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PhD thesis

"Innovative techniques for the extraction and quantification of bioactive compounds from food and agroindustrial by-products"

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Introduction

The present PhD thesis is the result of my three-year research in the fields of green extraction and analytical chemistry, carried out mainly in the laboratory of Food and Dietary Products of the Department of Pharmacy (DIFAR) of Genoa University, and taking advantage of collaborations among other research groups of Genoa University, the Chemometric and Qualimetry Group of University of Burgos and the Green Extraction Team of Avignon University.

The thread that runs through the entire thesis are innovative techniques both for the extraction and for the analysis of bioactive compounds, endowed with health-promoting activities or potentially harmful contaminants, occurring in different foods and agroindustrial by-products.

Several green extraction techniques have been performed, with a view towards environmental sustainability as well as economic and safety considerations, in order to optimize the extraction of antioxidant compounds from *Oryza sativa* L. 'Violet Nori' and pomegranate by-products.

'Violet Nori' rice is particularly rich in anthocyanins. The effect of different cooking conditions on this polyphenolic content have been also investigated.

Pomegranate peels and marcs can be valorised thanks to their high content in ellagic acid and ellagitannins. To improve ellagic acid bioavailability, its encapsulation in dendrimeric nanocarriers has been proposed too.

With respect to the analytical methods, the innovative second order calibration of excitation-emission fluorescence matrices (EEMs) coupled with the chemometric tool PARAFAC has been applied also to another food matrix. Thanks to this technique, in fact, it has been possible to evaluate the content of carcinogenic polycyclic aromatic hydrocarbons in a commercial smoked tuna sample.

CHAPTER 1

“Extraction of antioxidant phenolic compounds from food matrices with green techniques”

1. Green extraction

The interest in the development and exploitation of green extraction techniques has been successfully increasing over the past decade, due to the recent attention to operating in an environmentally sustainable way, as well as to safety and economic considerations.

Traditionally conventional extraction processes involve the use of large amounts of chemical solvents, representing a problem related to waste disposal and environmental impact, are time-consuming and not very efficient [1], and may also cause the degradation and loss of some target compounds [2].

Green extractions procedures, overcome these problems according to the twelve principles of green chemistry set by the Environmental Protection Agency of USA [3] and providing several advantages compared to conventional solid-liquid extraction techniques.

An exhaustive definition of green extraction of natural products was reported by Chemat et al., 2012 [4]:

"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 products, and ensure a safe and high-quality extract/product".

This definition is perfectly reflected by the "Six Principles of Green Extraction", that are directions to follow for establishing an innovative and green extractive process [4]:

- Principle 1: Innovation by selection of varieties and use of renewable plant resources.
- Principle 2: Use of alternative solvents and principally water or agro-solvents.
- Principle 3: Reduce energy consumption by energy recovery and using innovative technologies.
- Principle 4: Production of co-products instead of waste to include the bio- and agro-refining industry.
- Principle 5: Reduce unit operations and favour safe, robust and controlled processes.
- Principle 6: Aim for a non denatured and biodegradable extract without contaminants

The use of alternative GRAS (Generally Recognised As Safe) solvents is an essential condition for any innovative green technique.

The alternative eco-friendly solvents employed in green extractions are characterised by high solvency, low toxicity, low environmental impact and

biodegradability. They must be obtained from non-petrochemical renewable resources and must be easily recycled without any deleterious effect to the environment [5].

Several innovative extraction techniques are emerging in the field of green chemistry, exploiting different processes and mechanisms, but all aiming at providing faster, more efficient, safer, and sustainable alternatives to conventional extraction.

In particular, during my PhD, I had the opportunity to use and learn the following green extraction methodologies.

1.1. Ultrasound-assisted extraction

Ultrasound is a key-technology in achieving the goal of sustainable “green” chemistry and extraction.

Ultrasound-assisted extraction (UAE) is a relatively simple, cheap and efficient methodology, which is well known to have a significant impact on the rate of various processes in the chemical and food industry [6].

In fact, ultrasounds allow full extraction in few minutes of a great variety of bioactive compounds (like antioxidants, aromas, pigments, organic and mineral compounds) from several animal tissues, plants or food matrices [7], with high reproducibility both on small and large scale. The main benefits of UAE, in addition to faster kinetics and increased efficiency [8,9], involve reduction of solvents and processing costs, simplicity of manipulation and work-up, waste reduction, higher purity of the final product and the consumption of only a part of the fossil energy commonly used for a conventional extraction method such as Soxhlet extraction, Clevenger distillation or maceration [6,8].

Intensification of extraction efficacy using ultrasound has been attributed to the propagation of ultrasound waves in a solid/liquid media, thus originating the cavitation phenomena [10].

An acoustic pressure wave consists of a succession of different phases of compression and rarefaction in the transmitting medium along the wave propagation direction. When a large negative pressure is applied to a liquid, intermolecular van der Waals forces are not strong enough to maintain cohesion and micrometer-scale bubbles are formed. The rapid nucleation, growth and collapse of these microbubbles constitute the phenomenon of cavitation [11].

The implosion of cavitation bubbles in the proximity of a solid surface and their subsequent asymmetrical collapse generate fast micro-jets toward the sonicated surface, at very high temperature and pressure, which destroy the wall cells of the matrix, allowing the recovery of the intracellular content in the

extraction solvent [7]. Cavitation induces micro damages and structural modifications on the surface of the source material and causes impingement by micro-jets resulting in surface peeling, erosion and particle breakdown [8,11,12]. Additionally, implosion of cavitation bubbles in a liquid medium generates macro-turbulences and micro mixing [6].

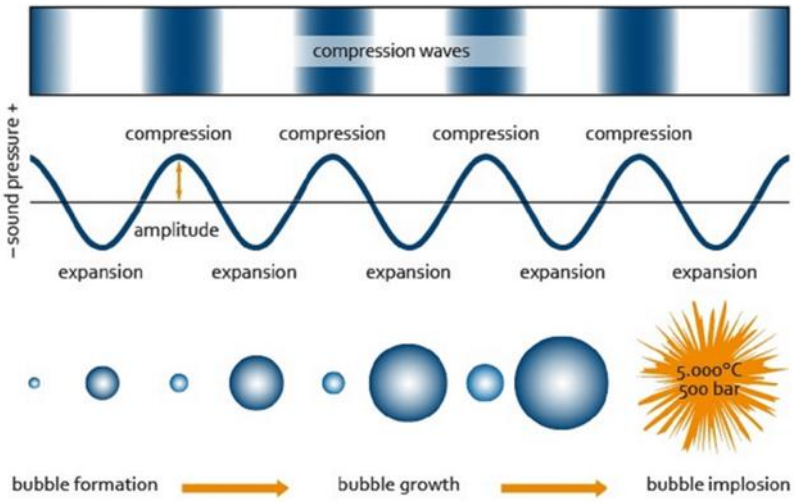

Figure 1. Principle of ultrasound cavitation [13].

There are several mechanisms involved in UAE which independently or in combination influence the final extraction yield, ranging from removing small particles or structures at the solid surface, to creating pores or even deep fractures within the raw material [14]. All these mechanical impacts (erosion, shear forces, sonoporation, fragmentation, capillarity, detexturation) have been reported by many authors.

Local erosion is a well-known ultrasound effect, shown for the first time by Degrois et al. on starch grains [15] and recently observed in the case of black tea leaves [16] and boldo leaves [17]. Ultrasounds are even used for their erosion capacity, for several purposes such as cleaning or for sonochemical reactions e.g. with metals [18].

In addition to the effect of erosion, the propagation of ultrasound waves generates strong shear forces within the liquid medium, also responsible for the surface damage and disintegration [19,20]. These forces, in fact, create oscillation and turbulence inside the liquid, impacting the nearby solid surface.

The sonoporation effect of ultrasound is mainly noticed in the field of biology and is applied to obtain the permeability of cell membranes [6]. Ultrasound-induced pores have been observed on wet yeast surface subjected to ultrasound [21].

A rapid fragmentation of the raw material, caused by inter-particle collisions and from shockwaves created from collapsing cavitation bubbles in the extraction solvent, can also be observed during application of ultrasound, as in the case of spinach leaves [6]. The reduction in particle size by ultrasound action leads to an increase of surface area of the solid resulting in higher mass transfer and increased extraction rate and yield [22].

The mechanism of ultrasonic capillary effect is not fully understood, but a relationship with cavitation has been established [23]. The effect of capillarity refers to the increase of depth and velocity of penetration of liquid into pores and canals under some conditions of sonication [24] and it has been observed in molten aluminium [25].

In some cases, destruction or detexturation of plant structures can be observed after UAE. This effect has been reported for the essential oil extraction from caraway seeds [26] and for the extraction of metabolites of rosemary leaves [14].

Two types of devices can be used to apply high power ultrasounds: an ultrasonic bath or a probe-type ultrasound equipment. Both systems are based on a transducer, generally a piezoelectric transducer, as a source of ultrasound power [6].

The ultrasonic bath is the most common type of ultrasonic device, usually consisting of a stainless-steel tank equipped with one or more ultrasonic transducers. Ultrasonic baths generally operate at a frequency of around 40 kHz and can be provided with temperature control. They are cheap, easily available in most laboratories, and they can process large numbers of samples simultaneously, but, compared with probe systems, they have the disadvantages of low reproducibility and of low ultrasound power directly delivered to the sample.

Recently a new bath system reactor has been developed by REUS (Contes, France): it consists of a stainless steel jug equipped with a transducer operating at a frequency of 25 kHz. It is provided with a double-layered mantle with water circulation to allow temperature control with cooling/heating systems. This kind of ultrasound apparatus is suitable for extraction in laboratory and in large scale [27], [28] and it can be considered a good alternative to ultrasonic probe also for industrial applications [29].

High power ultrasonic probes are generally preferred for extractions. Compared to the ultrasonic baths, the probe system is more powerful due to an ultrasonic intensity delivered through a smaller surface, represented by the tip of

the probe. They usually operate around 20 kHz and use a transducer bonded to the probe, which is immersed into the reactor, in order to directly convey ultrasounds to the extraction media with minimal ultrasonic energy loss. The intensity of the ultrasounds delivered by the probe system induces a rapid increase of temperature in the reactor. For this reason, the cooling of the reactor by a double-jacket mantle is required to conduct extraction, especially if a continuous reactor is used.

A specific operational feature of ultrasonic extraction involves, in fact, the ultrasound flow mode, which can be continuous or pulsed. When UAE is used in a pulsed mode (PUAE), the ultrasound processor works intermittently during the entire extraction process (active time vs. inactive time) [7]. This extraction mode allows to reduce the operating temperature, therefore in this case the cooling system is not necessary for a short time PUAE.

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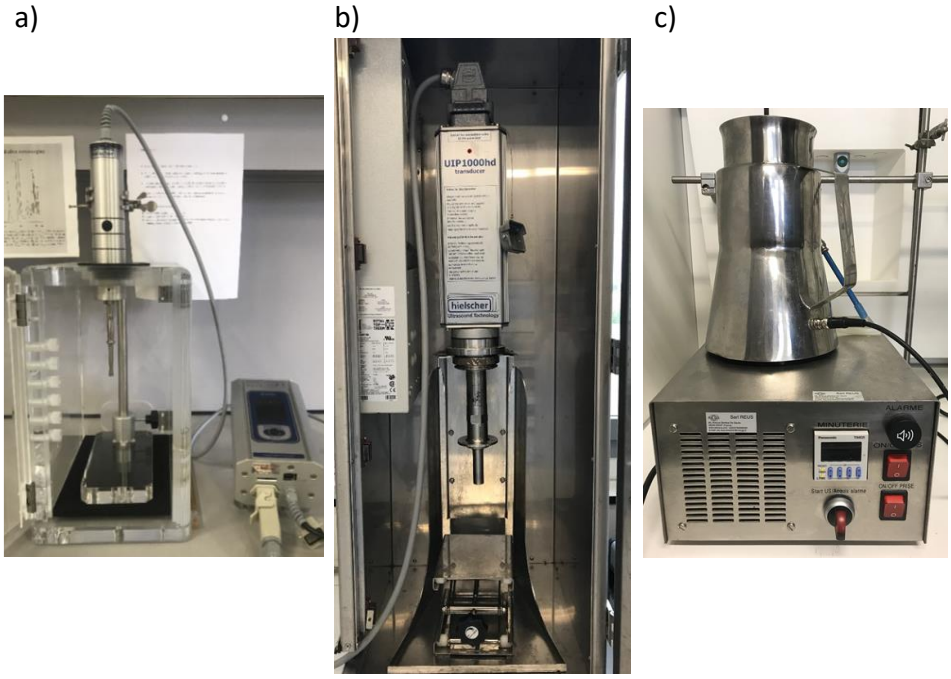

Figure 2*.* Devices for ultrasound-assisted extraction employed in the present thesis work.
a) Pulsed ultrasound probe (Hielscher UP200St, Teltow, Germany). University of Genoa, Italy.
b) Continuous ultrasound probe (1kW, 20 KHz, UIP 1000 hdt, Hielscher Ultrasonics GmbH, Germany). University of Avignon, France.
c) Ultrasound bath (PEX 1, R.E.U.S., Contes, France). University of Avignon, France.

1.2. Microwave-assisted extraction

In the last two decades, microwave assisted extraction (MAE) has been widely applied in the extraction of volatile and non-volatile compounds from vegetal matrices due to an increasing demand of more efficient extraction techniques, amenable to automation [30].

As green innovative technology, like UAE, shorter extraction times, reduced organic solvent consumption, energy and costs are the main goals pursued by MAE.

Microwave is a non-contact source, which has the peculiarity to instantly heat, and preferentially involves polar molecules [21].

Dielectric heating is the basis of microwaves mechanism, used to maximize the extraction of natural compounds, phytonutrients and functional food ingredients saving solvents and time. Belonging to non-ionizing radiation in the range of frequencies from 300 MHz up to 300 GHz, the heating induced by microwaves interacts with polar molecules in cells' cytoplasm and membranes leading to dipole rotation, ionic conduction and rapid diffusion [31].

Dipole rotation is caused by the alignment to the microwaves' swinging electric field of the molecules possessing a dipole moment (either permanent or induced by the electric field) in both the solvent and the solid sample [32]. This oscillation generates collisions with surrounding molecules and thus the liberation of thermal energy into the medium. Since this phenomenon is very fast, the resulting heating is also very fast; furthermore, the higher the dielectric constant of the solvent, the more effective the heating.

Consequently, unlike classical conductive heating methods, microwaves heat the whole sample simultaneously and, in the case of extraction, microwave heating leads to the disruption of weak hydrogen bonds promoted by the dipole rotation of the molecules.

So, in practice, the extraction solvent, under microwave irradiation, ensures a homogeneous and efficient heating and heat diffusion through the matrix, that is immediately stimulated and subjected to high stresses, which cause disintegration of cells' cytoplasm and membranes, promoting the release of the molecules of interest.

Several innovative extraction techniques based on microwaves have been recently developed, such as microwave assisted solvent extraction, microwave hydro-diffusion and gravity, vacuum microwave hydro-distillation, microwave-assisted Clevenger distillation, microwave Soxhlet extraction, compressed air microwave distillation, microwave headspace extraction and solvent-free microwave extraction [30].

The most common equipment employed at laboratory scale are of two types: the traditional Microwaves Assisted Extraction (a) can be performed in a closed device under high pressure and constant temperature, or, alternatively, in an open device at atmospheric pressure. Microwave Hydrodiffusion and Gravity (MHG) technique (b) is also commonly used in laboratory, mainly exploited for the extraction of essential oils, pigments and antioxidant compounds.

MHG system is an original combination of microwave heating and earth gravity at atmospheric pressure, based on placing plant material in a microwave reactor, without adding any solvent or water. Compared to the traditional system (a), MHG uses an "inverted" system (b) consisting of a container for the solid

sample, composed of a perforated pyrex disc, which holds in place the plant material, connected at the bottom to a condenser (placed outside the microwave oven) and to a collection vial [33].The internal heating of the in-situ water inside the plant material distends the plant cells and leads to the disruption of glands and oleiferous receptacles. This physical phenomenon is called hydrodiffusion and it allows the extract (water and essential oil) diffused outside the plant material to drop by earth gravity out of microwave reactor and fall through the perforated pyrex disc, while the condenser outside cools the extract continuously [34].

If compared to other green extraction techniques, MHG allows to extract essential oils without distillation and evaporation that are the most energy consuming between the unit operations.

