
20	85	3	0	10	9.35	0.23	14.50	37.07	19.5

Experimental factors					Response factors				
Run	**Temp.**	**Cycles**	**Modifier**	**Static time**	**5-CQA**	**1,3-diCQA**	**A-GLA**	**1,5-diCQA**	**Yield**
n°	**°C**	**n°**	**%**	**Min**	**area mAU*min**	**area mAU*min**	**area mAU*min**	**area mAU*min**	**%**
21	85	2	0	7	9.27	0.15	15.75	39.37	20.6
22	60	2	5	7	7.78	0.07	12.72	31.47	18.5
23	60	3	0	7	4.85	0.02	8.18	16.01	16.0
24	85	4	5	10	9.78	0.18	17.21	40.78	21.3
25	85	4	10	7	10.79	0.19	17.19	46.99	23.4
26	60	3	10	7	9.10	0.08	15.46	40.22	21.9
27	85	2	10	7	10.23	0.11	17.03	49.65	20.8
28	110	4	5	7	12.50	1.28	17.19	45.77	25.3

The chemometric analysis was performed using BBD, randomized, 2-factor model, with 28 experimental runs (Table 6), 4 center points and an error degree freedom of 13. The response factors were determined by UHPLC-UV analysis of PHWE extracts, after normalizing volume of extracts, and determination of the extraction yield (g/100 g of dry matter), following removal of ethanol with rotavapor and subsequent lyophilisation (Table 6).

The statistical significance of the influence of experimental factors on response factors was evaluated by analysis of variance (ANOVA). Values of p-value lower than 0.05 indicate that the effect is statistically significant on the determined response factor. The effects of each estimated experimental factors for the considered response factors are shown in Pareto charts (Figure 5 a-f) where the effects is in descending order of significance. The vertical line indicates the 95% confidence level (p-value <0.05). The size of the effects is proportional to the length of the bar. As shown in Figure 5 a-d, regarding all bioactive compounds content only two parameters are statistically significant at level of p = 0.05, temperature and modifier %, exerting a positive effect. It has observed the same trend for extraction yield % (Figure 5 e). The increase of temperature and modifier % determines an improvement in the efficiency of extraction (positive effect), in terms of yield and content of bioactive compounds.

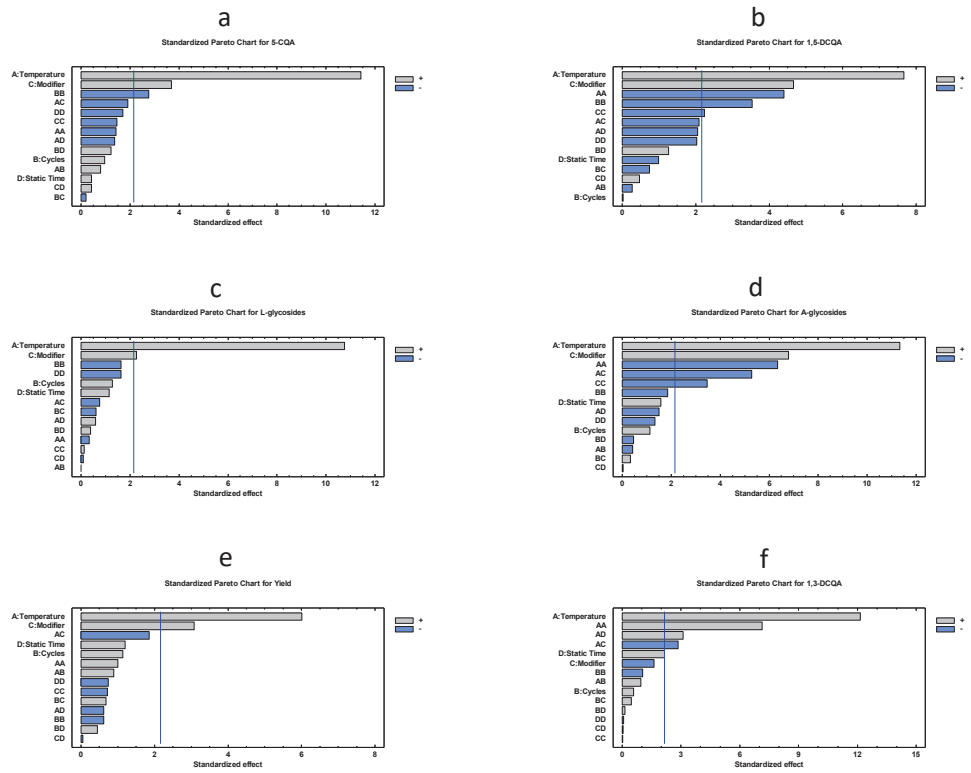

Figure 5 *Pareto charts and analysis of variance (ANOVA); a 5-CQA, b 1,5-diCQA, c L-glycosides, d A-glycosides, e Yield, f 1,3-diCQA.*

The positive influence of the temperature on the efficiency of extraction is related to the increase of the water solvent power at high temperatures. Similarly the solvent modifier has a positive influence, because it improves solubility of analytes and facilitates their desorption. As regards cynarin Figure 5 f, only temperature is statistically significant (p-value 0.0000) with an increase of its formation by isomerisation (increased chromatographic area) and positive effect. These results indicate that temperature is a crucial parameter because it simultaneously determines an increase in the efficiency of extraction (desired effect) and phenomena of alteration of the original compounds (side effect). Regarding other experimental factors no significance was observed. Analysis of interactions among experimental factors demonstrate for cynarin a positive effect between temperature and static time

and negative effect between temperature and modifier % (both effects statistically significant).

In Figure 6 desirability plot is shown obtained by considering simultaneously the effects of experimental parameters investigated on all response factors analyzed. Temperature exerts the most marked effect with initial increase, due to the increased efficiency of extraction, followed after 95-97 °C by a decrease, due to the beginning of isomerisation phenomena and to an increase of the amount of components co-extracted with target compounds (increased extraction yield). Instead, the effect of modifier %, although statistically significant, by ANOVA test, is less pronounced. The PHWE optimal extraction conditions extrapolated from BBD are: temperature: 97 °C; modifier: 10%; number of cycles: 3; static extraction time: 4 minutes. With these conditions, the design provides a degree of desirability equal to 71%.

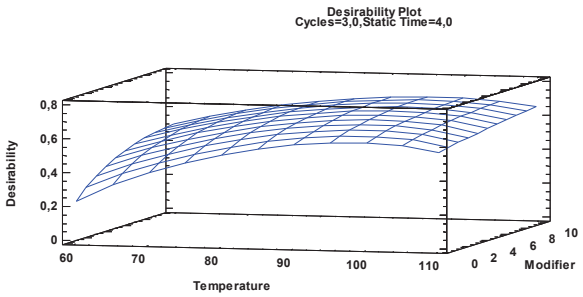

Figure 6 *Desirability plot.*

3B 2.3. PHWE validation

The optimal conditions PHWE extrapolated and recommended from BBD have been experimentally validated (quadruplicate extractions) by evaluating efficiency of extraction of the main bioactive compounds, expressed as recovery %. Recovery was determined by quantitative analysis UHPLC-UV, comparing the levels of each compound, expressed in terms of mg/100 g DM

in PHWE extracts and in exhaustive extracts obtained by solid-liquid extraction assisted by ultrasounds. In addition, to assess the applicability of the procedure, PHWE extraction was also applied to others artichoke by-products, bracts and leaves of two different cultivars, artichoke of Bianco di Pertosa and Tondo di Paestum (AB Pe-AL Pe/AB Pa-AL Pa). As shown in Figures 7-8, the developed PHWE procedure allows the exhaustive extraction of the main components from bracts (∑-CQAs 85.65-93.91%, ∑-DCQAs 88.74-92.89% and ∑-FLs 92.63-103.75%) and leaves (∑-CQAs 95.51-98.84%, ∑-DCQAs 104.00-105.00% and ∑-FLs 95.30-100.00%) of artichoke, showing that it can be used as an alternative, rapid, large-scale, economic to conventional extraction methods for the recovery of bioactive components of artichoke's by-products. Furthermore, the results are comparable for both cultivars (Pe and Pa) and types of artichoke by-products, highlighting the broad applicability of the developed technique. It was also assessed recovery % in optimal condition using only water as solvent and results (Figures 7-8) show only slight decrease efficiency, suggesting the possibility of using only water, resulting in lowered costs for the entire extraction procedure.

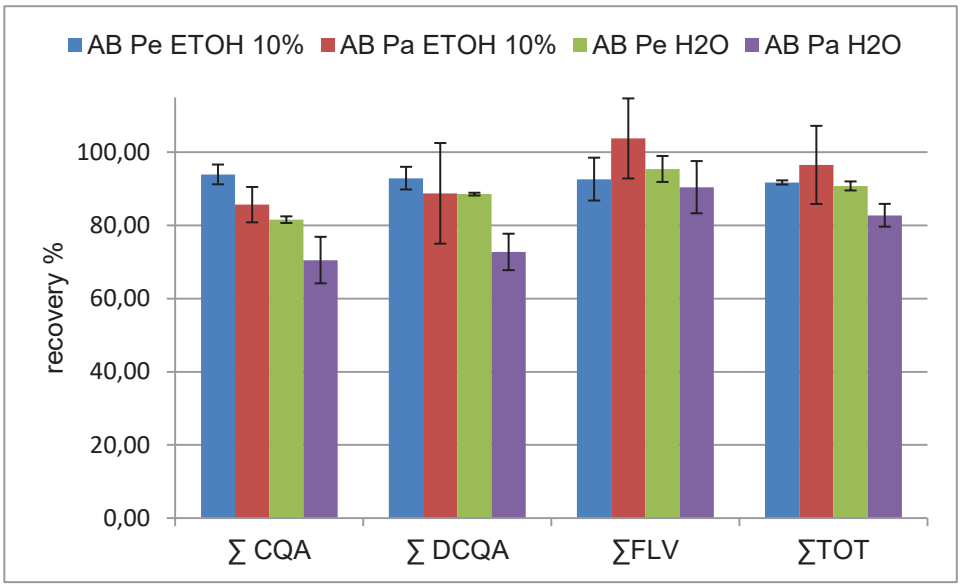

Figure 7 *PHWE recovery % of main compounds in AB Pe and AB Pa (n = 4) in optimal conditions using ethanol 10% and water.*

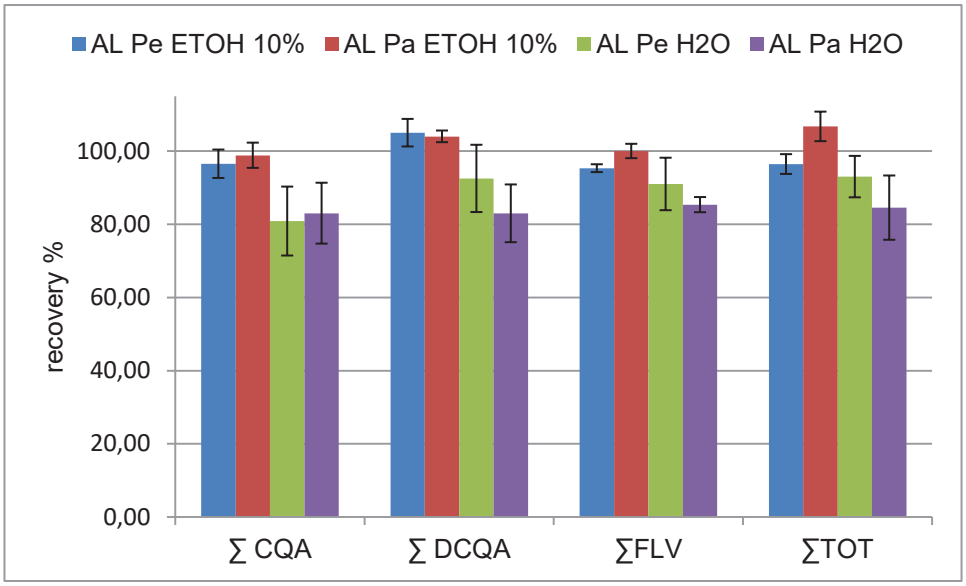

Figure 8 *PHWE recovery % of main compounds in AL Pe and AL Pa (n = 4) in optimal conditions using ethanol 10% and water.*

3B 2.4. Application of PHWE technique and comparison with commercial product

Finally PHWE extracts of ABs and ALs were characterized by quantitative analysis UHPLC-UV to determine CQAs and FLs content and comparing them to a commercial product (artichoke leaves extracts sold in pharmacy). In terms of quantity (Figure 9), the bracts have CQAs content and FLs respectively in the range of 12-25 mg CQAs/g of extract and 2-4 mg FLs/g of extract, while in the leaves were estimated CQAs and FLs content respectively in the range of 11-30 mg CQAs/g of extract and FLs 4-19 mg/g of extract. These contents are similar and / or superior to commercial product (CQAs 25 mg/g of extract and FLs 5 mg/g of extract).

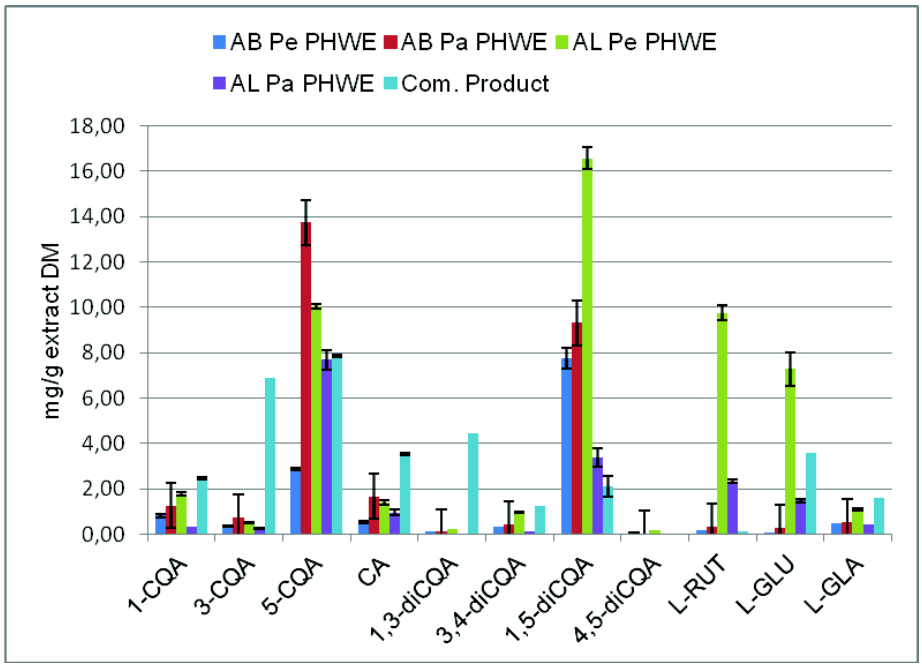

Figure 9 *Quantitative analysis by HPLC-UV of PHWE extracts in optimal conditions compared with commercial product, (n = 4).*
CQA, caffeoylquinic acid; diCQA, dicaffeoylquinic acid; CA, caffeic acid; RUT, rutinoside; GLU, glucoside; GLA, glucuronide.

Differences were also observed in the qualitative profile (Figure 10). In the latter (Figure 10 c), the main component of the CQAs class is represented by cynarin (1,3-diCQA), while in PHWE extracts the main CQA is 1,5-diCQA. Cynarin is not a natural constituent of the artichoke but an artefact of the isomerisation of the compound 1,5-diCQA during aqueous extractions performed at high temperatures (Schutz et al., 2006). To confirm this thesis there is also the highest content of caffeic acid in commercial products always arising from isomerisation and hydrolysis of the CQAs.

3B 2.5. Cellular antioxidant activity (CAA) of PHWE extracts

Cellular antioxidant activity of PHWE extracts was evaluated and compared with corresponding exhaustive extracts (EXH). Results are reported in Table 7 and values of EC50 of PHWE extracts are slightly higher than EXH extracts because of more concentrated and purified nature. PHWE procedure in optimal conditions provides selectively extraction of CQAs and FLs and the greatest activity was observed for by-product extracts with the highest content of phenolic compounds, confirming that they are responsible for the antioxidant activity.

Table 7 *Cellular antioxidant activity of PHWE extracts.*

	PHWE	**EXH**
Extract	EC_{50} (μg/mL)	EC_{50} (μg/mL)
AB Pe	33.6 ± 1.9	44.6 ± 5.5
AB Pa	10.5 ± 3.6	29.5 ± 3.3
AL Pe	20.5 ± 0.6	26.6 ± 3.5
AL Pa	83.2 ± 15.9	124.1 ± 9.4

Values are means of three replicates ± standard deviation (SD).

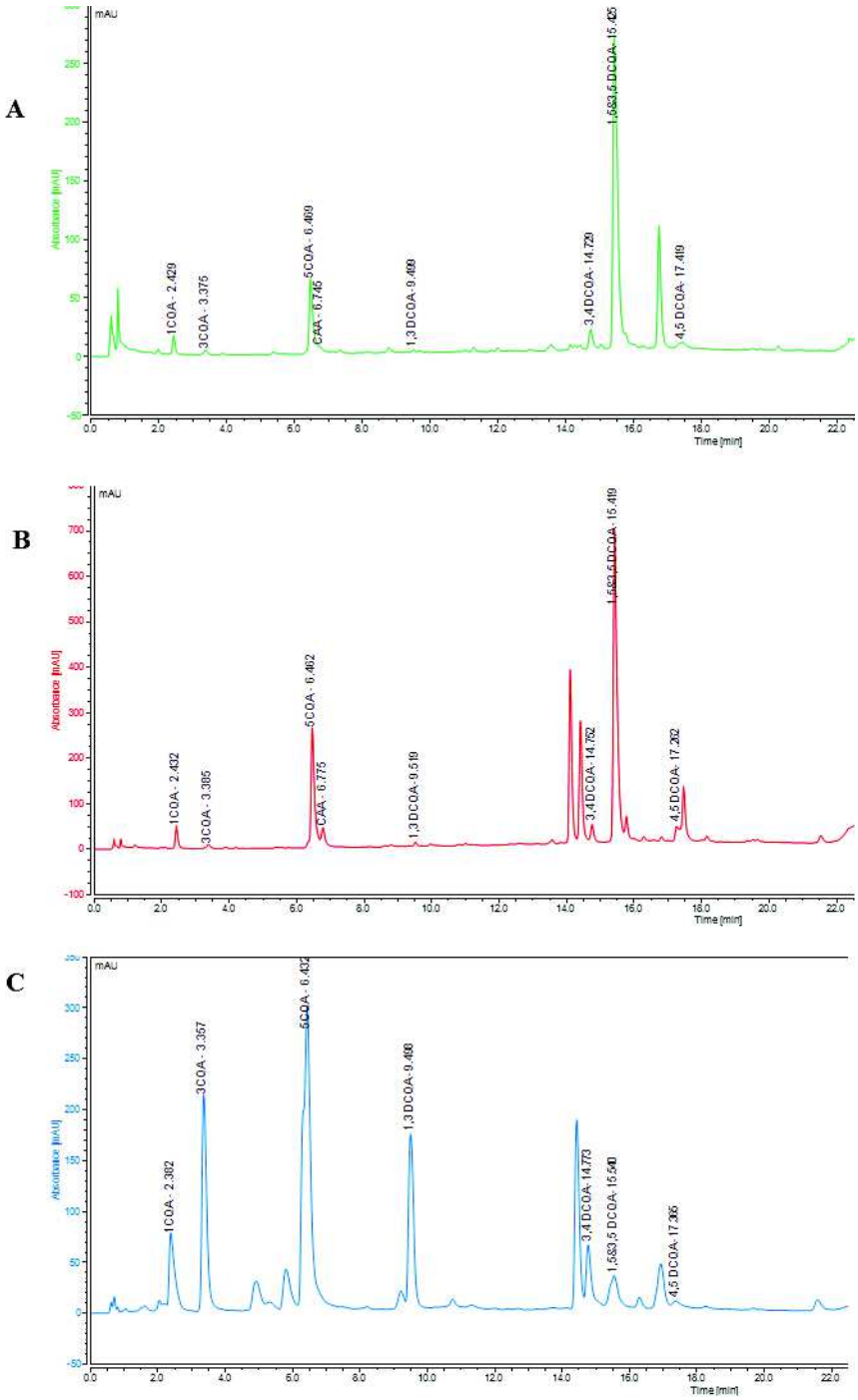

Figure 10 *HPLC-UV profiling (325 nm) of A AB Pe PHWE, B AL Pe PHWE and C commercial product.*

3B 3. Conclusions

This work shows that it is possible to recover bioactive compounds from artichoke by-products using PHWE, green, rapid, economic, environmentally friendly and potentially applicable on an industrial scale extraction technique. Artichoke by-products, with a negative impact on environment could be reused as a valuable source of phenolic compounds (caffeoylquinic acids and flavones glycosides). To find the best operating conditions chemometric analysis was performed that has allowed finding the optimal conditions for extraction, considering the influence of temperature, modifier %, number of cycles and static time and their interactions on response factors (area of the main chromatographic peaks, area of cynarin and yield of extraction %). The optimized conditions are temperature 97 °C, modifier (CH_3CH_2OH) 10% v/v, number of cycles 3, static extraction time 4 minutes. The PHWE extraction in optimized conditions allowed the exhaustive extraction of the main components of bracts and leaves. Finally, PHWE extracts were compared with a commercial extract of artichoke leaves and cynarin content is different. The results show that PHWE procedure is extremely suitable for recovery of bioactive compounds and it seems to be milder compared to other techniques, avoiding the production of artefacts. This research responds to the need of our society that requires a reduction of wastes and a valorisation of them, transforming waste into a resource.

3B 4. Materials and methods

3B 4.1.Solvents and reagents

Methanol (CH_3OH) and acetonitrile (MeCN) HPLC grade were purchased from Romil (Cambridge, UK) and ultrapure water (18 MΩ) was prepared by a Milli-Q purification system (Millipore, Bedford, USA). Acetonitrile ultrapure and water ultrapure were purchased by Romil (Cambridge, UK). Absolute ethanol (CH_3CH_2OH) and formic acid were purchased from Sigma-Aldrich (Milan, Italy). Reference standards luteolin (L), luteolin-7-O-glucoside (L-GLU), chlorogenic acid (5-CQA), cynarin (1,3-diCQA), apigenin 7-O-glucoside (A-GLU) were purchased by Extrasynthase (Lyon, France). Caffeic acid (CA) and apigenin (A) were obtained from Sigma-Aldrich (Milan, Italy). The stock solutions of analytes were prepared in methanol at concentration of 1 mg/mL and stored at dark (4 °C). A mixture containing all analytes was prepared at concentration of 100 µg/mL and was used to prepare calibration curves in MeOH/H_2O 1:9, v/v.

3B 4.2. Plant material

The bracts and leaves of "Bianco di Pertosa" (AB Pe and AL Pe) and "Tondo di Paestum" (AB Pa and AL Pa) samples were supplied by Cafaro Carmine (Caggiano, Vallo di Diano, Salerno, Italy) and Consorzio Meristema (Capaccio, Piana del Sele, Salerno, Italy), respectively. ABs-Pe were collected during the first ten days of May 2014 and ALs-Pe at the end of June 2014. ABs-Pa were recovered during the second ten days of March 2014 and ALs-Pa at the end of May 2014. The raw materials were dried in an oven at 60 °C until constant dry weight. The dry samples were finely blended using a knife mill Grindomix GM 200 (Retsch, Haan, Germany). The resulting ground samples

were sieved through a test sieve with a range of 300-600 µm to obtain powders with a homogeneous particle size distribution. Exhaustive reference extractions were performed by solid liquid extraction assisted by ultrasounds in triplicate, for 60 minutes (x 4) at 25 °C in a thermostat-controlled ultrasound bath (Labsonic LBS2, Treviglio, Italy) at the frequency of 20.0 kHz, using aqueous methanol (80% v/v) and a matrix/solvent ratio of 1:10. The extracts were pooled, filtered (Whatman No. 1 filter) and freeze-dried, after removal of the organic solvent under vacuum at 40 °C in a rotary evaporator (Rotavapor R-200, Buchi Italia s.r.l, Cornaredo, Italy).

3B 4.3. Pressurized hot water extraction (PHWE)

A Dionex Accelerated Solvent Extraction (ASE) 200 System (Dionex, Sunnyvale, CA) equipped with a solvent controller unit, with 11 mL stainless steel cells (Restek, Italy) were used for PHWE extractions. Briefly 1 g of sample was placed into cell after homogenization with 1 g diatomaceous earth (dispersant agent, *Hydromatrix*, Varian), matrix/dispersant ratio was 1:2. Fixed parameters were: purge 100 sec, flush 150%, pressure 1500 psi and for preliminary experiments temperature 100 °C, 2 cycles of extraction and static time of 5 minutes. Extracts obtained are reconstituted up to a final volume of 30 mL, (after removing organic solvent with rotary evaporator and 1 mL was utilized for chromatographic analysis (after filtration with syringe filter PTFE 0.45 µm, Phenomenex, Italy), the remaining part was lyophilised (freeze dryer Alpha 1-2 LD, Christ, Germany). All preliminary experiments were performed using AB Pe. Yield extraction % was calculated as mg PHWE lyophilized extract/mg sample DM x 100.

3B 4.4. HPLC analysis

HPLC-UV analyses were carried out with a Dionex Ultimate 3000 UHPLC system (ThermoFisher Scientific, Milan, Italy). A Kinetex PFP column (2.1x 100 mm, 2.6mm; Phenomenex, Bologna, Italy) was used at a flow rate of 400 µL/min. The column temperature was 25 °C, and the mobile phase was a gradient of water (A) and MeCN (B), both containing 0.1% formic acid. The gradient elution program is: 5–60% B in 0–22 min and 60–98% B in 22–29 min. After each injection (5µL), cleaning and re-equilibration column were performed. UV spectra were acquired in the range of 200-600 nm, and the wavelengths 280, 325 and 350 nm were employed for the detection. Mixtures of 7 reference standards (CA, 5-CQA, 1,3-diCQA, L-GLU, A-GLU, L and A), at different concentrations, were used to produce calibration curves. In the cases of the rutinoside and glucuronide derivatives of luteolin (L-RUT and L-GLA) and apigenin (A-RUT and A-GLA), L-GLU and A-GLU were used as reference standards for their quantification. CQAs and diCQAs were quantified using the calibration curves of 5-CQA and 1,3-diCQA, respectively. The amount of the compounds was expressed as mg per 100 g DM ± deviation standard (n = 3).

3B 4.5. Experimental of design (DoE)

The data were statistically analyzed using statistical software, Statgraphic Centurion XVI Version 16.1 from Statistical Graphics (Rockville, MD, USA). The whole DoE procedure was performed by response surface methodology and in particular using Box–Behnken design (BBD).

3B 4.6. Cell cultures, treatments and viability assay

Human hepatocellular carcinoma Hep-G2 cell line was obtained from the Interlab Cell Line Collection (IST, Genoa, Italy). Hep-G2 cells were routinely grown in MEM (EBSS) medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% antibiotic mixture, 1% Non-Essential amino acids. All cell cultures were maintained at 37 °C in humidified 5% CO_2 atmosphere. To evaluate cell viability, the colorimetric MTT metabolic activity assay was used as described by Gazzerro et al., 2010. Briefly, Hep-G2 ($3x10^4$ cells/well) were cultured in a 96-well plate at 37 °C and exposed to various concentrations of extract for 24 or 48 hours. All experiments were performed in triplicate, and the relative cell viability was expressed as a percentage comparison with the untreated control cells.