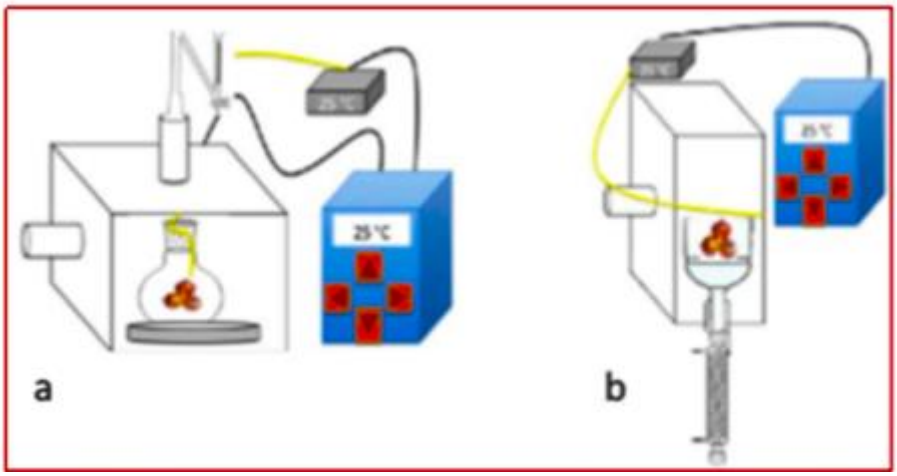

Figure 3. Scheme of the two microwave-assisted extraction methods used in the present thesis work: a) traditional MAE and b) MHG [35].

a) b)

Figure 4. Devices for microwave-assisted extraction employed in the present thesis work.
a) Milestone ETHOS X oven (Milestone, Italy). University of Avignon, France
b) CEM Discover® oven (CEM Corporation, Matthews, NC, USA) University of Genoa, Italy.

1.3. Bead milling extraction

Mechanochemistry is an interdisciplinary science based on chemistry and mechanical engineering, which investigates the physicochemical transformation of substances subjected to high energy mechanical force [31]. The main mechanism of the extraction by mechanochemistry is the cell disruption.

Studies have shown that mechanochemical treatment led to cell walls destruction and increasing of the total contact surface area, due to the transformation of the smooth surface to an open porous structure [36].

Cellular destruction can be defined as the loss of integrity of the cell wall or membrane and it leads to the release of intracellular contents. There are different levels of cell destruction depending on the degree of micronization of the debris and the selectivity of the release of the molecules, ranging from a simple permeabilization or perforation to a total disintegration of the membranes and cell walls [31].

The principle of the mechanochemical assisted extraction techniques is to obtain reduced particle size, destroy the cell wall, decompose cellulose and accelerate the dissolution kinetics in order to improve the extraction efficiency

and decrease the processing time. Through the mechanochemical treatment, the rigid cell walls are destroyed, thus eliminating the diffusion resistance between the matrix and the solvent, and increasing the release of bioactive compounds.

Bead milling, in particular, is a promising mechanochemical extraction technique with high potential for industrial application, due to its efficiency of cell disruption and the commercially available devices at large scale [37].

The most common design for this system is shown in Figure 5. The shaft carries agitators, consisting of concentric or eccentric disks or rings, that export kinetic energy to small ceramic, glass or steel beads in the chamber resulting in multiple collisions [38]. The grinding chambers, vertical or horizontal, can be equipped with a double jacketed device for cooling. The rotating beads, subjected to high centrifugal force, are believed to be responsible of the cellular structural damages: it is hypothesized that the suspended cells are disrupted in the bead collision zones by compaction or shear forces, with energy transfer from the beads to the cells [39,40].

High disruption efficiency in single-pass operations, high biomass loading, high throughput, good temperature control, easy scale up procedures and low labour intensity are the primary factors that make bead milling an interesting extraction method [41].

Moreover, this approach contributes to the development of green and sustainable chemistry because it allows to avoid or considerably reduce the use of organic solvents.

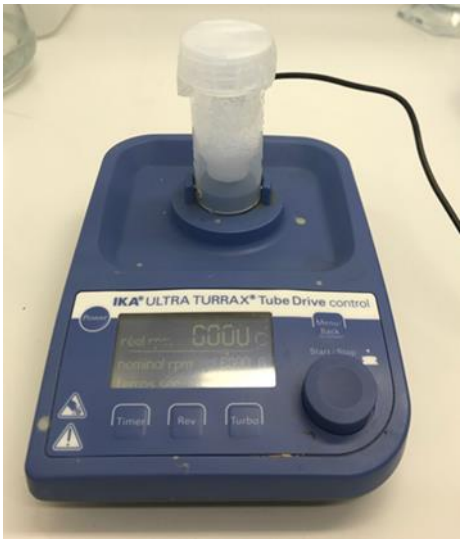

Figure 5. ULTRA-TURRAX® Tube Drive (UTTD, Ika, Germany) employed for Bead Milling Extraction. University of Avignon, France.

1.4. Accelerated-solvent extraction

Accelerated solvent extraction (ASE) is an automated extraction technique based on percolation at elevated temperatures and pressures.

With ASE, a solid or semisolid sample is enclosed in a sample cartridge that is filled with the extraction solvent and it is statically extract under elevated temperature (50−200 °C) and pressure (500−3000 psi) conditions for short time periods (5−10 min). Compressed gas is used to purge the sample extract from the cell into a collection vessel.

The use of liquid solvents at high temperature and pressure conditions should enhance performance compared to extractions at or near room temperature and atmospheric pressure, not only because applying pressure it is possible to use temperatures above the boiling point of the solvents. Elevated temperature and pressure, in fact, lead to increase solubility and mass transfer effects, since the capacity of the solvent to solubilize analytes increases and faster diffusion rates occur [42].

Moreover, ASE promotes the disruption of surface equilibria. Higher temperature can disrupt the strong solute-matrix interactions caused by van der Waals forces, hydrogen bondings, and dipole attractions and it can decrease the viscosity of liquid solvents, thus allowing a better penetration of matrix particles

and enhancing extraction. Increased temperatures can also decrease the surface tension of the solvent, solutes, and matrix, facilitating a better contact of the sample matrix with the solvent.

At the same time, increased pressure should facilitate the extraction of compounds that could be trapped in matrix pores, since the pressure forces the solvent into areas of the matrix that would not normally be contacted by the solvent using atmospheric conditions. The use of elevated pressures (along with elevated temperatures and the reduced solvent surface tensions) helps to force the solvent into the pore to contact the analytes [42].

ASE offers several advantages including easy automation of process, short time extraction, good repeatability, low required solvent volume and low risk of exposure to solvents. This technique allows to maintain samples in an oxygen and light-free environment, which makes it preferable for use in the nutraceutical industry [43,44].

Furthermore, accelerated extraction systems also allow the operator to control temperature, pressure, extraction time and number of extractions, which can increase the amount of compounds extracted when optimized [45].

Evaluations of the performance of ASE for extraction of bioactive natural compounds have been reported in the last years. When ASE was investigated in medicinal plant analysis, the extraction yield of plant constituents in some herbs was found equivalent or higher compared with extracts obtained according to Pharmacopoeia monographs, with a great reduction in extraction in time and solvent consumption [46].

When compared to Soxhlet extraction technique, a drastic decrease in solvent consumption and extraction time for ASE was reported [42], as well as ASE was considered a potential alternative technique to supercritical fluid extraction (SFE) to extract polar compounds [47].

Figure 6. SpeedExtractor (E-916/E-914, BUTCHI, Switzerland) employed for Accelerated Solvent Extraction. University of Avignon, France.

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CHAPTER 1

PART A

“Extraction from colored brown rice: a new Italian purple cultivar”

1. Introduction

1.1. Rice: the most common cereal in the world

Rice is the fruit of the grass species *Oryza sativa* L., of Asian origin, grown worldwide, and *Oryza glaberrima* L., of African origin, grown in parts of West Africa.

Rice (*Oryza sativa* L.), as a cereal grain, is the most widely consumed staple food for a large part of the global population: more than 3.5 billion people depend on it for more than one-fifth of their daily calories [1]. Rice is in fact one of the best sources of complex carbohydrates, which should provide more than a half of the total daily energy intake for human body.

It represents the most important cereal crop in the world, with approximately 158 million hectares of harvested areas, producing more than 700 million tons annually. It is the agricultural output with the third-highest worldwide production, after sugarcane and maize [2]. Since substantial quantities of sugarcane and maize crops are used for purposes different from human consumption, rice is the most important grain regarding human nutrition and caloric intake.

The rice plant (*Oryza sativa* L.) is an annual grass, which belongs to the family of Gramineae, classified as Monocotyledons. This plant can grow to almost 2 m tall, depending on the variety and soil fertility. It has long and slender leaves covered with short hairs and its panicle-shaped inflorescence is composed of spikelets containing small wind-pollinated flowers that produce the fruit. The edible fruit consists of a grain, named caryopsis, of oblong and ellipsoidal shape, that when ripe is released with a husk of glumellae, which strongly adhere to the caryopsis. The pericarp of the seed can be white or pigmented: the law prohibits any treatment that may alter its natural colour or composition.

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Figure 7. The rice plant (*Oryza sativa* L.)

Based on different genomes and geographical origins, *Oryza sativa* L. contains three major subspecies:

- *Oryza sativa* japonica, whose caryopsis are round and thick.
  Japonica rice is extensively cultivated and consumed in China, from which originated, Japan and western countries (Europe and United States). This plant stands out for its high productivity.
- *Oryza sativa* indica, endowed with elongated and thin caryopsis.
  Indica rice is mainly cultivated in India and surrounding area and it represents the most of Asian crops. The plant features medium productivity but strong resistance to climatic adversities.
- *Oryza sativa* javanica, bearing elongated and wide caryopsis.
  Recently known as tropical japonica, it is cultivated exclusively in Indonesia.

Rice cultivation is well-suited to constant warm and humid climates, typical of tropical and equatorial areas. However, rice can also be grown in countries and regions with drier climates, even if with low yields, and practically anywhere, with the use of water-controlling terrace systems.

The traditional procedure for cultivating rice is in fact flooding the fields while, or after, setting the young seedlings, because water acts as a thermal storage system, accumulating solar heat during the day and releasing it at night.

Rice cultivation dates back from several thousand years before the Christian era, when it spread in Asia.

Nowadays over 90% of the world's rice is cultivated and consumed in the Asian continent, where China is the world's largest producer in the global economy, followed by India [3]. In south-east Asia, rice represents the principal staple food for population, with an annual pro-capita consumption in many countries between 120 kg and 170 kg, compared to the 54 kg of global pro-capita use and to only 4-5 kg in Europe [4], where the large consumption of wheat and derivative products generates a strong competition.

However, rice is cultivated all over the world, at every altitude and in every continent (except Antarctica). USA and Brazil are the main producer in the Americas and Egypt holds the record in Africa.

The leading European producer is Italy, where the cultivated areas are concentrated in four provinces: 35% in Pavia, 30% in Vercelli, 15% in Novara, 5% in Milan; the remaining 15% is distributed in the rest of the country.

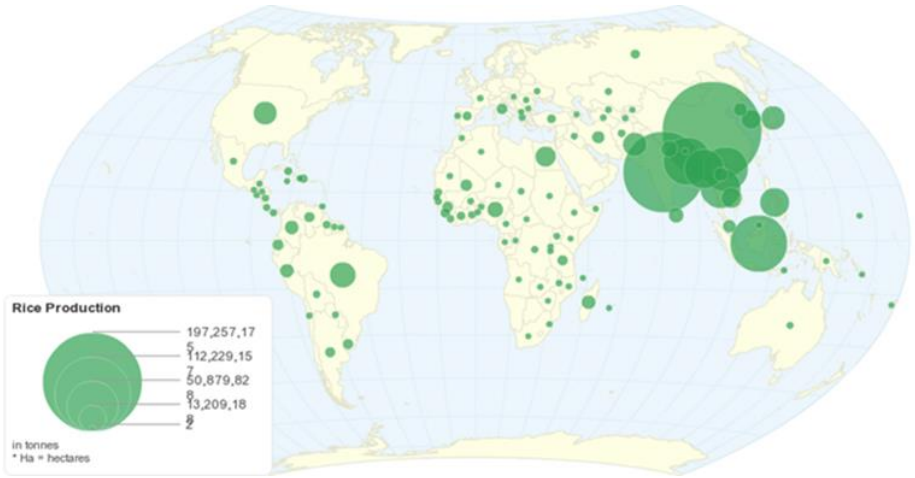

Figure 8. Worldwide Rice Production [5]

1.2. Nutritional and chemical composition of rice

Rice is a rich source of complex carbohydrates, occurring at levels higher than in any other cereal.

The main component of rice carbohydrates is starch, which alone accounts for 75-80% of the total nutrients in the caryopsis [6]. Starch is composed of amylose and amylopectin: the second one is considerably more abundant in rice

and this explains the fast release of glucose, since the branched structure of amylopectin facilitates the enzymatic attack and the consequent release of glucose.

Rice grain contains a low amount of protein (only the 7%). However, its proteins are highly digestible and contain higher concentration of lysine compared to other cereals, though even rice proteins are deficient in this essential amino acid: the amount of lysine corresponds to an amino acid score of 59% in milled rice based on the amino acid pattern of 5.8% lysine as 100%, edited by the Food and Agriculture Organization/World Health Organization/United Nations University (FAO/WHO/UNU) [7].

The soluble fractions of protein are about 15% of albumin–globulin, 65% glutelin and 20% prolamin in milled rice, while bran proteins are 66–98% albumins [7]. Prolamins are limited, so that the formation of gluten is impossible: in fact rice is, together with maize, the most popular gluten-free grain for people with celiac disease.

Rice is also a source of vitamin-B complex: thiamin, riboflavin and niacin [8]. Lipid and lipophilic vitamins are instead present in small quantities (less than 3 mg/100 g in brown rice and less than 0,5 mg/100 g in milled rice) [7].

Minerals like magnesium, calcium and phosphorus occur along with some traces of zinc, copper, iron and manganese [9]. Rice caryopsis have a very low content in sodium, compared to potassium.

Compared with other cereals, rice is characterized by low fat content after the removal of the bran, low protein content and higher digestibility of protein [7]. Thanks to its nutritional value and higher digestibility, rice deserves a primary position in a balanced human diet.

1.2.1. Rice antioxidants

Since in 2000 a positive correlation between the lower incidence of breast and colon cancers in Asian populations and rice consumption has been found by Hudson et al. [10], a great number of papers have been published dealing with the antioxidant potential of rice and its pharmacological properties. In fact, based on epidemiological studies, the low incidence of some chronic diseases in rice-consuming areas of the world might be related to the antioxidant compounds contained in rice.

The main molecules with antioxidant activity identified in rice include [11]:

- Phenolic acids

The principal phenolic acid present in the endosperm, bran and whole grain is ferulic acid, a hydroxycinnamic acid derivative which alone accounts for 56–

77% of total phenolic acids. The other most abundant compounds found in rice are in the following decreasing order: p-coumaric acid, sinapic acid, gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid and syringic acid.

Minor constituents are caffeic, chlorogenic, ellagic and cinnamic acids, each representing less than 1% of total phenolic acids.

- Flavonoids

The most abundant flavonoids occurring in rice belong to the class of flavones, whose major compounds are luteolin, apigenin and tricin, and to the class of flavonols, mainly represented by quercetin, isorhamnetin, kaempferol and myricetin.

Anthocyanins, another class of flavonoids, are present in large amount and indeed represent the predominant class in pigmented rice. Among the 18 anthocyanins identified in rice, only four have been quantified: the most abundant is cyanidin-3-O-glucoside, accounting for 51–84% of the total anthocyanin content, followed by peonidin-3-O-glucoside (6-16% of the total content), cyanidin-3-O-rutinoside (3-5%) and cyanidin-3-O-galactoside (1-2%).

- Tocopherols and tocotrienols

Tocotrienols, with the major compound γ-tocotrienol, account for 47–80% of the total vitamin E content of rice, and tocopherols for 20–53%, in which the most abundant is α-tocopherol.

However, these lipophilic compounds are sparcely present in rice (51,0 mg/kg) compared to other cereals like rye (108,0 mg/kg), oats (104,2 mg/kg) and wheat (102,2 mg/kg).

- γ-oryzanol

Gamma-oryzanol is a mixture of antioxidant compounds occurring in rice bran and consisting of steryl ferulates, which are formed by esterification of ferulic acid with phytosterols and triterpenoids.

The major constituents of the total γ-oryzanol content are in the following decreasing order: 24-methylenecycloartanyl trans-ferulate, cycloartenyl trans-ferulate, campesteryl trans-ferulate, β-sitosteryl trans-ferulate and stigmasteryl trans-ferulate, which together represent the 80% of γ-oryzanol.

- Phytic acid

Also known as phytate phosphorus or myo-inositol-1.2.3.4.5.6-hexakisphosphate, phytic acid is the most abundant form of phosphorus in the bran and in the whole grain, accounting for 65–73% of the total phosphorus content, which include also inorganic phosphorus and cellular phosphorus.

Phytic acid in rice suppresses Fe-catalyzed oxidative reactions thanks to its tendency to chelate Fe^{2+} or to keep iron in its inert form Fe^{3+}.

However, when compared with the seven most common cereals, namely maize, wheat, oat, barley, rye, sorghum and millet, rice does not result to be a

rich source of antioxidant compounds [12–16]. The contents of all the antioxidants is in fact lower in rice, except for γ-oryzanol [17] and anthocyanins, which, limited to colored rice [13], are considerably more abundant in rice than in the other not colored cereals.

Colored rice includes indeed the varieties of rice richest in antioxidant compounds, such as the main variety of black rice, followed by purple and red rice varieties [11].

Moreover, there is a great difference between the antioxidant content of white rice (endosperm) and brown rice (whole grain), as the major compounds provided with antioxidant activity are located in rice bran [11]. Therefore, brown rice appears to be the most important product in terms of antioxidant intake and nutritional value.

1.3. Brown rice

Once it is harvested, after threshing, the fruit of the rice plant is called paddy or rough rice, consisting in the caryopsis enclosed in a rigid non-edible hull, which accounts for about 20% of rice weight.

Hence, before it can be consumed as food, rice must be subjected to a careful processing.

Freshly harvested paddy rice has a humidity of 20% and it must be quickly dried with warm air until it reaches a humidity of 12-13% which ensures its shelf life.

Then, paddy rice must be cleaned to avoid contaminations, and then milled using a rice huller to remove the chaff, namely the outer husks of the grain that are made up of a high concentration of silica, making rice not still edible. This initial milling process of dehusking, to remove the hull from the caryopsis, results in ‘brown’ or ‘unpolished’ rice, which is already suitable for human consumption.

Brown rice preserves its bran layer intact: removing it by further milling or polishing, ‘white’ or ‘polished’ rice is obtained.

All rice varieties can be post-harvest processed to obtain either brown/unpolished or white/polished rice.

The transition from brown rice to white rice causes a great loss of vitamins, minerals and fibers mainly contained in the bran, and an enrichment in starch.

Brown rice has a higher nutritional value and it is considered healthier than white rice, since polishing removes the external layer of the caryopsis which is rich in important nutrients.

Brown rice has a different texture and taste than white rice, but brown rice is not as popular because it requires long time for cooking, its storage is difficult,

since the oil in the bran layer tends to turn rancid even at moderate temperatures, and the taste of white rice is preferred in many rice-consuming countries.

As a matter of fact, increasing use is being made of parboiled rice, which mostly preserves the nutritional value of brown rice. Parboiling is a hydrothermal process, which consists of soaking, steaming and drying of paddy rice, thus promoting the migration and diffusion of water-soluble compounds, such as vitamins and inorganic elements, towards the endosperm, the inner part of the grain. In this way, parboiling allows the enrichment of white rice with nutrients otherwise lost during the subsequent stages of processing.

Property	*Amounts per 100 g*		
	Brown rice	**Milled rice**	**Rice Bran**
Moisture (g)	14.0	14.0	14.0
Energy content (KJ)	1520–1610	1460–1560	1670–1990
Energy content (kcal)	363–385	349–373	399–476
Crude protein (g)	7.1–8.3	6.3–7.1	11.3–14.9
Crude fat (g)	1.6–2.8	0.3–0.5	15.0–19.7
Crude fiber (g)	0.6–1.0	0.2–0.5	7.0–11.4
Crude ash (g)	1.0–1.5	0.3–0.8	6.6–9.9
Available carbohydrates (g)	73–87	77–89	34–62
Total dietary fiber (g)	2.9–4.0	0.7–2.3	17–29
Water-insoluble fiber (g)	2.0	0.5	15–27
Sugars (g)	1.9	0.2–0.5	0.2–0.5
Thiamin (mg)	0.3–0.6	0.02–0.11	1.2–2.5
Riboflavin (mg)	0.04–0.14	0.02–0.06	0.18–0.43
Niacin (mg)	3.5–5.3	1.3–2.4	26.7–49.9
Pantothenic acid (mg)	1.4	1.0	6.8
Vitamin B6 (mg)	0.5	0.2	3.7
Folate (mg)	19	8	58
Vitamin E, α-tocopherol (mg)	0.8–2.5	<0.01–0.30	3–15
Calcium(mg)	10–50	10–30	30–120
Phosphorus (g)	0.17–0.43	0.08–0.15	1.1–2.5
Phytic acid P (g)	0.13–0.27	0.02–0.07	0.9–2.2
Iron (mg)	0.2–5.2	0.2–2.8	8.6–43.0
Zinc (mg)	0.6–2.8	0.6–2.3	4.3–25.8

Table 1. Comparison of nutrient composition of brown rice, milled rice and rice bran [7].

1.4. Colored rice

In the last few years, considerable evidences have been accumulated showing that the varieties of pigmented rice (black, purple and red) possess higher antioxidant activities compared with nonpigmented rice varieties [18–29].

The antioxidant composition of pigmented rice, responsible for their healthy properties, such as anticancer [30,31] and anti-inflammatory activities [32], includes several bioactive compounds, most belonging to the class of phenolic compounds.

Anthocyanins are the most abundant antioxidant phenolic compounds identified in pigmented rice, since they are the pigments responsible for the characteristic colour of the caryopsis, accumulating into them, especially in the pericarp layer, during maturation.

Based on in vitro and in vivo assays, cyanidin-3-O-glucoside, the major anthocyanin in rice, proved to possess an anti-proliferative effect against different types of cancer cell, even if its effect was reported when used in amounts often higher than those achievable from food sources [33].

Anthocyanins showed also an anti-inflammatory effect both in vitro and in vivo, especially due to their antioxidant capacity to downregulate the redox-sensitive nuclear factor-κB signaling pathway, strightly associate to the occurrence of inflammation, but the mechanisms are not fully understood and still need to be clarified [34].

Moreover, many other health-promoting activities of dietary anthocyanins have been investigated and reported in literature, such as cardiovascular diseases prevention [35], neuroprotective effect [36,37], retinal protection activity and the ability to regulate lipid profile and inhibit cholesterol adsorption [38].

Other classes of flavonoids contribute to the beneficial biological effects of colored rice varieties too, especially the main groups of flavonols and flavones [11].

In 2012, Chen et al. [23] even stated that the antioxidant activity of pigmented rice is primarily due to flavonols and flavan-3-ols, thanks to their radical scavenging capacity and prevention against cardiovascular diseases, rather than anthocyanins. Anyways, a positive interaction between anthocyanins and flavonols and flavan-3-ols has now been clearly established [37].

Phenolic acids represent the other class of phenolic compounds extensively occurring in coloured caryopsis [38], which gives an important contribution to the health benefits of whole grains.

It has been reported that phenolic acids, due to the antioxidant activity, have positive effects against human chronic diseases, such as cancer, especially the

gastrointestinal colonic and gastric cancers, cardiovascular diseases, diabetes and obesity [39,40].

The interest aroused by the beneficial effects of pigmented rice consumption is continuously expanding all over the world. Until few years ago, only limited areas of the world were devoted to the cultivation of pigmented rice varieties, which were merely used for decorative purposes or for making special foods and alcoholic beverages, but nowadays there are more and more cultivated lands and the global population is gaining new appreciation for colored rice consumption.

In Italy, for instance, pigmented rice crop areas have increased by more than 700% in the last decade [41] and black and red rice consumption is increasing every year.

The primary position of rice in a balance human diet makes pigmented rice interesting functional foods, by virtue of their content in anthocyanins and other phenolic compounds. Pigmented rice, in fact, can be used in human nutrition as alternatives to foods generally considered the best sources of dietary anthocyanins and polyphenols, but whose consumption is restricted by different factors. For example, the availability of fresh fruits and vegetables containing anthocyanins, such as grapes, blueberries, black plums or eggplants, is often limited to the harvest period as well as fruit consumption is limited due to its content of simple carbohydrates. Moreover, given the increasing alarm about the negative health effect of wine and ethanol [42], red wine consumption must be severely limited.

On the contrary, rice is a quite cheap food, easily available all the year round, and it can be conveniently ingested every day as source of complex carbohydrates.

1.5. ‘Violet Nori’ rice

Oryza Sativa L. ‘Violet Nori’ is a native variety of aromatic purple-colored rice, spontaneously growing in Piedmont (Italy) and recently registered at the Community Plant Variety Office (CDVO) [43].

‘Violet Nori’, originated by a natural intersection, is cultivated in a rice farm (‘Azienda Agricola Eleonora Bertolone’) located in Collobiano, in the countryside of Vercelli, recognized as one of the best rice production areas in Italy. The farm claims and underlines its commitment to the excellence of the products and the passion of the historic heritage, developing innovative technologies and sustainable production processes and defending the biodiversity of agricultural soil.

Figure 9. ‘Violet Nori’ rice crop of ‘Azienda Agricola Eleonora Bertolone’ in the plain near Vercelli.

‘Violet Nori’ rice is indeed one of the wonders of the Italian biodiversity, representing a unique variety due to its vegetative cycle which is entirely characterized by the presence of anthocyanins, responsible of the purple colour: in fact, the distinctive feature of its plant is that each part of it has an intense violet colour, both its fresh leaves and its caryopses.

Violet caryopses also stand out for their texture and size as well as for their intense aromatic taste and cooking resistance, which makes ‘Violet Nori’ rice suitable for the preparation of many different recipes, leading to the assumption of a high content of anthocyanins and other antioxidant compounds.

Oryza sativa L. ‘Violet Nori’ presents an anthocyanins content generally higher or at least comparable with that in other Italian black rice varieties, such as the best known ‘Venere’, ‘Nerone’ and ‘Artemide’ [44].

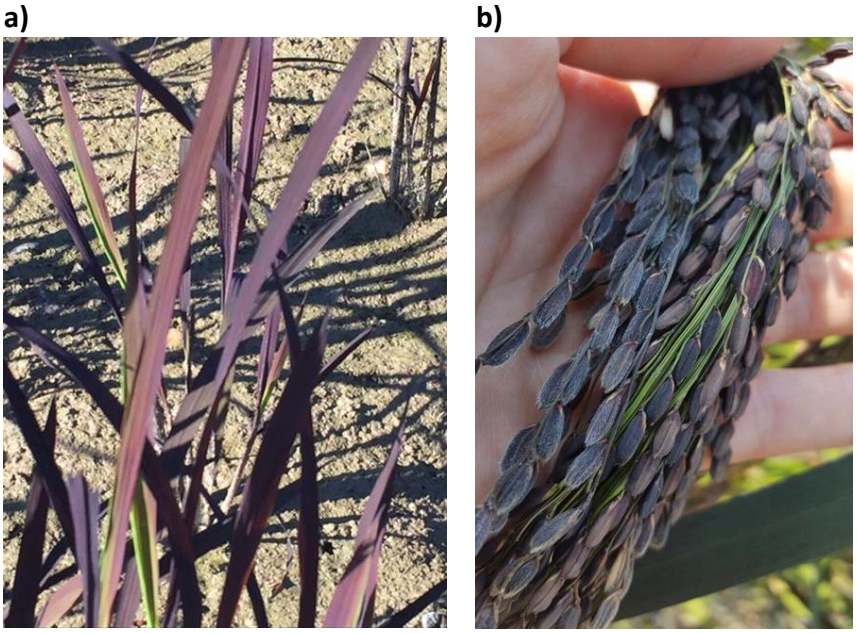

Figure 10. 'Violet Nori' rice's a) plant and b) ear.

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2. Solubility study and intensification of extraction of phenolic and anthocyanin compounds from *Oryza sativa* L. 'Violet Nori'

2.1. The present work

The present work is the result of my three-month research period at Avignon University, in the Green Extraction Team, made possible by a collaboration with the research Group of Chemistry of Food and Dietary Products of Genoa University.

The aim of the study was to increase the extraction yields of the antioxidant compounds (flavonoids and phenolic acids) of *Oryza sativa* L. 'Violet Nori' rice by means of green strategies. For this purpose, the work involved several steps.

At the beginning, a comparative kinetic study between the antioxidant extraction from 'Violet Nori' rice grains and from 'Violet Nori' rice powder was performed. In this way, it was possible to identify the best rice form to be exploited in the following steps of solubility study and intensification and comparison of the green extraction techniques.

The first main part concerned the solubility study of the selected principal polyphenols of 'Violet Nori' rice in GRAS hydroalcoholic mixtures composed by different percentages of ethanol and water.

The simulation program COMSO-RS was used to predict theoretical values of solubility index of the targeted polyphenols in the ethanol:water mixtures, in addition to supply the analysis of σ-surfaces, σ-profiles and σ-potentials of each investigated molecule, including ethanol and water.

Then, the computational results obtained by COSMO-RS were validated by practical experiments, involving conventional macerations of rice powders in all the different EtOH/H_2O mixtures.

Once the best extraction mixture was identified, the following step of the work was performed, with the purpose of intensifying the extraction yield.

Several innovative green extraction techniques, including continuous ultrasound, using both the probe system (UAE probe) and the ultrasonic bath (UAE bath), bead milling (BM), microwave (MAE) and accelerated solvent (ASE) extractions were tested on rice grains and compared to a conventional maceration in the same condition.

Finally, a comprehension of the extraction mechanism was proposed, based on the macroscopic images of rice grains subjected to conventional maceration and to the most promising ultrasonic and bead milling extractions.

Analytical methods to evaluate the extraction recovery and antioxidant activity of the obtained 'Violet Nori' rice extracts involved the spectrophotometric Folin-Ciocalteu test (for total phenolic content) and pH differential method (for total monomeric anthocyanin content) and the quantification of the total anthocyanins by HPLC, using an internal standard of cyanidin-3-O-glucoside.

Results showed that the best solvent to solubilise and extract 'Violet Nori' rice polyphenols was the mixture EtOH/H_2O (60:40 v/v). COSMO-RS computational predictions were found to be in perfect correlation with the experimental results, until the threshold of the selected best hydroalcoholic mixture.

Moreover, the best green extraction techniques to intensify the antioxidant compounds yield resulted to be both UAE probe and BM, providing in only 5 minutes the same extractive efficiency of 3 hours conventional maceration. This may be due to the capacity of ultrasound and bead milling to erode the rice grain's surface, with their direct mechanical impact, compared to the other performed extraction techniques.

2.2. Scientific paper

Please find the article at the following link:

https://doi.org/10.1016/j.ultsonch.2020.105231

S. Catena, N. Rakotomanomana, P. Zunin, R. Boggia, F. Turrini and F. Chemat, Solubility study and intensification of extraction of antioxidant compounds from Oryza Sativa L. 'Violet Nori', Ultrason. Sonochem. 68 (2020) 105231.

CHAPTER 1

PART B

"Extraction from pomegranate's by-product: investigation of marcs and external peels"

1. Introduction

1.1. Pomegranate: an ancient fruit

Pomegranate (*Punica granatum* L.) is one of the oldest known edible fruits, originating in Central Asia, consumed since the dawn of civilization. The pomegranate plant was one of the first fruit plants to be domesticated and is believed to have been planted for the first time around 4000 and 3000 BC [1].

The scientific name *Punica granatum* derives from the Latin "pomum" (apple) and "granatus" (grainy), which means "apple with grains". Romans also called it "Punicum malum", literally "Punic apple", in allusion to the ancient Phoenicia in which large pomegranate plantations were grown and the name of the genus Punica originates from this terminology.

The genus *Punica* is often associated to the Punicaceae family, although the taxonomy currently accepted is in accordance with that one of the APG-III (Angiosperm Phylogeny Group III), which included the *Punica* genus in the Lythraceae family (subfamily Punicoideae).

Within the *Punica* genus, two species can be distinguished, namely *Punica granatum* L. and *Punica protopunica*, also called Socotra pomegranate as it is endemic to this island in Yemen. A particular form of *Punica granatum* known as *Punica nana* is sometimes considered a third species of the genus [1].

Pomegranate plants are fruit-bearing shrubs or small trees that grow between 5 and 10 m tall.

The stem is smooth with dark gray bark, often quadrangular, and the branches are sometimes spiny.

The leaves are opposite or sub-opposite, often crowded on short lateral shoots, short-petioled, oblong or obovate, bright green, glabrous and glandular, with variable length between 2-8 cm.

Flowering occurs about 1 month after the bud breaks on newly developed annual branches. Flowers can appear solitary, in pairs or in clusters and they are regular, actinomorphic, bisexual, terminal or axillary. Calix is persistent and tubular and it is divided into 5-8 intense red triangular lobes, between which are inserted 5-7 petals, imbricate, brilliant orange-red and lanceolate.