3B 4.7. Cellular antioxidant activity (CAA) assay

CAA assay was performed according to the manufacturer's instructions (OxiSelect™ Cellular Antioxidant Activity Assay Kit, Cell Biolabs, San Diego, USA) and Wolfe's method (Wolfe and Liu, 2007) Experiments were performed using passages 4-8 of Hep-G2 cells. 2',7'-Dichlorodihydrofluorescin diacetate (DCFH-DA) probe, pure compound and extract solutions were prepared from stock solution in cell culture medium (MEM without FBS). Free radical initiator (ROS) was made in sterile PBS and diluted before use following the manufacturer's instructions. Briefly, 6×10^4/well were placed in a 96-well microplate in 100 µL complete medium, excluding the outside wells, and incubated for 24 h. After removal of medium, each well was treated with 50 µL of DCFH-DA probe solution. Then 50 µL of a range of concentrations of pure compounds and samples were added to the wells (at least in triplicate for each experimental point) and incubated at 37 °C

for 1 h in humidified 5% CO_2 atmosphere. Subsequently, the solution was gently removed and the cells were treated with 100 µL of free radical initiator (ROS). Emission fluorescence intensity at 530 nm with excitation at 485 nm was recorded every 5 min for 1 h at 37 °C using a multimode plate reader EnSpire 2300 (Perkin Elmer, Waltham, Massachusetts). A negative control without ROS (blank) and a positive control (PC) without quercetin or samples were tested in triplicate wells. The concentration range tested were: artichoke by-product extracts, 5-40 µg/mL; quercetin, 2-25 µM; 1,3-diCQA, 1-12 µM; 5-CQA, 2-20 µM; L-GLU and L, 7-30 µM.

CAA values were calculated by the area under-the curve (AUC) for the plot of fluorescence intensity against time, according to the equation:

$$CAA = 100 \times [1 - (AUC - AUC_{blank})/(AUC_{PC} - AUC_{blank})]$$

Subsequently, Fa/Fu (Fa = CAA and Fu = 100 – CAA) was plotted against the concentration of compounds on double logarithmic scale, and EC_{50} values were determined mathematically by linear regression of the data on the plot as the concentration where Fa/Fu = 1. EC_{50} values of quercetin and pure compounds were expressed in µM instead for extracts in µg/mL.

CHAPTER IV
SECTION A

CHEMICAL PROFILE OF ROASTED HAZELNUT SKINS BY-PRODUCT

Piccinelli, A.L., **Pagano**, **I**., Esposito T., Mencherini T., Porta A., Petrone A., Gazzerro P., Picerno P., Sansone F., Rastrelli L., & Aquino, R.P. (2016). HRMS profile of a hazelnut skins proanthocyanidin-rich fraction with antioxidant and anti-Candida albicans activities. *Journal of Agricultural and Food Chemistry*, *64*(3), 585-595.

4A 1. Introduction

Hazelnut (*Corylus avellana* L.) is a fruit belonging to the family *Betulaceae* (Shahidi et al., 2007). The plant is cultivated in many countries such as Turkey, Italy, Spain and United States, but Turkey detains around 70% of the world market, followed by Italy (20% of the market) with a production of 12000 tonnes per year.

The hazelnut market represents a substantial source of income for Italian agricultural economy, and hazelnut industry is mainly concentrated in four regions: Campania (37%), Lazio (35%), Piedmont (14%) and Sicily (13%) (Briamonte, 2001). Hazelnut is rich in oils, essential elements, vitamin E and B, sterols and various phenolic compounds, which are responsible for organoleptic properties, such as astringency and bitterness (Schmitzer et al., 2011). Moreover, it is a good source of tocopherols and bioactive compounds and makes a beneficial effect on health by reducing oxidative stress, risk of cancer, stroke and inflammation (Schmitzer et al., 2011). In food industry, pastry and confectionery, hazelnut is used in various forms (toasted or natural, whole or chopped, cut into flakes or ground) and for the production of creams, ice creams, cereals, biscuits, nougat and also for cooking oil production (Platteau et al, 2011). The fruits are constituted by external green leaves, which are removed mechanically immediately after harvest, hard and woody shell, seed, which is the edible portion and seed-coat, a dark film, (perisperm) (Shahidi et al., 2007). Perisperm, shell and leaves can be used as by-products, with or without treatments. Currently the hard shells are used as fuel, while the leaves as organic fertilizer. However, these by-products have been defined as potential sources of antioxidants and functional food ingredients (Shahidi et al., 2007). Among the various treatments roasting process improves the flavour of the fruit. During this process perisperm, which represents about 2.5% of the total weight (Shahidi et al., 2007), tends to absorb fat by allowing

the separation of the skins from the seed of hazelnut and is discarded upon roasting. Some studies have shown that compared to other hazelnut by-products, perisperm has a high antioxidant power due to the presence of polyphenolic compounds (Alasalvar et al., 2009). Perisperm appears to provide protection against harmful effects: it reduces the risk of various diseases such as type 2 diabetes, inflammation, certain types of cancer and coronary heart disease (CHD) (Ternus et al., 2009). Several studies have shown that bioactive secondary metabolites in seeds, such as peanuts, almonds and hazelnuts, are main concentrated in perisperm (Shahidi et al., 2007; Schmitzer et al., 2011). In addition, some studies conducted on different cultivars of hazelnut show a correlation between the polyphenolic content and antioxidant activity. It was a significant difference between hazelnut with and without perisperm. Hazelnut with perisperm shows greater antioxidant activity than the one without perisperm, and roasting process does not induce a significant difference in antioxidant activity (Schmitzer et al., 2011). These aspects allowed concluding that phenolic content of perisperm is responsible for antioxidant activity and which does not undergo changes during the processes of transformation, such as roasting or blanching (Schmitzer et al., 2011). The perisperm is extremely rich in polyphenolic components as flavan-3-ols, proanthocyanidins, and phenolic acids, and fiber (Alasalvar et al., 2009; Locatelli et al., 2010; Shahidi et al., 2007), and also contains about 65% of dietary fiber, 8% protein and 9% of oil (Anil, 2007). In particular proanthocyanidins (PAs) are oligomers or polymers, also known as condensed tannins, classified in procyanidins (PCs), propelargonidins (PPs), or prodelfinidins (PDs) depending on the unit flavan-3-ol, units (epi)-catechin (eC), (epi)-afzelechin (eA), or (epi)-gallocatechin (eG), respectively (Monagas et al., 2009). PAs contained in hazelnut skins are mainly PAs B-type, linked at C4-C6 or C4-C8 positions, or doubly linked through an additional bond between C2-O7 (A-type) and with an average degree of polymerization (DP)

of 7-11 (Gu et al., 2003a; Monagas et al., 2009) but also heteropolymers of procyanidins and prodelfinidins. PAs B-type are more common in foods than A-type (Figure 11). From commercial point of view, roasted hazelnut skins (RHS), thanks to their brown colour, can be used as natural food colorant, replacing the synthetic dyes (Özdemir et al., 2014). Nowadays RHS doesn't possess important commercial value, thus valorisation of this by-product is very important for hazelnut industry, because the disposal of this biodegradable waste material represents a serious environmental and expensive problem.

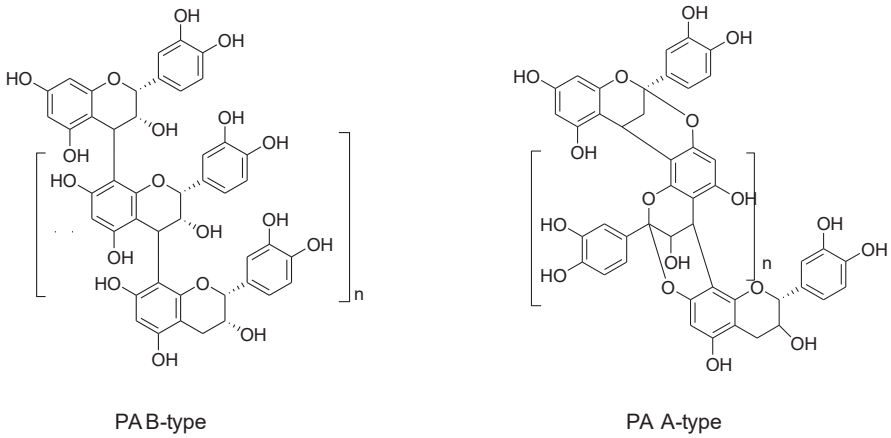

Figure 11 *Proanthocyanidins B-type and A-type structures.*

Recent studies have shown that RHS possesses high content of total polyphenols (Folin-Ciocalteu assay), 502-701 catechin equivalent mg CE/g of extract, proportionally correlated to high antioxidant activity (Piccinelli et al., 2016; Shahidi et al., 2007; Alasalvar et al., 2009; Gu et al., 2003b; Monagas et al., 2009). A RHS extract and its fraction RHS-M-F3 enriched in PAs were characterized in terms of total phenolic compound and PA contents, chemical profile by HPLC-HRMS technique and showed antioxidant and antifungal properties against *Candida albicans* (Piccinelli et al., 2016).

Therefore, due to the high amounts of proanthocyanidins, hazelnut skins could be a valuable by-product that could find applications in different sectors: pharmaceutical, nutraceutical and cosmetic (Alasalvar et al., 2009; Montella et al., 2013).

Determination qualitative and quantitative of crude extracts with PAs is very complex. For the determination there are some colorimetric methods that exploit reaction of A-ring with aromatic aldehydes in acidic conditions to obtain coloured products and depolymerisation methods, with disruption of C4-C8 or C4-C6 bonds in alcoholic and acidic media to yield red-coloured anthocyanidins. The identification of oligomeric and polymeric PAs with conventional HPLC techniques is complex because it is obtained the characteristic "hump", a single broad peak with a low resolution in reverse phase HPLC and unresolved peaks divided by growing degree of polymerization in direct phase HPLC due to presence of many isomers with the same polarity. Acidic depolymerisation in presence of nucleophile (benzilmercaptan, thiolysis, or phloroglucinol, phloroglucinolysis) followed by HPLC-UV analysis is currently employed for PAs mDP value (mean degree of polymerization) determination and to study flavonol molar distribution as terminal or extension units. Also mass spectrometry is a technique utilized in order to analyze PAs complex matrices (alone or coupled to HPLC).

Aim of this study was to evaluate the added value of RHS extract through chemical characterization using thiolysis followed by HPLC-UV-HRMS analysis and a combination of HRMS techniques, HRMS-FIA and HPLC-$HRMS^n$, in order to determine nature and proportions of the flavonol units, mDP, mass distribution, type of linkage, sequence of flavonol units in oligomers. Quantification of PAs was performed by vanillin assay, a colorimetric method.

4A 2. Results and discussion

4A 2.1. RHS extraction

The first extraction step with hexane allowed removing lipophilic compounds from RHS. Fat content resulted very high (18.4±0.01%) due to fats transfer from hazelnut to perisperm during roasting process at high temperatures. PAs are polar compounds and according to literature data (Contini et al., 2008) aqueous acetone (70%, v/v) was used for their extraction. PAs in plant material are located in vacuoles and are strongly linked to insoluble cell wall material through hydrogen and hydrophobic bonds. Water-acetone mixtures are able to destroy these linkages and are very efficient to extract oligomeric and polymeric PAs.

The exhaustive extraction of metabolites from a vegetable matrix requires the choice of an appropriate extraction technique. In this study, the solid-liquid extraction assisted by ultrasounds was selected as exhaustive extraction technique. It is faster than traditional extraction methods such as, maceration, and moreover, has the advantage of providing higher yields in a short time (Vinatoru, 2001). Overheating caused by ultrasounds was monitored and for this purpose, all extractions were performed at a controlled temperature of 25 °C. Furthermore the sonication induces the formation of micro-jets of solvent on solid walls of plant cells, providing the rupture and the release of compounds in the external solvent (Toma et al., 2001).

4A 2.2. PAs quantification

For the determination of the PAs content of RHS, a quantitative analysis was carried out by colorimetric method vanillin assay. The extract exhaustive RHS shows a content of 163.7 ± 4.34 mg CE/g of extract, corresponding to 4.15 ±

0.1 g CE/100 g DM (dry matter), comparable to the most well-known commercial extract rich in PAs (Pycnogenol® 126.1 ± 2.00 mg CE/g of extract, obtained from the bark of the French maritime pine *Pinus maritime*). These results suggest that RHS appears to be richer matrix than commercial product reference and support the thesis of the potential use of this by-product as a source of PAs. Vanillin assay is highly specific for flavanols: in the presence of a mineral acid, the condensation of vanillin with proanthocyanidins occurs in position 6 or 8 of A- ring of each subunit, to give a chromophore that absorbs at λ max wavelength of 510 nm. Method was validated in terms of linearity and accuracy of the response. Butler suggests use of acetic acid instead of methanol in vanillin assay, because absorbance produced is higher and method is more sensitive (Butler et al., 1982).

4A 2.3. RHS chemical profiling by mass spectrometry

To determine chemical profile of RHS it is important to thoroughly characterize their bioactive compounds. To obtain a complete chromatographic resolution of PAs in complex samples by HPLC is difficult because of great PAs diversity or polymers (DP > 6) (Guyot, 2012). Acidic depolymerisation of PAs with nucleophiles is currently the method most used to determine mDP value and the average flavonol composition of complex PA mixtures. Moreover, mass spectrometry, alone or coupled to HPLC, has been successfully employed to analyze complex PAs mixtures from various plant sources (Guyot, 2012), in particular, flow injection ESI-MS (Hayasaka et al., 2003; Li et al., 2012) and MALDI-TOF-MS (Monagas et al., 2010). HPLC-MS/MS has been applied for the structural characterization of PA oligomers (DP ≤ 6) by the study of fragmentation patterns (Gu et al., 2003b; Li and Deinzer, 2007; Li and Deinzer, 2008; Lin et al., 2014).

In this work, RHS chemical characterization was performed by thiolysis followed by HPLC-UV-HRMS analysis and a combination of HRMS techniques, HRMS-FIA and HPLC-HRMS^n, to define the nature and the proportion of the flavanol units, mDP and PA mass distributions, type of linkages, the presence of derivatives and the sequence of flavanol units in PA oligomers.

4A 2.4. Thiolysis

Firstly, the acid-catalyzed depolymerisation in the presence of benzyl mercaptan as nucleophilic reagent, coupled to HPLC-UV-HRMS analysis, was applied to characterize PAs of RHS extract Thiolysis is a useful tool for the characterization of PAs, as it able to distinguishes between extension (released as adducts with nucleophile) and terminal units (released as free flavanols). This method allows the determination of the nature and the proportion of the flavanol units and mDP of PA mixtures (Gu et al., 2002). The flavanol molar proportions of thiolytically-degraded PAs in RHS are reported in Table 8, and a representative chromatogram of thiolytically-degraded PAs is shown in Figure 12. Results were: mDP values of 8.3 ± 0.15. Flavonol units of RHS consisted of mostly catechin (78.5 ± 0.7%) and epicatechin (50.4 ± 0.1%). Catechin-3-*O*-gallate (13.6 ± 0.3%), epigallocatechin (3.5 ± 0.2%), gallocatechin (1.4 ± 0.0%) and gallocatechin-3-*O*-gallate (3.4 ± 0.2 %) were also found as flavonol units. Catechin and epicatechin occurred as both terminal and extension units and catechin was the main terminal unit (about 78%), while epicatechin was the major extension unit (50%).

Table 8 *Flavanol molar proportions, average DP and galloylation degree of PAs in RHS.*

	RHS
Extension units	**%**
catechin	30.2 ± 0.2
epicatechin	50.4 ± 0.1
gallocatechin	1.4 ± 0.1
epigallocatechin	3.5 ± 0.2
gallocatechin-gallate	3.4 ± 0.2
epicatechin-gallate	11.1 ± 0.4
Terminal units	%
gallocatechin	0.3 ± 0.2
catechin	78.5 ± 0.7
epicatechin	7.6 ± 0.1
catechin-gallate	13.6 ± 0.3
mDP	8.3 ± 0.1
galloylation degree	14.4 ± 0.2

mean ± SD of triplicate analysis

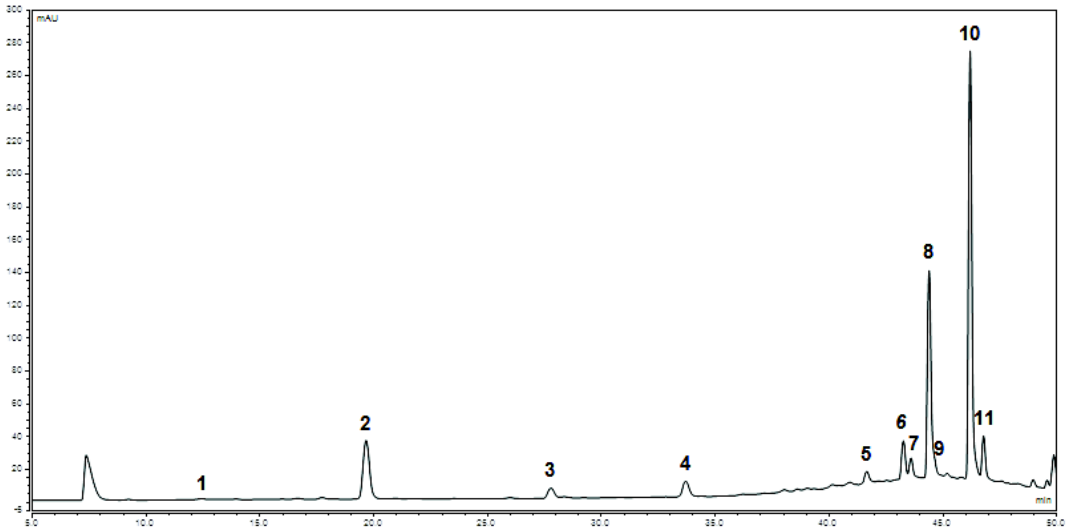

Figure 12 *HPLC-UV (280 nm) chromatogram of thiolytically-degraded PAs in RHS.* **1**, gallocatechin; **2**, catechin; **3**, epicatechin; **4**, catechin-3-gallate; **5**, gallocatechin benzylthioether; **6**, *trans*-catechin benzylthioether; **7**, epigallocatechin benzylthioether; **8**, *cis*-catechin benzylthioether; **9**, gallocatechin-3-gallate benzylthioether; **10**, epicatechin benzylthioether; **11**, catechin-3-gallate benzylthioether.

4A 2.5. HRMS-FIA

After thiolysis, HRMS-FIA, with ESI source operating in negative ion mode, was applied to investigate the metabolite profiling of RHS.

ESI-MS-FIA results with the complementary information of thiolysis provide a detailed identification of the composition of PAs. HRMS-FIA rapidly enabled detection of signals corresponding to monocharged ions ($[M–H]^-$ PAs DP up to 5-6), and to doubly charged ions ($[M_2–H]^{2-}$ for DP > 4). The *m/z* values allowed establishing the high-resolution molecular weight and exact molecular formula, but also the determination of the type of flavanol units, the degree of polymerization (DP), the presence of galloyl groups, and the number of A- or B-type linkages (Table 9). Furthermore, with ESI-FT-MS instrumentation it was possible to determine the charge state of the molecular species by the analysis of the isotopic distribution of the mass signals (Lin et al., 2014). Table 9 reports PA composition of RHS. The main peaks corresponded to $[M–H]^-$ ions of B-type PCs up to DP 6: a series of signals with a peak occurring every 288.06 mass units starting at m/z 577.1356 (2). $[M_2–H]^{2-}$ ions allowed identifying PAs with a higher DP, up to DP 10 (Table 9), whereas $[M_3–H]^{3-}$ ions were not detected. The HRMS spectrum revealed also peaks of PAs with one (Table 9) or more eG units (Table 9) and gallate derivatives up to DP 4 (Table 9). Moreover, the signals with 2.02 or 4.03 Da lower in mass than the B-type indicated the presence of A-type PAs with one and two extra interflavanic linkages (Table 9). Results are comparable to literature data regarding as hazelnut skin PAs composition (Esatbeyoglu et al., 2013; Esatbeyoglu et al., 2014; Del Rio et al., 2011; Monagas et al., 2009).

Table 9 *High-Resolution Masses, molecular formula and composition of PAs found in HRMS-FIA spectra of RHS.*

$[M–H]^-$ m/z (ppm)	$[M_2–H]^{2-}$ m/z (ppm)	Molecular formula	DP[a]	A-bond	eC[b]	eG[c]	g[d]
575.1197 (2.2)	–	$C_{30}H_{24}O_{12}$	2	1	2	–	–
577.1356 (2.7)	–	$C_{30}H_{26}O_{12}$	2	–	2	–	–
591.1147 (2.3)	–	$C_{30}H_{24}O_{13}$	2	1	1	1	–
593.1301 (2.0)	–	$C_{30}H_{26}O_{13}$	2	–	1	1	–
607.1096 (2.3)	–	$C_{30}H_{24}O_{14}$	2	1	–	2	–
609.1251 (1.9)	–	$C_{30}H_{26}O_{14}$	2	–	–	2	–
727.1316 (3.1)	–	$C_{37}H_{28}O_{16}$	2	1	2	–	1
729.1468 (2.4)	–	$C_{37}H_{30}O_{16}$	2	–	2	–	1
743.1262 (2.6)	–	$C_{37}H_{28}O_{17}$	2	1	1	1	1
745.1425 (3.5)	–	$C_{37}H_{30}O_{17}$	2	–	1	1	1
863.1841 (2.7)	–	$C_{45}H_{36}O_{18}$	3	1	3	–	–
865.1995 (2.4)	–	$C_{45}H_{38}O_{18}$	3	–	3	–	–
877.1631 (2.3)	–	$C_{45}H_{34}O_{19}$	3	2	2	1	–
879.1786 (2.1)	–	$C_{45}H_{36}O_{19}$	3	1	2	1	–
881.1960 (4.1)	–	$C_{45}H_{38}O_{19}$	3	–	2	1	–
893.1577 (1.9)	–	$C_{45}H_{34}O_{20}$	3	2	1	2	–
895.1741 (2.7)	–	$C_{45}H_{36}O_{20}$	3	1	1	2	–
897.1903 (3.4)	–	$C_{45}H_{38}O_{20}$	3	–	1	2	–
1017.2100 (1.6)	–	$C_{52}H_{42}O_{22}$	3	–	3	–	1
1151.2477 (2.2)	–	$C_{60}H_{48}O_{24}$	4	1	4	–	–
1153.2633 (2.1)	–	$C_{60}H_{50}O_{24}$	4	–	4	–	–
1167.2424 (2.0)	–	$C_{60}H_{48}O_{25}$	4	1	3	1	–
1169.2579 (1.9)	–	$C_{60}H_{50}O_{25}$	4	–	3	1	–
1181.2203 (0.8)	–	$C_{60}H_{46}O_{26}$	4	2	2	2	–
1183.2374 (2.0)	–	$C_{60}H_{48}O_{26}$	4	1	2	2	–
1185.2480 (–2.2)	–	$C_{60}H_{50}O_{26}$	4	–	2	2	–
1199.2330 (2.6)	–	$C_{60}H_{48}O_{27}$	4	1	1	3	–
1201.2504 (4.0)	–	$C_{60}H_{50}O_{27}$	4	–	1	3	–
1305.2787 (5.3)	–	$C_{67}H_{54}O_{28}$	4	–	4	–	1
1439.3124 (2.6)	719.1515 (1.9)	$C_{75}H_{60}O_{30}$	5	1	5	–	–
1441.3260 (1.3)	720.1596 (2.3)	$C_{75}H_{62}O_{30}$	5	–	5	–	–
1453.2927 (3.4)	726.1412 (2.0)	$C_{75}H_{58}O_{31}$	5	2	4	1	–
1455.3044 (0.7)	727.1491 (2.1)	$C_{75}H_{60}O_{31}$	5	1	4	1	–
1457.3156 (–3.6)	728.1572 (2.4)	$C_{75}H_{62}O_{31}$	5	–	4	1	–
–	734.1392 (2.7)	$C_{75}H_{58}O_{32}$	5	2	3	2	–
1471.3015 (3.5)	735.1470 (2.7)	$C_{75}H_{60}O_{32}$	5	1	3	2	–
1485.2782 (0.4)	742.1348 (0.3)	$C_{75}H_{58}O_{33}$	5	2	2	3	–
1487.2963 (2.0)	–	$C_{75}H_{60}O_{33}$	5	1	2	3	–
1489.3111 (1.4)	–	$C_{75}H_{62}O_{33}$	5	–	2	3	–
–	863.1841 (2.6)	$C_{90}H_{72}O_{36}$	6	1	6	–	–
1729.3919 (2.5)	864.1883 (–1.5)	$C_{90}H_{74}O_{36}$	6	–	6	–	–
1743.3720 (2.9)	871.1812 (2.2)	$C_{90}H_{72}O_{37}$	6	1	5	1	–
1745.3856 (1.8)	872.1849 (–2.4)	$C_{90}H_{74}O_{37}$	6	0	5	1	–

$[M–H]^-$ m/z (ppm)	**$[M_2–H]^{2-}$ m/z (ppm)**	**Molecular formula**	**DP**[a]	**A-bond**	**eC**[b]	**eG**[c]	**g**[d]
–	878.1680 (–1.0)	$C_{90}H_{70}O_{38}$	6	2	4	2	–
1759.3662 (2.5)	879.1786 (2.1)	$C_{90}H_{72}O_{38}$	6	1	4	2	–
1761.3816 (2.3)	880.1823 (–2.5)	$C_{90}H_{74}O_{38}$	6	0	4	2	–
1175.3529 (–2.1)	887.1761 (2.2)	$C_{90}H_{72}O_{39}$	6	1	3	3	–
1777.3685 (–2.1)	888.1789 (–3.5)	$C_{90}H_{74}O_{39}$	6	0	3	3	–
–	1008.2227 (1.4)	$C_{105}H_{86}O_{42}$	7	–	7	–	–
–	1015.2142 (3.2)	$C_{105}H_{84}O_{43}$	7	1	6	1	–
–	1022.2021 (1.5)	$C_{105}H_{82}O_{44}$	7	2	5	2	–
–	1023.2073 (–1.0)	$C_{105}H_{84}O_{44}$	7	1	5	2	–
–	1024.2172 (0.9)	$C_{105}H_{86}O_{44}$	7	–	5	2	–
–	1152.2526 (–0.4)	$C_{120}H_{98}O_{48}$	8	–	8	–	–
–	1159.2437 (0.9)	$C_{120}H_{96}O_{49}$	8	1	7	1	–
–	1160.2518 (1.2)	$C_{120}H_{98}O_{49}$	8	–	7	1	–
–	1167.2424 (2.0)	$C_{120}H_{96}O_{50}$	8	1	6	2	–
–	1296.2918 (5.5)	$C_{135}H_{110}O_{54}$	9	–	9	–	–
–	1304.2794 (–2.1)	$C_{135}H_{110}O_{55}$	9	–	8	1	–
–	1312.2851 (4.1)	$C_{135}H_{110}O_{56}$	9	–	7	2	–
–	1440.3177 (0.9)	$C_{150}H_{122}O_{60}$	10	–	10	–	–
–	1448.3144 (0.4)	$C_{150}H_{122}O_{61}$	10	–	9	1	–

[a]degree of polymerization; [b](epi)catechin; [c](epi)gallocatechin; [d]galloyl.