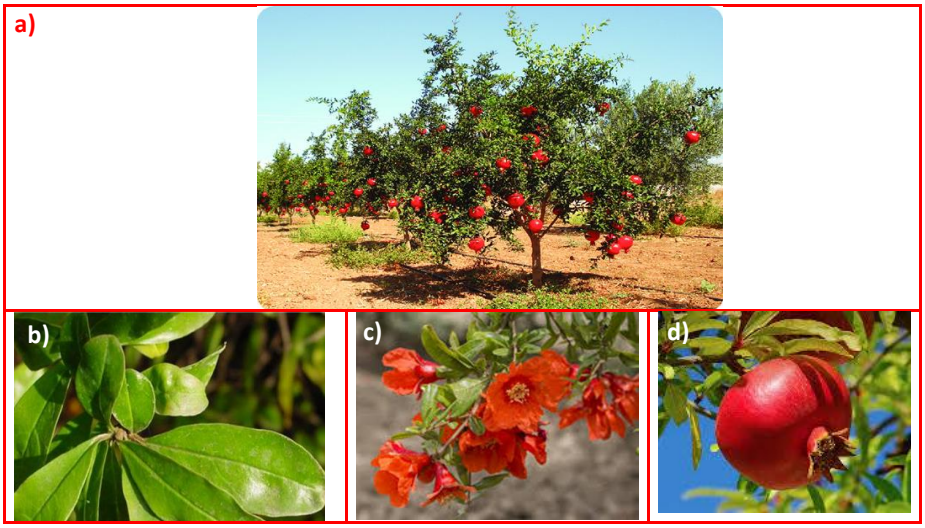

Figure 11. Pomegranate: a) Plant, b) Leaf, c) Flower, d) Fruit.

The pomegranate fruit ripens about 6-7 months after flowering.

It is a fleshy berry, also called “balausta”, non-climacteric, globular or slightly flattened and between 5-12 cm in diameter. A feature distinctive of the pomegranate fruit is the maintenance of the prominent calyx until ripe.

The husk is composed of two parts: the pericarp and the mesocarp.

The pericarp (rind) is smooth and leathery (or leathery-woody) of variable thickness and color (from brownish-yellow to red when ripe) and provides an external protective cuticle layer.

The mesocarp (or albedo) is made up of spongy tissue and is divided into several internal portions by a horizontal diaphragm and cartilaginous tissue membranes called endocarp.

Endocarp is occupied by the edible part of the fruit, which is called sarcotesta and consists of arils containing the seeds of the fruit and a juicy episperm. Arils color range from deep red to almost colourless according to the different cultivars, while the enclosed seed varies in content of sclerenchyma tissue, which affects its softness. The number of arils is variable and may be as high as 1300 per single fruit [1].

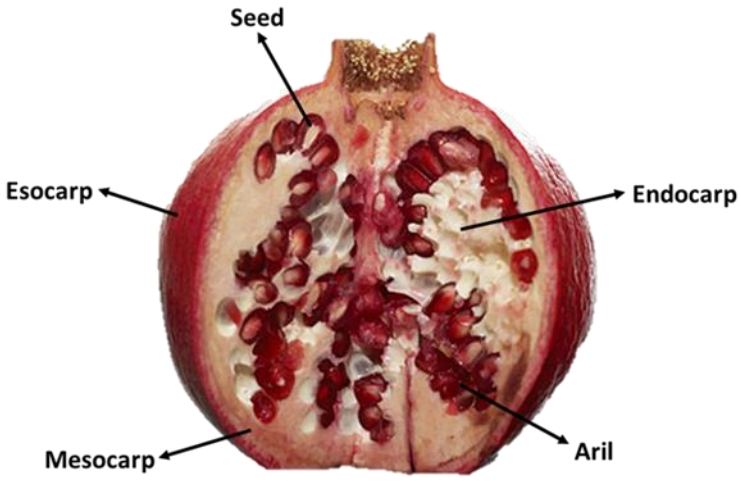

Figure 12. Different portions of pomegranate fruit.

Pomegranate is believed to be native to Central Asia, particularly parts of Iran, from where it quickly spread to the rest of the world.

Several authors have discussed the presence of three mega-centers (primary, secondary and tertiary) and five macro-centers (Middle Eastern, Mediterranean, Asian Eastern, American and South African) of origin and genetic diversity of pomegranate [2].

This plant was initially cultivated in ancient Egypt, Greece, Italy and Iraq. Subsequently, it spread to Asian countries such as Turkmenistan, Iran, Afghanistan, India and China, North Africa and Mediterranean countries of Europe.

Today, pomegranate has a wide global geographical distribution that reflects its easy adaptation to various climatic conditions and large range of genetic diversity.

Exact data are not available about world production of pomegranate as this is rapidly increasing, although it is estimated that about 1.5 million tons of pomegranates are produced annually worldwide.

India represents the largest area of pomegranate cultivation and production worldwide, while it ranks second, after Iran, in the list of main exporters: Iran exports 60,000 tons of pomegranates annually against 35,176 exported from India.

In terms of productivity, Spain ranks first (18.5 tons per year) followed by the United States (18.3 tons per year).

Although Spain has a very small production area (2000 hectares), it exports the 37.8% of total pomegranate production (37,000 tons), followed by Israel (23.5%) and the States United (15.5%) [1].

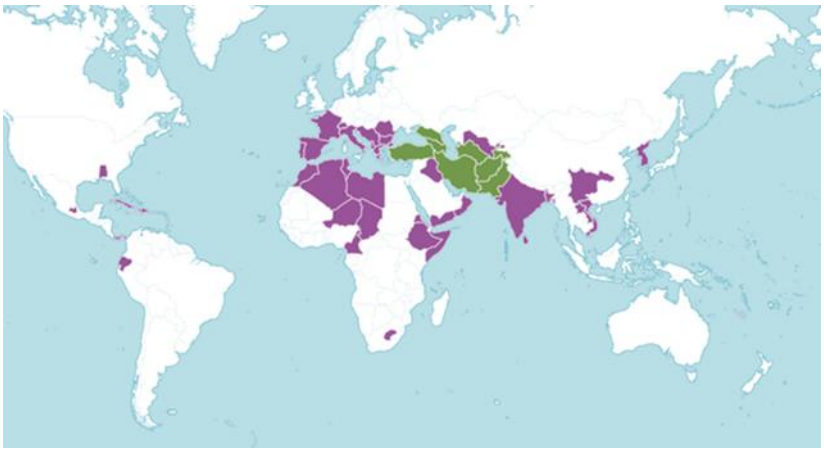

Figure 13. Pomegranate worldwide distribution (green: native, purple: introduced) [3].

1.2. Uses of pomegranate

Pomegranate is an important fruit by virtue of its nutritional, medicinal and ornamental properties which justify its high consumption worldwide and therefore its important industrial value.

A great amount of evidence reveals the multifaced use of pomegranate in different cultures and mythologies.

In addition to its traditional use in nutrition, in fact, pomegranate has a plurality of symbolic meanings: mentioned in the Bible and in the Koran, it was considered a sacred plant since the ancient Egyptians, a symbol of friendship by the Jews, a symbol of immortality and fertility according to Christian symbology. Even today pomegranate continues to be the emblem of fertility, prosperity, brotherhood and vital energy among many populations [4].

Pomegranate has been employed since ancient times for dyeing textiles. This has been proved in the case of a yellow dye in wool threads found in the Royal Tumulus of In Aghelachem, Libyan Sahara, from the second or third century AC [5]. It has also been reported that pomegranate fruit extracts were used to make dye, while the leaf extract to make ink in antiquity in a region of India and that pomegranate peels extracts could dye the cotton textile [6].

Cosmetic uses of many parts of pomegranate plant and related extracts are also known.

Aqueous extracts (especially of pomegranate peel) proved to promote the regeneration of dermis, while pomegranate seed oil the regeneration of epidermis, suggesting the potential of pomegranate fractions for facilitating skin repair [7]. Pomegranate extracts and juice showed a protective effect against damage by UV-B radiation in human reconstituted skin [8]. Pomegranate formulations were recently included in a review of herbal cosmeceuticals [9].

The medicinal properties of pomegranate were described for the first time by Hippocrates, who magnified its anti-inflammatory, anti-diarrheal, antibacterial and anthelmintic activities in his works. The same ancient literature of Pliny, Sorano and Dioscorides reports different uses of pomegranate as a medicine.

Its use in folk medicine of many cultures is documented as an antiparasitic, anthelmintic, antipyretic, antimicrobial, in the treatment of canker sores, acidosis, ulcers, and respiratory diseases [10].

Despite its well-known properties, pomegranate has long remained a forgotten and unused plant, often relegated to a predominantly ornamental use. The healing properties of the fruit found confirmation many centuries later in medical research. In the last ten years, in fact, pomegranate has been the subject of many studies that proved its multiple health-promoting effects.

1.2.1. Health-promoting activities

The pharmaceutical, pharmacological and medicinal bioactivities of different portions of pomegranate plant are due to their high content of antioxidant compounds, molecules that prevent body's oxidative stress, thus promoting health and reducing risk of disease.

The antioxidant potential of pomegranate juice has been shown to be superior to that of red wine and green tea due to its high content in ellagitannins and hydrosable tannins [11].

Studies conducted both in vitro and in vivo on animals, have shown that pomegranate has a strong antioxidant and antitumor activity: in fact, its beneficial effects in the prevention and treatment of various tumor pathologies are reported, including breast carcinoma, prostate, lung, stomach, colon and skin cancer [12].

It has also been demonstrated a preventive activity in the oxidation of lipoproteins (high and low density), in inflammation, hypertension, process of atherosclerosis, platelet aggregation and cardiovascular diseases. Many of these studies have been conducted not only in vitro but also on in vivo animal models and in some cases also on humans [12].

Several studies also confirmed pomegranate's antibacterial, antifungal and antiviral properties [12].

Furthermore, its potential beneficial effects on the health of the oral cavity have been evaluated, especially in the prevention of stomatitis, gingivitis and periodontal disease, as well as its capacity to improve the skin health and to prevent problems related to the male reproductive system including erectile dysfunction [12].

Finally, the neuroprotective properties of pomegranate in the treatment of several degenerative pathologies are reported in various scientific papers [13].

In particular, it is believed that the consumption of this fruit or products made by its derivatives (such as juice, peel and seeds) can be adjuvant and supportive in the treatment of senile dementia and / or Alzheimer's disease, although more in-depth clinical investigations and studies are necessary to evaluate their effective efficacy and safety [14].

1.3. Chemical composition of pomegranate

The chemical composition of pomegranate fruit varies greatly depending on several factors including cultivar, growing region, cultivation practice, climate, maturity index and storage conditions.

Several authors have in fact reported significant variations in the content of organic acids, phenolic compounds, sugars, water-soluble vitamins and mineral elements within pomegranate. Moreover, the different parts of the fruit have their characteristic content of nutritional and bioactive compounds [12].

The edible part of pomegranate fruit, which represents about the 50 - 52% of the weight of the fruit (the other 50% corresponds to the peels), consists of 40% of the arils and 10% of the seeds.

The arils contain 85% water, 10% carbohydrates (mainly fructose, glucose), about 1.5% pectin, vitamins (such as ascorbic acid), organic acids (such as citric acid, malic acid, succinic , oxalic, tartaric, etc.) and other bioactive compounds including mainly anthocyanins and hydrolysable tannins.

The seeds of pomegranate represent a rich source of lipids and their oil, which constitutes 12 - 20% of the total weight of the seed, contains a unique profile of fatty acids characterized by a high concentration of polyunsaturated (n-3) fatty acids such as linoleic, linolenic and oleic. It is also very important the presence of punicic acid [C18: 3 (c9, t11, c13)], polyunsaturated fatty acid isomer of linolenic acid characteristic of pomegranate oil [12]. Other important components of pomegranate seed oil are phytosterols, including β–sitosterol, which represents 77.94% of the total sterol content, followed by Δ5-avenasterol (7.45%) and campesterol (6.35%) [15].

Property	*Amounts per 100 g*
Water	77.93 g
Energy	83 kcal
Protein	1.67 g
Total lipid (fat)	1.17 g
Carbohydrate	18.7 g
Fiber	4 g
Sugars	13.67 g
Calcium, Ca	10 mg
Iron, Fe	0.3 mg
Magnesium, Mg	12 mg
Phosphorus, P	36 mg
Potassium, K	236 mg
Sodium, Na	3 mg
Zinc, Zn	0.35 mg
Copper, Cu	0.158 mg
Manganese, Mn	0.119 mg
Selenium, Se	0.5 µg
Vitamin C, total ascorbic acid	10.2 mg
Thiamin	0.067 mg
Riboflavin	0.053 mg
Niacin	0.293 mg
Pantothenic acid	0.377 mg
Vitamin B-6	0.075 mg
Folic acid	38 µg
Vitamin E (α-tocopherol)	0.6 mg
Vitamin K (phylloquinone)	16.4 µg

***Table 2*.** Nutritional composition of 100 g of pomegranate's edible part [16].

1.3.1. Phenolic compounds

Phenolic compounds are considered the main responsible for most of the functional properties of many foods, including the pomegranate fruit, thanks to their high antioxidant activity.

The presence of these bioactive compounds, including phenolic acids, flavonoids and tannins ensures a considerable nutritional value of pomegranate fruit [17].

Phenolic acids occurring in pomegranate juice can be divided into two major groups:

- Hydroxybenzoic acids: mainly gallic acid and ellagic acid;
- Hydroxycinnamic acids: mainly caffeic acid, chlorogenic acid and p-coumaric acid.

Among flavonoids, the main compounds in pomegranate are anthocyanins, the pigments responsible for the characteristic red color of the fruit and its juice. The main occurring anthocyanins are cyanidin3-O-glucoside, cyanidin-3,5-di-O-glucoside, delphinidin-3-O-glucoside, delphinidin-3,5-di-O-glucoside, pelargonidin-3 -O-glucoside and pelargonidin-3,5-di-O-glucoside.

Ellagitannins (ETs) are very abundant in pomegranate, primarily in the peels. They belong to the category of hydrolyzable tannins and they are esters of hexahydroxydiphenic acid and a sugar, generally glucose. The main ETs are punicalin, pedunculagin and punicalagin which is a characteristic compound of the genus Punica.

1.4. Pomegranate by-products

The main product obtained from pomegranate processing is the juice, which can be obtained by direct pressing of the fruit or after separation of the arils.

The industrial transformation of the fruit mainly aims obtaining and marketing clear juices, although the processing of intact arils has recently been spreading, to be marketed as fresh-cut fruit. In both cases pomegranate manufacturing generates a considerable amount of waste, the disposal of which is very expensive for industries.

In Europe, the amount of food-processing by-products and wastes corresponds to almost 250 million ton/year, among which 0.5 million ton derives from fruit and vegetable industrial processes [18].

As a fruit waste, pomegranate is responsible for a big impact, since about 40–50% of the whole fruit is usually discarded.

Two types of by-products are mainly obtained from the pomegranate juice industrial chain:

a) a non-edible by-product consisting of the almost intact exocarp with an associated part of mesocarp, both characterized by variable thickness and color depending on fruit cultivar, which represent the ‘external by-product’, or external peels;
b) a theoretically still "edible" by-product deriving from the chopped endocarp and from the post-pressing residues of arils, consisting mainly of seeds deprived of the juicy coating, which represent the ‘internal by-product’.

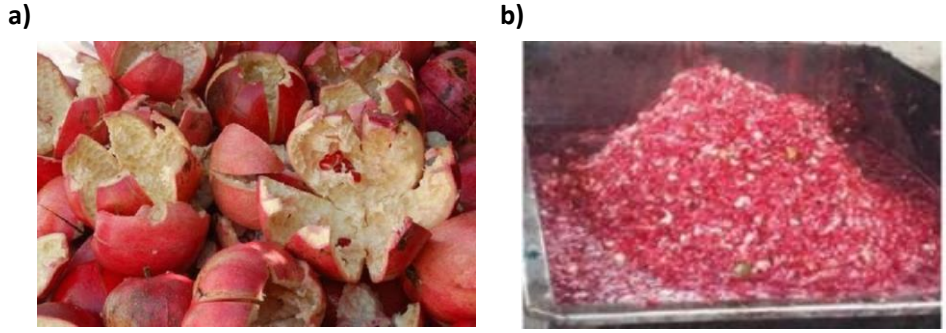

Figure 14. Pomegranate by-products obtained from juice production.

It is interesting to note that the nutraceutical properties of pomegranate are not limited to its edible part, but different parts of the fruit (i.e. the above mentioned peels and seeds) and of the tree (i.e. flowers, bark, buds and leaves) contain even higher amounts of bioactive compounds with high nutritional value than the fruit itself [19].

The pomegranate ‘external by-product’ or external peels corresponds to about 48 - 50% of the total weight of the fruit and it is an important source of bioactive phenolic compounds (flavonoids and ellagitannins), mineral compounds and complex polysaccharides.

In particular, pomegranate exocarp is rich of ellagitannins, which are hydrolysable tannins containing ellagic acid and could be involved in the

reduction of risk factors of different chronic diseases, such as colorectal cancer, prostate cancer and Alzheimer's disease [20-23].

In addition to their nutraceutical relevance, pomegranate peels and their extracts boast important technical functions (as antimicrobial, colourant and flavouring) and could also be employed as natural additives for food preservation and quality enhancement [24–26].

The green extraction of bioactive compounds from pomegranate peels has become an important research topic. Especially the whole exocarp phyto-complex looks promising for its possible nutraceutical use, thanks to the synergistical effects of the phyto-complex bioactive compounds [27].

The pomegranate 'internal by-product' or marcs is another waste product that deserves to be valorized, since being "edible", it is free of regulatory constraints.