4A 2.6. HPLC-HRMS^n analysis

Finally, HPLC-HRMS^n analysis of RHS was performed to elucidate the detailed structures of oligomer isomers and galloylated derivatives.

HPLC-MS/MS is a valid technique to obtain important information as the sequence of flavanol units, the type and position of linkages, the hydroxylation scheme, and the galloylation sites of oligomeric PAs (DP < 5). Based on the proposed fragmentation patterns in literature (Gu et al., 2003b; Li and Deinzer, 2007; Li and Deinzer, 2008; Lin et al., 2014) information about the hydroxylation pattern and type of interflavan bond were obtained by the fragment ions derived from retro Diels-Alder (RDA) reaction (loss of 152, 136, and 168 Da for eC, eA and eG, respectively) and from heterocyclic ring fission (HRF) of the extension unit (loss of 126 Da for eC, eA or eG). Quinone methide (QM) cleavage of interflavan bonds produces diagnostic fragment ions ($[M_t-H]^-$ and $[M_{EX}-3H]^-$ ions for B-type PAs and $[M_T-5H]^-$ ions for A-type PAs, where EX = extension unit and T = terminal unit) useful to identify the connection sequence of the oligomers. Using the information from HPLC-HRMS and MS/MS experiments, 7 monomers were identified and a total of 59 PA oligomers (14 isomeric groups), were structurally sequenced from RHS. Table 10 shows the retention times, $[M-H]^-$ ions, molecular formulas and diagnostic MS^2 product ions of the identified monomers and oligomeric PAs. Figure 13 shows representative MS^2 spectra of RHS. The natural occurrence of identified PA oligomers was confirmed by HPLC-HRMS^n analysis of the filtrate obtained after precipitation of polymers (Esatbeyoglu et al., 2014) enabling to exclude the origin of $[M-H]^-$ ions from the insource fragmentation of higher PAs. Isomers of B-type PC dimers (7 $C_{30}H_{25}O_{12}$), trimers (11 $C_{45}H_{37}O_{18}$) and tetramers (6 $C_{60}H_{49}O_{24}$) were successfully identified (Table 10). Also, the flavanol sequence of B-type dimers (5 eG-eC, 2 eC-eG and 2 eG-eG) and trimers containing eG (3 eG-eC-eC, 1 eC-eG-eC and 1 eG-eG-eC)

were well established by HRMS and MS/MS data (Table 10). Regarding A-type PAs, both dimers and trimers were detected as minor compounds (Table 10). A fully characterization of A-type dimer isomers (3 eC-A-eC, 6 eG-A-eC) was obtained (Table 10), while in the case of dimers eG-A-eG and A-type trimers, due to their low abundance, it has not been possible to carry out MS/MS experiments. Also, the galloylated PC dimers (2 eC-eCg and 1 eCg-eC) and trimer (eC-eC-eC-g) and galloylated dimers containing eG (eC-eGg and eG-eCg) were structurally sequenced through the diagnostic QM, RDA and HRF ions. In particular, the galloylation positions in the isomers eC-eCg and eCg-eC were established based on the product ions corresponding to QM_{EX} (*m/z* at 287 and 439, respectively) and QM_T (*m/z* at 441 and 289, respectively). The extension units of isomers eC-eGg and eG-eCg were identified by the diagnostic product ions of RDA_{EX} (*m/z* at 593 and 577, respectively) and $RDA_{EX} - H_2O$ (*m/z* at 575 and 559, respectively) and corroborated from QM_T ion at *m/z* 457 and 441, respectively (Table 10). HRMS-FIA and HPLC-$HRMS^n$ data were fully consistent with thiolysis results. The results showed that condensed tannins present in RHS are PCs, PC-PD heteroligomers and galloyl derivatives.

Table 10 *Retention times, [M−H]⁻ ions, molecular formulas, sequences and diagnostic MS^2 product ions of the identified monomers and oligomeric PAs in RHS.*

T_R (min)	[M–H]⁻ (*m/z*)	Error ppm	Molecular formula	Compound[a-f]	Diagnostic MS^2 ions (*m/z*)[g]
21.3	289.0713	2.3	$C_{15}H_{13}O_6$	catechin	175, 179, 205, 231, 245
29.7	289.0716	3.3	$C_{15}H_{13}O_6$	epicatechin	
12.5	305.0664	2.8	$C_{15}H_{13}O_7$	gallocatechin	165, 175, 179, 219, 221, 247, 261, 287
20.8	305.0668	3.9	$C_{15}H_{13}O_7$	epigallocatechin	
35.1	441.0820	0.3	$C_{22}H_{17}O_{10}$	catechin-3-g	169, 193, 271, 289, 331
36.8	441.0823	1.5	$C_{22}H_{17}O_{10}$	epicatechin-3-g	
28.0	457.0773	1.7	$C_{22}H_{17}O_{11}$	eG-3-g	169, 193, 225, 305, 331, 385, 389, 429
29.7	575.1189	0.9	$C_{30}H_{23}O_{12}$	eC-A-eC	285 (QM_{EX}) 289 (QM_T) 407 (RDA – H_2O) 423 (RDA)
34.1	575.1189	0.9	$C_{30}H_{23}O_{12}$	eC-A-eC (A_2)	449 (HRF)
36.6	575.1191	1.2	$C_{30}H_{23}O_{12}$	eC-A-eC	
15.5	577.1348	1.2	$C_{30}H_{25}O_{12}$	eC-eC	287 (QM_{EX}) 289 (QM_T) 299 (RDA + HRF) 407 (RDA
16.5	577.1346	0.9	$C_{30}H_{25}O_{12}$	eC-eC	– H_2O) 425 (RDA) 451 (HRF)
18.5	577.1347	1.1	$C_{30}H_{25}O_{12}$	eC-eC	
19.6	577.1349	1.5	$C_{30}H_{25}O_{12}$	eC-eC	
22.1	577.1350	1.6	$C_{30}H_{25}O_{12}$	eC-eC	
24.0	577.1350	1.7	$C_{30}H_{25}O_{12}$	eC-eC (B_2)	
28.3	577.1353	2.1	$C_{30}H_{25}O_{12}$	eC-eC	

T_R (min)	$[M–H]^-$ (*m/z*)	Error ppm	Molecular formula	Compound[a-f]	Diagnostic MS^2 ions (*m/z*)[g]
22.0	591.1141	1.4	$C_{30}H_{23}O_{13}$	eG-A-eC	301 (QM_{EX}) 289 (QM_T) 407 (RDA – H_2O) 439 (RDA_T) 465 (HRF)
23.3	591.1139	0.9	$C_{30}H_{23}O_{13}$	eG-A-eC	
25.2	591.1140	1.2	$C_{30}H_{23}O_{13}$	eG-A-eC	
27.2	591.1137	0.6	$C_{30}H_{23}O_{13}$	eG-A-eC	
28.6	591.1143	1.7	$C_{30}H_{23}O_{13}$	eG-A-eC	
29.9	591.1139	1.0	$C_{30}H_{23}O_{13}$	eG-A-eC	
10.1	593.1290	0.2	$C_{30}H_{25}O_{13}$	eG-eC	303 (QM_{EX}) 289 (QM_T) 407 (RDA_{EX} – H_2O) 425 (RDA_{EX}) 441 (RDA_T) 467 (HRF)
10.4	593.1298	1.4	$C_{30}H_{25}O_{13}$	eG-eC	
11.4	593.1296	1.5	$C_{30}H_{25}O_{13}$	eG-eC	
14.7	593.1296	1.1	$C_{30}H_{25}O_{13}$	eG-eC	
14.9	593.1296	1.1	$C_{30}H_{25}O_{13}$	eG-eC	
12.0	593.1298	1.5	$C_{30}H_{25}O_{13}$	eC-eG	305 (QM_{EX}) 287 (QM_T) 423 (RDA_{EX} – H_2O) 425 (RDA_T) 441 (RDA_{EX}) 467 (HRF)
12.4	593.1236	1.4	$C_{30}H_{25}O_{13}$	eC-eG	
12.7	607.1088	1.0	$C_{30}H_{23}O_{14}$	eG-A-eG	Not recorded
19.6	607.1088	0.9	$C_{30}H_{23}O_{14}$	eG-A-eG	
26.3	607.1088	1.0	$C_{30}H_{23}O_{14}$	eG-A-eG	
8.1	609.1241	0.3	$C_{30}H_{25}O_{14}$	eG-eG	305 (QM_T) 423 (RDA – H_2O) 441 (RDA) 483 (HRF)
9.2	609.1243	0.7	$C_{30}H_{25}O_{14}$	eG-eG	
24.5	729.1458	1.1	$C_{37}H_{29}O_{16}$	eC-eCg	287 (QM_{EX}) 441 (QM_T) 289 (QM_T – gal) 407 (RDA – H_2O – gal) 451 (HRF – gallate) 559 (RDA – H_2O) 577 (RDA/– gal) 603 (HRF)
27.5	729.1460	1.4	$C_{37}H_{29}O_{16}$	eC-eCg	

T_R (min)	$[M–H]^-$ (*m/z*)	Error ppm	Molecular formula	Compound[a-f]	Diagnostic MS^2 ions (*m/z*)[g]
26.1	729.1460	1.4	$C_{37}H_{29}O_{16}$	eCgal-eC	439 (QM_{EX}) 289 (QM_T) 287 (QM_{EX} – gal) 407 (RDA – H_2O – gal) 425 (RDA – gal) 451 (HRF – gal) 559 (RDA – H_2O) 577 (RDA/– gal) 603 (HRF)
22.9	745.1406	1.0	$C_{37}H_{29}O_{17}$	eC-eGg	457 (QM_T) 305 (QM_T – gal) 423 (RDA_{EX} – H_2O – gal) 467 (HRF – gal) 575 (RDA_{EX} – H_2O) 593 (RDA_{EX}/– gal) 619 (HRF)
23.8	745.1402	0.3	$C_{37}H_{29}O_{17}$	eG-eCg	303 (QM_{EX}) 441 (QM_T) 289 (QM_T – gal) 407 (RDA_{EX} – H_2O – gal) 467 (HRF – gal) 559 (RDA_{EX} – H_2O) 577 (RDA_{EX}) 593 (– gal) 619 (HRF)
9.4	863.1827	1.1	$C_{45}H_{35}O_{18}$		Not recorded
8.7	865.1976	0.2	$C_{45}H_{37}O_{18}$	eC-eC-eC	287 ($QM_{EX(CD)}$) 577 ($QM_{T(CD)}$) 575 ($QM_{EX(FG)}$) 289 ($QM_{T(FG)}$) 407 ($QM_{T(CD)}$ + RDA – H_2O) 425 ($QM_{T(CD)}$ + RDA) 449 ($QM_{EX(FG)}$ + HRF) 451 ($QM_{T(CD)}$ + HRF) 695 (RDA – H_2O) 713 (RDA) 739 (HRF)
15.4	865.1978	0.4	$C_{45}H_{37}O_{18}$	eC-eC-eC	
17.0	865.1978	0.4	$C_{45}H_{37}O_{18}$	eC-eC-eC	
17.8	865.1981	0.8	$C_{45}H_{37}O_{18}$	eC-eC-eC	
18.6	865.1978	0.4	$C_{45}H_{37}O_{18}$	eC-eC-eC	
19.0	865.1978	0.4	$C_{45}H_{37}O_{18}$	eC-eC-eC	
20.0	865.1975	0.1	$C_{45}H_{37}O_{18}$	eC-eC-eC	
24.6	865.1977	0.3	$C_{45}H_{37}O_{18}$	eC-eC-eC	
25.9	865.1978	0.4	$C_{45}H_{37}O_{18}$	eC-eC-eC	
27.7	865.1980	0.6	$C_{45}H_{37}O_{18}$	eC-eC-eC	
29.1	865.1979	0.6	$C_{45}H_{37}O_{18}$	eC-eC-eC	

T_R (min)	$[M–H]^-$ (*m/z*)	Error ppm	Molecular formula	Compound[a-f]	Diagnostic MS^2 ions (*m/z*)[g]
7.8	881.1922	-0.1	$C_{45}H_{37}O_{19}$	eG-eC-eC	303 ($QM_{EX(CD)}$) 577 ($QM_{T(CD)}$) 591 ($QM_{EX(FG)}$) 289 ($QM_{T(FG)}$) 695 ($RDA_{eG} – H_2O$) 711 ($RDA_{eC} – H_2O$) 713 (RDA_{eG}) 729 (RDA_{eC}) 755 (HRF)
12.0	881.1921	-0.3	$C_{45}H_{37}O_{19}$	eG-eC-eC	
13.9	881.1932	1.0	$C_{45}H_{37}O_{19}$	eG-eC-eC	
12.8	881.1928	0.5	$C_{45}H_{37}O_{19}$	mixture	
14.5	881.1932	1.0	$C_{45}H_{37}O_{19}$	mixture	
8.3	881.1925	0.2	$C_{45}H_{37}O_{19}$	eC-eG-eC	287 ($QM_{EX(CD)}$) 593 ($QM_{T(CD)}$) 591 ($QM_{EX(FG)}$) 289 ($QM_{T(FG)}$) 407 ($QM_{T(CD)} + RDA_{eG} – H_2O$) 425 ($QM_{T(CD)} + RDA_{eG}$) 695 ($RDA_{eG} – H_2O$) 711 ($RDA_{eC} – H_2O$) 713 ($RDA_{eG}$) 729 ($RDA_{eC}$) 755 (HRF)
10.4	897.1872	-0.1	$C_{45}H_{37}O_{20}$	eG-eG-eC	303 ($QM_{EX(CD)}$) 593 ($QM_{T(CD)}$) 607 ($QM_{EX(FG)}$) 543 ($2RDA_{eG} – H_2O$) 603 (RDA_{eG} + HRF) 711 ($RDA_{eG} – H_2O$) 729 (RDA_{eG}) 771 (HRF)
32.8	1017.2092	0.8	$C_{52}H_{41}O_{22}$	eC-eC-eCg	729 ($QM_{T(CD)}$) 575 ($QM_{EX(FG)}$) 441 ($QM_{T(FG)}$) 577 ($QM_{T(CD)}$ + RDA/– gal) 603 ($QM_{T(CD)}$ + HRF) 847 ($RDA – H_2O$) 865 (RDA/– gal) 891 (HRF)

T_R (min)	$[M–H]^-$ (*m/z*)	Error ppm	Molecular formula	Compound[a-f]	Diagnostic MS^2 ions (*m/z*)[g]
13.1	1153.2619	0.9	$C_{60}H_{49}O_{24}$	eC-eC-eC-eC	865 ($QM_{T(CD)}$) 575 ($QM_{EX(FG)}$) 577 ($QM_{T(FG)}$) 863 ($QM_{EX(IL)}$) 739 ($QM_{T(CD)}$ + HRF) 983 (RDA – H_2O) 1001 (RDA) 1027 (HRF)
16.4	1153.2621	1.1	$C_{60}H_{49}O_{24}$	eC-eC-eC-eC	
17.1	1153.2616	0.7	$C_{60}H_{49}O_{24}$	eC-eC-eC-eC	
22.4	1153.2618	0.8	$C_{60}H_{49}O_{24}$	eC-eC-eC-eC	
21.5	1153.2614	0.5	$C_{60}H_{49}O_{24}$	eC-eC-eC-eC	
23.0	1153.2618	0.8	$C_{60}H_{49}O_{24}$	eC-eC-eC-eC	

[a]eG, (epi); [b]eC, (epi)catechin; [c]A_2, procyanidin A-type dimer A_2; [d]B_2, procyanidin B-type dimer B_2; [e]eCg, (epi)catechin-3-*O*-gallate; [f]eGg, (epi)gallocatechin-3-*O*-gallate; [g]EX, extension unit; T, terminal unit; CD, FG and IL, nomenclature of flavanol ring by Li and Deinzer, 2007.

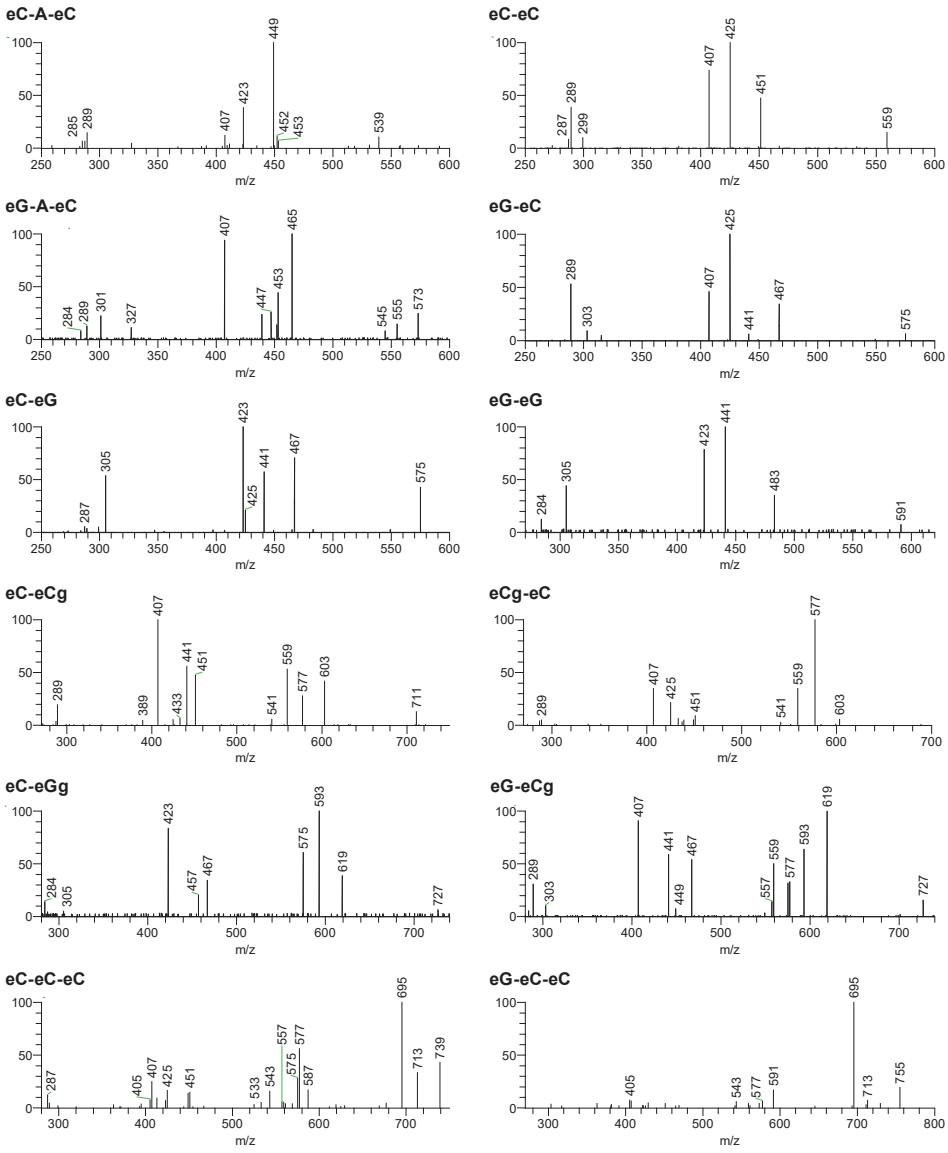

Figure 13 *Representative MS^2 spectra of PA oligomers detected in RHS.*

4A 3. Conclusions

In this work chemical profile of RHS extract was determined by thiolysis followed by HPLC-UV-HRMS analysis and HRMS-FIA and HPLC-$HRMS^n$. Valorisation of a food by-product can only be explicated if it is a matrix

interesting from the point of view of secondary metabolites and their contents. RHS is rich in oligomeric and polymeric PAs and PAs content is very high. Results indicated that hazelnut skin by-products possess added value and are suitable, potential matrices for pharmaceutical, cosmetic and nutraceutical applications.

4A 4. Materials and methods

4A 4.1. Materials

Methanol (CH_3OH) and acetone $(CH_3)_2CO$ HPLC grade, water ultrapure and methanol ultrapure were purchased from Romil (Cambridge, UK) and water grade milli-Q (18MΩ) was obtained using a Milli Q apparatus (Millipore, Bedford, USA). N-hexane was purchased from Merck (Germany) and hydrochloric acid HCl 37% and acetic acid from Carlo Erba (Italy). The standard used (+)-catechin hydrate ≥98% HPLC, vanillin reagent plus 99%, ammonium formate, formic acid and benzilmercaptan were purchased from Sigma Aldrich (Milan, Italy). The stock standard solution (+)-catechin hydrate was prepared in methanol a concentration of 1 mg/mL and stored at 4 °C.

4A 4.2. Hazelnut skin samples

RHS samples were kindly supplied by an Italian hazelnut processing industry (Hazelnuts South Italy Manufacturing S.r.l., Baiano, Avellino). RHS is waste of daily industrial processing, carried out on Campania hazelnut varieties (90% Mortarella and 10% Lunga San Giovanni). RHS material was finely blended using a knife mill Grindomix GM 200 (Retsch, Haan, Germany).
The resulting ground samples were sieved through a test sieve with a range of 300-600 μm to obtain powders with a homogeneous particle size distribution.

4A 4.3. Extraction procedure

Exhaustive reference extraction was performed by solid liquid extraction assisted by ultrasounds in triplicate, after defatting matrix with hexane (liquid-liquid extraction), for 30 minutes (x 2) at 25 °C in a thermostat-controlled ultrasound bath (Labsonic LBS2, Treviglio, Italy) at the frequency of 20.0 kHz, using aqueous acetone (70% v/v) and a matrix/solvent ratio of 1:10. The extracts were pooled, filtered (Whatman No. 1 filter) and freeze-dried, after removal of the organic solvent under vacuum at 40 °C in a rotary evaporator (Rotavapor R-200, Buchi Italia s.r.l, Cornaredo, Italy). Yield of extraction was 21.2±1.2%.

4A 4.4. HRMS-FIA

HRMS-FIA experiments were performed with linear ion trap–Orbitrap hybrid mass spectrometer (LTQ OrbiTrap XL, ThermoFisher Scientific, Milan, Italy) using electrospray ionization (ESI) in negative ion mode acquisition. RHS (50 and 100 µg/mL in methanol/water 1:1, v/v) was pumped at 5 µL/min and the MS data were acquired in two range (normal mass range, m/z 500-2000 and high mass range, m/z 1000-3000) with a resolution of 60000 and maximum ion injection time of 100 ms. The following conditions were used: sheath and auxiliary gas, 30 and 5 (arbitrary units), respectively; spray voltage, 4.0 kV, capillary temperature, 280 °C; capillary voltage, −49 V; tube lens, 146.5 V. Xcalibur 2.2 software (ThermoFisher Scientific, Milan, Italy) was used for data interpretation.

4A 4.5. HPLC-HRMS analysis

HPLC analysis were carried out with a Dionex Ultimate 3000 UHPLC system (ThermoFisher Scientific, Milan, Italy) using a Hibar Purospher STAR RP-18 endcapped column (3 µm, 150x3 mm) (Merck, Darmstadt, Germany). A linear gradient (5 to 75% B in 45 min) of water (A) and methanol (B), both with 2mM ammonium formate and 0.1% formic acid, was applied. The flow rate was 300 µL min-1 and the injection volume 5 µL. The column was thermostated at 25 °C. The detection was performed at the wavelength of 280 nm. HPLC-HRMS analysis was performed with LTQ OrbiTrap XL connected to an Accela system (ThermoFisher Scientific, Milan, Italy) using the above chromatographic conditions. The mass spectrometer, equipped with ESI source, was operated in negative mode. Instrumental parameters were: source voltage 4.0 kV, capillary voltage −49 V, tube lens voltage −146.5 V, capillary temperature 300 °C, sheath and auxiliary gas flow (N_2) 35 and 10 (arbitrary units), respectively. MS spectra were acquired by full range acquisition (250–2000 m/z). For fragmentation study, data dependent scan was performed by deploying the collision-induced dissociation (CID). The normalized collision energy of the collision-induced dissociation (CID) cell was set at 30 eV. PAs were identified according to the corresponding spectral characteristics: mass spectra, accurate mass, characteristic fragmentation, and retention time. Xcalibur 2.2 software was used for instrument control, data acquisition and data analysis.

4A 4.6. Thiolysis

Thiolysis was performed according to a procedure previously reported (Gu et al., 2002), with some modifications. In particular, 50 µL methanol acidified with concentrated HCl (3.3%, v/v) and 100 µL of benzyl mercaptan solution

(5%, v/v, in methanol) were added to 50 µL of the sample (4 mg/mL in methanol). The mixture was vortexed and the vial was sealed. The reaction was carried out for 30 min at 40 °C and was stopped placing the vial in an ice bath. Immediately 200 µL of water were added to the samples and 10 µL of the reaction mixture was injected for HPLC analysis (same chromatographic method of HPLC-HRMS analysis). Also pure catechin (1 mg/mL) was analyzed. Triplicate experiments for sample were performed. The proportions of constituent flavan-3-ol and degree of polymerization (mDP) were calculated according to a published method (Gu et al., 2002).

4A 4.7. Determination of condensed tannins, vanillin assay

For determination of condensed tannins, PAs, vanillin assay was employed as reported by Butler with some modifications (Butler et al., 1982). The vanillin solution (0.5% vanillin reagent, w/v, in acetic acid, plus 4% of concentrated HCl) was prepared immediately before use. In brief, 40 µL of extracts or fraction (200 µg/mL in acetic acid) were mixed with 200 µL of vanillin solution in a well of 96-well microplate (96-Well Microtiter Microplates, Thermo Fisher Scientific, Milan, Italy). After 5 minutes at room temperature, absorbance was read at 510 nm using a microplate spectrophotometer reader Multiskan Go (ThermoFisher Scientific, Milan, Italy). A control without vanillin reagent and a blank with acetic acid instead of sample were included in the assay. The concentration of total PAs was estimated from a calibration curve using catechin (range 1-100 µg/mL, prepared in acetic acid) and the data were expressed as catechin equivalent (CE g/100 g DM, means ± S.D. of three determinations).

CHAPTER IV
SECTION B

PRESSURIZED HOT WATER EXTRACTION OF PROANTHOCYANIDINS FROM ROASTED HAZELNUT SKINS BY-PRODUCT

4B 1. Introduction

Subsequently to qualitative and quantitative analysis of hazelnut skin by-products and their evaluation as potential raw materials for nutraceutical, cosmetic and pharmaceutical sectors, the second part of project regarded the development and optimization of pressurized hot water extraction (PHWE) procedure. PHWE – a new non-conventional extraction method, rapid, economic and environmentally friendly – was employed in order to recover proanthocyanidins (PAs) from perisperm of roasted hazelnuts (Roasted Hazelnut Skins, RHS) using response surface methodology (RSM). PHWE is an extraction technique that employs water as extraction solvent at temperature above its atmospheric boiling point but below its critical point and at elevated pressures; it provides some advantages over other traditional extractions: is green, fast and more efficient (Plaza and Turner, 2015). Many authors reported extraction of phenolic compounds from hazelnut skins using conventional extraction techniques (Contini et al., 2008; Alasalvar et al., 2009; Locatelli et al., 2010); instead few authors studied novel extraction methods. A recent study reports recovery of phenolic compounds from hazelnut skin using different non-conventional extraction methods (ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE) and supercritical carbon dioxide extraction (SCE)) and response surface methodogy to estimate the optimal conditions. UAE was the best technique with the highest total phenolic content and antioxidant activity values (Odabaş and Koca, 2016). In addition, to the best of our knowledge, no work reported PHWE for the extraction of PAs from RHS. As for extraction of proanthocyanidins, one of the earliest papers reports online coupling of pressurized liquid extraction, solid-phase extraction and high-performance liquid chromatography for automated analysis of PAs, using aqueous acetone solution (40%, v/v), for 10 minutes, 60 °C, 100 bar (Papagiannopoulos et al., 2002). A further article describes determination of

proanthocyanidins and some phenolic compounds using pressurized conditions: methanol, 40 °C and 2 cycles of 5 min extraction (Alonso-Salces et al., 2001). Pressurized water extraction was also applied to recover polyphenols (evaluated as total phenolics, total flavonoids, hydrolysable tannins, condensed tannins) from pomegranate peels, one of the most valuable by-products of food industry and water proved to be effective like methanol in the extraction of target analytes (Çam and Hışıl, 2010).

4B 2. Results and discussion

4B 2.1. Preliminary experiments

For the development of PHWE technique preliminary experiments were carried out to set some specific parameters (particle size, matrix / dispersant ratio, modifier %, maximum operating temperature), in order to reduce number of independent variables (experimental factors) in chemometric analysis. For each parameter optimal value were selected considering the efficiency and selectivity of PHWE and good instrumental operation. Particle size of matrix more suitable was range 300-600 microns. Regarding the dispersant material, irreversible interaction has been observed between analytes and diatomaceous earths (most commonly dispersant used in PHWE). Therefore, glass balls were employed to pack homogeneously plant material in the cell extraction. Moreover different volumes of the steel extraction cell were evaluated (5, 11, 33 mL). Comparable results were obtained with all kind of cells and so 11 mL was selected for all subsequent experiments. The parameters flush volume % and pressure have been set to 150% and 1500 psi, respectively, based on previous experience. Ideal pressure recommended for extractions with pressurized water is 1500 psi (Teo et al., 2010). Regarding

modifier, to develop a green procedure and increase extraction efficiency the addition has been considered of organic solvent in small percentages, as it may exert a positive effect on solubility of the analytes and on their desorption (Mustafa and Turner, 2011). As modifiers were selected ethanol (GRAS) and acetone, considered good green solvent (American Chemical Society Pharmaceutical). Ethanol and acetone proved to be efficient in the extraction of proanthocyanidins (Guyot, 2012). Subsequently, some experiments were performed to evaluate the effect of modifier (aqueous solution of acetone and ethanol, 20% v/v) and temperature on the efficiency and selectivity of PHWE. Figure 14 shows the content of PAs, expressed as catechin equivalent g CE/100g DM (dry matrix), by vanillin assay at different temperatures (80, 100, 125 and 150 °C) with different solvents. The use of only water as extraction solvent, also increasing the temperature, it does not allow an exhaustive extraction of PAs from the matrix (compared to exhaustive extract, RHS EXH). However presence of modifiers (acetone and ethanol) increases content making it much more exhaustive and efficacious. Ethanol is a better modifier than acetone but without water is ineffective for extraction of proanthocyanidins.

Regarding the influence of temperature, it has been observed, for both modifiers, an increase of PAs content proportional to the increase of the extraction temperature. However, this increase is linear up to 125 °C, while at temperature of 150 °C is observed a slight decrease. Subsequent experiments performed at temperatures above 150 °C (175 and 200 °C) confirm the collapse of the PAs content extracted. Probably and in agreement with the literature data, in these conditions phenomena of degradation of proanthocyanidins begin to occur, such as rupture of interflavanoidic bond (depolymerisation) and oxidation (Guyot, 2012; Teo et al., 2010).

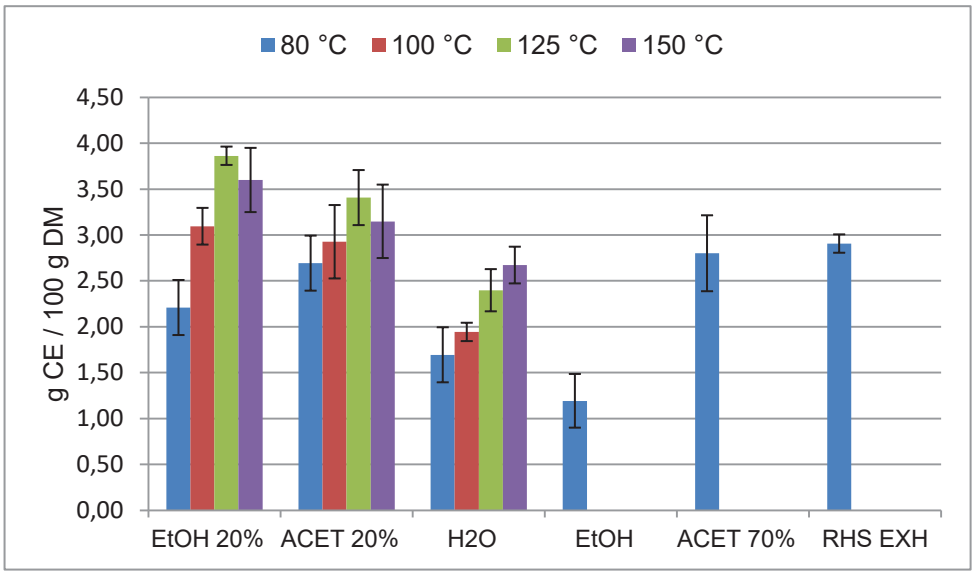

Figure 14 *Determination condensed tannins, PAs content, vanillin assay.* Catechin equivalent g CE/100 g DM, PHWE extractions at different temperatures with different solvents. RHS EXH exhaustive extract at 25 °C, values are means of three replicates ± standard deviation (SD).

To assess these depolymerisation phenomena, thiolysis followed by HPLC-UV-HRMS analysis and HPLC-HRMS analysis were carried out. Thiolysis results show degradation phenomena at 150 °C: the main degree of polymerization (mDP) decreases (Figure 15) from 100 to 150 °C and changes in the molar proportions of main monomers as terminal and extension units were observed. Catechin as terminal unit decreases and as extension unit increases. The reverse occurs for epi-catechin (Figure 16). The degree of polymerization decreases and high-resolution mass experiments have confirmed this at 150 °C for both modifiers: the percentage of the monomers increases (evaluating area of monomers and oligomers up to DP <5), while the percentage of oligomers decreases because of extension units start to break away (Figure 17). Variation of temperatures up to 125 °C does not involve changes of mDP but at 150 °C is observed decrease of mDP (from 7.3 to 6.6 for ethanol and from 7.6 to 6.4 for acetone -100 to 150 °C).

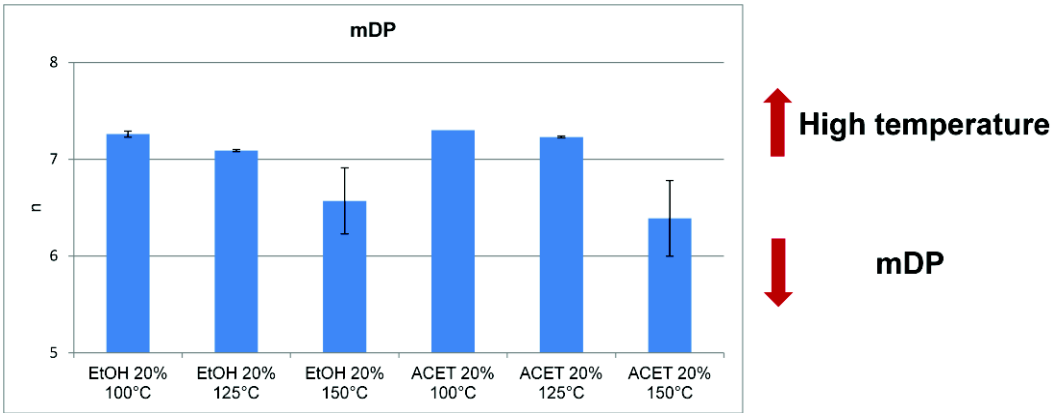

Figure 15 *Degree of polymerization at different temperatures, thiolysis assay.*

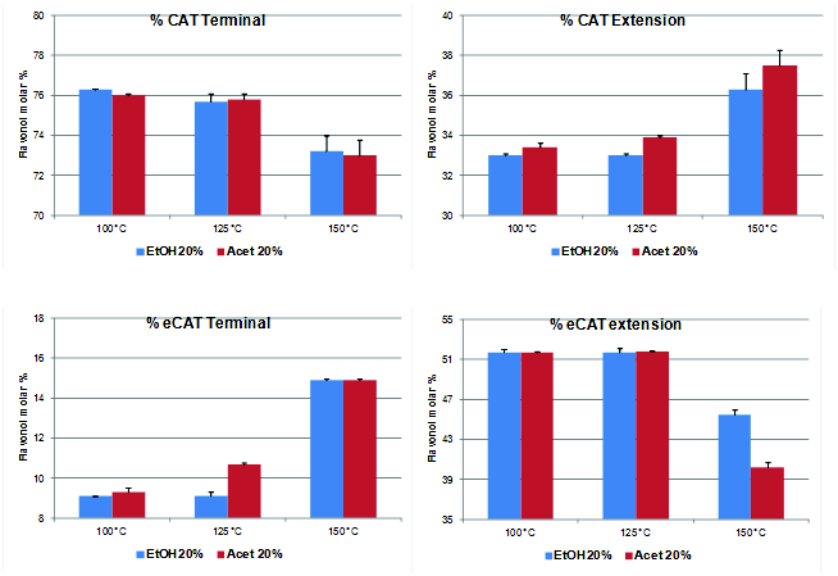

Figure 16 *Variation of flavonol molar % distribution at different temperature, thiolysis assay.*

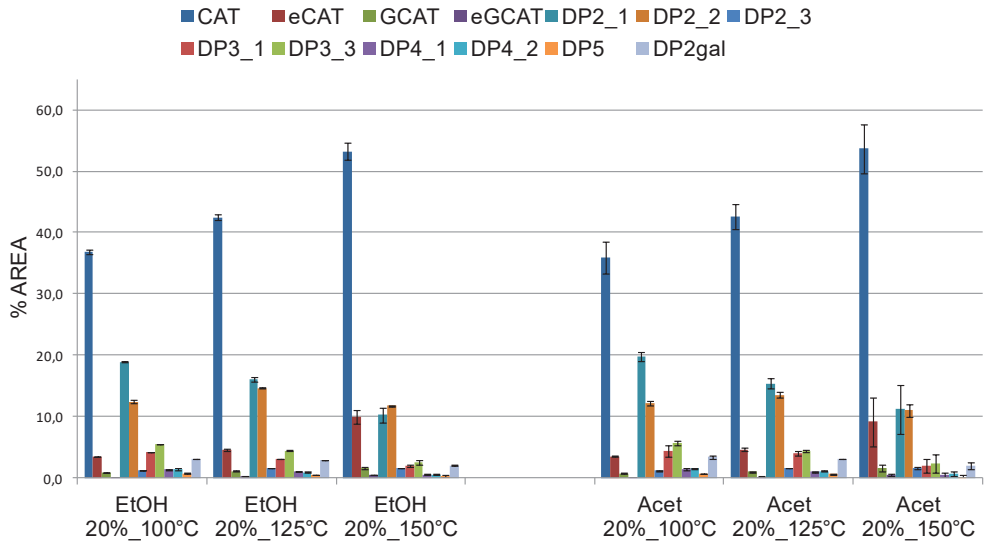

Figure 17 *HPLC-HRMS analysis of preliminary experiments at different temperatures.*
CAT, catechin, eCAT, epi-catechin, GCAT, gallocatechin, eGCAT, epi-gallocatechin, DP, degree of polymerization, oligomers from DP 1-5.

PHWE solvent selectivity was studied because of high fat content of RHS exhaustive extract (18.4±0.10 g/100 g DM). Fat content of PHWE extracts obtained with different solvents at different temperatures is significantly lower (<1.6 g/100g RHS DM), demonstrating the high selectivity of PHWE technique only for target analytes. Presence of modifier does not influence water selectivity, despite the change of its dielectric constant that occurs during pressurized conditions.

4B 2.2. Optimization design

Afterward, the effects of experimental factors temperature, modifier % and number of cycles have been attentively studied by chemometric analysis. In fact, these parameters are the most influential in PHWE extraction (Teo et al., 2010) and they may exercise a powerful effect on the efficiency of extraction. These parameters can influence the process directly or indirectly, by

interacting among them. For these reasons it is important to simultaneously evaluate their effects on response factors, in order to minimize costs of the whole process too. Regarding modifier, ethanol was chosen, in small percentages, because results of preliminary experiments indicated a power solvent higher than acetone for PAs extraction. Low and high values of experimental parameters have been selected on the basis of preliminary experiments as: A. temperature: 75-125 °C; B. number of cycles: 1-5; C.% modifier: 10-30%, v/v. Response factor was PAs content evaluated by vanillin assay and expressed as g catechin equivalent CE/100g DM. For response factor the desired effect has been set (maximize PAs content) in order to obtain an enriched extracts.

The chemometric analysis was performed using Box–Behnken design (BBD), randomized, linear model, with 30 experimental runs (Table 11) divided in 2 blocks, 3 center points and error degree freedom of 19. The response factor was determined by vanillin assay of PHWE processed extracts. In fact, after extraction, the percentage of ethanol was removed under nitrogen flow and a liquid-liquid extraction with hexane (2 times) was performed in order to eliminate the small fat content (<2%). The presence of fat could interfere with subsequent lyophilisation and vanillin assay. Extraction yield %, calculated as mg PHWE lyophilized extract/mg dry matrix x 100, fat content, expressed as g/100 g DM and PAs content, expressed as g CE/100 g DM, for all experimental runs of BBD are reported in Table 11.

Table 11 *BBD design.*

Experimental factors				Response factor		
Run	Temperature °C	Cycles n°	Modifier %	Yield %	Fat content	PAs content
1_1	100	5	30	29.52	0.88	5.77
1_2	75	1	20	22.04	0.82	4.23
1_3	75	3	10	21.42	0.56	4.24
1_4	125	3	30	32.60	2.00	5.74
1_5	125	1	20	27.00	0.96	5.06
1_6	100	1	30	25.40	1.04	5.18
1_7	75	5	20	24.52	0.60	5.13
1_8	100	5	10	26.66	0.24	4.78
1_9	125	5	20	29.86	0.94	5.09
1_10	100	3	20	26.22	0.76	4.94
1_11	100	3	20	26.44	0.84	4.96
1_12	75	3	30	25.88	0.94	5.18
1_13	125	3	10	27.02	0.48	5.21
1_14	100	3	20	26.34	0.58	5.05
1_15	100	1	10	23.54	0.54	4.12
2_1	100	5	30	28.04	1.22	5.25
2_2	75	1	20	22.02	0.92	4.28
2_3	75	3	10	22.00	0.56	4.19
2_4	125	3	30	30.40	1.38	5.41
2_5	125	1	20	27.48	1.08	4.89
2_6	100	1	30	26.38	0.96	4.92
2_7	75	5	20	25.76	0.82	4.82
2_8	100	5	10	24.46	0.36	4.49
2_9	125	5	20	32.28	0.98	5.30
2_10	100	3	20	26.96	0.80	5.58
2_11	100	3	20	27.36	1.30	4.99
2_12	75	3	30	24.56	1.02	4.51
2_13	125	3	10	27.44	0.56	4.79
2_14	100	3	20	26.32	1.48	4.52
2_15	100	1	10	22.26	0.54	4.01

Extraction yield %, as mg PHWE lyophilized extract/mg dry matrix x 100, fat content as g/100 g DM and PAs content as g CE/100 g DM.

Table 12 shows each of the estimated effects and interactions and the standard error of each effect, which measures their sampling error. It's possible to note the largest variance inflation factor (V.I.F.) equal to 1.0. For a perfectly orthogonal design, all of the factors would equal to 1.

Table 12 *Estimated effects for PAs content.*

Effect	Estimate	Stnd. Error	V.I.F.
average	4.88767	0.044178	
A: temperature	0.6375	0.120986	1.0
B: cycles	0.4925	0.120986	1.0
C: modifier	0.76625	0.120986	1.0
block	-0.182	0.0883559	1.0

Standard errors are based in total error with 25 degree freedom.

The statistical significance of the influence of experimental factors on response factor was evaluated by analysis of variance (ANOVA). The ANOVA table partitions the variability of PAs content into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. Values of p-value lower than 0.05 indicate that the effect is statistically significant on the determined response factor. Table 13 reports analysis of variance. In this case, 4 effects have p-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level.

Table 13 *Analysis of Variance for PAs content.*

Source	Sum of squares	Df	Mean Square	F-Ratio	P-Value
A: temperature	1.50676	1	1.50676	25.73	0.0000
B: cycles	0.970225	1	0.970225	16.57	0.0004
C: modifier	2.34856	1	2.34856	40.11	0.0000
blocks	0.24843	1	0.24843	4.24	0.0500
Total error	1.46377	25	0.0585508		
Total (corr.)	6.53774	29			

The R-squared statistic indicates that the model as fitted explains 77.61% of the variability in PAs content. The adjusted R-squared statistic, which is more suitable for comparing models with different number of independent variables is 74.03%. The effects of each estimated experimental factors for the considered response factor are shown in Pareto charts (Figure 18) in which the effects are in descending order of significance. The vertical line indicates the 95% confidence level (p-value <0.05). The size of the effects is proportional to the length of the bar.

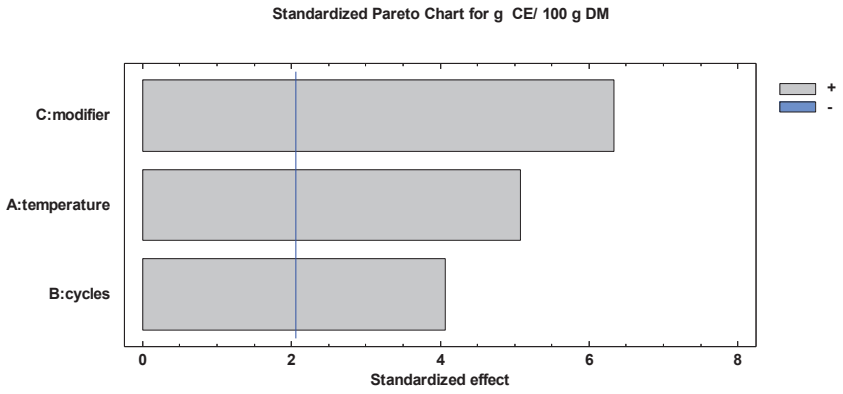

Figure 18 *Pareto chart for PAs content.*

As shown also in Figure 18, all parameters are statistically significant at level of p = 0.05, exerting a positive effect. The increase of temperature modifier % and cycles determine an improvement in the efficiency of extraction (positive effect), in terms of yield and content of bioactive compounds.

The positive influence of the temperature on the efficiency of extraction is related to the increase of the water solvent power at high temperatures and to breakage of bonds between analyte and matrix. Similarly the solvent modifier has a positive influence (the most pronounced), because it improves solubility of analytes and facilitates their desorption. These observations are shown also in Figure 19 which reports the main effects on PAs content in the range between the lowest value and the highest value for each experimental parameter.

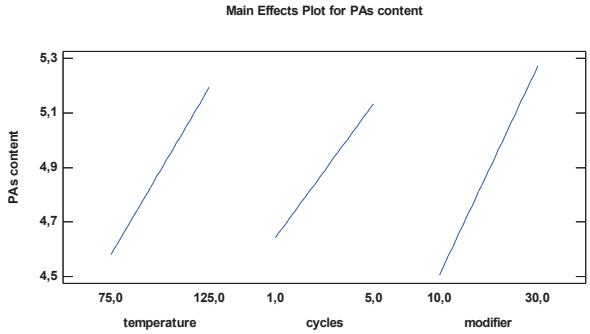

Figure 19 *Main effects of experimental factors.*

Table 14 displays the calculated desirability of the responses at each run in the experiments. Based on the observed responses, the most desirable results were obtained for run 1. Based on the predicted responses from the fitted model, the most desirable results correspond to run 4.

Table 14 *Calculated desirability, observed and predicted value.*

	Observed	Predicted	Observed	Predicted
Run	**PAs content**	**PAs content**	**Desirability**	**Desirability**
1	5.77	5.61	0.9712	0.8847
2	4.23	4.43	0.1488	0.2532
3	4.24	4.29	0.1541	0.1801
4	5.74	5.67	0.9552	0.9171
5	5.06	5.04	0.5920	0.5810
6	5.18	5.12	0.6561	0.6217
7	5.13	4.92	0.6294	0.5162
8	4.78	4.84	0.4425	0.4755
9	5.09	5.53	0.6081	0.8440
10	4.94	4.98	0.5279	0.5486
11	4.96	4.98	0.5386	0.5486
12	5.18	5.05	0.6561	0.5893
13	5.21	4.90	0.6721	0.5079
14	5.05	4.98	0.5867	0.5486
15	4.12	4.35	0.0900	0.2125
16	5.25	5.43	0.6935	0.7875
17	4.28	4.24	0.1755	0.1560
18	4.19	4.11	0.1274	0.0829
19	5.41	5.49	0.7790	0.8199
20	4.89	4.86	0.5012	0.4838
21	4.92	4.93	0.5173	0.5245
22	4.82	4.74	0.4639	0.4190
23	4.49	4.66	0.2876	0.3783
24	5.30	5.35	0.7202	0.7468
25	5.58	4.80	0.8697	0.4514
26	4.99	4.80	0.5547	0.4514
27	4.51	4.87	0.2983	0.4921
28	4.79	4.72	0.4478	0.4107
29	4.52	4.80	0.3036	0.4514
30	4.01	4.17	0.0313	0.1153

In Figure 20 desirability plot is shown obtained by considering simultaneously the effects of experimental parameters investigated on response factor analyzed. Modifier exerts the most marked effect. The PHWE optimal extraction conditions extrapolated from BBD are: temperature: 125 °C;

modifier: 30%; number of cycles: 5. With these conditions, the design provides a degree of desirability equal to 94%.

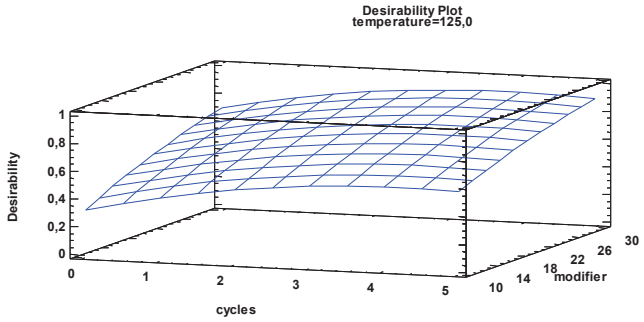

Figure 20 *Desirability plot.*

4B 2.3. PHWE validation and comparison with exhaustive extraction

The optimized conditions suggested by BBD were applied and PHWE extractions were carried out in triplicate. Final PHWE extracts were processed and evaluated in terms of yield extraction %, fat content, PAs content and also by thiolysis experiments. Yield % is higher than RHS EXH (33.2±0.7- 21.2±1.2, % ±SD) due to high pressure of PHWE instrument. In pressurized conditions swelling of matrix with water occurs and solvent can penetrate easier into pores of the hazelnut skins. Water selectivity is maintained for fat extraction (1.4±0.4- 18.4±0.1, % ±SD). As for PAs content, an enrichment of the extract was observed (4.1±0.1- 5.1±0.3, g CE/100 g DM ±SD). Thiolysis experiments were carried out in order to control changes in molar distribution of main monomers and mDP.

The basic aim of work is an extraction better than exhaustive extraction but without affecting the originality of the vegetable matrix, with regard to the degree of polymerization and the molar distribution of its constituents. Figure 21 shows results of thiolysis experiments. As regards molar distribution of catechin (CAT) is not apparent an increase or a decrease as terminal unit or as

extension unit in PHWE extract. The same trend was observed for epi-catechin (eCAT). These data confirm results of preliminary experiments, in fact at optimal temperature of 125 °C depolymerisation phenomena don't occur. So PHWE procedure doesn't modify characteristics and originality of matrix, even if mDP was considered (RHS mDP 8.3±0.1 and PHWE 8.6±0.6).

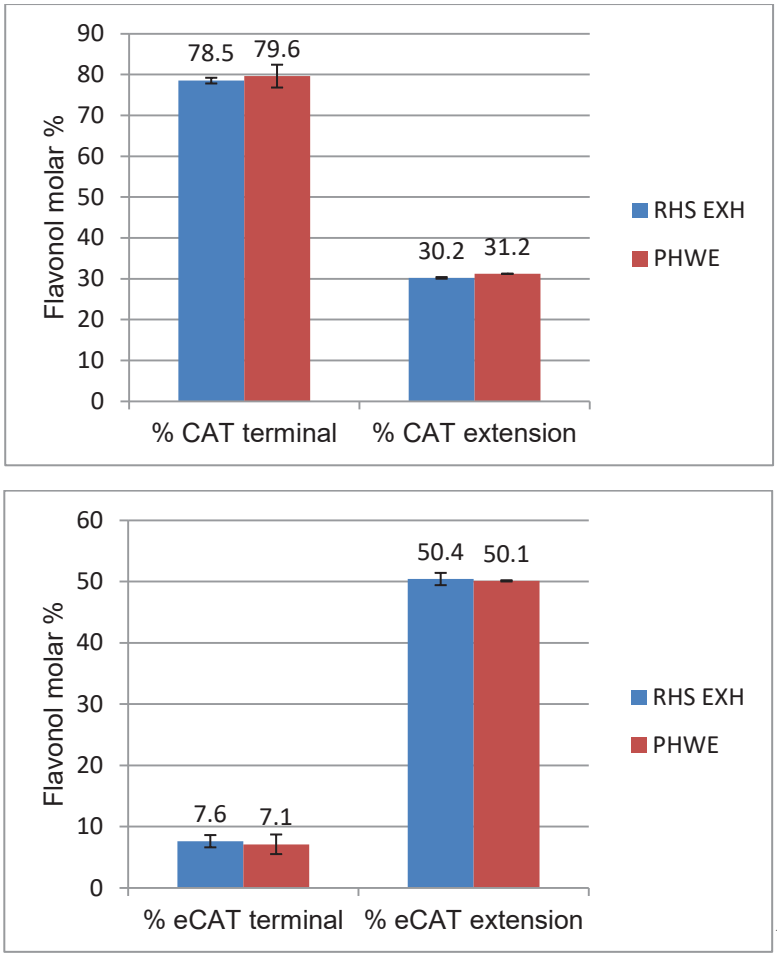

Figure 21 *CAT terminal and extension units flavonol molar % in RHS EXH and in PHWE at optimal conditions (a), eCAT terminal and extension units flavonol molar % in RHS EXH and in PHWE at optimal conditions (b).*

4B 3. Conclusions

Response surface methodology has allowed developing and optimizing a model in order to recover PAs from RHS by-product by pressurized hot water extraction. Temperature, number of cycles and modifier % were significant parameters that can influence positively PAs content. The extraction procedure possesses potential industrial applications: PHWE extraction in optimal conditions permits to obtain extracts with low fat content and high PAs content. Furthermore natural distribution of molecules in matrix doesn't change and mDP doesn't decrease, avoiding depolymerisation phenomena that occur at high temperatures. PHWE procedure is fast, economic, environmentally friendly and more efficient than conventional extraction methods. On the other hand, PHWE could be used for recovery of other bioactive compounds from agricultural and food by-products.