The endocarp contains a high amount of hydrolysable tannins, phenolic acids (especially ellagic acid and gallic acid) and flavonoids that can be exploited for nutraceutical and cosmeceutical applications, such as the production of functional foods or cosmetics [28].

Although the seeds of pomegranate, which account for about 3% of the whole fruit weight, have a low content of polyphenols and a poor capacity antioxidant in vitro, they contain other important components, as previously described (section 1.3, Chapter 1-Part B), that may contribute to health-promoting effects [29].

1.5. References

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2. Traditional or hydro-diffusion and gravity microwave coupled with ultrasound as green technologies for the valorization of pomegranate external peels

2.1. The present work

The present work is the fruit of a collaboration between the research Group of Chemistry of Food and Dietary Products and the Group of Cosmetic Chemistry of Genoa University, which performed the microwaves treatments.

The aim of this study was to exploit the full potential of pomegranate external by-product, enhancing the green extraction of its ellagitannins and other bioactive phenolic compounds.

Two different certified cultivars of pomegranate were investigated, namely Akko and Wonderful, both harvested in Apulia, a southern Italy region, and collected at full maturity at the end of September and of November, respectively.

Before extraction, the external peels were dried by means of different methodologies: drying with traditional heating oven, microwave drying by a single-mode microwave oven (MH) or by a hydro-diffusion and gravity (MHG) oven.

Then, the different samples of dried peels were finely ground and subjected to a direct ultrasound-assisted extraction in pulsed mode (PUAE), using a mixture EtOH/H_2O (70:30 v/v).

Analytical methods to evaluate the antioxidant capacity of the obtained peels' extracts involved the spectrophotometric Folin-Ciocalteu and DPPH tests and the quantification of ellagic acid by HPLC.

The total ellagitannins content of the extracts was expressed as total ellagic acid, after acid hydrolysis of ellagitannins to ellagic acid. The completion of hydrolysis was monitored by FT-IR spectroscopy, made available by the Organic Chemistry Group of Genoa University.

Results showed that coupling the microwave assisted drying (both MH or MGH treatment) to PUAE, allowed to significantly increase the recovery of ellagitannins, free ellagic acid and antioxidant compounds from pomegranate peels, and to greatly reduce the processing time, compared to a traditional drying technique.

2.2. Scientific paper

Please find the article at the following link:

https://doi.org/10.1016/j.fbp.2019.06.014

F. Turrini, P. Zunin, S. Catena, C. Villa, S. Alfei and R. Boggia, Traditional or hydro-diffusion and gravity microwave coupled with ultrasound as green technologies for the valorization of pomegranate external peels, Food Bioprod. Process. 117 (2019) 30-37.

3. Pomegranate marcs and external peels as potential bioactive ingredients

Aqueous extracts of pomegranate marcs (*P. granatum* cv. Dente di Cavallo) and pomegranate external peels (*P. granatum* cv. Akko and Wonderful) obtained by the research Group of Chemistry of Food and Dietary Products have been tested for different purposes.

These studies were carried out thanks to the collaboration of various research groups: Group of Pharmaceutical Technology, Group of Biochemistry and Group of Plant Biology of Genoa University and Department of Agriculture, Forestry and Food Science of Turin University.

Squeezing marcs coming from juice processing of the pomegranate cultivar Dente di Cavallo (harvested and collected in Sicily) were subjected to pulsed ultrasound-assisted extraction (PUAE), using just water as extractive solvent, obtaining promising extracts with high antioxidant activity and higher content in vitamin C compared to the corresponding pomegranate juice.

The aqueous extracts were then microdispersed in a polymeric matrix of low methoxyl pectins, using the spray-drying technology. In this way it was possible to achieve a water-soluble formulation with a good encapsulation efficiency (about 50%) and able to maintain the functional properties of the bioactive compounds occurring in the corresponding marcs extracts.

Therefore, the extracts, both before and after the microparticles powder production, were tested ex vivo on human platelets for the inhibition of thrombin-induced platelets aggregation. The microdispersion of the pomegranate marcs extract resulted to be significantly able to inhibit human platelets aggregation (about 60% of inhibition compared to 30% of the extracts not subjected to microencapsulation).

Finally, this promising formulated extract was tentatively exploited as potential novel food ingredient, by enriching fresh-cut apple wedges using the vacuum impregnation technique. The enzymatic browning of the fresh-cut wedges was monitored spectrophotometrically, confirming the achievement of “polyphenols-enriched” apples.

For a more detailed discussion about the present work, please find the scientific paper **2** (*From pomegranate marcs to a potential bioactive ingredient: a recycling proposal for pomegranate-squeezed marcs)* in section “Other publications”.

External peels of the two pomegranate cultivars Akko and Wonderful were extracted using two different techniques: a traditional decoction and a pulsed ultrasound assisted extraction (PUAE), using for both just water as extraction solvent and 10 minutes as time process.

The obtained aqueous extracts were tested as potential anti-tyrosinase ingredients, by evaluating their inhibition property of a mushroom tyrosinase enzyme in cell culture.

In particular, the decoctions showed slightly higher radical scavenging activity, total phenolic content and free ellagic acid content, while PUAE extracts had higher content in ellagitannins (ETs) and then a lower EC50.

By means of the chemometric tool principal component analysis (PCA), in fact, the ETs content was found to be directly correlated to the capacity of tyrosinase inhibition and then to lower values of EC50.

Although these water extracts may need a further specific formulation for their preservation, these results suggest the potential use of both these pomegranate peels extracts as anti-browning and/or lightening ingredients exploitable in several formulations, even extemporarly.

For a more detailed discussion about the present work, please find the scientific paper **1** *(Traditional Decoction and PUAE Aqueous Extracts of Pomegranate Peels as Potential Low-Cost Anti-Tyrosinase Ingredient)* in section “Other publications”.

Conclusions

Chapter 1 of the present thesis deals with the biggest topic that characterised my doctoral research: the green extraction of antioxidant compounds from the food ‘Violet Nori’ rice and from the agroindustrial by-products peels and marcs of pomegranate.

The studies reported in this chapter have been carried out at the University of Genoa, especially the ones related to pomegranate, which has been one of the main subject of research of the group of Food and Dietary Products for several years and at Avignon University, where I deeply investigated the most effective way to extract polyphenols from ‘Violet Nori’ rice, studying both the best extraction solvent and the best technique.

In the GREEN laboratory of Avignon I had the opportunity to test many different green extraction methodologies: continuous ultrasound assisted extraction, performed both with the ultrasonic bath and the probe system, traditional microwave assisted extraction, bead milling and accelerated-solvent extraction. On the other hand, at the department of Pharmacy of Genoa I could extensively employ the pulsed ultrasound assisted extraction performed with probe system and I could also indirectly study the microwave hydro-diffusion and gravity technique.

The most effective green extraction methodology for both the investigated food matrices resulted to be ultrasound assisted extraction performed with probe system, which provides a fast, efficient, safe and sustainable method of extraction.

For what concern the food subjects of the research ‘Violet Nori’ rice and pomegranate, interesting finds have been reported.

Oryza sativa L. ‘Violet Nori’ has been investigated for its outstanding content of anthocyanins, responsible for its characteristic purple color. The amount of these beneficial bioactive compounds resulted to be higher in ‘Violet Nori’ compared to the other Italian black rice varieties, such as the best known ‘Venere’, ‘Nerone’ and ‘Artemide’.

Pomegranate peels and marcs have been studied as valuable agroindustrial by-products. Thanks to their high content in antioxidant compounds ellagic acid and ellagitannins, pomegranate by-products could be further exploited for their nutraceutical properties as well as natural additives. This would allow industries to solve the serious problem of the disposal of waste deriving from pomegranate juice processing, with a view to recycling and sustainability.

CHAPTER 2

“Preserving or increasing the activity of antioxidant phenolic compounds”

1. Introduction

The second chapter of the present PhD thesis work is dedicated to the main antioxidant bioactive compounds occurring in the two food matrices analysed in Chapter 1:

- Anthocyanins, responsible for the purple colour of *Oryza sativa* L. 'Violet Nori';
- Ellagic acid, which confers to pomegranate (*Punica granatum* L.) its antioxidant power.

In both cases, given their promising health promoting activities, investigations and considerations have been made related to their real efficacy inside the human body.

The effect of cooking conditions on the anthocyanins content of 'Violet Nori' rice has been investigated and many different cooking techniques have been tested, in order to establish the best operative conditions for cooking Violet rice that allow effectively preserving the highest possible amount of total anthocyanins contained in a portion usually consumed by humans.

A strategy to improve ellagic acid bioavailability has been investigated. Dendrimeric nanocarriers have been employed to encapsulate ellagic acid, thus enhancing its very low water solubility which has a considerable negative effect on its pharmacokinetic properties. Thanks to these polymeric scaffolds, dendrimer/ellagic acid complexes have been achieved characterised by water solubility higher than free ellagic acid, that could potentially vehiculate the natural polyphenol within the human body.

CHAPTER 2

PART A

“Anthocyanins content of ‘Violet Nori’ rice and its preservation after cooking”

1. Introduction

1.1. Anthocyanins

Anthocyanins are naturally occurring water-soluble pigments found in all plant tissues belonging to the class of flavonoids and possess many different colour hues like blue, purple, red, pink, orange and red, depending on the environmental pH.

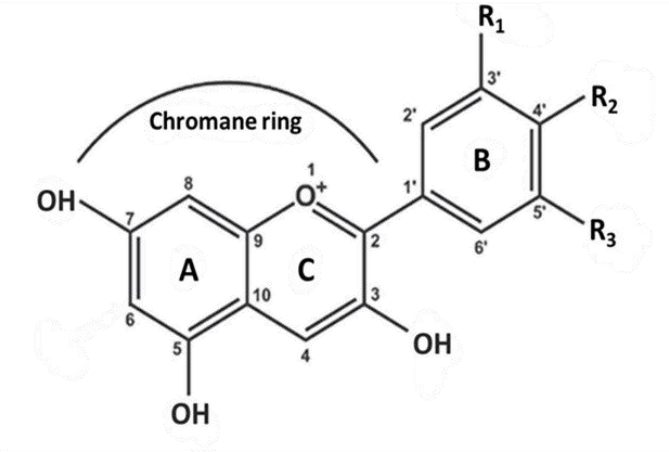
Chemically, anthocyanins can be defined as the glycosides of an aglycone unit, represented by anthocyanidins (the sugar-free counterparts of anthocyanins), whose skeleton structure is based on the 2-phenylbenzopyrilium or flavylium cation (2-phenylchromenylium).

Anthocyanins exist in nature as glycosylated polyhydroxyl and polymethoxyl derivatives of 2-phenylbenzopyrylium salts, for which they are medium-size biomolecules with weight ranging from 400 to 1200. The general structure is composed of two aromatic rings (A and B) linked by three carbons in an oxygenated heterocycle (C), namely a chromane ring (A and C) with a second aromatic ring (B) attached in position 2. In all structures the structural skeleton presents hydroxylation at the C3, C5, and C7 positions [1].

Sugar moieties are always found attached by an α or β linkage to the free anomeric hydroxy at position 3 of the anthocyanidin. When additional sugars are linked to the aglycon structure, they occupy positions 5 and/or 7, and less often 3′ and 5′. Sugar groups may be bounded to by aliphatic, hydroxycinnamic or hydroxybenzoic acids, the most common of which are malonic, acetic, caffeic and p-coumaric acids [2].

According to literature, approximately 30 different anthocyanidins have been fully described, but only 6 are widespread and commonly found in nature. They are: cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin.

Their structures can vary by conjugation with sugars and organic acids to generate a large variety of anthocyanins of different colors. The most common sugars moieties of these anthocyanins are glucose and galactose among hexoses and xylose, arabinose and rhamnose among pentoses. Di- and trisaccharide functional groups are also common (like rutinose, sophorose, 2G xylosylrutinose and glucosylrutinose) [3].



Anthocyanidins	R_1	R_2	R_3
Cyanidin	-OH	-OH	-H
Delphinidin	-OH	-OH	-OH
Pelargolidin	-H	-OH	-H
Malvidin	$-OCH_3$	-OH	$-OCH_3$
Peonidin	$-OCH_3$	-OH	-H
Petunidin	-OH	-OH	$-OCH_3$

Figure 15. Chemical structure of anthocyanidins.

The chemical structure of anthocyanins influences their physicochemical properties like color, stability and aqueous equilibrium, and it is responsible for their antioxidant activity [4,5].

The color varies from the blue end of the UV-Vis spectrum, when the B ring possesses more hydroxyl groups, to the red end of the UV-Vis spectrum when the B ring possesses more methoxyl groups [6]: for instance, cyanidin, delphinidin and pelargonidin, showed red to magenta, violet to blue and orange to red color hues, respectively [7].

The presence of hydroxyl groups and sugar(s) on the rings is responsible for the solubility of anthocyanins in water and ethanol [3].

The chromane ring of the structures is associated with their aromatic properties [4].

The structural characteristic based on overall rings and conjugated double bonds makes anthocyanins highly reactive toward reactive oxygen species: their multi-

functions have been proposed as antioxidants against the stable organic free radical DPPH· and against the damaging active oxygen species $O_2^{\cdot -}$, OH·, $^1O_2$ and H_2O_2 [8,9] as inhibitors of lipid peroxidation [10] and as potential protectors from light stress thanks to their capacity of photoinhibition [11].
Thanks to these beneficial activities anthocyanins play a fundamental role in plants, where they act also as protection against mechanical damage from the attacks by pathogens, insect and artificial wounding treatment [12], as well as osmotic adjusters [13]. Since they impart the characteristic color to different parts of plants, they also have a role in reproductive mechanisms: in flowers they lead to attract pollinators and in fruits and fruits to attract seed disseminators [3].
As far as their presence in the plant kingdom is concerned, anthocyanins are widely distributed in several plant, floral and fruit tissues, but they are also located in roots, shoots and leaves of both gymnosperms and angiosperms [13]. Anthocyanins are secondary metabolites produced in plants via the shikimic acid pathway in cytoplasm and then transported into the vacuole [14]. These phytopigments are in fact present in vacuole of epidermis/coat and mesophyll/flesh cells of the colorful plants parts [15].

1.2. Dietary anthocyanins

Since anthocyanins can be found in nature in all kinds of vascular plants (tracheophytes) and in any plant tissue, especially in flowers and fruits, they are widely distributed in the human diet through plant-based foods.

Natural edible sources of dietary anthocyanins include colored fruits such as all types of red and black berries, dark-colored vegetables, such as eggplant, red radish, black bean and red cabbage, pigmented cereals, such as purple corn, blue wheat and black rice, and others [3].

Food	mg/100 g (of fresh weight or form consumed) **Total Anthocyanins**
Fruits	
Blackberry	245 ± 68
Blueberry	386.6 ± 77.7
Cherry, sweet	122 ± 21.3
Cranberry	140 ± 28.5
Black currant	476 ± 115
Red grape	26.7 ± 10.9
Black plum	124.5 ± 21.6
Black raspberry	687*
Red raspberry	92.1 ± 19.7
Strawberry	21.2 ± 3.3
Vegetables	
Black bean	44.5*
Eggplant	85.7*
Red cabbage	322 ± 40.8
Red onion	48.5*
Red radish	100.1 ± 30.0

Table 3. Concentration of dietetic anthocyanins in common foods generally considered as the best sources.
* Data available only for one sample (no SD provided) [16].

1.2.1. Anthocyanins in rice

As previously described, rice shows genetic diversity in the colour of the kernels [17]. Anthocyanins are found in pigmented rice.

Different parts of a rice grain contain different amounts of anthocyanins, that are mostly concentrate in the bran of de-husked kernels.

About eighteen anthocyanins have been identified in rice, but only four are the predominant ones: cyanidin-3-O-glucoside, peonidin-3-O-glucoside, cyanidin-3-O-rutinoside and cyanidin-3-O-galactoside. The total amount of these four anthocyanins in pigmented rice varieties is 1252.7 mg/100 g and 345.8 mg/ 100 g (average values) in the bran and in the whole grain, respectively [18].

The total anthocyanin content (TAC) in pigmented rice varies greatly depending on the different species. However, in every cases, cyanidin-3-O-glucoside is the major anthocyanin in rice, accounting for 51-84% of the TAC (depending on rice fraction and rice bran color), followed by the second most abundant peonidin-3-O-glucoside, which accounts for 6–16% of the TAC.

The other two most common anthocyanins in pigmented rice are cyanidin-3-O-rutinoside and cyanidin-3-O-galactoside accounting for 3-5% and 1-2% of the TAC respectively [18].

Other minor anthocyanins occurring in small quantities in pigmented rice are cyanidin-3,5-O-diglucoside, cyanidin-3-gentiobioside, cyanidin-3-rhamnoside, cyanidin-3-rhamnoglucoside [3], cyanidin-3-O-sophoroside, delphinidin-3-O-glucoside, delphinidin-3-O-galactoside, delphinidin-3-O-arabinoside, malvidin-3-O-glucoside, malvidin-3-O-galactoside, peonidin-3-O-rutinoside, peonidin-3,5-O-diglucoside, pelargonidin-3-O-glucoside, pelargonidin-3,5-O-diglucoside, petunidin-3-O-glucoside, petunidin-3-O-galactoside and petunidin-3-O-arabinoside. The anthocyanidins cyanidin, delphinidin, peonidin and malvidin have also been detected in pigmented rice[18].