4B 4. Materials and methods

4B 4.1. Materials

Methanol (CH_3OH) and acetone $(CH_3)_2CO$ HPLC grade, absolute ethanol (CH_3CH_2OH), water ultrapure and methanol ultrapure were purchased from Romil (Cambridge, UK) and water grade milli-Q (18MΩ) was obtained using a Milli Q apparatus (Millipore, Bedford, USA). N-hexane was purchased from Merck (Germany) and hydrochloric acid HCl 37% and acetic acid from Carlo Erba (Italy). The standard used (+)-catechin hydrate ≥98% HPLC, vanillin reagent plus 99%, ammonium formate, formic acid and benzilmercaptane were purchased from Sigma Aldrich (Milan, Italy). The stock standard solution (+)-catechin hydrate was prepared in methanol a concentration of 1 mg/mL and stored at 4 °C.

4B 4.2. Hazelnut skin samples

RHS samples were kindly supplied by an Italian hazelnut processing industry (Hazelnuts South Italy Manufacturing S.r.l., Baiano, Avellino). RHS is waste of daily industrial processing, carried out on Campania hazelnut varieties (90% Mortarella and 10% Lunga San Giovanni). RHS material was finely blended using a knife mill Grindomix GM 200 (Retsch, Haan, Germany).
The resulting ground samples were sieved through a test sieve with a range of 300-600 μm to obtain powders with a homogeneous particle size distribution. Exhaustive reference extractions were performed by solid liquid extraction assisted by ultrasounds in triplicate, after defatting matrix with hexane, for 30 minutes (x 3) at 25 °C in a thermostat-controlled ultrasound bath (Labsonic LBS2, Treviglio, Italy) at the frequency of 20.0 kHz, using aqueous acetone (70% v/v) and a matrix/solvent ratio of 1:10. The extracts were pooled, filtered (Whatman No. 1 filter) and freeze-dried, after the removal of the organic solvent under vacuum at 40 °C in a rotary evaporator (Rotavapor R-200, Buchi Italia s.r.l, Cornaredo, Italy).

4B 4.3. Pressurized hot water extraction (PHWE)

A Dionex Accelerated Solvent Extraction (ASE) 200 System (Dionex, Sunnyvale, CA) equipped with a solvent controller unit, with 11 mL stainless steel cells (Restek, Italy) were used for PHWE extractions. Briefly 1 g of sample was placed into cell after homogenization with glass beads. Fixed parameters were: purge 100 sec, flush 150%, pressure 1500 psi and for preliminary experiments 1 cycle of extraction and static time of 5 minutes. Organic solvent from PHWE extracts obtained is removed under nitrogen and a liquid-liquid extraction with hexane (2 times) is performed. At last PHWE extracts were lyophilized (freeze dryer Alpha 1-2 LD, Christ, Germany). Yield

extraction % was calculated as mg PHWE lyophilized extract/mg sample DM x 100, PAs content as catechin equivalent g CE/100 g DM, fat content as g/100 g DM.

4B 4.4. HPLC-HRMS analysis

HPLC analysis were carried out with a Dionex Ultimate 3000 UHPLC system (ThermoFisher Scientific, Milan, Italy) using a Hibar Purospher STAR RP-18 endcapped column (3 µm, 150x3 mm) (Merck, Darmstadt, Germany). A linear gradient (5 to 75% B in 45 min) of water (A) and methanol (B), both with 2mM ammonium formate and 0.1% formic acid, was applied. The flow rate was 300 µL/min and the injection volume 5 µL. The column was thermostated at 25 °C. The detection was performed at the wavelength of 280 nm. HPLC-HRMS analysis were performed with LTQ OrbiTrap XL connected to an Accela system (ThermoFisher Scientific, Milan, Italy) using the same chromatographic conditions. The mass spectrometer, equipped with ESI source, was operated in negative mode. Instrumental parameters were set as follows: source voltage 4.0 kV, capillary voltage −49 V, tube lens voltage −146.5 V, capillary temperature 300 °C, sheath and auxiliary gas flow (N_2) 35 and 10 (arbitrary units), respectively. MS spectra were acquired by full range acquisition covering 250–2000 m/z. For fragmentation study, a data dependent scan was performed by deploying the collision-induced dissociation (CID). The normalized collision energy of the collision-induced dissociation (CID) cell was set at 30 eV. PAs were identified according to the corresponding spectral characteristics: mass spectra, accurate mass, characteristic fragmentation, and retention time. Xcalibur 2.2 software was used for instrument control, data acquisition and data analysis.

4B 4.5. Thiolysis

Thiolysis was performed according to a procedure previously reported (Gu et al., 2002), with some modifications. Briefly, 50 µL methanol acidified with concentrated HCl (3.3%, v/v) and 100 µL of benzyl mercaptan solution (5%, v/v, in methanol) were added to 50 µL of the sample (4 mg/mL in methanol). The mixture was vortexed and the vial was sealed. The reaction was carried out for 30 min at 40 °C and was stopped placing the vial in an ice bath. Immediately 200 µL of water were added to the samples and 10 µL of the reaction mixture was injected for HPLC analysis. Also pure catechin (1 mg/mL) was thiolyzed. Triplicate experiments for sample were performed. The proportions of constituent flavan-3-ol and degree of polymerization (mDP) were calculated according to a published method (Gu et al., 2002).

4B 4.6. Vanillin assay

For determination of condensed tannins, PAs, vanillin assay was employed as reported by Butler with some modifications (Butler et al., 1982). The vanillin solution (0.5% vanillin reagent, w/v, in acetic acid, plus 4% of concentrated HCl) was prepared immediately before use. In brief, 40 µL of extracts or fraction (200 µg/mL in acetic acid) were mixed with 200 µL of vanillin solution in a well of 96-well microplate (96-Well Microtiter Microplates, Thermo Fisher Scientific, Milan, Italy). After 5 minutes at room temperature, absorbance was read at 510 nm using a microplate spectrophotometer reader Multiskan Go (ThermoFisher Scientific, Milan, Italy). A control without vanillin reagent and a blank with acetic acid instead of sample were included in the assay. The concentration of total PAs was estimated from a calibration curve using catechin (range 1-100 µg/mL, prepared in acetic acid) and the data

were expressed as catechin equivalents (CE g/100 g DM, means ± S.D. of three determinations).

4B 4.7. Experimental of design (DoE)

The data were statistically analyzed using statistical software, Statgraphic Centurion XVI Version 16.1 from Statistical Graphics (Rockville, MD, USA). The whole DoE procedure was performed by response surface methodology and in particular using Box–Behnken design (BBD).

CHAPTER V
SECTION A

CHEMICAL PROFILE AND ANTIOXIDANT ACTIVITY OF ESSENTIAL OIL DISTILLATION WASTEWATERS FROM AROMATIC HERBS

5A 1. Introduction

Aromatic herbs are usually used for their colour, aroma and flavour in the preparation of foods and cosmetics. Those belonging to the *Lamiaceae* family are also a source of secondary metabolites with well recognized pharmacologically activities and recently have been exploited as promising ingredients to develop novel products in pharmaceutical, cosmetic and food sectors (Trivellini et al. 2016). Particularly, there is a growing interest in the food industry to replace synthetic antioxidants and additives with natural ones. One of the most effective approaches employs the extracts of aromatic herbs as an affordable and valuable alternative to synthetic additives. In fact, numerous studies demonstrated that herbs of *Lamiaceae* family, mainly rosemary, oregano, sage, basil, mint and thyme, have food-preserving properties because of the presence of antioxidant and antimicrobial phenolic constituents (Trivellini et al. 2016; Embuscado 2015). In addition, the consumption of aromatic herbs is growing related to their value as functional foods which allow a reduction in the need for salt and fatty condiments (Viuda-Martos et al., 2010).

Thus, the improved interest of researchers, consumers and food industries broadened the request and the typology of products based on aromatic plants. In recent years the production of aromatic herbs destined to packaged fresh products for Mass Market Retailers (MMRs) had a sharp increase. This type of destination requires very high quality standards as regards the characteristics of the products, such as color, leaf integrity, absence of yellowing or pest damage and inflorescences, fruit and lignified parts. Therefore, a careful selection of material during packaging and particular cultivation techniques (frequent pruning of perennial crops or periodic elimination of plant at flowering stage) are needed to reach these quality standards. The wastes resulting from processing, packaging and cultivation of packaged fresh herbs

may be useful biomasses for the recovery of high-value products, in line with the concept of biorefinery and green extraction (Lin et al. 2014). A possible use can be the production of essential oils and aromatic waters, by steam distillation processes, to obtain quality products in a traceable supply chain. However, the essential oil steam distillation generates two main by-products: the residual plant materials and the wastewaters of the oil distillation process (distillation wastewaters, DWWs). The latter are generated, during the steam distillation, by the partial condensation of hot water that passes through the vegetable matrix and is collected in the distillation chamber (Wollinger et al., 2016). After the steam distillation process, the non-volatile compounds of aromatic herbs remain in the distillation by-products and the hydrophilic water-soluble fraction can be dissolved in the DWWs followed to the extraction of plant material with condensed hot water. Valorization of the by-products generated from whole chain of production of packaged fresh aromatic herbs, via integrated biorefinery schemes, could target the production of high-value products such as essential oils, aromatic waters and natural food additives and/or functional ingredients for cosmetic, nutraceutical and food applications. Furthermore, the recovery of bioactive compounds from agricultural residues contributes to an improvement in the environmental sustainability of the whole production chain (Taticchi et al., 2013).

In this contest, the potential of aromatic herb DWWs as source of compounds with antioxidant and antimicrobial activity should be evaluated. DWWs are an unexplored by-product and very limited data are available on their chemical characterization. DWWs from some essential-oil crops were proposed as growth promoters and modifiers of the essential oil composition of spearmint (Zheljazkov and Astatkie, 2012). Recently, a chemical study revealed that rose oil DWW is a rich source of flavonoids with strong anti-tyrosinase activities (Rusanov et al. 2014; Solimine et al. 2016). DWWs of rosemary, obtained with two different methods of distillation and characterized in terms of

rosmarinic acid, carnosic acid and antioxidant activity, have been identified as a possible source of the natural antioxidant rosmarinic acid, which is transferred to DWWs during essential oils production (Wollinger et al., 2016). Thus, the aim of the present study was to determine the qualitative and quantitative chemical profiles and antioxidant activity of DWWs of three common culinary herbs, basil (*Ocimum basilicum* L.) (B), rosemary (*Rosmarinus officinalis* L.) (R) and sage (*Salvia officinalis* L.) (S), obtained from the steam distillation of the waste materials produced during the processing of packaged fresh aromatic herbs destined to MMRs and the common cultivation procedures. Moreover, DWWs arising from two different systems of essential oil distillation have been taken into consideration: the distiller Albrigi (DWW1) and a distiller prototype (EOE-10) developed (DWW2).

5A 2. Results and discussion

5A 2.1. Qualitative analysis

The chemical profile of DWWs basil, rosemary and sage was determined by the development of an analytical method UHPLC-DAD-HRMS, coupling the high efficiency of ultra high performance liquid chromatography (UHPLC) separation to two detectors, a photodiode detector and a high resolution mass spectrometer (LTQ-Orbitrap). For the development of chromatographic method different chromatographic conditions have been carefully applied and optimized. Mass spectrometry experiments were conducted in positive and negative ionization mode, and the latter showed better sensitivity and was chosen. Bioactive compounds of DWWs were characterized on the basis of

UV/Vis, HRMS and MS/MS spectra and comparing data with reference standards, when available, or with literature data. In Table 15, are listed identified compounds in DWWs and their chromatographic values (Tr), UV (λ max) and MS (accurate mass, molecular formula, error mass and diagnostic product ions). The UHPLC-DAD-HRMS^n analysis allowed obtaining identification of 76 compounds (36 in B-DWW, 52 in R-DWW and 45 in S-DWW) which can be grouped into three major classes of secondary metabolites: phenolic acids, flavonoids and phenolic diterpenes. Phenolic acids are the main constituents of DWWs. Most of the identified phenolic acids are caffeoyl derivatives. Caffeic acid (CA) plays a central role in the biochemistry of the *Lamiaceae*, and is present mainly in dimeric form as rosmarinic acid (Lu and Foo, 2002). However, CA is the building block of numerous metabolites, derived from the condensation of CA and dihydroxyphenyl lactic acid (danshensu, DSS) or Diels Alder reaction between the double bond of CA and phenolic rings (Lu and Foo, 2002). Caffeoyl derivatives identified were classified into monomers, dimers, trimers, tetramers, based on the number of C6-C3 units.

Table 15 *Characterization by UHPLC-DAD-HRMS^n of B-DWWs, R-DWWs e S-DWWs.*

Peak	t_R MS min	Measured m/z	Error (ppm)	Formula	λmax (nm)	MS/MS fragments	Proposed metabolite	B	R	S
Hydroxybenzoic acids and derivatives										
3	8.6	447.1125	-1.9	$C_{18}H_{24}O_{13}$	254	315 [M-H-pentose-H_2O]$^-$; 297 [M-H-pentose]$^-$; 271[M-H-$C_6H_8O_6$]$^-$	Unknown	X	X	X
7	9.4	521.1493	-1.4	$C_{21}H_{30}O_{15}$	Nr	341(100) [M-H-hexose-H_2O]$^-$; 269(25) [M-H-$C_9H_{16}O_8$]$^-$; 197(90) [M-H-hexose-2H_2O]	Syringoyl dihexoside	X		
9	9.6	417.1018	-1.9	$C_{17}H_{22}O_{12}$	325	285(35) [M-H-pentose-H_2O]$^-$; 241(100) [M-H-$C_6H_8O_6$]$^-$	Unknown	X		
Hydroxycinnamic acid and derivatives										
Monomers										
1	8.3	197.0449	2.6	$C_9H_{10}O_5$	280	179(100) [M-H-H_2O]$^-$; 135(30) [M-H-H_2O-CO_2]	Dihydroxyphenyllactic acid, Danshensu (DSS)	X	X	X
4	8.9	353.0868	0.3	$C_{16}H_{18}O_9$	325	309(10) [M-H-CO_2]$^-$; 191(100) [M-H-CA-H_2O]$^-$; 179(52) [M-H-$C_7H_{10}O_5$]$^-$; 135(20) [M-H-$C_8H_{10}O_7$]$^-$	5-caffeoyl quinic acid		X	X
6	9.3	311.0399	0.3	$C_{13}H_{12}O_9$	325	293(65) [M-H_2O]$^-$; 179(55) [M-H-$C_4H_4O_5$]$^-$;149(100) [M-H-CA]$^-$	Caftaric acid	X		
8	9.4	503.1385	-2.5	$C_{21}H_{28}O_{14}$	325	341(100) [M-H-hexose-H_2O]$^-$; 323(45) [M-H-hexose]$^-$; 281(70) [M-H-$C_8H_{14}O_7$]$^-$; 251(35) [M-H-$C_9H_{16}O_8$]$^-$; 221(10) [M-H-$C_{10}H_{18}O_9$]$^-$;179(25) [M-H-2hexose-2H_2O]$^-$	1-caffeoyl-hexosyl-hexose			X

Peak	t_R MS min	Measured m/z	Error (ppm)	Formula	λmax (nm)	MS/MS fragments	Proposed metabolite	B	R	S
Monomers										
10	9.7	341.0862	-1.3	$C_{15}H_{18}O_9$	325	281(100) [M-H-$C_8H_{14}O_7$]$^-$; 251(70) [M-H-$C_9H_{16}O_8$]$^-$; 221(25) [M-H-$C_{10}H_{18}O_9$]$^-$; 179(55) [M-H-2hexose-2H_2O]$^-$	1-caffeoyl hexose		X	X
11	10.0	353.0867	-0.1	$C_{16}H_{18}O_9$	325	309(15) [M-H-CO_2]$^-$; 191(85) [M-H-CA-H_2O]$^-$; 179(65) [M-H-$C_7H_{10}O_5$]$^-$; 173(100) [M-H-CA]$^-$	4-caffeoyl quinic acid		X	X
17	11.0	325.0554	-0.03	$C_{14}H_{14}O_9$	325	193(100) [M-H-$C_4H_4O_5$]$^-$	Fertaric acid	X	X	
20	11.3	179.0344	2.7	$C_9H_8O_4$	325	135(100) [M-H-CO_2]$^-$	Caffeic acid	X	X	X
Dimers										
13	10.5	355.04458	-0.7	$C_{18}H_{11}O_8$	254	311(100) [M-H-CO_2]$^-$; 287(10) [M-H-CO_2-H_2O]$^-$; 267(25) [M-H-2CO_2]$^-$	Schizotenuin D	X		
18	11.1	377.0866	-0.1	$C_{18}H_{18}O_9$	280	359(100) [M-H$_2$O]$^-$; 197(15) [M-H-DSS]$^-$;	Danshensuan C	X	X	X
25	12.5	357.0601	-1.0	$C_{18}H_{14}O_8$	350	313(60) [M-H-CO_2]$^-$; 269(100) [M-H-2CO_2]$^-$	Prolithospermic acid(isomer)	X	X	X
32	14.6	473.0722	1.6	$C_{22}H_{18}O_{12}$	325	311(100) [M-H-CA-H_2O]$^-$; 293(45) [M-H-CA]$^-$; 179 (20) [M-H-CA-$C_4H_6O_6$]$^-$	Chicoric acid	X		
33	14.8	521.1283	-1.2	$C_{24}H_{26}O_{13}$	325	503(20) [M-H-H_2O]$^-$;35 (100) [M-H-hexose -H_2O]$^-$	Rosmarinic acid hexoside		X	
47	19.7	359.0761	-0.2	$C_{18}H_{16}O_8$	325	197(30) [M-H-CA-H_2O]$^-$; 179(25) [M-H-DSS-H_2O]$^-$; 161(100) [M-H-DSS]$^-$	Rosmarinic acid	X	X	X
Trimers										
19	11.3	537.1014	-2.3	$C_{27}H_{22}O_{12}$	280	519(100) [M-H-H_2O]$^-$	Trimer			X

Peak	t_R MS min	Measured m/z	Error (ppm)	Formula	λmax (nm)	MS/MS fragments	Proposed metabolite	B	R	S
Trimers										
24	12.3	537.1015	-2.4	$C_{27}H_{22}O_{12}$	Nr	493(100) [M-H-CO_2]$^-$; 359(25) [M-H-$C_9H_6O_4$]$^-$; 357(20) [M-H-CA]$^-$; 295(45) [M-H-DSS-CO_2]$^-$	Salvianolic acid J/Clinopodic acid E			X
26	12.9	537.1017	-1.9	$C_{27}H_{22}O_{12}$	254	493(42) [M-H-CO_2]$^-$; 339(100) [M-H-DSS]$^-$; 295(10) [M-H-DSS-CO_2]$^-$	Salvianolic acid H/I	X	X	
27	13.1	555.1124	-1.7	$C_{27}H_{24}O_{13}$	325	537(10) [M-H-H_2O]$^-$; 493(100) [M-H-H_2O-CO_2]$^-$; 359(55) [M-H-$C_9H_8O_5$]$^-$	Salvianolic acid K isomer	X	X	
29	13.9	537.1015	-2.2	$C_{27}H_{22}O_{12}$	350	493(100) [M-H-CO_2]$^-$; 359(25) [M-H-$C_9H_6O_4$]$^-$; 357(20) [M-H-CA]$^-$; 295(45) [M-H-DSS-CO_2]$^-$	Salvianolic acid J/clinopodic acid E	X	X	
30	14.1	597.1224	-2.4	$C_{29}H_{26}O_{14}$	280	329(55) [M-H-$C_{15}H_8O_5$]$^-$; 311(100) [M-H-$C_{15}H_{10}O_6$]$^-$	Yunnaneic acid F	X	X	X
44	18.5	537.1253		$C_{27}H_{22}O_{12}$	Nr	519(100) [M-H-H_2O]$^-$	Trimer			X
46	19.2	555.1124	-1.5	$C_{27}H_{24}O_{13}$	325	537(10) [M-H-H_2O]$^-$; 493(100) [M-H-H_2O-CO_2]$^-$; 359(55) [M-H-$C_9H_8O_5$]$^-$	Salvianolic acid K isomer			X
48	20.0	537.1017	-1.9	$C_{27}H_{22}O_{12}$	254	493(100) [M-H-CO_2]$^-$; 313(25) [M-H-CA]$^-$; 295(45) [M-H-DSS-CO_2]$^-$	Lithospermic acid	X	X	
64	29.9	491.0980	1.5	$C_{26}H_{20}O_{10}$	254	311(70) [M-H-DSS-H_2O]$^-$; 293(100) [M-H-DSS]$^-$	Iso/Salvianolic acid C		X	
66	31.5	491.0983	2.2	$C_{26}H_{20}O_{10}$	254	311(70) [M-H-DSS-H_2O]$^-$; 293(100) [M-H-DSS]$^-$	Iso/Salvianolic acid C		X	

Peak	t_R MS min	Measured m/z	Error (ppm)	Formula	λmax (nm)	MS/MS fragments	Proposed metabolite	B	R	S
Tetramers										
37	16.4	717.1424	1.2	$C_{36}H_{30}O_{16}$	254	519(100) [M-H-DSS]$^-$; 475(38) [M-H-DSS-CO_2]$^-$; 339(8) [M-H-DSS-CA]$^-$	Salvianolic acid B/salvianolic acid E	X	X	X
38	16.9	719.1589	-2.4	$C_{36}H_{32}O_{16}$	280	539(100) [M-H-DSS-H_2O]$^-$; 521(25) [M-H-DSS]$^-$; 359(98) [M-H-DSS-CA-H_2O]$^-$; 341(35) [M-H-DSS-CA]$^-$	Nc6	X	X	X
40	17.8	717.1436	-1.9	$C_{36}H_{30}O_{16}$	325	519(100) [M-H-DSS]$^-$; 475(38) [M-H-DSS-CO_2]$^-$; 339(8) [M-H-DSS-CA]$^-$	Salvianolic acid B/Salvianolic acid E	X		X
42	18.4	717.1436	-1.9	$C_{36}H_{30}O_{16}$	325	555(100) [M-H-CA-H_2O]$^-$; 537(15) [M-H-CA]$^-$; 519(50) [M-H-DSS]$^-$; 375(15) [M-H-DSS-CO_2]$^-$	Clinopodic acid I	X		
52	23.9	717.1436	-1.9	$C_{36}H_{30}O_{16}$	254	519(100) [M-H-DSS]$^-$; 475(38) [M-H-DSS-CO_2]$^-$; 339(8) [M-H-DSS-CA]$^-$	Salvianolic acid B/Salvianolic acid E	X		
67	32.1	717.1437	-1.9	$C_{36}H_{30}O_{16}$	325	519(100) [M-H-DSS]$^-$; 475(38) [M-H-DSS-CO_2]$^-$; 339(8) [M-H-DSS-CA]$^-$	Salvianolic acid B/Salvianolic acid E			X
69	32.6	717.1436	-1.9	$C_{36}H_{30}O_{16}$	325	519(100) [M-H-DSS]$^-$; 475(38) [M-H-DSS-CO_2]$^-$; 339(8) [M-H-DSS-CA]$^-$	Salvianolic acid B/Salvianolic acid E	X	X	

Peak	t_R MS min	Measured m/z	Error (ppm)	Formula	λmax (nm)	MS/MS fragments	Proposed metabolite	B	R	S
Esamers										
50	21.1	1075.2153 (537.1036)*	1.6	$C_{54}H_{44}O_{24}$	325	877(100) [M-H-DSS]$^-$; 679(20) [M-H-2DSS]$^-$; 197(30) [M-H-2DSS-3CA]$^-$	Clinopodic Acid O isomer	X		
63	28.7	1075.21513 (537.1036)*	1.6	$C_{54}H_{44}O_{24}$	325	877(100) [M-H-DSS]$^-$; 679(20) [M-H-2DSS]$^-$; 197(30) [M-H-2DSS-3CA]$^-$	Clinopodic Acid O isomer	X		
Eptamers										
65	31.2	1253.240833 (626.1172)*		$C_{63}H_{50}O_{28}$	325		Eptamer	X		
Ottamers										
68	32.3	1433.28441 (716.1339)*	3.5	$C_{72}H_{58}O_{32}$	325	1235(15) [M-H-DSS]$^-$; 1037(30) [M-H-2DSS]$^-$; 877(10) [M-H-2DSS]$^-$; 679(20) [M-H-3DSS]$^-$; 617(100) [M-H-2H-DSS]$^-$; 518(70) [M-H-2H-2DSS]$^-$; 357(20) [RA-2H]$^-$	Clinopodic Acid P	X		

Flavonoids										
Flavanones derivatives										
41	17.9	609.1802	-1.9	$C_{28}H_{34}O_{15}$	280	301(100) ([$C_{16}H_{14}O_6$] 2.72 ppm) [M-H-deoxyhexose-hexose-2H_2O]$^-$	Hesperidin		X	X
Flavones derivatives										
12	10.2	593.1495	-0.9	$C_{27}H_{30}O_{15}$	325	575(15) [M-H-H_2O]$^-$; 503(30) [M-H-$C_3H_6O_3$]$^-$; 473(100) [M-H-$C_4H_8O_4$]$^-$; 383(35) [$C_8H_{16}O_8$]$^-$;	Vicenin 2	X	X	X
28	13.3	593.1491	-1.6	$C_{27}H_{30}O_{15}$	350	285(100) ($C_{15}H_{10}O_6$. 2.05 ppm) [M-H-dexoyhexose-hexose-2H_2O]$^-$	Luteolin-O-pentosyl-hexoside		X	X
31	14.6	447.0913	-1.8	$C_{21}H_{20}O_{11}$	350	285(100) ($C_{15}H_{10}O_6$. 1.55 ppm) [M-H-hexose]$^-$	Luteolin-O-glucoside		X	X
34	14.9	461.0707	-1.6	$C_{21}H_{18}O_{12}$	350	285(100) ($C_{15}H_{10}O_6$. 1.55 ppm) [M-H-$C_6H_8O_6$]$^-$	Luteolin-O-glucuronide		X	X
35	15.4	477.1020	-1.6	$C_{22}H_{22}O_{12}$	350	462(8) [M-H-CH_3]$^-$; 315(100) [M-H-hexose-H_2O]$^-$; 300(10) [M-H-hexose-H_2O-CH_3]$^-$	Nepitrin		X	X
39	17.6	607.1646	-1.9	$C_{28}H_{32}O_{15}$	350	299(100) [M-H-dexoyhexose-hexose-2H_2O]$^-$; 284(28) [M-H-dexoyhexose-hexose-2H_2O-CH_3]$^-$	Diosmin		X	X
43	18.5	533.0917	-1.6	$C_{24}H_{22}O_{14}$	350	515(25) [M-H-H_2O]$^-$; 489(100) [M-H-CO_2]$^-$; 285(45) [M-H-$C_9H_{12}O_8$]$^-$	Luteolin-O-malonylhexoside			X
45	19.0	461.1071	-1.4	$C_{22}H_{22}O_{11}$	350	446(30) [M-H-CH_3]$^-$; 299(100) [M-H-hexose-H_2O]$^-$; 285(45) [M-H-hexose-H_2O-CH_3]$^-$;	Homoplantaginin		X	X
49	20.3	461.0707	-1.5	$C_{21}H_{18}O_{12}$	350	285(100) [M-H-$C_6H_8O_6$]$^-$	Luteolin-O-glucuronide		X	
53	25.3	503.0832	2.5	$C_{23}H_{20}O_{13}$	350	443(35) [M-H-$C_2H_4O_2$]$^-$; 399(45) [M-H-$C_3H_4O_4$]$^-$; 285(100) [M-H-$C_8H_{10}O_7$]$^-$;	Luteolin-O-acetylglucuronide		X	