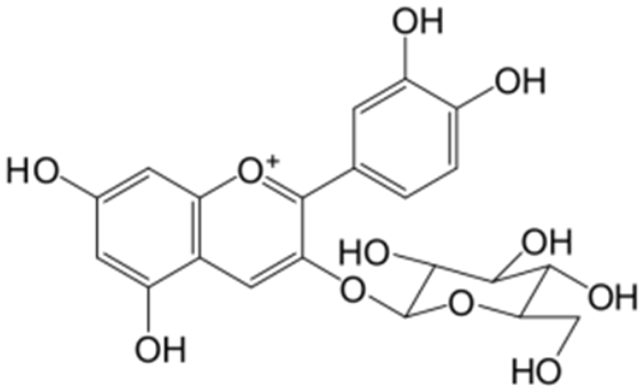


Figure 16. Chemical structure of cyanidin-3-O-glucoside, the major anthocyanin in rice.

1.3. Metabolism of anthocyanins

Despite metabolism is a key aspect of the biological activity in vivo, to the present there are still few specific studies on the bioavailability of anthocyanins and their metabolites in colored grains.

Some systematic literature review studies [19-20] have shown that anthocyanins from fruit and wine and their conjugated metabolites, glucuronates and sulphates, are poorly absorbed within the human body. Olivas-Aguirre et al. in 2016 [21] studied the gastric absorption of cyanidin-3-O-glucoside and reported that it undergoes a series of steps. Firstly, food is minced in the oral cavity and cyanidin-3-O-glucoside and other anthocyanins are partially released from the food matrix. Therefore, anthocyanins can bind to proteins and turn into protocatechuic acid and related glucuronides. Under the acidic conditions of stomach anthocyanins are completely released from the food matrix and partially absorbed by the epithelium: the high gastric absorption of intact anthocyanins and related compounds may be partially responsible for their observed biological effects.

Then the physico-chemical conditions of the small intestine drastically reduce the bioavailability of anthocyanins by more than 50%. Finally, a large portion of anthocyanins comes into the colon to be fermented.

In vitro studies have shown that malvidin-3-glucoside and its metabolites greatly increase the growth of *Lactobacillus enterococcus* and *Bifidobacterius*, thus positively improving the intestinal bacterial population [22]. However, further in vivo studies are needed to better understand the role of intestinal metabolites of anthocyanins in human health.

Considering the above, the potential beneficial effects of cereal anthocyanins are clearly indicated.

However, most of the studies in literature have investigated pure anthocyanin extracts and not real extracts, and this limits the possibility of extending the results to real food matrices. Foodstuff made from colored grains contain not only anthocyanins but also a variety of other components such as dietary fiber, starch, protein and other polyphenols. The interactions of anthocyanins with these components during food processing and digestion can have a great impact on the beneficial effects.

Moreover, the impact of food preparation before eating, particularly cooking, on anthocyanins must also be considered, since they are more susceptible to

degradation in presence of different factors such as high temperature or luminous radiation.

1.4. Health-promoting activities of anthocyanins

Anthocyanins-rich foods have proved to possess biological efficacy in several acute and chronic human diseases, despite their apparent chemical instability and low bioavailability.

Anthocyanins are principally known for their antioxidant activity [21], that seems to be responsible for the other health-promoting activities, such as their anti-inflammatory properties [23], their cancer preventive activity [24] and their possible role in cardiovascular diseases (CVD) prevention [17,25].

Some epidemiological studies showed that a higher intake of dietary anthocyanins is associated to a decrease in many inflammatory biomarkers in population of US adults [26] and to a lower incidence of CVD events, CV non-fatal events and all-cause mortality in middle-aged adults [25].

With respect to the anticancer activity, a pilot study on patients with colorectal adenocarcinomas provided evidence of the ability of anthocyanins extract (from black raspberries) to demethylate tumor suppressor genes and to modulate other biomarkers related to the development of tumor in human colon and rectum [27].

Humans studies also confirmed the ability of anthocyanins to inhibit cholesterol adsorption and to regulate lipid profile. A randomized controlled trial of adults with hypercholesterolemia, taking an anthocyanin supplementation for 24 weeks, showed a significant increase of HDL- and decrease of LDL-cholesterol in serum. Anthocyanin consumption also decreased the levels of serum high sensitivity C-reactive protein and soluble vascular cell adhesion molecule-1 [28].

In vitro and in vivo studies verified the retinal protection activity of anthocyanins. In particular, one study evaluated the protective effects of purple rice bran extract against light-induced retinal damage. The in vitro retinal-protective effect of the extract of purple rice bran was confirmed in mice exposed to light, where the extract suppressed the photoreceptor degeneration [29].

Other interesting biological activities of dietary anthocyanins are their neuroprotective effect, probably due to the capacity of modulate the gut microbiota composition of animals, suggesting that anthocyanins can attenuate the neurologic complications of obesity [30], and their glycaemic regulation activity, at present confirmed only by in vitro tests [17].

Cyanidin-3-O-glucoside, in particular, is by far the most investigated anthocyanin in literature, thanks to its beneficial properties [21].

Several in vivo studies proved the health-promoting activities of cyanidin-3-O-glucoside precisely extracted and purified from black and/or purple rice in retinal protection, lipid profile regulation, body fat reduction, hepatoprotection, enhancing immune response, extending lifespan and anti-ageing [17].

In addition, in vitro tests showed the neuroprotective effect of purple rice extract and cyanidin-3-O-glucoside against amyloid beta-induced neuronal cell death and reported the antimetastatic property of the black rice extract in human oral cancer cells [17].

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2. Effects of different cooking conditions on the anthocyanin content of a black rice (*Oryza sativa* L. 'Violet Nori')

2.1. The present work

This study has been carried out in collaboration with the Hotel School "Marco Polo" of Genova, which performed the cooking tests through professional cooking equipment.

The aim of the work was to investigate the effect of different cooking conditions on the decrease of anthocyanins content of 'Violet Nori' rice.

For this purpose, ten cooking tests were applied to 100 g of whole rice, cooked in pan or oven, in order to evaluate the effect of boiling, oven cooking, risotto cooking, oriental cooking and roasting.

Four boiling cooking tests were performed investigating a different rice/water ratio (100 g rice in 650 mL or in 1 L of water) and a different initial cooking temperature (room temperature or T=100°C).

Three oven cooking (called "Pilaf") tests differed from each other for the different rice/water ratio (500 mL or 650 L of water) and for the initial pretreatment (roasting, boiling or no one).

Two risotto cooking tests were carried out in 750 mL or in 1 L of water and with or without an initial roasting.

One oriental cooking test involved the use of 300 mL of water boiled without covering for 14 min and then, with a tight lid on the pot, for other 14 min.

The anthocyanin extraction was performed on the cooked rice samples, oven dried at 40 °C for 48 hours and then finely ground, by means of pulsed ultrasounds (PUAE) with an hydroalcoholic mixture (EtOH:H_2O) as extraction solvent.

Then, the total monomeric anthocyanins content of each sample was evaluated by both a spectrophotometric pH differential method and by HPLC using an internal standard of cyanidin-3-O-glucoside. Folin-Ciocalteu test (for total phenolic content) and DPPH test (for the radical scavenging activity) were also performed to evaluate the samples.

Results showed that despite cooking necessarily decreases the rice content of anthocyanins, a careful choice of the operating conditions allows effectively preserving at least a part of these valuable polyphenols.

In particular, oriental cooking and boiling with a low water amount (100 g rice/650 mL water) allowed ‘Violet Nori’ rice to preserve a total anthocyanins content higher than 100 mg/100 g, which is a value comparable or even superior to the ones found in other well-known sources of dietetic anthocyanins (see Table 3, section 1.2, Chapter 2-Part A).

2.2. Scientific paper

Please find the article at the following link:

https://doi.org/10.1007/s00217-019-03337-6

S. Catena, F. Turrini, R. Boggia, M. Borriello, M. Gardella and P. Zunin, Effects of different cooking conditions on the antioxidant content of a violet rice (Oryza Sativa L. ‘Violet Nori’), Eur. Food Res. Technol. 245 (2019) 2303–2310.

CHAPTER 2

PART B

“Increase of ellagic acid bioavailability by its encapsulation in dendrimeric nanocarriers”

1. Introduction

1.1. Ellagic acid (EA)

Ellagic acid (EA) is a dietary antioxidant polyphenol occurring in many plant species, particularly fruits and nuts, like in pomegranate, the ancient fruit already described in the present thesis work (Chapter 1-Part B).

Chemically, EA can be defined as a dimeric derivative of gallic acid. It is a chromene-dione derivative, whose structure is named 2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione.

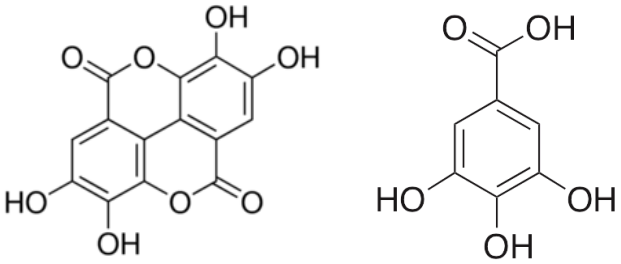

Figure 17. Chemical structure of ellagic acid: a dimeric derivative of gallic acid

Ellagic acid is present as odourless yellow powder or cream-colored needles and it is a weak acid incompatible with strong reducing agents. EA is a high thermostable compound with melting point of 450 °C and boiling point of 796.5 °C [1]. The structure of EA is responsible for its powerful antioxidant property, which has been reported to be similar to that of well-known antioxidant vitamins ascorbic acid and α-tocopherol [2]. In addition to a lipophilic planar moiety composed by two aromatic hydrocarbon rings, the presence of a hydrophilic moiety consisting of four hydroxyl and two lactones groups confers to ellagic acid a potent scavenging activity against free radical ROS and reactive nitrogen species. The hydroxyl groups and lactone systems in fact can accept electrons from different

substrates, form hydrogen bonds as well as participate to oxidation-reduction reactions [3].

On the other hand, its planar and symmetrical structure and its large hydrogen bonding network resulting in a high degree of crystallinity are responsible for the low solubility problems of ellagic acid. Its water solubility is inferior to 1 mg/mL at 21 °C and the solubility in alcohols is very poor too [4].

Ellagic acid can be considered a secondary metabolite of many plants and vegetables, where it naturally occurs in three different forms: free EA, EA derivatives and polymeric ellagitannins (ETs) [5]. Numerous derivatives of EA are present, formed by glycosylation (with a saccharide unit such as glucose, rhamnose or arabinose), methylation and metoxylation of its hydroxyl groups[5]. Furthermore, ETs change to free EA and EA derivatives during food processing [6].

These structural diversities influence ellagic acid solubility and then its bioavailability and bioactivity.

The amount of EA in food is determined as free and/or total EA after acid hydrolysis of its derivatives and ellagitannins.

1.1.2. Ellagitannins (ETs)

Ellagitannins are an important group of polyphenolic compounds characterised by one or more moieties of hexahydroxydiphenic acid (HHDP) esterified to a sugar residue, generally β-d-glucose, and they are classified under ‘hydrolyzable tannins’ (HTs).

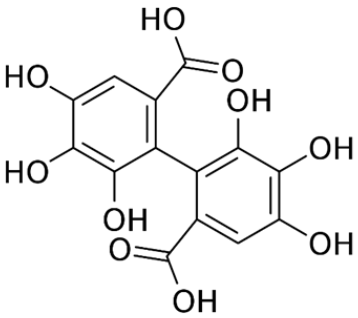

Figure 18. Chemical structure of hexahydroxydiphenic acid (HHDP).

HTs are tannins that can be fractionated into their components by hydrolysis, such as treatment with acids, bases, hot water or with enzymes tannases [7]. This category includes both ellagitannins, that release ellagic acid (EA) after hydrolysis, and gallotannins, which release gallic acid (GA).

While the other class of the phytochemicals tannins is represented by ‘nonhydrolyzable’ or ‘condensed tannins’, also known as the oligomeric proanthocyanidins [7].

Ellagitannins could be C-glycosidic or O-glycosidic compounds[8]. It should be mentioned here that ETs with C–C glycosidic bound are not hydrolyzable, due to further C—C coupling of their polyphenolic moieties with the polyol unit, but they are still classified as ‘hydrolyzable’ tannins for historical reasons, such as vescalagin[7]. Differently the O-glycosidic ETs are C–O–C sugar-bonded compounds and they exist in monomeric, dimeric or oligomeric forms. An example of O-glycosidic ET in monomeric form is punicalagin, the largest polyphenol with a molecular weight of greater than 1000, which is the most abundant ellagitannin occurring in pomegranate and unique to this fruit[9].

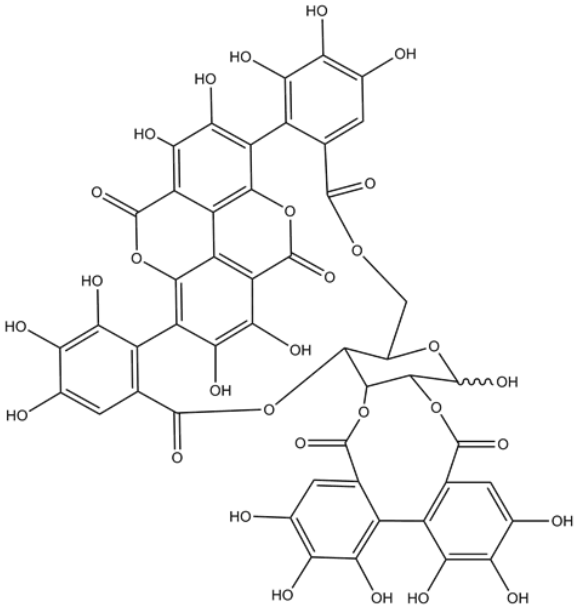

Figure 19. Example of ETs chemical structure: punicalagin.

ETs present an enormous structural variability due to the different possibilities of linkage of hexahydroxydiphenoil moieties with the glucose residue, and especially due to their high tendency to form dimeric and oligomeric derivatives.

These hydrolyzable tannins are high molecular weight amorphous materials that feature astringent flavor caused by their interactions with salivary proline-rich proteins [10].

As ellagic acid, ETs are strong antioxidant compounds due to their chemical structure. The presence of several hydroxyl groups in position ortho leads to the capacity of ETs of donating a hydrogen atom and supporting the unpaired electron[9]. The antioxidant power of ETs is directly related to their degree of hydroxylation and decreases with the presence of a sugar moiety.

ETs are hydrolyzed in vivo under physiological conditions of the gastrointestinal tract to HHDP and GA and then to EA, since HDDP spontaneously lactonized to EA and GA becomes EA after dimerization and lactonization[9].

The minor fraction that succeed in escaping hydrolysis in small intestine undergo a metabolism through the gut microbiota action. Only a negligible part of ETs that reach the gastrointestinal tract pass in the systemic circle and arrive to the tissues, where their detection actually lacks completely of documentation. Thus, it seems more correct supposing that ETs administered by foods or food supplements are totally converted into EA.

The susceptibility to hydrolysis of ellagitannins is influenced by their structure and provides health benefits mainly due to the release of the EA and/or GA molecules depending on the ETs structure[9].

1.2. Ellagic acid most common sources

ETs and EA are constantly consumed in many fruits, in seeds such as berries seeds, in some nuts and vegetables and in the foods or beverages based on fruit juices and jams [11].

Food	Content
Fresh fruits	
Raspberry	51–330 mg/100 g f.w.
Strawberry	25–85 mg/100 g f.w.
Cloudberry	56–360 mg/100 g f.w.
Blackberry	1.5–2.0 mg/g d.w.
Arctic bramble	69–320 mg/100 g f.w.
Pomegranates	35–75 mg/100 g f.w. arils
Muscadine grapes	36–91 mg/100 g f.w.
Nuts	
Walnut	802 mg/50 g (8 nuts)
Pecan	20.96–86.2 mg/g (EA)
Chestnut	1.61–24.9 mg/kg d.w. (EA)
Processed fruits	
Pomegranate juice (wonderful)	2020–2660 mg/L ETs and EA
Pomegranate juice (Mollar)	5700 mg/L Ets and EA
Raspberry jam	76 mg/100 g f.w.
Strawberry jam	24 mg/100 g f.w.
Muscadine grape juice	8–84 mg/L
Wines	
Oak-aged red wine	9.4-50 mg/L
Muscadine grape wine	2–65 mg/L
Whiskey	1–2 mg/L
Cognac	31–55 mg/L

***Table 4*.** Content of ETs and EA in fruits, nuts, processed fruits and wines [12].

Pomegranate is a rich source of ETs and EA: these compounds, responsible for its important health benefits, are very abundant not only in the edible part (arils) but especially in the external and internal peels of the fruit [13].

Significative levels of EA equivalents are also present in many berries of the *Rosaceae* family, including strawberries, cranberry, blueberry, blackberry, cloudberry and red and black raspberries [14–16].

Other important sources are nuts, including walnuts [17], chestnuts[18], pecans [19], pistachio, oak acorns, and cashew nut [20]. Moreover, ETs are

important constituents of wood, especially oak wood [20]. By migration from wood to the food matrix during ageing processes, ETs can be incorporated into different foodstuff such as wines and whiskey [12].

EA has also been detected in several types of honey [21] and in the mushroom beefsteak fungus (*Fistulina hepatica*) [22]. Recently, tea (*Camellia sinensis* L.) proved to be another important dietary source of ETS and EA [23].

The main dietary ETs are punicalagin, occurring in pomegranate, sanguiin H-6 contained in raspberry and strawberry, along with several EA derivatives. Walnut extract contains pedunculagin, followed by casuarictin and valoneic acid dilactone, while sanguiin H-5 derive from muscadine grapes and castalagin, vescalagin and roburin E from oak wood [12].

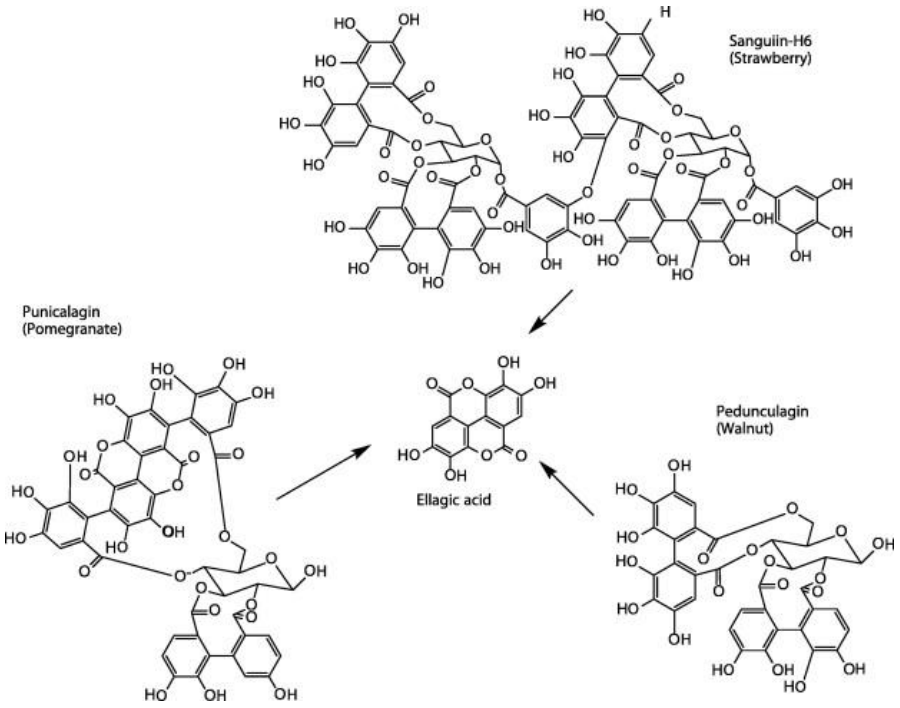

***Figure* 20.** Dietary ellagitannins (ETs) and their transformation to ellagic acid (EA) [12].

1.2.1. EA and ETs dietary intake

The limited knowledge of the EA and ETs content in foods makes it difficult to evaluate accurately their actual dietary intake and only few estimations are reported in the scientific literature.

The major contributors to ETs dietary intake in Western countries are red fruits such as the berries strawberries, blackberries and raspberries [11].

In France people consume about 1.7 kg of fresh strawberries per year and the same quantity of processed products (sweets, yogurts, pastries, syrups, preserves), which would correspond to a daily intake of about 0.3-0.4 mg of total EA [11].

Bavarian men and women have been estimated to daily consume 4.9 and 5.4 mg/day of EA, respectively [11]. While in the Finnish diet the daily consumption of ETs has been evaluated to be 12 mg/day[24], higher than in Germany [11].

The contribution of wine consumption is even more difficult to evaluate, since the ETs intake depends on its aging in oak barrels. In France 60 L yearly on average are consumed, mainly as red wine [5].

Recently, Tomas-Barberan et al. suggested that the intake of dietary ETs could be much higher than previously estimated (5 mg/day) [25]. This was stated taking into account that a glass of pomegranate juice (corresponding to 200 mL) can provide about 1 g of ETs, a serving of raspberry (100 g raspberries) around 300 mg, a strawberry serving 70 mg and four walnuts 400 mg of ETs, especially if these ETs-rich foods are regularly consumed in the diet [25].

1.2.1. Bioavailability and metabolism of ellagic acid

In order to evaluate the health-promoting activity of EA in the human body is essential to consider the absorption and metabolism of EA is essential. The poor water solubility of ellagic acid associated with its metabolism in the gastrointestinal tract, the first pass effect and irreversible binding to cellular DNA and proteins, cause its very poor bioavailability.

EA concentration was evaluated in rat plasma and tissues after administration of a single dose (50 mg/kg, *per os*) [26]. Its plasma level peaked after about 30 min from its ingestion and it was detectable in several tissues including lung, heart and brain, with the highest levels in kidney and liver. The EA

Cmax value in plasma was about 94 ng/mL, showing a low diffusion of this polyphenol in the body. The concentration detected in the different tissues appeared insufficient to provide an effective therapeutic activity [27]. EA availability is negatively influenced by several kinetic variables, including the short half-life of the molecule (about 8.4 h), its ionization at physiological pH with the formation of insoluble complexes with Ca and Mg ions, and the extensive binding of EA to the plasma proteins (about 50% of EA) and to the intestinal epithelium, that significantly reduce the molecule diffusion [28,29].

Ellagic acid once ingested is poorly absorbed in stomach and small intestine, and largely metabolized by the bacteria of the intestinal lumen to produce urolithins, its microflora-derived metabolites. Microbial metabolism starts in the small intestine where the first produced metabolites preserve four phenolic hydroxyls that are further metabolized in the intestinal tract by removing hydroxyl units to generate urolithin A (with two hydroxyls) and B (one hydroxyl) in the colon distal parts [12]. The absorbed metabolites are then conjugated with glucuronic acid and/or methyl ethers. Urolithin A and B are the main metabolites of EA detected in plasma and urine, even if hydroxyl-urolithin A, urolithin A-glucuronide and dimethyl ellagic acid-glucuronide have also been identified in smaller amounts [30].

For what concern ET, *in vitro* digestion simulation studies have shown that they are quite stable under the stomach's physiological conditions. The acidic pH conditions and the stomach enzymes do not hydrolyze the ETs and no degradation of these compounds has been observed [25]. Although the stomach seems to be the first major place for the absorption of free EA, ETs are not absorbed here and do not release free EA. However, under the physiological conditions of the small intestine release of free EA from ETs has been detected. This hydrolysis seems to be caused by the neutral to mild alkaline pH conditions rather than to the action of bile salts and pancreatic enzymes [31].

Seeram et al. investigated the pharmacokinetics of pomegranate ETs in humans [31]: 18 healthy volunteers consumed 180 mL of pomegranate concentrate juice (with ETs content of 318 mg as punicalagins). EA, and not ETs, was found in the plasma of all subjects. 18 ng/mL was the maximum concentration detected but it rapidly declined within few hours. EA metabolites, including dimethyl ellagic acid glucuronide and urolithins were also detected in plasma and urine both in conjugated and in free forms and, differently from EA, they remain longer in blood and were excreted in the urine after 48 h from the pomegranate juice administration [31].

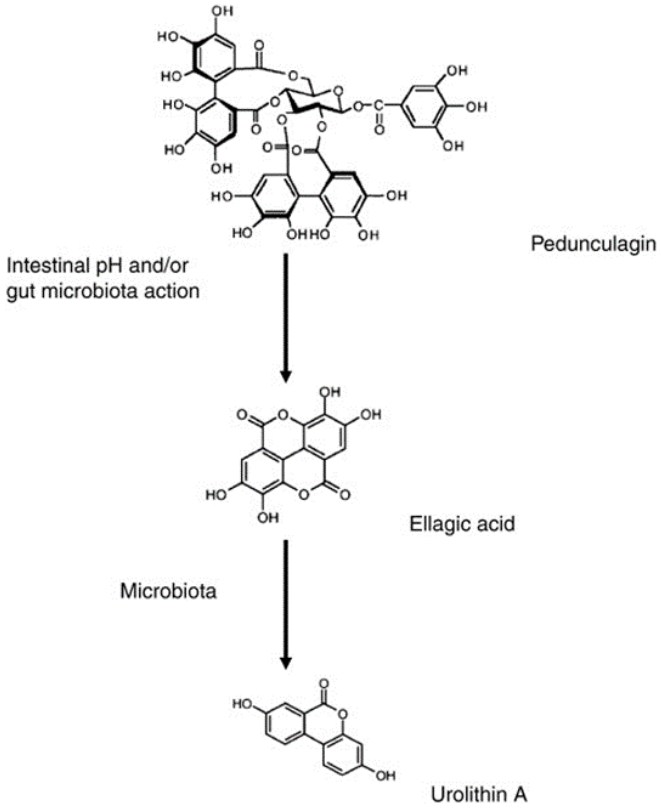

Figure 21. Metabolism of ellagitannins and ellagic acid [5].

The principal problem of ellagic acid bioavailability is its poor solubility in water, which has an important negative effect on its pharmacokinetic properties. For this reason, some strategies have been developed to improve it, from natural or semisynthetic derivatives to nanotechnological approaches, like encapsulation within nano-microspheres and molecular dispersion in polymer matrices [32].

1.3. Healthy properties

As already described in section 1.2.1, Chapter 1-Part B, EA is endowed with many beneficial properties especially thanks to its high antioxidant activity.

Ellagic acid and ellagitannins have shown considerable biological effects in animal models and human studies suggesting their potential preventive effects against chronic diseases such as cardiovascular diseases, diabetes, neurodegenerative diseases and cancer: these effects are associated with a multitarget action involving antioxidant, anti-inflammatory and anticarcinogenic effects [12,32]

However, the poor bioavailability of EA and ETs and the extensive metabolism of the unabsorbed compounds to urolithins by the gut microbiota may suggest that urolithins rather than ellagitannins or ellagic acid could be the actual bioactive molecules [33].

Recent research, mainly based on *in vitro* tests, have shown preliminary evidence of the antioxidant, anti-inflammatory, antiglycative, anticarcinogenic, and antimicrobial effects of urolithins, thus supporting their potential contribution to the healthy properties attributed to pomegranate and ellagitannin-rich foods. *In vivo* studies are still limited, but they reveal preventive effects of urolithins on gut and systemic inflammation. This encourages further research in order to clarify the health effects of these metabolites [33].

- In this research, two strategies have been developed with the aim at increasing ellagic acid water solubility and bioavailability, achieving water-soluble drug formulations for the administration of EA at therapeutically effective doses.

The first strategy involved the pharmaceutical technology. EA microdispersion in a pectin food-compatible matrix were obtained by means of the spray drying technique. This EA formulation is suitable both to produce fine suspensions for EA oral administration in dietary supplements and to be used as potential ingredient in functional food preparations.

The second method, discussed in the present thesis, exploited the knowledge of organic chemistry. Two dendrimeric polymers, one hydrophilic and one amphiphilic, were synthetized in order to physically encapsulate EA and vehiculate it inside the human body. The two achieved dendriplexes are nanoparticle drug formulations that could be suitable for effective therapeutic parenteral administration of EA.

2. Dendrimers

Dendrimers are a class of dendritic polymers that can be constructed with a well-defined, globular-shaped, homogeneous and monodisperse molecular structure [34,35].

They are nanostructured “architectural motifs” consisting of repetitively branched molecules (or tree-like arms), built around a small polyfunctional “kernel”, usually named *core.* Dendrimers in fact consist of three different domains, involving a core, layers of branched repeat units emerging from the core and functional end-groups on the outside periphery, which can be functionalized, thus modifying their biological or physicochemical properties [36,37].

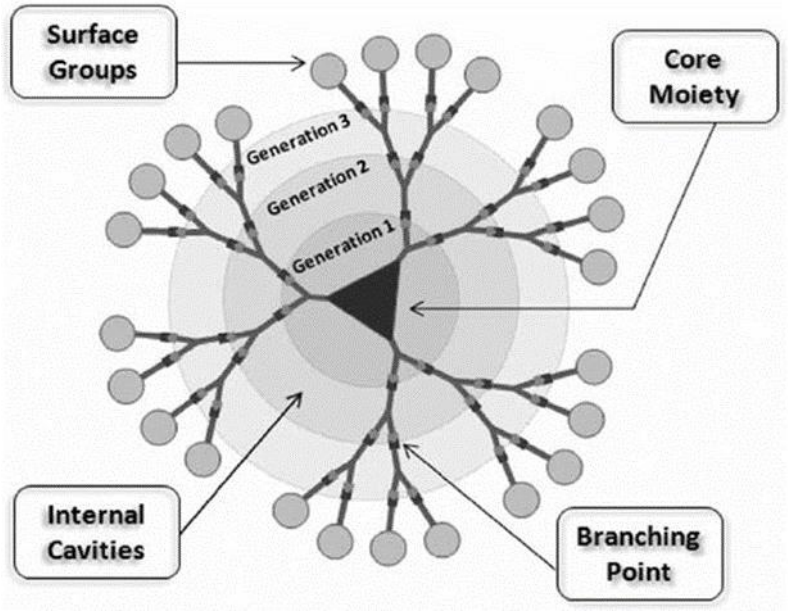

Figure 22. The dendrimer’s structure [35].

These hyperbranched globular macromolecules are able to covalently link drugs or target molecules thanks to the peripheral functions, while their inner cavities can lodge small natural or synthetic bioactive molecules in order to

protect them from premature degradation, decrease their toxicity, increase their solubility in biological fluids, thus favouring their bioavailability [38,39].

These features confer dendrimers the role of winning materials for several biomedical applications such as drug delivery nanocarriers[40], bio-imaging agents [41], biosensors [42] and theranostics [43]. Moreover, when equipped with nitrogen atoms that can be protonated at physiological pH, dendrimers could be suitable as non-viral polymeric vectors for conveying plasmid DNA, RNA or antisense oligonucleotides into specific defective cells to treat several diseases, including cancer [44–47]. Dendrimers are considered the newest class of macromolecular nanosized delivery devices.

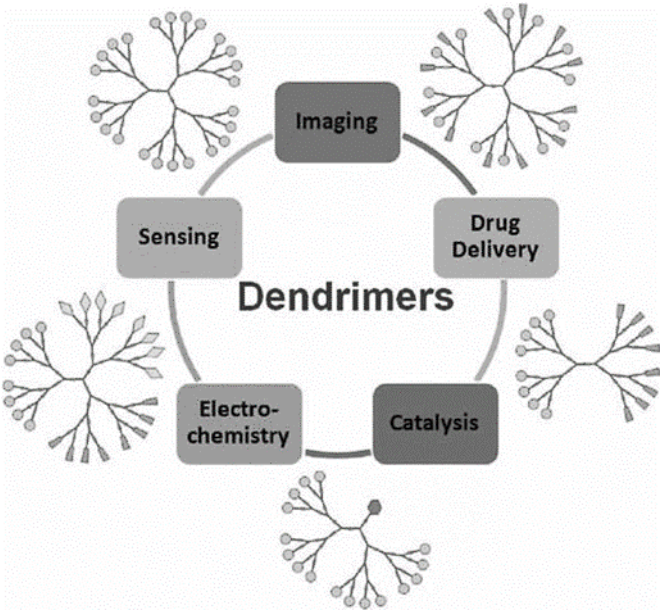

***Figure* 23.** Dendrimers and their applications [35].

2.1. Dendrimer-based drug delivery

Commercially available polyamidoamines (PAMAM) [48,49] and polyethylenimine (PEI) [50] are considered the gold standard reference among dendrimer and polymer vectors in the field of gene and drug delivery.

PAMAM and PEI are strongly cationic systems, characterised by high charge density that leads to a powerful transfection activity but also high cytotoxicity.

Cationic dendrimers are able to condense nucleic acids or encapsulate drugs by electrostatic interactions and the net positive charge of the dendriplexes (i.e. the complexes of dendrimers and drugs) contributes to cellular uptake and transfection efficiency, being cell membranes negatively charged.

However, an excessive cationic character of the dendrimer scaffolds, caused by too high density of protonated amino groups, could cause permanent damages to cell membranes up to elicit cell death and prevent a proper hydrophilic lipophilic balance (HLB), which is one of the crucial features for a good, safe and efficient delivery system.