Peak	t_R MS min	Measured m/z	Error (ppm)	Formula	λmax (nm)	MS/MS fragments	Proposed metabolite	B	R	S
Flavones derivatives										
54	25.7	491.1199	3.1	$C_{23}H_{24}O_{12}$	254	329(100) $[M-H-hexose-H_2O]^-$	Trihydroxy-dimethoxyflavone-O-hexoside		X	
55	25.7	653.1515	2.2	$C_{32}H_{30}O_{15}$	325	329(55) $[M-H-hexose-CA-2H_2O]^-$; 323(100) $[M-H-Aglicone]^-$; 315(68) $[M-H-hexose-CA-CH_3-2H_2O]^-$; 300(48) $[M-H-hexose-CA-2CH_3]^-$	Gnaphaloside A		X	
57	25.8	491.1182	-0.3	$C_{23}H_{24}O_{12}$	254	329(100) $[M-H-hexose-H_2O]^-$	Trihydroxy-dimethoxyflavone-O-hexoside		X	
58	26.3	475.1244	1.9	$C_{23}H_{24}O_{11}$	280	313(100) $[M-H-hexose-H_2O]^-$	Cirsimarin		X	
59	26.4	503.0824	0.1	$C_{23}H_{20}O_{13}$	350	443(35) $[M-H-C_2H_4O_2]^-$; 399(45) $[M-H-C_3H_4O_4]^-$; 285(100) $[M-H-C_8H_{10}O_7]^-$	Luteolin-O-acetyl-glucuronide		X	
70	35.6	313.0712	1.6	$C_{17}H_{14}O_6$	325	298(100) $[M-H-CH_3]^-$; 283(10) $[M-H-2CH_3]^-$;	Cirsimaritin		X	X
Flavonol derivatives										
21	12.0	477.0656	-1.7	$C_{21}H_{18}O_{13}$	350	301(100) $[M-H-C_6H_8O_6]^-$	Quercetin-O-glucuronide		X	X
22	12.0	463.0861	-2.2	$C_{21}H_{20}O_{12}$	350	301(100) $[M-H-hexose-H_2O]^-$	Quercetina-O-hexose		X	X
36	15.7	491.0812	-1.6	$C_{22}H_{20}O_{13}$	350	476(60) $[M-H-CH_3]^-$; 315(100) $[M-H-hexose-CH_3]^-$	Isorhamnetin-O-hexose		X	X
Ditherpene phenols										
71	36.0	345.1698	0.5	$C_{20}H_{26}O_5$	254	283(100) $[M-H-CO_2]^-$; 301(25) $[M-H-H_2O]^-$	Epi/Rosmanol	X	X	X
72	36.5	345.172	4.5	$C_{20}H_{26}O_5$	Nr	283(100) $[M-H-CO_2]^-$; 301(25) $[M-H-_{H_2O}]^-$	Epi/Rosmanol	X	X	X

Peak	t_R MS min	Measured m/z	Error (ppm)	Formula	λmax (nm)	MS/MS fragments	Proposed metabolite	B	R	S
Ditherpene phenols										
73	38.7	343.1539	-0.1	$C_{20}H_{24}O_5$	254	299(100) $[M-H-CO_2]^-$; 315(35) $[M-H-CO]^-$	Rosmadial	X	X	X
74	39.3	329.1747	-0.02	$C_{20}H_{26}O_4$	254	285(100) $[M-H-CO_2]^-$	carnosol	X	X	X
75	41.0	331.1902	-0.6	$C_{20}H_{28}O_4$	280	287(100) $[M-H-CO_2]^-$	carnosic acid		X	X
76	41.9	345.2058	-0.7	$C_{21}H_{30}O_4$	280	283(100) $[M-H-CO_2]^-$; 301(25) $[M-H-H_2O]^-$; 286(55) $[M-H-CO_2-CH_3]^-$	methyl carnosate	X	X	X
Other compounds										
2	8.7	447.1489	-1.9	$C_{19}H_{28}O_{12}$	254	293(100) $[M-H-C_7H_{10}O_3]^-$; 233(20) $[M-H-C_{10}H_{14}O_6]^-$; 153(15) $[M-H-C_{11}H_{18}O_9]^-$	Unknow		X	
5	9.3	449.2006	-0.2	$C_{20}H_{34}O_{11}$	Nr	223(100) $[M-H-hexose-H_2O]^-$	Ionol-hexoside		X	
14	10.5	387.1644	-1.6	$C_{18}H_{28}O_9$	Nr	369(20) $[M-H-H_2O]^-$	Tuberonic acid hexoside	X	X	X
15	10.5	401.1436	-1.5	$C_{18}H_{26}O_{10}$	Nr	269(100) $[M-H-pentose-H_2O]^-$; 161(20) $[M-H-C_9H_{16}O_5]^-$	Icariside F2		X	X
16	10.7	385.1853	-1.1	$C_{19}H_{30}O_8$	Nr	223(100) $[M-H-hexose-H_2O]^-$; 205(25) $[M-H-hexose]^-$	Drovomifoliol-O-B-D-glucopyranoside (roseoside A)	X	X	X
23	12.3	377.1803	-0.8	$C_{17}H_{30}O_9$	Nr	359(100) $[M-H-H_2O]^-$;	Unknow	X		

Peak	t_R MS min	Measured m/z	Error (ppm)	Formula	λmax (nm)	MS/MS fragments	Proposed metabolite	B	R	S
Other compounds										
51	23.1	999.1803 (499.0860)*	-0,2	$C_{48}H_{40}O_{24}$	254	823(20) [M-H-$C_6H_8O_6$]$^-$;801(35) [M-H-DSS]$^-$, 757(55) [M-H-DSS-CO_2]$^-$; 581(100) [M-H-$C_6H_8O_6$-DSS-CO_2]	Unknow		X	
56	25.7	593.2216	-2.1	$C_{29}H_{38}O_{13}$	Nr	561(20) [M-H-CH_3OH]$^-$; 519(10) [M-$HC_3H_6O_2$]$^-$; 387(100) [$C_{11}H_{10}O_3$]$^-$	Unknow			X
60	26.6	563.2111	-2.0	$C_{28}H_{36}O_{12}$	Nr	531(20) [M-H-CH_3OH]$^-$; 489(10) [M-$HC_3H_6O_2$]$^-$; 387(100) [$C_{10}H_8O_3$]$^-$	Unknow			X
61	27.1	651.2271	-1.9	$C_{31}H_{40}O_{15}$	Nr	505(75); [M-H-deoxyhexose-H_2O]$^-$; 475(100) [M-H-ferulic acid-H_2O]$^-$	Martynoside			X
62	27.9	435.0914	-1.7	$C_{20}H_{20}O_{11}$	Nr	315(20) [M-H-salicilic acid-H2O]$^-$; 297(100) [M-H-Salicilic acid]$^-$	Shimobashiraside C			X

The caffeic acid (CA, 20) and DSS (1) are the main monomers present in all DWWs studied. Other derivatives are: esters with quinic acid (acid 5- and 4-caffeoylquinic, 4 and 11), glycosidic esters (1-caffeoyl-hexosyl-hexose, 8 and 1 caffeoyl-hexose, 10), and esters with tartaric acid (caftaric acid, 6; fertaric acid, 17). Fragmentation MS/MS characteristics of CA oligomers are represented by product ions at m/z 197.0444 (DSS) and 179.0339 (CA) and the residue loss of DSS ($C_9H_{10}O_5$), CA ($C_9H_8O_4$), CA-H_2O ($C_9H_6O_3$), generated by the cleavage of ester bond, and CO_2. Moreover, the relative abundance of the fragment $[M\text{-}H\text{-}CO_2]^-$ is diagnostic to discriminate oligomers according to the presence of cyclic ether structure (Liu et al., 2007). For example, the characterization of 68 is described, an octamer of CA. In Figure 22 spectra are shown (-) - HRMS and MS/MS of 68, which was assigned the formula $C_{72}H_{58}O_{32}$ according to ions at m/z 716.1391 ($[M\text{-}2H]^{2-}$) and 1433.2834 ($[M\text{-}H]^-$) (Figure 22 A). This molecular formula corresponds in databases to acids clinopodic L and P. The analysis of fragmentation MS/MS (Figure 22 B) has allowed discriminating between the two isomers and to assign to peak 68 the structure of clinopodic acid P. In fact, its spectrum is characterized by sequential loss of 2 units of DSS ($[M\text{-}H\text{-}DSS]^-$ in 1235.2351 $[M\text{-}2H\text{-}DSS]^{2-}$ to 617.1117 m/z; ($[M\text{-}H\text{-}2DSS]^-$ in 1037.1794 $[M\text{-}2H\text{-}2DSS]^{2-}$ to 518.0855 m/z), while it is absent the fragment relative to the loss of a unit of CA terminal ($[M\text{-}H\text{-}CA]^-$ or ($[M\text{-}H\text{-}CA\text{-}H_2O]^-$) present in clinopodic acid L. Figure 23 shows the fragmentation pathway of the peak 68 derived from the study of its spectrum MS/MS.

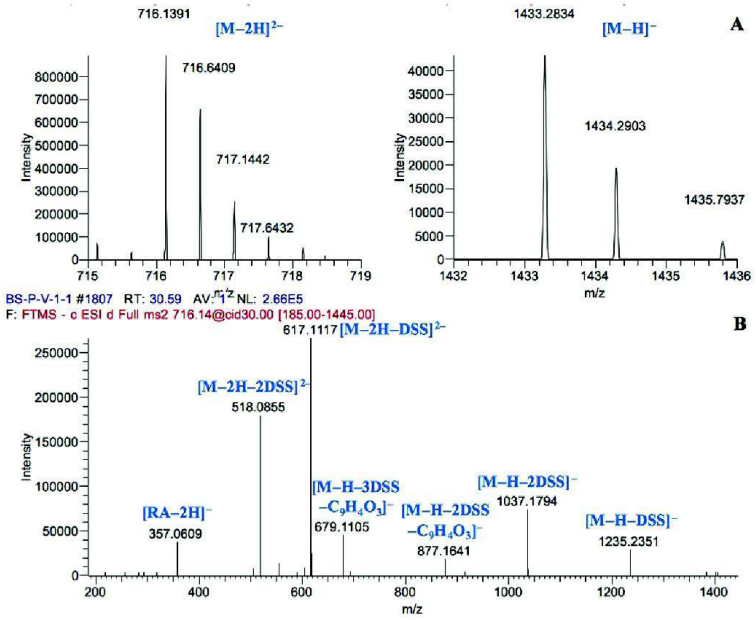

Figure 22 *(-)-HRMS (A) and MS/MS (B) spectra of clinopodic acid P (68).*

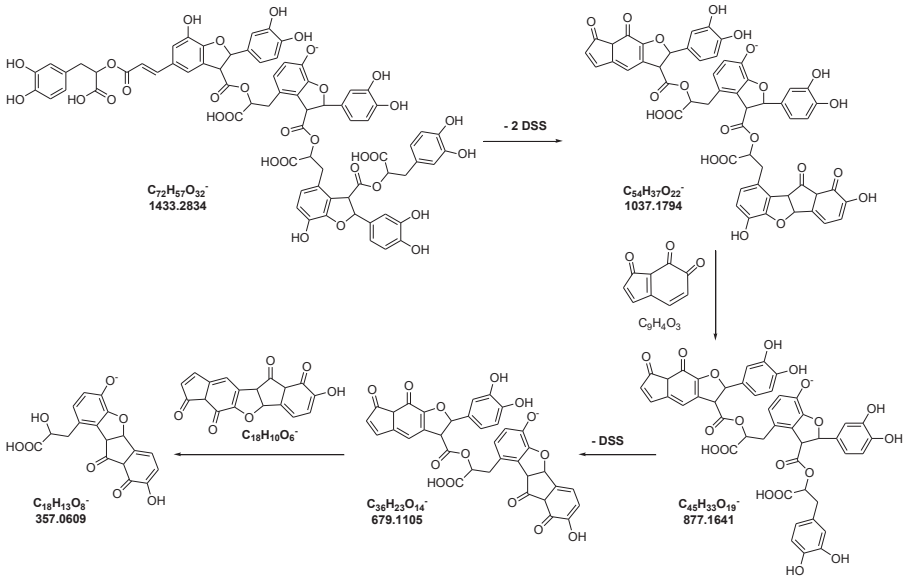

Figure 23 *Fragmentation pathway of clinopodic acid P (68).*

Similarly, it was possible to discriminate some trimers CA isomers ($C_{27}H_{22}O_{12}$). The fragmentation spectra of lithospermic acid (48), salvianolic acid J (24, 29) and H/I (26) (Figure 24) showed a different relative abundance of the fragment ($[M\text{-}H\text{-}CO_2]^-$ 493 m/z that is related to position of carboxylic function. In the case of lithospermic acid (48) and salvianolic acid J (24, 29), the presence of the COOH group on a heterocycle strongly favors the elimination of CO_2 for electron-withdrawing effect of the oxygen. In fact, ion ($[M\text{-}H\text{-}CO_2]^-$ is the base peak of MS/MS spectra of 48 and 24 (Figure 24 A and B). In salvianolic acid H/I structure (26), instead, the loss of CO_2 is not so promoted by the absence of heterocycle, and the base peak corresponds to the direct loss of DSS ($[M\text{-}H\text{-}DSS]^-$339.0502 m/z), unlike 48, 24 and 29 that exclusively showed fragment ($[M\text{-}H\text{-}CO_2\text{-}DSS]^-$, 295.0603 m/z).

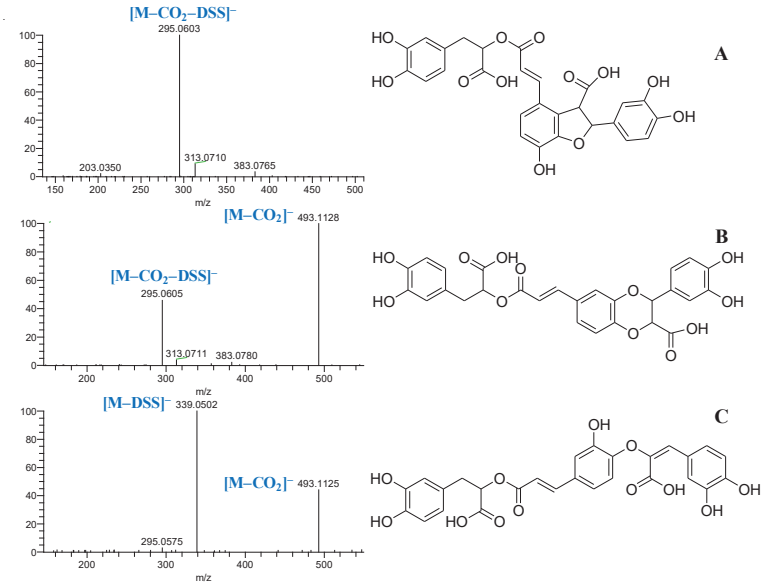

Figure 24 *MS^3 spectrum of 48 (A), and MS/MS spectra of 24 (B) and 26 (C).*

Based on these special fragmentations of the CA oligomers dimers, trimers, tetramers, hexamers, octamers and eptamers were characterized. The main dimer results rosmarinic acid (RA, 47). Other analogues similar to RA were

schizotenuin D (13), danshensuan C (18) and prolithospermic acid (25). In addition other two compounds were characterized as rosmarinic acid hexose (33) and chicoric acid (32). 11 trimers and 7 tetramers have also been identified as characteristic of caffeoyl derivatives. In the case of trimers, have been identified also salvianolic acid K (27) and its isomer (46) (RA + DSS), 2 decarboxylated trimers, salvianolic acid (64) and isosalvianolic acid (66), and yunnaneic acid F (30). Regarding as tetramers, all of them are dimers of RA with the molecular formula $C_{36}H_{30}O_{16}$ (37, 40, 42, 52, 67, 69), except the peak 38 which results from the condensation of RA with two DSS units.

The second class of secondary metabolites identified in DWWs consists of glycosidic flavonoids. The main constituents of DWWs were flavones, of which nine are flavones (28, 31, 34, 43, 45, 49, 53, 55 and 59) mainly derivatives of luteolin (product ion $[Agl–H\ ]^-$ at m/z 285.0400, $C_{15}H_{10}O_6$,. Among these, in addition to those glycosylated form with one (31) or two (28) sugar units, two derivatives were identified as glucuronides (34 and 49), methoxylated derivatives, nepitrin (35) and diosmin (39), two acetylated derivatives, the isomers of luteolin-O-acetilglucuronide (53, 59), and a malonyl derivative (43). Furthermore, among the detected flavones were also two apigenin derivatives: vincenin II (12), a flavone C-glycoside (spectrum MS/MS characterized by characteristic neutral losses of C-glycosides, –30, –60 , –90 and –120 Da), and omoplantaginin (45) product ion $[M–H–CH_3]^-$ and $[M–H–hexose]^-$).

In Figure 25 the MS/MS spectrum of the compound identified as 55 gnafaloside A is shown, a caffeoyl derivative of dimethoxy-luteolin-hexoside. The main product ions observed were 323.0766 and 329.0659 m/z respectively correspond to the fragments $[Caffeoyl\text{-}hexose–2H_2O–H]^-$ and $[Agl–H]^-$ generated by the cleavage of the glycoside bond. The 315.0504 and 300.0270 m/z ions, however, correspond to successive loss of methoxyl group.

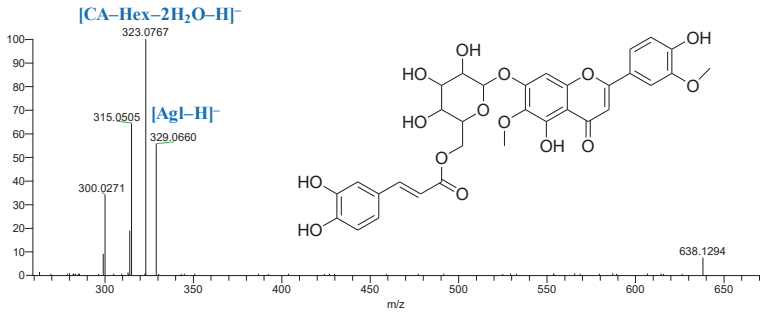

Figure 25 *MS/MS spectrum of gnafaloside A (55).*

As third class of DWWs metabolites have been identified phenolic diterpenes as minority constituents. All these compounds exhibit losses of H_2O and CO_2, diagnostic for this class. Methyl carnosate (76), in addition to the loss of CO_2, presents also the subsequent loss of the methyl $[M–H–CO_2–CH_3]^–$. Some identified compounds are carnosic acid (75), carnosol (74), rosmadial (73), rosmanol and epirosmanol (71 and 74).

5A 2.2. Profiling DWWs

Figure 26 shows the UHPLC-UV profile of BL-, RL- and SL-DWW1s. The qualitative analysis reveals that these by-products contain as majors components the characteristic water-soluble compounds of the *Lamiaceae*. In particular, DSS, CA and RA are the main compounds, in agreement with the literature data regarding as the water-soluble component of basil, rosemary and sage (Shan et al., 2005). B-WWS presents the most distinctive chemical profile (Figure 26 A). Unlike rosemary and sage, it exclusively consists of derivative of CA, while the flavonoids are absent, with the exception of vincenin II that like some phenolic diterpenes, is present only in traces. Other

main constituents of B-WWS are caftaric acid (6), chicoric acid (32) and fertaric acid (17) that were not detected in DWWs of rosemary and sage. Therefore, 6, 32 and 17 can be considered markers of *O. basilicum*, at least from a quantitative point of view. Other typical compounds of B-WWS are clinopodic acid I (42) and oligomers with CA units > 4 (isomers of clinopodic acid O, 50 and 63, and P, 68). This type of caffeoyl derivatives are very rare and have only recently been isolated from 2 species of *Lamiaceae* (Aoshima et al. 2012; Moghadam et al., 2015). Instead, chemical profiles of R- and S-DWWs (Figure 26 B-C) are more complex and heterogeneous. In fact, in both is well represented the flavonoid component in the water-soluble fraction, and there is also a valuable lipophilic component constituted by phenolic diterpenes, mainly rosmanol (71) and methylcarnosate (76). In R-DWW1 (Figure 26 B) the most abundant compounds, after RA and CA, is luteolin-O-glucuronide (49). Luteolin-O-hexoside (31), luteolin-O-glucuronide (34) and salvianolic acid K (46) are typical of S-DWWs (Figure 26 C).

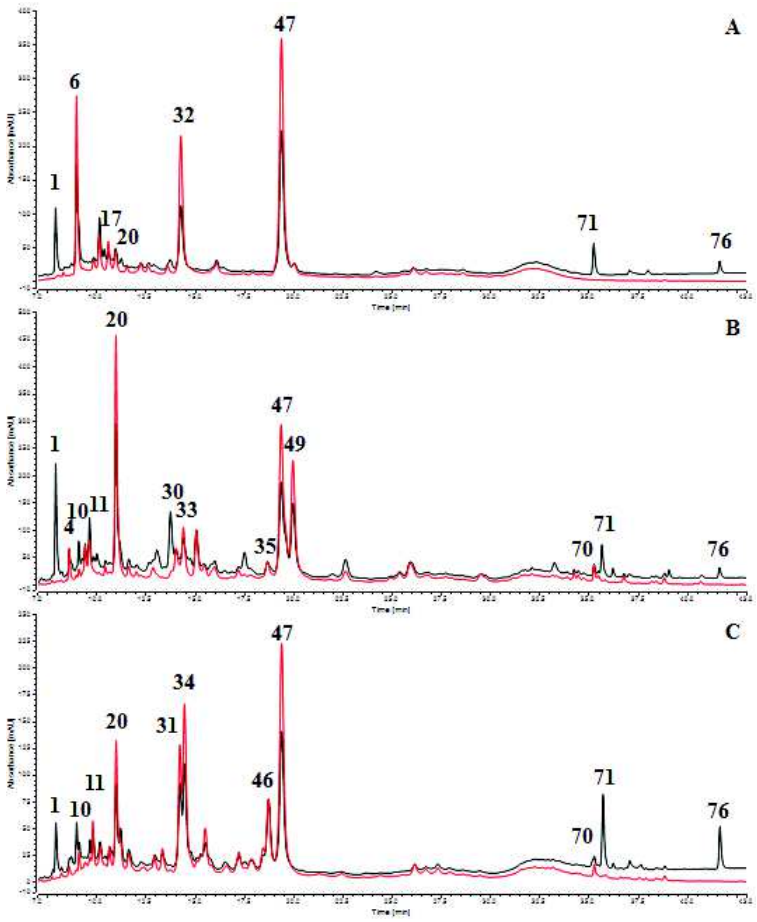

Figure 26 *HPLC-UV profile BL-DWW1 (A), RL-DWW1 (B) e SL-DWW1 (C).*
Black line: 280 nm, Red: 325 nm

5A 2.3. Quantitative analysis by UHPLC-UV

Qualitative analysis data showed that RA is the most abundant compound of DWWs and its content may be characteristic for this type of new by-products. Therefore, a determination of RA by UHPLC-UV was done. Moreover, due to chemical nature and the complexity shown by every sample, total phenolic content (Total Phenolic Content, TPC) was determined by Folin-Ciocalteu colorimetric method, a tool widely used in the characterization of plant extracts and commercial products. The quantitative determination of RA was

carried out at 325 nm, λ max typical of the hydroxycinnamic acids, and using the external standard method. (Figure 27). The results obtained showed a certain variability depending on the species, part of the plant used to do distillation and distillation method. In particular, SL-DWW2 showed the highest content of RA (135 mg/100 mL) and TPC (442 mg GAE/100 mL), while SP-DWW1 the lowest (RA: 1 mg/100 mL; TPC: 11 mg GAE/100 mL). However, the levels of RA and TPC in DWW1s are not negligible, in fact, except for SP, it is possible to observe for DWW1 samples valuable results (RA: 12-20 mg/100 mL; TPC: 112-163 mg GAE/100 mL). In general, the DWWs showed a significant content of RA (12-135 mg/100 mL) and TPC (112-442 mg GAE/100 mL). Regarding TPC data are higher than those reported for infusions of rosemary (19-36 mg GAE/100 mL) and sage (14-73 and 43 mg GAE/100 mL), and in the same range found for infusions of leaves of various aromatic and medicinal plants (488-14 and 55-0.5 mg GAE/100 mL) (Tahirović et al., 2014; Gião et al., 2007). These results indicate that DWWs generated during distillation process of essential oils from aromatic plants are a good source of RA and phenolic compounds.

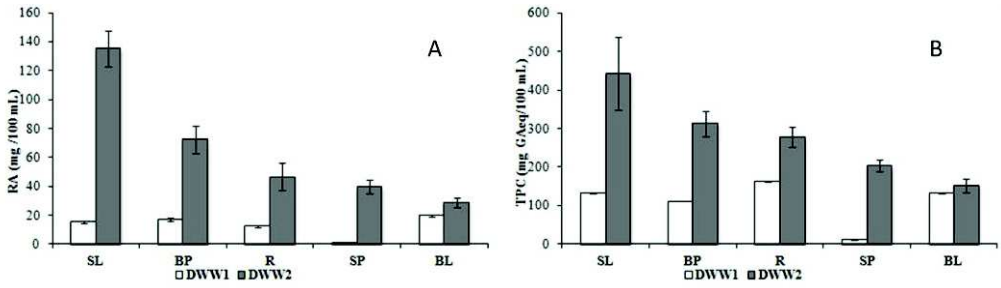

Figure 27 *RA content (A) e TPC (B) of DWW1s and DWW2s.*

Related to various distillers, it was possible to observe that samples obtained with the new prototype (DWW2s) are richer in RA (Figure 27 A) compared to those obtained by the distiller Albrigi (DWW1s). The same trend was observed for TPC (Figure 27 B). This difference is mainly due to the different extractor parameters (load sample 1.5 kg Albrigi and 5 kg EOE-10) and volume of DWW obtained (2-2.6 L for Albrigi and 3.7-4.6 L for EOE-10). There are some differences in the extraction capacity of the water-soluble components for the two different extractors. Supporting this hypothesis there are also relevant differences observed in DWW1 and DWW2 related to chemical profiles (Figure 28) for complexity and for relative distribution of the various constituents.

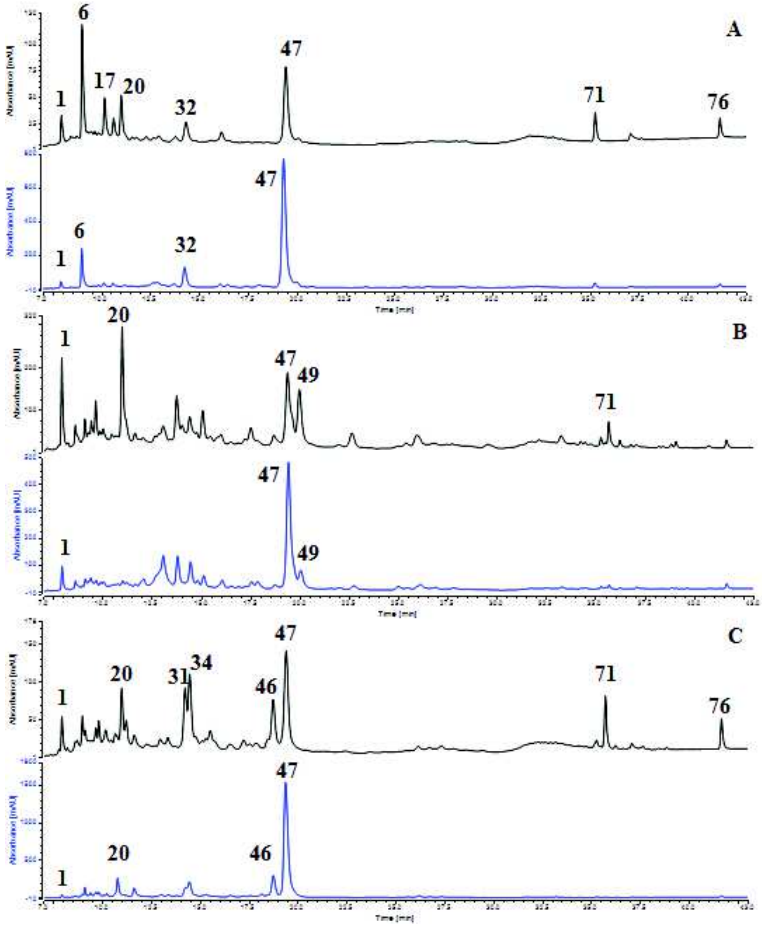

Figure 28 *UHPLC-UV profile (280 nm) of B-DWWs (A), R-DWWs (B) and SL DWWs (C).* Black line: DWW1; blue line: DWW2

The greater complexity of the DWW1 profile is probably related to higher temperature employed of the distiller Albrigi (100 °C instead of 75 °C) which increases the extraction efficiency of condensed water. These data suggest that in the distiller Albrigi, due to the highest working temperature, occurs a degradation of RA (47) as CA (20) and DSS (1) as a result of hydrolysis and / or dehydration reactions. In fact, it is well known instability of RA at high temperatures (Hossain et al., 2011). A similar trend was observed for chicoric acid (32) / caftaric acid (6) (Figure 29), suggesting hydrolysis of these

compounds in distiller Albrigi. These evidences indicate that distiller EOE-10 is superior system for the essential oil quality, and moreover doesn't generate extraction artefacts, due to degradation phenomena.

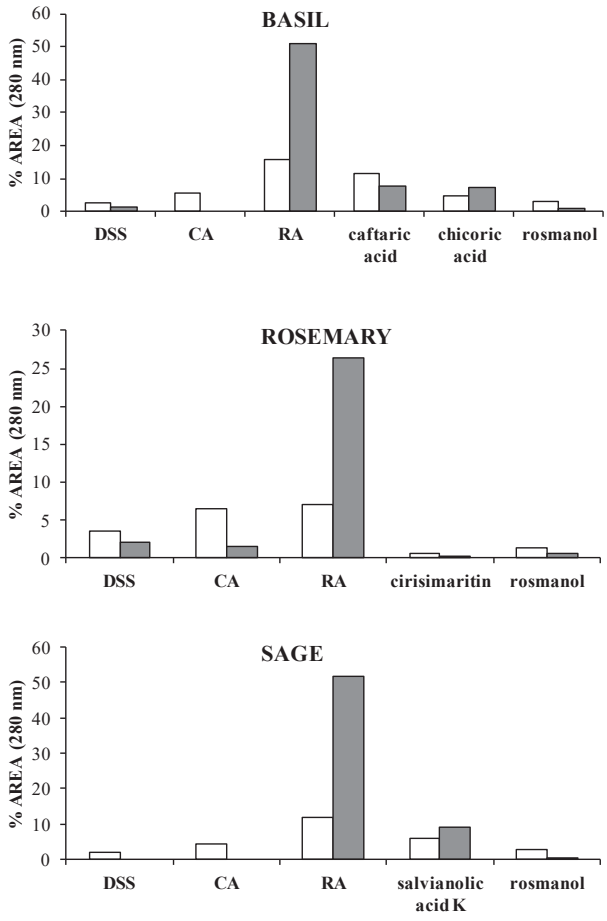

Figure 29 *Relative abundance (% chromatographic area) of main constituents in DWW1s and DWW2s.*

5A 2.4. Antioxidant activities measurement

A compound or an extract can exert antioxidant activity by different mechanisms of action; therefore it is not possible to use a single method to evaluate this capacity. Furthermore, to investigate the behaviour of the compounds in complex matrices is not possible to use a single assay but is necessary a critical selection of different methods based on different chemical mechanisms (single electron transfer- hydrogen atom transfer, SET-HAT). For these reasons, the evaluation of the antioxidant activity of DWWs was performed by three different chemical methods, such as ORAC (HAT), ABTS and DPPH (SET-HAT) assays, which are those most used (Prior et al., 2005) and validated as Official Methods of Analysis of AOAC (Association of Official Agricultural Chemists) (Ou et al., 2013; Brand-Williams et al., 1995). For all three methods were determined firstly the kinetic reaction and the most appropriate range of dilutions, and then the validation of the standard determinations (RA, CA and BHA) was done. The antioxidant capacity of RA, CA and BHA determined are in agreement with data reported in literature (Brand-Williams et al., 1995; Ou et al., 2001). In Table 16 are reported antioxidant capacity DWWs, determined by different methods and expressed as micromol Trolox equivalent (TE)/100 mL. Also there were the values determined for RA and CA, the main constituents of DWWs, and BHA, a common synthetic antioxidant authorized as food additive (EU Regulation No 1129/2011).

Table 16 *Antioxidant capacity of DWWs and standards.*

DWW	DPPH	ABTS	ORAC
	(µmol TE/100 mL)		
SL-DWW1	491.4 ± 40.3	687.3 ± 43.3	833.3 ± 47.4
SL-DWW2	4244.2 ± 825.8	3145.6 ± 522.8	4719.9 ± 870.4
BP-DWW1	494.5 ± 24.5	723.4 ± 59.9	975.8 ± 318.3
BP-DWW2	2250.4 ± 235.1	1444.6 ± 218.6	2212.6 ± 262.2
R-DWW1	1077.9 ± 11.9	1459.4 ± 10.9	871.4 ± 62.0
R-DWW2	2307.4 ± 231.8	1440.6 ± 221.1	3174.6 ± 374.1
SP-DWW1	Nd	Nd	Nd
SP-DWW2	1263.5 ± 140.5	1217.8 ± 201.7	1686.1 ± 217.8
BL-DWW1	952.7 ± 79.0	911.8 ± 11.5	1292.8 ± 58.9
BL-DWW2	635.3 ± 74.0	570.6 ± 58.0	1100.9 ± 121.6
Standards		(µmol TE/µmol)	
RA	1.77 ± 0.16	2.21 ± 0.02	5.86 ± 0.41
CA	1.12 ± 0.17	1.00 ± 0.02	4.52 ± 0.29
BHA	1.05 ± 0.20	0.65 ± 0.01	2.09 ± 0.17

Nd: not detected; n = 3, ± SD, standard deviation.

Antioxidant capacity determined by DPPH and ABTS assays showed the same trend: DWW2s possess greater antioxidant activity than DWW1s, with the following descending order SL> BP ≥ R-> SP> BL-DWW2. Regarding ORAC assay, however, R-DWW2 showed greater activity than BP-DWW2, and SP-DWW2 comparable activity. These differences are related to different antioxidant mechanism evaluated by three methods. ORAC assay is able to measure the ability of an antioxidant to terminate the propagation reactions triggered by free radicals through the transfer of a hydrogen atom (HAT method). Instead, DPPH and ABTS assays allow simultaneously determining the antioxidant capacity of compounds able to neutralize the radicals by transferring a hydrogen atom (HAT) and at same time transferring an electron (SET) (Prior et al., 2005). Therefore, it is possible to deduce that rosemary is richer in type HAT antioxidants. These compounds, also based on quantitative results, probably belong to the flavonoid class, almost absent in basil. However, the relevant activity shown by B-DWWs in ORAC assay indicates that the CA derivatives act as antioxidants using both mechanisms (HAT and

SET). The antioxidant capacity of DWWs is very significant when compared to the activity reported for aqueous liquid matrices. As of DPPH assay, DWWs show an activity higher than green tea (644 µmol TE/100 mL), red wine (1935 µmol TE/100 mL) in the case of SL, BP- and R-DWW2, and comparable to the pomegranate juice (3901 µmol TE/100 mL) (Plank et al., 2012). Even the ORAC values, indicated that DWWs are superior if compared to infusions of various *Lamiaceae* plants (303-1322 µmol TE/100 mL) (Tahirović et al., 2014). These findings further on support the quantitative data and so it's possible to assert that DWWs represent a potential and interesting source of compounds with antioxidant activity. The antioxidant capacity of certain DWWs with the three different assays showed a trend related to the levels of their main constituents. In fact, as shown in Figure 27 and Table 16, samples with high antioxidant activity also have the highest content of RA and TPC. Therefore, in order to validate this thesis and to identify the constituents of DWWs responsible of the antioxidant activity, it was carried out a statistical analysis (Pearson correlation) to assess the significant correlation among RA and TPC and TEAC. In Figure 30 matrix statistical analysis results are shown in the form of the scatterplot. Both TPC and RA show statistical significant correlation with the TEAC data of all three antioxidant evaluation methods: Pearson coefficient > 0.884 and p values <0.0015. These results may determined that antioxidant capacity shown by DWWs is mainly due to their high content RA, and in general the presence of water-soluble phenolic compounds (TPC), indicating that RA and TPC are valid quantitative indexes for the characterization of basil, rosemary and sage DWWs, and the standardization of future products derived from them (freeze-dried, purified fractions and formulations).

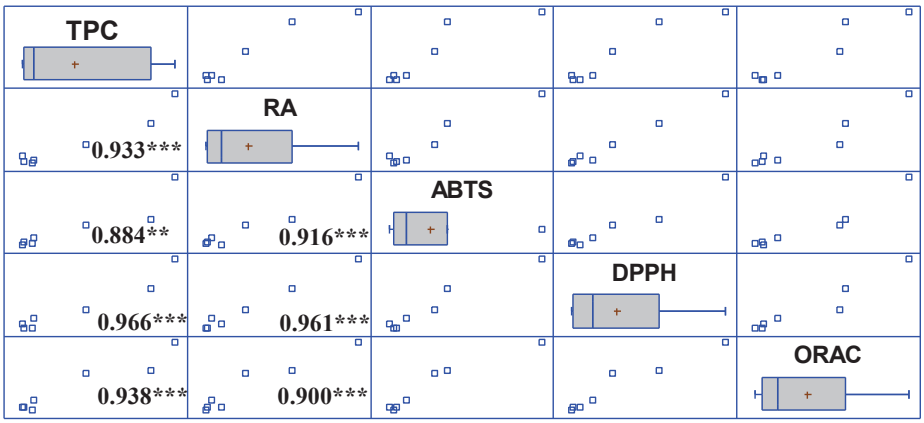

Figure 30 *Scatterplot matrix for correlation TPC and RA and antioxidant capacity (DPPH, ABTS, ORAC).*
Pairs of data = 9; ** $p < 0.01$; *** $p < 0.001$

5A 3. Conclusions

This study about chemical composition and antioxidant activity of DWWs of herbs (basil, rosemary and sage) has shown that DWW represents a new type of by-product to be used as a source of cheap bioactive phenolic compounds with high antioxidant power. In fact, the UHPLC-DAD-HRMS^n analysis revealed the presence of phenolic acids, flavonoids and phenolic diterpenes. Caffeoyl derivatives are main compounds present in all DWWs analyzed. Instead flavonoids are mainly present in glycosidic form of flavones in significant quantities in DWWs of rosemary and sage. The phenolic diterpenes represent minority constituents. In general, the distribution of the identified compounds differs between R-DWWs, B-DWWs and S-DWWs while other compounds appear to be as common, as rosmarinic acid, caffeic acid and danshensu. Rosmarinic acid is the most abundant compound in all DWWs (12-135 mg/100 mL) and can be considered a useful marker for the quantitative characterization of this type of by-products, together with the total phenolic

content (TPC 112-442 mg GAE/100 mL). The levels of RA and TPC indicate that the DWWs generated by the distillation process of essential oils from aromatic plants are rich sources of RA and phenolic constituents. The antioxidant capacity of DWWs, determined by several chemical methods (DPPH, ABTS and ORAC), is very significant if compared to the activity reported for aqueous liquid matrices. A very good statistically significant correlation among antioxidant capacity, RA and TPC was observed in order to confirm that antioxidant activity of DWWs is due mainly to their high content of RA, and in general to the presence of water-soluble phenolic compounds. Finally, regarding the different distiller, the results obtained show that EOE-10 prototype provides DWWs with the highest content of bioactive compounds and the greatest antioxidant capacity, avoiding the formation of artefacts caused by extractions at high temperatures employed in traditional systems (distiller Albrigi). In conclusion, this work encourages future studies about the use of DWWs as natural antioxidants and / or functional ingredients. In fact, their use as substitutes of synthetic antioxidants, in addition to increasing food security, would contribute greatly to raise their added value. In this perspective DWWs become co-distillation products. However to investigate the ability of DWWs to increase the shelf-life of perishable products or their action as functional ingredients, further studies will be necessary.

5A 4. Materials and methods

5A 4.1. Materials

MS-grade acetonitrile (MeCN) and water were supplied by Romil (Cambridge, UK). Ultrapure water (18 MΩ) was prepared by a Milli-Q purification system (Millipore, Bedford, USA). Analytical-grade methanol and

ethanol, MS-grade formic acid, caffeic acid (CA), gallic acid (GA), rosmarinic acid (RA), butylhydroxyanisole (BHA), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), Folin and Ciocalteu's phenol reagent, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), fluorescein sodium salt, potassium persulfate ($K_2S_2O_8$) were obtained from Sigma-Aldrich (Milan, Italy).

5A 4.2. DWW samples

The fresh aromatic herb wastes of *Ocimum basilicum* L. type "Genovese", *Rosmarinus officinalis* L. and *Salvia officinalis* L. were collected at Azienda Agricola Nicola Palma (Capaccio, Salerno, Italy), a farm specialized in the production of packaged fresh herbs for retail chains.

DWWs were obtained from the distillation of the waste material resulting from cultivation (mostly created by common pruning techniques), processing and packaging of aromatic herbs. In the case of basil and sage, DWWs derive from the cultivation waste (BP and SP), twigs and pruning residues, that represent the non-edible parts, and from leaves generated during the processing and packaging (BL and SL). In the case of rosemary, instead, DWWs obtained from the processing and packaging waste material (R) were analyzed.

Steam distillations of waste materials were performed with two different extractors: an industrial scale essential oils extractor EOE-10 (Tred Technology, Campobasso, Italy) operating to low processing temperatures (75 °C) obtained with an integrated vacuum system. This extractor system was loaded with 5 kg of each waste material (three independent extractions), homogeneously distributed and compacted on a perforated grid to ensure the spreading of steam over the entire load and 6 L of water. The steam

distillations were carried out for 1 hour from the appearance of the first drop of distillate. The second distiller (Albrigi) employed operated at 100 °C, with 1.5 kg of waste materials and 3L of water. The cooled DWW samples were filtered immediately through 1.0 μm glass fiber filters (circles size 4.7 cm, Millipore, Bedford, USA), added with ethanol (1%, v/v) and stored at 4 °C until analysis.

5A 4.3. HPLC-DAD-HRMS analysis

Chromatographic analyses were performed using a Platin Blue UHPLC system (Knauer, Labservice Analytica, Bologna, Italy), consisting of two Ultra High-Pressure Pumps, an autosampler, a column temperature manager and a diode array detector, coupled to a LTQ OrbiTrap XL mass spectrometer (ThermoFisher Scientific, Milan, Italy). A Hibar Purospher STAR, RP-18 endcapped (3 X 150 mm, 3 μm; Merck) column was used at a flow rate of 300 μL/min and at temperature of 25 °C. The mobile phase was a binary gradient of water (A) and MeCN (B), both containing 0.1%, v/v, formic acid. The gradient elution program is as follows: 0-22 min, 5-60% B; 22-29 min, 60-98% B. After each injection (5 μL), cleaning (98% B, 6 min) and re-equilibration column (6 min) were performed. UV spectra were acquired in the range of 200-600 nm, and the wavelengths 245, 280, 325 and 350 nm were employed for the detection. The mass spectrometer, equipped with ESI source, operating in negative mode. High purity nitrogen (N_2) was used as sheath gas (30 arbitrary units) and auxiliary gas (10 arbitrary units). High purity helium (He) was used as collision gas. Mass spectrometer parameters were set as follows: source voltage 4.0 kV, capillary voltage –33 V, tube lens voltage –41.5 V, capillary temperature 300ºC. MS spectra were acquired by full range acquisition covering 140–1500 *m/z*. For fragmentation study, a data dependent scan was performed and the normalized collision energy of the collision-

induced dissociation (CID) cell was set at 30 eV and the isolation width of precursor ions was set at *m/z* 2.0. The resolution was 60000 and 7500 for the full mass and the data dependant MS scan, respectively. Phenolic compounds were characterized according to the corresponding spectral characteristics: UV and mass spectra, accurate mass, characteristic fragmentation, and retention time. Xcalibur software (version 2.2) was used for instrument control, data acquisition and data analysis.

5A 4.4. Determination of RA by HPLC-UV

The quantitative analyses were performed using a Dionex Ultimate 3000 UHPLC system (ThermoFisher Scientific) constituted of an Ultimate 3000 RS Pump, an Ultimate 3000 RS autosampler, an Ultimate 3000 RS column compartment and Ultimate 3000 RS variable wavelength detector. The chromatographic conditions were the same as those used for HPLC–DAD-HRMS^n analysis. The UV chromatograms were recorded at 325 nm and calibration external standard method was used to quantify RA in DWWs. Seven different RA concentration levels were prepared diluting with water appropriate volumes of RA stock solution (4 mg/mL, MeOH). Linearity of calibration curve was evaluated in the concentration range of 2-300 µg/mL (triplicate injections for each level). UV peak areas of the external standards (at each concentration) were plotted against the corresponding standard concentrations (µg/mL). The regression curve was tested with the analysis of variance (ANOVA) and linear model was found appropriate over the tested concentration range ($y = 0.9072\ x - 5.0669$; $R^2 = 0.9989$). DWWs were diluted with water for quantification analyses, and the RA amount was expressed as mg/100 mL ± SD (deviation standard, three independent steam distillations).

5A 4.5. Folin-Ciocalteau assay

Total phenolic content (TPC) of DWWs were determined using the Folin-Ciocalteu (FC) colorimetric method according to the literature (Singleton et al. 1999). Briefly, 20 µL of diluted DWW (200 µg/mL) and 5 µL of FC reagent were added to 145 µL of ultrapure water in a 96-well microplate. Then 30 µL of Na_2CO_3 (20%, w/v) were added at each well and finally absorbances were read after 45 min at 725 nm and 25 °C with a microplate spectrophotometer reader Multiskan Go (Thermo Scientific). A control without FC reagent and a blank with water instead of sample were included in the assay. Gallic acid (GA) was used as reference standard and TPC was estimated from the calibration curve (range 5-200 µg/mL, 7 levels; $y = 0.0037\ x + 0.0014$; $R^2 = 0.9992$). Data, expressed as mg gallic acid equivalents mg GAE/100 mL of DWW, were the mean of three independent steam distillations.

5A 4.6. DPPH assay

DPPH assay was performed according to the procedure of Brand-Williams and co-authors (Brand-Williams et al., 1995) adapted for use in 96-well plate. Briefly, 5 µL of diluted DWW samples, pure compounds or MeOH (blank) were added to 195 µL of DPPH 120 µM. After 45 min at dark, the absorbances were read at 515 nm and 25 °C with a microplate spectrophotometer reader Multiskan Go (ThermoFisher, Milan, Italy). All DWW samples and standard solutions were diluted approximately to provide 20–80% of DPPH remaining at the steady state, previously established by reaction kinetics (0-240 min). Compound concentration ranges tested were: RA, 1-8 µg/mL, 5 levels; CA, 1-10 µg/mL, 6 levels; BHA, 2-14 µg/mL, 7 levels. Trolox was used as reference standard (range 2-10 µg/mL, 6 levels, $y = 2.1784\ x – 10.795$; $R^2 = 0.9901$), and the results (mean ± SD, 3 independent

extraction replicates) were expressed as Trolox equivalent antioxidant capacity (TEAC): μmol TE/100 mL of DWW or μmol TE/μmol of pure compound.

5A 4.7. ABTS assay

The ABTS assay was conducted according to the procedure described by Re and co-authors with some modifications (Re et al., 1999). The assay was conducted by adding 5μL of sample, standard or 5 mM PBS (control), all diluted with 5 mM PBS and analyzed in triplicate, to 500 μL of $ABTS^{\cdot+}$ radical (about 0.1 mM). 300 μL of mixture were transferred into the wells of 96-well microplate and the absorbances were read at 734 nm after 1 hour at 30 °C (Thermo Scientific Multiskan go, ThermoFisher, Milan, Italy). The reagent (ABTS radical, approximately 0.1 mM) was prepared by adding 2.5 mL of 7 mM ABTS (ABTS 9.6 mg dissolved in 2.5 mL of milli-Q H_2O) to 44 μL of $K_2S_2O_8$ 139.8 mM (final concentration $K_2S_2O_8$ 2.45 mM). To obtain the formation ABTS radical, the solution was left at dark for 16 hours before use. Then 1 mL of the working solution was diluted with about 70 mL of 5 mM PBS at pH 7.4 up to have an absorbance at 734 nm equal to 0.7 ± 0.02 at 30 °C. For the calibration curve of Trolox (reference antioxidant) 6 levels were prepared in the concentration range of 2-25 μM ($y = 4.0565\ x + 0.0785$; $R^2 = 0.9992$), diluting with PBS 5 mM its stock solution 2.5 mM (3.2 mg of Trolox dissolved in 1 mL of MeOH and diluted to 5 mL with PBS 5 mM). The standards are tested were RA (1-10 μM, 5 levels), CA (2-20 μM, 6 levels) and BHA (2-20 μM, 7 levels). The results were expressed as TEAC, in terms of μmol of Trolox equivalents per 100 mL of sample (μmol TE/100 mL) or TE μmol/μmol for the standards.

5A 4.8. ORAC assay

The ORAC assay was conducted as described by Ou and co-workers (Ou et al., 2013). The assay was conducted in a 96-well microplate adding 150 µL of sodium fluorescein (11.12 x 10^{-2} µM) and 25 µL of sample, 75 mM PBS (control) or standard, all diluted with PBS 75 mM and analyzed in triplicate. The plate was then thermostated at 37 °C for 30 minutes. Subsequently 25 µL of AAPH were added and the plate was immediately placed in a fluorescence reader (Enspire-2300, PerkinElmer, Waltham, MA, USA). The fluorescence was measured every minute for 60 minutes using 485 nm and 530 nm as the wavelength of excitation and emission, respectively. The AAPH solution (0.414 g in 10 mL of PBS 75 mM) was daily prepared and stored at 4 °C before use. The sodium fluorescein (11.12 x 10^{-2} µM) work solution was prepared from a stock solution 5.70 µM, in turn obtained by diluting with PBS 75 mM a stock solution 1.14 x 10^{-3} M. The calibration curve of Trolox (reference antioxidant) was determined in the concentration range of 0.5-7.5 µM (5 levels), plotting the net area under the curve (net AUC = AUCsample - AUC control) as function of the tested concentrations y = 1 X 10^{7} x - 738411; R^2 = 0.9914). Standard RA (0.25-2 µM, 6 levels), CA (0.125-2 µM, 7 levels) and BHA (1-3 µM, 5 levels) also were analyzed. The results were expressed as TEAC, in terms of µmol of Trolox equivalents per 100 mL of sample (µmol TE/100 mL) or TE µmol/µmol for the standards.

5A 4.9. Trolox Equivalent Antioxidant Capacity (TEAC)

In DPPH and ABTS assays, for Trolox (antioxidant reference), DWW samples and standards (RA, CA and BHA) were determined curves of discoloration of DPPH and ABTS radicals (range 20-80% I), plotting the % inhibition of the

radical (% I) against the concentration, expressed as mL/L for DWWs and μM for Trolox and standards. % I is calculated according to equation:

$$\%\ \mathrm{I}\ = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

$Abs_{control}$ absorbance of DPPH or ABTS without sample or standard, Abs_{sample} absorbance of sample or standard (blank subtracted).

From the linear equations of both inhibition curves (Trolox and samples) were extrapolated concentrations corresponding to % I equal to 50, and TEAC was calculated according to equation:

$$\mathrm{TEAC} = \frac{[\text{Trolox } (\mu\mathrm{M})]50\%\ \mathrm{I}}{\left[\text{Sample } \left(\frac{\mathrm{mL}}{\mathrm{L}}\right)\right] 50\%\ \mathrm{I}} \times 100$$

In ORAC assay, the decay of the fluorescence was expressed graphically as net AUC against the concentration. Therefore TEAC was calculated according to equation:

$$\mathrm{TEAC} = \frac{[\text{Trolox } (\mu\mathrm{M})]\text{net AUC}}{\left[\text{Sample } \left(\frac{\mathrm{mL}}{\mathrm{L}}\right)\right] \text{net AUC}} \times 100$$

CHAPTER V
SECTION B

SELECTIVE EXTRACTION OF BIOACTIVE COMPOUNDS OF DISTILLATION WASTEWATER FROM *OCIMUM BASILICUM* L. BY SUPERCRITICAL ANTISOLVENT FRACTIONATION

5B 1. Introduction

In previous work distillation wastewaters (DWWs) have been studied as liquid matrices. For by-products valorisation, it might take a different approach removing water from matrix by drying processes and evaluating potential applications on dry material. In fact, the dry powder may be used as functional ingredient. Moreover dried material is not subjected to microbial attack. Antioxidant capacity measurements further corroborate this hypothesis, showing that DWWs are extracts with high antioxidant activities correlated to high bioactive compounds content. At the same time, purification and concentration processes (green applications), always in line with the concept of recovery and biorefinery, would allow obtaining purified and enriched fractions of bioactive compounds for industrial applications. Basic aims of the whole research line are always the utilization of by-products, with high value added and the development of extraction green methods, environmentally friendly, with low costs, high yields and potential industrial applications.

In this work basil DWW was chosen among different DWWs (basil, rosemary and sage) for the interesting chemical profile and for rosmarinic acid and total phenolic content (RA:72.3±9.4 mg/100 mL; TPC: 313.2±32.5 mg GAE/100 mL) reported in previous study. Water from matrix was removed by lyophilisation. The aim has been to obtain a purified fraction of B-DWW enriched in rosmarinic acid by the innovative technique of extraction supercritical antisolvent fractionation (SAF).

Then in this work the experimental plan was divided as follows:

1. Hansen solubility parameters study for solvent extraction selection;
2. Pressurized liquid extraction (PLE) to confirm experimentally solvent extraction selection;
3. Purification and concentration by SAF.

Basil is important aromatic plant belonging to the *Lamiaceae* family. It is cultivated worldwide, especially in Mediterranean area. Basil leaves were used like a spice to give flavour to food and in traditional medicine (Chanwitheesuk et al., 2005) as carminative, stomachic and antispasmodic (Simon et al., 1999) and its extracts show antibacterial, antifungal and antioxidant activities (Vlase et al., 2014). Basil is characterized by high levels of phenolic compounds. The most important compounds are rosmarinic acid – the ester between caffeic acid and acid 3,4-dihydroxyfenillattic – and caffeic acid. Rosmarinic acid (RA) shows antibacterial, anti-inflammatory, antiviral and antioxidant activities (Petersen and Simmonds, 2003). Caffeic acid (CA) is a hydroxycinnamic acid widely distributed in the plant kingdom with antioxidant and free radical scavenging properties (El-Seedi et al., 2012). The criterion employed in this study was to use Hansen solubility parameters in order to select the most suitable mixture solvent for SAF for obtaining of enriched RA fraction.

SAF was employed to fractionate phenolic compounds from plant extracts (Gonzalez-Coloma et al., 2012; Catchpole et al., 2004; Visentin et al., 2011). SAF provides the continuous contact between a supercritical carbon dioxide (sc-CO_2) flow and a polar liquid mixture flow in a pressurized chamber. The polar mixture is an extract usually obtained with ethanol/water mixtures. This contact allows the precipitation of polar components from the liquid mixture insoluble in sc-CO_2, while less polar components and ethanol remain dissolved in the SC-CO_2 flow and were recovered by downstream pressure reduction system (Catchpole et al., 2004; Catchpole et al., 2009). Studies of technical parameters are critical, such as pressure, mass percentage of water in feed and feed/sc-CO_2 mass ratio (Durling et al., 2007). The main advantage of SAF is the lipophilic character of CO_2 that allows separation and concentration of polar compounds from organic extracts. SAF permits to work at reduced temperatures, in absence of oxygen and light; furthermore CO_2 is inexpensive,

recyclable and non toxic. The fractionation of propolis tincture by SAF allowed obtaining flavonoid enriched fractions (Catchpole et al., 2004). Moreover SAF was employed to obtain high flavonoid content fractions from lyophilized grape residues (Floris et al., 2010).

5B 2. Results and discussion

5B 2.1. Hansen solubility parameters estimation

It is possible to study the selectivity of different solvents in order to predict the dissolving power for different types of compounds using the predictive computational methods. The theoretical modeling of the Hansen solubility parameters could provide an accurate estimation for the solvent selection and prediction of the solubility of bioactive compounds.

The Hansen solubility parameters have been estimated for different types of solvents for RA and CA. The dissolution energy is given by three contributions: ED, dispersion energy, EP, polarity energy and EH, hydrogen bonds energy. It draws a three-dimensional space (Hansen space, "solubility sphere") and is studied the solubility of a solute in a determined solvent by measuring the distance of the point of the solvent from the sphere which represents the solute. Smaller is the distance, better is dissolving solvent power (Hansen, 2000). In Table 17 are reported the estimated values (Simplified Molecular Input Line Entry Syntax (SMILES) HSPiP version 5.0) for some solvents. In recent years there is a need of the use of environmentally solvents in extraction applications in order to perform green extractions of polar bioactive compounds (Golmakani et al., 2012). The Hansen solubility parameters (HSPs) were employed to study the dissolution factors of a solute in a specific solvent and to determine solvents selectivity.

From calculations obtained, ethanol appears to be the best solvent for CA (distance 1.12) while ethyl lactate the most suitable for RA (distance 1.10).

Table 17 *Hansen solubility parameters.*

	Caffeic acid CA						Rosmarinic acid RA					
	δD	δP	δH	δT	MVol	Distance	δD	δP	δH	δT	MVol	Distance
Target	20.7	8.3	19	29.30	136.6	0.00	20.8	6.3	12.4	25.02	258.6	0.00
Ethanol	15.8	8.8	19.4	26.52	58.6	1.12	15.8	8.8	19.4	26.52	58.6	1.42
2-propanol	15.8	6.1	16.4	23.58	76.9	1.18	15.8	6.1	16.4	23.58	76.9	1.22
Ethyl lactate	16	7.6	12.5	21.68	115	1.30	16	7.6	12.5	21.68	115	1.10
Dimethyl sulfoxide	18.4	16.4	10.2	26.68	71.3	1.46	18.4	16.4	10.2	26.68	71.3	1.30

δD dispersive interaction, δP polar interaction, δH hydrogen bonds, δT total, molar volume MVol = $cm^3\ mol^{-1}$ T = 25 °C δ = $MPA^{1/2}$.

5B 2.2. PLE extraction

HSPs calculations can suggest the possible interactions solute-solvent. Therefore, among the various green solvents were chosen ethanol and ethyl lactate to proceed to the experimental assays of theoretical conditions with PLE extractions. PLE is an extraction technique which uses liquid solvents at elevated temperature (above their atmospheric boiling point) and elevated pressure (Mustafa and Turner, 2011). The solvents normally used to extract polar compounds are water, ethanol or mixture of both. Ethanol is recognized as safe (GRAS) but also ethyl lactate, a renewable solvent, can be considered food grade solvent. In literature there are many papers regards PLE using ethanol and water but only few papers with ethyl lactate. Lores and collaborators evaluated the use of ethyl lactate to extract polyphenols from *Cytisus scoparius* by PLE (Lores et al., 2015).

Due to the polarity of CA and RA, the influence has been studied of the addition of a percentage of water. Ethanol and ethyl lactate are both miscible in water, so it's possible to confirm predictive data with experimental results.

Furthermore the effect of temperature was evaluated to study the efficiency of extraction. In particular, PLE extractions were performed with mixture of

water and ethanol or ethyl lactate (100:0-75:25-50:50-25:75-0:100, v/v) at three different temperatures (50-100-150 °C).

PLE extraction using aqueous ethanol solutions (Figures 31-32) results show that absolute ethanol at low temperature (50 °C) permits 1.65-fold higher CA content than mixture 25:75, v/v (2.42±0.04 -1.46±0.08 mg/g extract). In the case of RA is observed that the best mixture is 25:75, v/v, with a decremental content when the temperature was increased (23.90±2.06 mg/g extract). No substantial differences are observed with the other mixtures. Therefore for CA and RA mixture 25:75, v/v aqueous ethanol solutions could be a suitable solution.

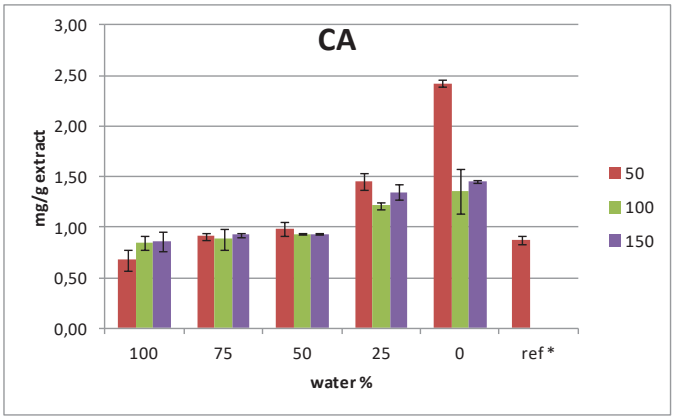

Figure 31 *PLE extraction efficiency using aqueous ethanol solutions, CA content expressed as mg/g extract.* (n = 3) ref* B-DWW reference extract

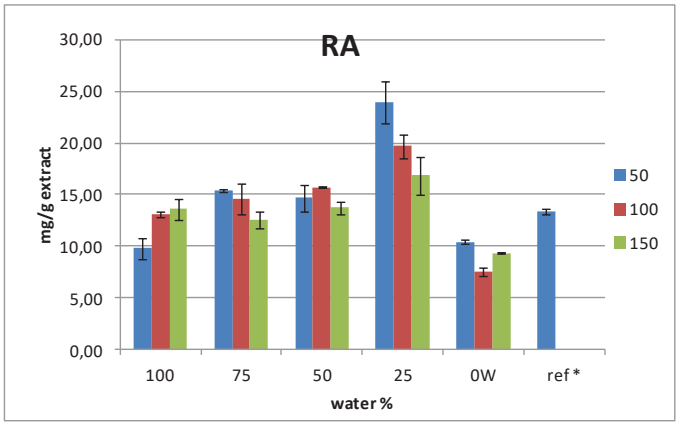

Figure 32 *PLE extraction efficiency using aqueous ethanol solutions, RA content expressed as mg/g extract*. (n = 3) ref* B-DWW reference extract

PLE extraction using aqueous ethyl lactate solutions (Figures 33-34) results show that the best mixture is 25:75, v/v for CA (1.04±0.04 mg/g extract) and RA (17.78±0.19 mg/g extract). The higher content is obtained at 150 °C. This fact is due to viscosity of ethyl lactate. The viscosity of ethyl lactate is high, a temperature increase may enhance mass transfer coefficients and allow obtaining an increasing content for CA and RA at 25:75, v/v. Definitely only 25% water is sufficient to confer the correct polarity to the mixture with organic solvent allowing extraction of polar compounds CA and RA. Green solvent ethanol and ethyl lactate are both capable of extracting CA and RA. Moreover aqueous ethanol solutions are more efficient in extracting CA and RA, giving 1.34-fold higher content for RA and enrichment % CA 167.75±9.52%, enrichment % RA 178.37±15.37%. Therefore for DWW basil lyophilized sample aqueous ethanol mixture 25:75, v/v could be the choice in order to obtain maximum efficiency of extraction.

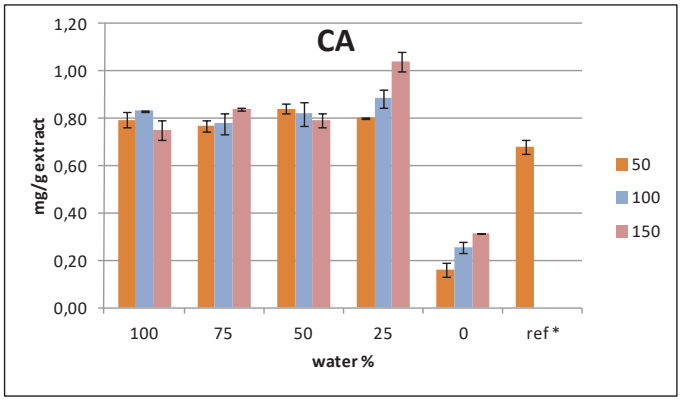

Figure 33 *PLE extraction efficiency using aqueous ethyl lactate solutions, CA content expressed as mg/g extract.* (n = 3) ref* B-DWW reference extract

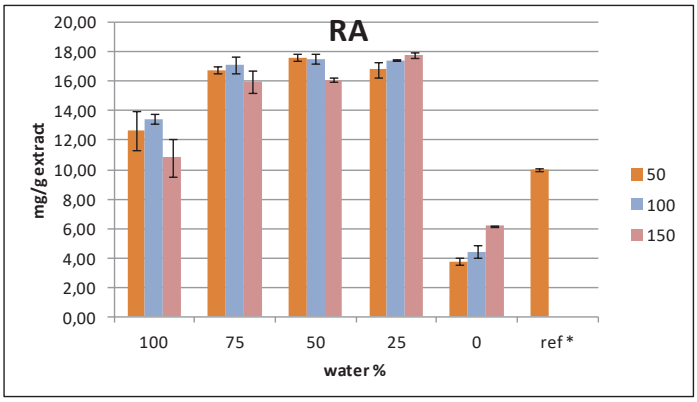

Figure 34 *PLE extraction efficiency using aqueous ethyl lactate solutions, RA content expressed as mg/g extract.* (n = 3) ref* B-DWW reference extract

Regarding to ratio RA/CA to determine solvent selectivity results confirmed predictive data from HSPs program. In fact, for RA the best solvent is ethyl lactate: PLE extractions with 100% ethyl lactate give a ratio RA/CA of 24.17±0.13 at low temperature (50 °C), 1.42-fold higher than aqueous ethanol solutions 75:25 v/v at 50 °C.

5B 2.3. SAF experiments

Water and CO_2 are immiscible, ethanol and CO_2 are completely miscible at determined pressures and water and ethanol are completely miscible. In this technique CO_2 is the antisolvent, because polar and high molecular weight compounds insoluble in CO_2 can be recovered in separator chamber and separated from non polar components of matrix. The effects of technical parameters are studied and enrichment % of RA was monitored. Firstly influence of percentage of water in feed was studied. HSPs prediction and PLE results showed that mixture 25:75, v/v is suitable for RA and CA extraction. Different mixture solutions were tested in order to study the influence of water in the three-phase system ethanol-water-scCO_2. As shown in Figure 35, the best mixture was aqueous ethanol 10:90, v/v, indicating that if percentage of water increase, the recovery of RA decrease (fixed parameters are 100 bar, T 40 °C, ratio feed/scCO_2 0.025).

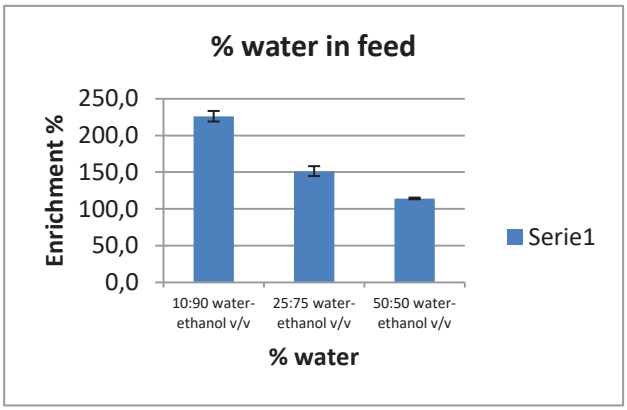

Figure 35 *Influence of % water in feed n = 3.*

Subsequently influence of pressure was evaluated: experiments conducted at three different pressure values (100-200-300 bar) revealed that this parameter plays an important role in SAF. Figure 36 showed results. At 200 bar is

obtained the highest enrichment % RA (246.8±14.1) (fixed parameters are T 40 °C, ratio feed/scCO$_2$ 0.025, aqueous ethanol 10:90, v/v).

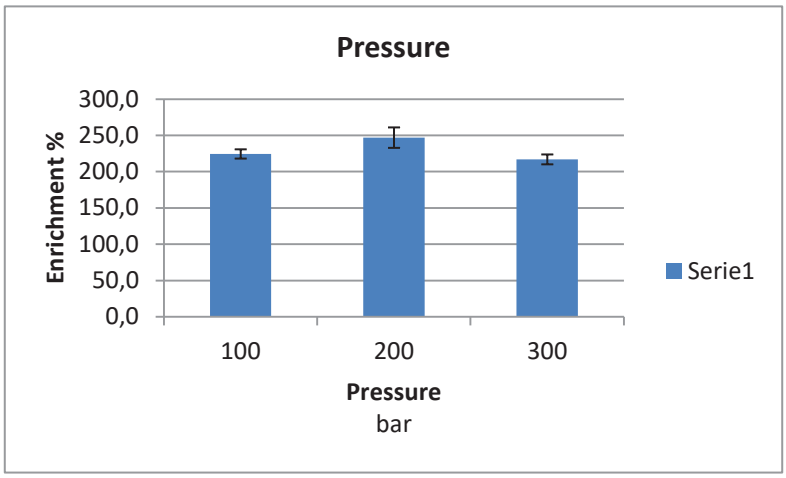

Figure 36 *Influence of pressure n = 3.*

Further experiments regarded sample concentration. B-DWW for SAF experiments was reconstituted at initial concentration and the effect of this parameter was studied. In particular runs at halved and doubled concentration were carried out. Dissolved solids concentration in feed solution don't influence SAF system and the results showed the broad applicability of technique at different sample concentration (Figure 37) (fixed parameters are 100 bar, T 40 °C, ratio feed/scCO$_2$ 0.025, aqueous ethanol 10:90, v/v).

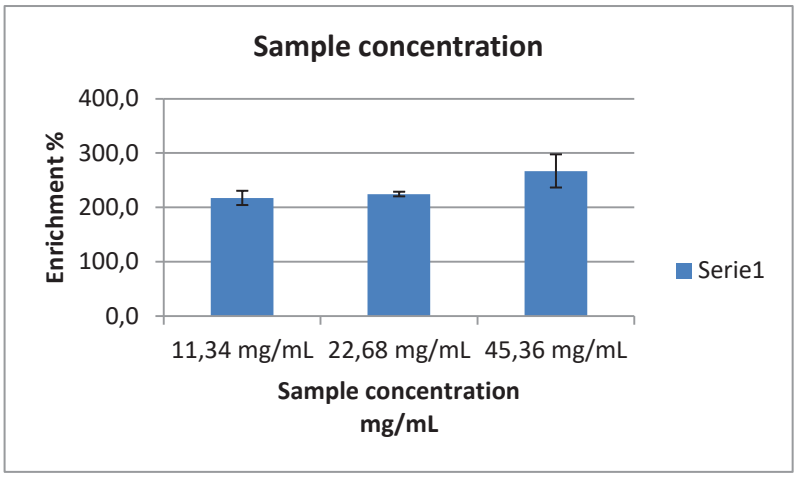

Figure 37 *Influence of sample concentration n = 3.*

Finally quantity of CO_2 employed was evaluated in order to decrease more process costs. Quantity of CO_2 was changed setting different ratio feed/scCO_2 and pumps flow. Results showed that it possible to use only 1 g of CO_2 (1.030 mL/min) corresponding to ratio feed/sc-CO_2 of 0.050 (fixed parameters are 100 bar, T 40 °C, aqueous ethanol 10:90, v/v) (Figure 38).

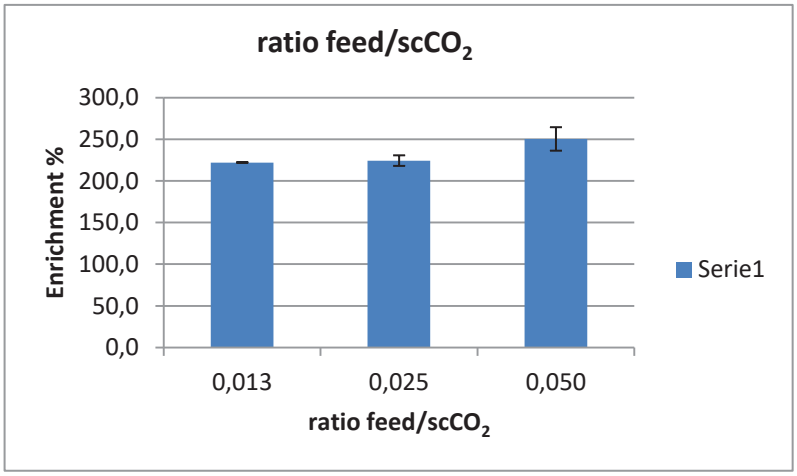

Figure 38 *Influence of ratio feed/scCO_2 n = 3.*

Then the best conditions optimized step by step were validated (% water in feed aqueous solution 10:90, v/v, 200 bar, ratio feed/scCO_2 0.050, temperature

40 °C, time 2 hours). Optimal conditions for RA were applied also for calculations CA enrichment %. Results are: enrichment % RA 253.69 ±7.82 and CA 263.61 ±28.53 and also RA content 64.59±1.99 mg/g extract and CA content 3.37±0.37 mg/g extract compared to B-DWWs used as reference extract. In our case RA and also CA are completely recovered and enriched in raffinate phase. Figure 39 shows chromatographic profile (HPLC-UV) of raffinate fraction in optimal conditions.

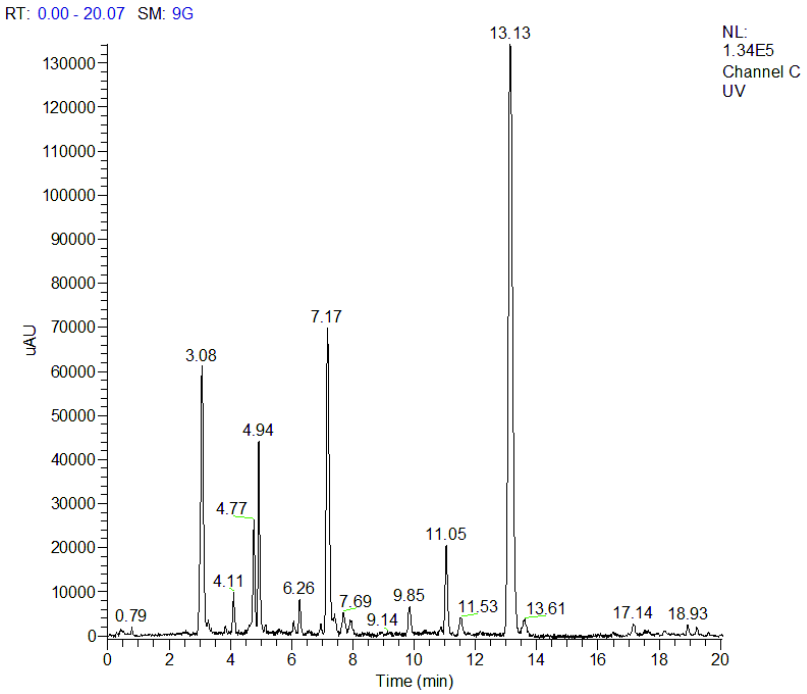

Figure 39 *Chromatographic profile (HPLC-UV) of raffinate fraction in optimal conditions. Peak retention time 13.13 min corresponds to RA.*

5B 3. Conclusions

Mentioned extraction procedure represents a valid tool to give further added value to the distillation wastewaters. SAF provides an enriched RA fraction in the optimal conditions, with low costs, low environment impact, high yield for future industrial applications.

5B 4. Materials and methods

5B 4.1. Samples and reagents

Dried basil leaves (*Ocimum basilicum* L.) are obtained from local farms (Capaccio, Salerno, Italy). Ultrapure water obtained using a Millipore system (Billerica, MA, USA), ethanol and ethyl lactate provided from WVR chemicals (Fontenay-sous-Bois, France) were utilized for PLE extractions. Rosmarinic acid (RA ≥98%), caffeic acid (CA ≥98%) and sea sand were purchased from Sigma Aldrich (Madrid, Spain). Water ultra gradient, methanol and formic acid (≥95%) for HPLC were provided from WVR chemicals (Fontenay-sous-Bois, France).

5B 4.2. Steam distillation

Steam distillation was performed using a semi-industrial apparatus. 5 Kg of basil leaves are loaded on a perforated grid with 6 L of water at temperature of 75 °C in steam distillation apparatus. Time extraction was 60 min. The DWWs were filtered after extraction with glass fiber filter (Millipore® glass-fiber filters, type 2, pore size 1 μm) and stored at -20 °C until lyophilisation.

5B 4.3. Pressurized liquid extraction

All the extractions were performed using an Accelerated Solvent Extraction system ASE 200 (Dionex, Sunnyvale, CA, USA), equipped with a solvent controller unit. Each extraction was carried out loading 250 mg of lyophilized sample and 4 g of sea sand (dispersive agent) into an 11 mL stainless steel extraction cell. The extraction time (static mode) was selected at 20 min after

preliminary experiments. PLE extractions were performed using different percentage of water in ethanol and ethyl lactate, in particular (100:0, 75:25, 50:50, 25:75, 0:100, v/v) at three different temperatures 50-100-150 °C, in duplicate experiments. Extraction fixed conditions were: pressure 1500 psi, flush volume 60%, purge 100 s.

All the experiments were carried out in order to study solubility factors of bioactive compounds (CA and RA) in different conditions. Extracts obtained with water and ethanol or mixtures of both are lyophilized (after removing organic solvent with rotary evaporator) before chromatographic analysis. Extracts obtained with water and ethyl lactate or mixture of both are reconstituted up to a final volume of 30 mL with water and then injected in chromatographic apparatus.

5B 4.4. Quantitative analysis by HPLC-UV

Chromatographic analyses were performed using an ACCELA UHPLC system (Thermo Scientific, San Jose, CA, USA) using a Zorbax Eclipse Plus C18 Rapid -resolution HD column (1.8 μm, 2.1x 50 mm), (Agilent Technologies, Waldbronn, Germany). A linear gradient (5-60% B in 20 min) of water (A) and methanol (B), both with 0.1% formic acid, was applied. The flow rate was 400 μL/min and the injection volume 10 μL. The column was thermostated at 30 °C. The detection was performed at the selective wavelength of 324 nm for CA and 330 nm for RA, but also spectra are recorded in the range 200-600 nm. Calibration curve were constructed using external calibration method and linearity were evaluated by ANOVA test (CA $y = 141467x+12810$ $R^2 = 0.9993$; RA $y = 68232x-22892$ $R^2 = 0.9998$). Stock solutions of CA and RA are prepared in methanol at 5 mg/mL and stored at dark and 4 °C. Calibration curves are obtained with appropriate dilutions of the stock solutions in water (CA range 0.5-30 μg/mL, 11 levels; RA range 1-

50 μg/mL, 11 levels). Each calibration level was injected in triplicate. All the extracts (PLE and SAF) are analyzed at 1 mg/mL (filtered before injection). For each experiment set are calculated RA/CA expressed as mg/g extract RA/mg/g extract CA in order to determine selectivity; CA and RA content expressed as mg/g extract and finally enrichment % defined as mg/g extract CA or RA in PLE extraction / mg/g extract CA or RA in DWW basil sample. For SAF experiments CA and RA content expressed as mg/g extract and enrichment % defined as mg/g extract CA or RA in SAF extraction / mg/g extract CA or RA in DWW basil sample.

5B 4.5. Supercritical antisolvent fractionation

Instrument was built in lab. CO_2 was supplied from a pressurized cylinder and cooled in a heat exchanger placed within the high pressure pump (Jasco, PU-2080 CO_2 Plus, Tokyo, Japan), it was compressed and continuously pumped at a constant flow rate (1.030 mL/min), and just before reaching the separation chamber, CO_2 was mixed in a T-tube device with the feed solution (B-DWWs), which was pumped by another high pressure pump (Jasco, PU-2080Plus, Tokyo,Japan; 0.055 mL/min. After mixing, the B-DWW components that resulted insoluble in sc–CO_2+EtOH mixture precipitated and were collected in separator (this fraction is called raffinate). The temperature was controlled by an oven and was maintained constant at 40 °C for all the experiments. Pressure was also maintained constant by regulation of back pressure valve (LF40, Pressure Tech, Houston, TX, USA). The non polar fraction soluble in sc–CO_2+EtOH was recovered after reducing pressure (the soluble fraction is called extract). The experiments were performed for 120 min. After obtaining raffinate and extract fractions, ethanol was removed by rotary evaporator and water was eliminated by freeze-drying (Labconco Corporation, Missouri, USA). B-DWW from dry state (after lyophilisation)

was reconstituted to initial concentration using mixture water-ethanol (22.68 mg/mL). A typical experiment consists of this step: pressure, CO_2 flow and temperature stabilization, feed flow stabilization and finally after extraction injection of pure sc-CO_2 to wash system. Triplicate experiments were performed. Sc-CO_2 and feed solution are injected at the same time at fixed feed/CO_2 ratio.

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Publications related to PhD project

Pagano, I., Piccinelli, A. L., Celano, R., Campone, L., Gazzerro, P., De Falco, E., & Rastrelli, L. (2016). Chemical profile and cellular antioxidant activity of artichoke by-products. *Food & Function*, *7*(12), 4841-4850.

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Publication

Campone, L., Piccinelli, A. L., Celano, R., Pagano, I., Russo, M., & Rastrelli, L. (2016). Rapid and automated analysis of aflatoxin M1 in milk and dairy products by online solid phase extraction coupled to ultra-high-pressure-liquid-chromatography tandem mass spectrometry. *Journal of Chromatography A, 1428*, 212-219.