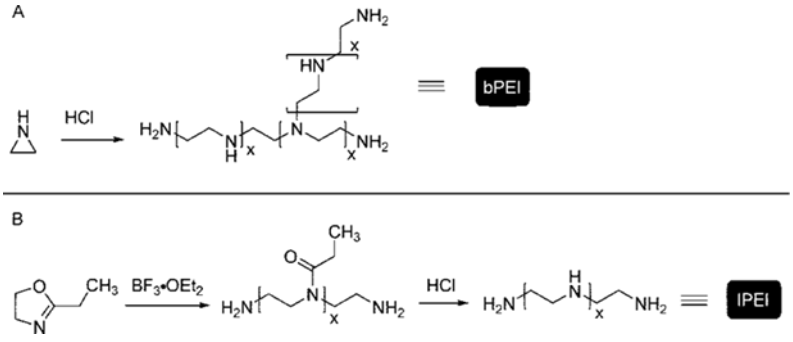

Figure 24. Synthesis of a) branched PEI and b) linear PEI [51].

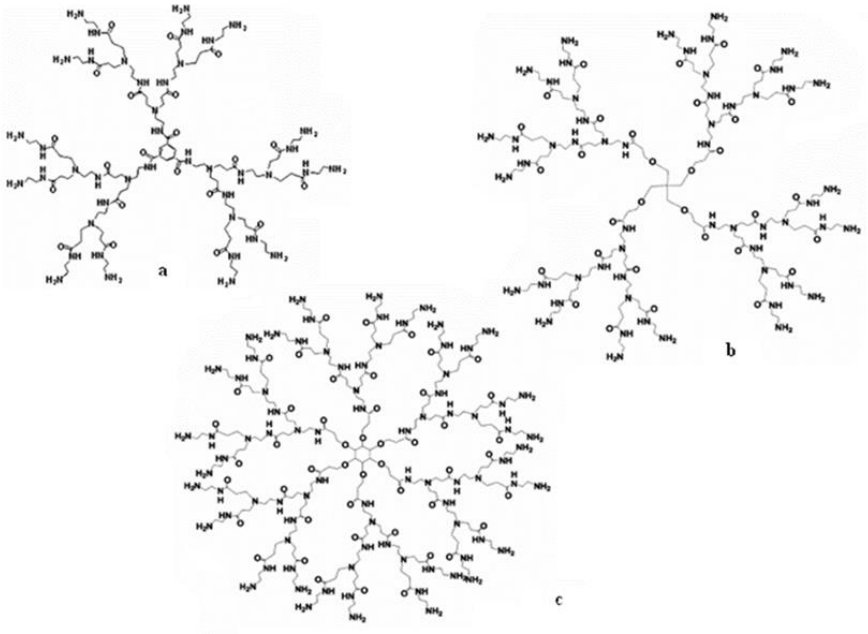

***Figure* 25.** Example of PAMAMs with starting monomer of a) trymesil, b) pentaerythritol and c) inositol [51].

Chemical modification or other strategies, such as the introduction of hydrolysable linkages [52,53] or lipophilic segments [54], are usually performed to overcome these problems, thus improving biodegradability and HLB and decreasing toxicity. PAMAMs modified in periphery by PEGylation [55], acetylation [56], introduction of saccharide residues [57] or conjugation with targeted moieties [58] have been successfully employed to covalently bind or encapsulate therapeutic compounds.

Neutral dendrimer matrices containing protonatable amino acid residues [52,59] are gaining more and more interest and seemed to be the best solution. The variety of amino acid residues can be exploited to regulate the buffer capacity of the vectors, thus aiding their escape from endosomal acidic compartments through membrane disruption of endosomes (the so called “proton sponge” effect) [60]. For an effective drug transfection, in fact, the delivery system should

be able to preserve the transported material from lysosome attack escaping from the endosome as soon as possible.

In addition to l-histidine [61], l-arginine [62,63] is particularly suitable for the functionalization of synthetic vectors since the presence of the guanidine residue in its structure allows to strongly reduce the dendrimers toxicity and to improve DNA-packaging ability, cellular uptake and transfection [64].

2.2. Hydrophilic and amphiphilic polyester dendrimers based on bis‑HMPA

The research group of Organic Chemistry at University of Genoa, where I developed my master thesis, is working since several years on the synthesis and characterisation of dendrimeric structures with internal polyester and biodegradable matrix derived from a starting monomer of 2,2-bis(hydroxymethyl)propanoic acid (bis-HMPA), and outer periphery with many hydroxyl groups exploited for further esterifications with amino acids, to explore their efficacy as carriers of drugs for the targeted delivery [65].

bis-HMPA is a versatile monomer widely used for the construction of polyester dendrimeric systems. The inner polyester matrix of this type of dendrimers make them easily biodegradable through chemical or enzymatic hydrolysis, while the external surface functionalised with amino acids, that are naturally biocompatible and protonatable under physiological pH, confer to these structures the fundamental characteristic of not being cytotoxic, as opposed to PAMAM and PEI [65].

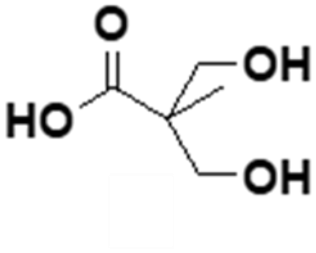

Figure 26. The starting monomer 2,2-bis(hydroxymethyl)propanoic acid (bis-HMPA)

In particular, two new synthesised polyester dendrimers scaffolds have been employed to encapsulate and transport ellagic acid in the present work, starting from the same monomer bis-HMPA:

1) Hydrophilic fourth generation dendrimers built on a trifunctional core (2,2-bis (hydroxymethyl) propanol) endowed with 48 free hydroxyl groups in periphery that have been further functionalised with amino acids l-arginine and l-lysine;
2) A third generation dendrimer built on a tetrafunctional core (2,2-bis (hydroxymethyl)-1,3-propanediol), which is amphiphilic due to the presence of an hydrocarbon chain with 18 atoms of C, which improves the interaction with membrane phospholipids and the transfection efficiency.

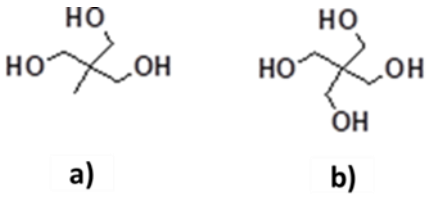

***Figure* 27.** a) Trifuncional and b) tetrafunctional core [51].

The drug/dendrimer complexes showed high water solubility, thus allowing the dissolution in water of the incorporated molecules. For this reason, they may be useful for clinical applications.

Figures of the hydrophilic fourth generation amino acid-modified (1) and amphiphilic third generation amino acid-modified (2) hetero dendrimers are reported in the following scientific paper (section 4, Chapter 2-Part B).

To learn more about the molecule of ellagic acid and its therapeutic properties, with a special focus on the central disorders, please find the scientific review **3** *(Ellagic acid a multi-target bioactive compound for drug discovery in CNS? A narrative review)* in section “Other publications”.

For a more detailed discussion about dendrimers, please find the scientific papers **8** (*Synthesis and characterization of fourth generation polyester-based dendrimers with cationic amino acids-modified crown as promising water soluble biomedical devices*) and **9** (*Synthesis and characterization of versatile amphiphilic dendrimers peripherally decorated with positively charged amino acids)* in section “Other publications”.

To learn more about the potential use of dendrimers as drug delivery nanocarriers please find the scientific papers **4** *(Biodegradable and biocompatible spherical dendrimer nanoparticles with a gallic acid shell and a double-acting strong antioxidant activity as potential device to fight diseases from oxidative stress),* **5** *(Reshaped as polyester-based nanoparticles, gallic acid inhibits platelet aggregation, reactive oxygen species production and multi-resistant Gram-positive bacteria with an ever-achieved efficiency),* **6** (*Synthesis of Water-soluble, Polyester-based Dendrimer Prodrugs for Exploiting Therapeutic Properties of Two Triterpenoid Acids)* and **7** (*Hydrophilic and amphiphilic water-soluble dendrimer prodrugs suitable for parenteral administration of a non-soluble non-nucleoside HIV-1 reverse transcriptase inhibitor thiocarbamate derivative)* in section “Other publications”.

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4. Ellagic Acids micro and nano formulations with amazingly increased water solubility by its entrapment in pectin or non-PAMAM dendrimers eligible for clinical applications

4.1. The present work

The present work is the result of a collaboration among the research groups of Chemistry of Food and Dietary Product (where I carried out my PhD), Pharmaceutical Technology and Organic Chemistry (where I developed my master thesis) of the department of Pharmacy of Genoa University.

The aim of the study was to increase EA solubility, thus improving its bioavailability. For this purpose, two strategies have been adopted.

An EA solid microdispersion was realized employing only water and low methoxylated pectin as food compatible excipient by means of the spray drying technology and its optimization by experimental design. The drug loading of ellagic acid in the obtained solid microdispersion was equal to about 22%. realizing a solid microdispersion by a spray drying technique optimized by a 2^3 full factorial design.

Subsequently, the previously described hydrophilic and amphiphilic dendrimers have been employed as nanocarriers, realizing two EA nanodipersions (60-70 nm) endowed with 46 and 53% (w/w) Drug Loading.

The sprayed-dried EA microdispersion provided a 30-fold increase in the water solubility of ellagic acid and preserves a strong radical scavenging activity.

Dendrimers resulted in a 300 or 1000 fold increase of free EA solubility (300 with the amphiphilic dendrimer and 1000 times with the hydrophilic one) and they showed high antioxidant power too.

The prepared micro and nanodispersions represent ellagic acid carriers suitable for food and biomedical applications.

4.2. Scientific paper

Please find the article at the following link:

https://doi.org/10.1039/C8NJ05657A

S. Alfei, F. Turrini, S. Catena, P. Zunin, B. Parodi, G. Zuccari, A.M. Pittaluga and R. Boggia, Preparation of ellagic acid micro and nano formulations with amazingly increased water solubility by its entrapment in pectin or nonPAMAM dendrimers eligible for clinical applications. New J. Chem. 43 (2019) 2438-2448.

Conclusions

Chapter 2 is dedicated to the bioactive compounds anthocyanins and ellagic acid that characterise the two main food subjects under study in the PhD thesis, namely 'Violet Nori' rice and pomegranate respectively, conferring them promising health promoting activities.

The studies described in this chapter have been entirely developed at the University of Genoa, taking advantages of internal collaborations established by the group of Food and Dietary Products, especially the one with the Organic Chemistry group, where I previously carried out my master thesis.

The goal of this research has been to preserve the outstanding antioxidant properties of both 'Violet Nori' rice's anthocyanins and pomegranate's ellagic acid, in order to exploit them inside the human body making it possible to achieve effective health benefits.

For this reason, chapter 2 has been focused on the chemical structures of anthocyanins and ellagic acid, considering their stability, solubility and physicochemical properties as well as their bioavailability and metabolism.

Anthocyanins are thermolabile compounds. Thus the effect of cooking 'Violet Nori' rice before consumption and the consequently exposure to high temperature of anthocyanins could dramatically decrease their content, resulting in a reduction of the beneficial apport in humans. For this reason, many different cooking conditions have been tested in order to find the best solution to preserve the anthocyanins amount in cooked 'Violet Nori' rice.

Oriental cooking as well as boiling with a low amount of water allowed effectively saving at least part of the valuable anthocyanins content, keeping an amount of these polyphenols in 'Violet Nori' rice still comparable or even superior to the one contained in the vegetables and fruits recognised as the most important sources of dietetic anthocyanins

Due to its poor water solubility, ellagic acid has a very low bioavailability and this obviously affects its capacity to effectively exert health benefits in the human body. For this reason, two strategies have been evaluated to enhance ellagic acid solubility, thus improving its pharmacokinetic properties.

After a first successful outcome achieved by developing an ellagic acid solid microdispersion through spray drying technique, ellagic acid has been encapsulated in dendrimeric nanocarriers obtaining a nanodispersion which resulted in a 300 or 1000 fold increase of free ellagic acid solubility.

CHAPTER 3

“Fluorescence spectroscopy coupled with chemometric tools for the identification of bioactive compounds”

1. Fluorescence

Fluorescence is a form of photoluminescence (photo = light and luminescence = emission of light) [1,2]. In simple terms, it is the emission of light by a substance that has been exposed and then has absorbed light or other electromagnetic radiation: the exposure and the absorption of light is called excitation. Generally, the emitted light has a longer wavelength and therefore lower energy compared to the absorbed radiation.

Photoluminescence can be often observed in daily life in form of phosphorescence, for instance in glow-in-the-dark toys. This type of photoluminescence occurs when there is a long delay (about 10^{-6} seconds or longer) between the excitation and the emission of light.

On the contrary, when the delay between excitation and emission is shorter (between 10^{-6} and 10^{-8} seconds), the result is fluorescence [3].

After the radiation source stops, fluorescent materials immediately cease to glow, generating a quick flash of emission (about 10 nanoseconds, but sometimes as short as 1 nanosecond). On the contrary, phosphorescent materials continue to emit light for some time afterwards [1].

The first observation and description of fluorescence was reported by Sir John Frederick William Herschel in 1845. He prepared a mixture of quinine and tartaric acid in water into a glass cylinder, which stood near a window in bright sunlight. Looking at it from all angles, Sir Herschel saw “an extremely vivid and beautiful celestial blue color”: the observed blue color was generated by the aromatic organic molecule or fluorophore quinine, occurring in tonic water [2].

1.1. Mechanism

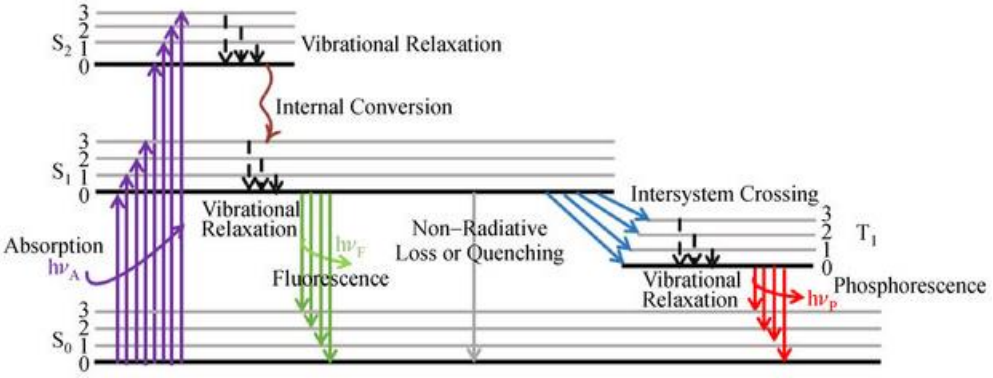

Figure 28. Jablosnki diagram: the luminescence mechanisms [4].

The mechanism of luminescence is illustrated using Jablonski diagrams (being Alexander Jablonski the father of fluorescence spectroscopy), as the one reported in Figure 28 of the present work.

The physics behind fluorescence involves the different electronic and vibrational states in which fluorophores can exist. The π-electrons of molecules exist into two major states: the ground state (S_0), or state of resting, and the excited states (S_1, S_2 and T_1) or the state of higher energy [5]. The energy states S_0, S_1 and S_2 are singlet states (S_0 is a singlet ground state and S_1 and S_2 singlet excited states) while T_1 is a triplet excited state. Fluorescence is emitted when S_1 returns to S_0 whereas phosphorescence is emitted when T_1 returns to S_0 [4]. Each of these energy states can be further divided into smaller energy levels represented by vibrational energy levels (as indicated by the grey horizontal lines in Jablonski diagram).

Fluorescence occurs when an excited molecule relaxes to a lower energy state through emission of a photon. This is possible when the light energy is incident on certain aromatic-conjugated molecules capable of fluorescence, whose π electron systems are able to absorb the incident light passing to the excited energy states and therefore to come back to the ground state via the emission of specific quantums of energy in the form of light [6].

Generally, the fluorescence process involves the excitation of the fluorophore from the ground state S_0 to a singlet state S_2 through absorption of a photon of energy and subsequently the emission of a photon of lower energy as it relaxes to state S_1:

Excitation: $S_0 + h\nu_{ex} \rightarrow S_2$

Fluorescence (emission): $S_2 \rightarrow S_1 + h\nu_{em}$

In each case the photon energy E is proportional to its frequency v according to $E=h\nu$ where h=Planck's constant [7]. The final state S_1, if not the ground state, may then lose its remaining energy by further fluorescent emission and non-radiative relaxation in which the energy is dissipated as heat.

Photons, that possess energies in the ultraviolet to blue-green range of the spectrum, can generate an electronic transition from a lowest vibration in the ground state to one of the vibrational levels in a higher electronic excited state [5]. When the excitation stops, the fluorophore molecule relaxes into the lowest vibrational level of the excited electronic state. As previously mentioned, the fluorophore remains in this state for about 10 nanoseconds, which is known as the fluorescence lifetime and then it returns to the electronic ground state. This return is associated with a release of energy, namely the fluorescence emission [5].

Vibrational energy is subjected to rapid dissipation, and the lowering of the energy level in the same electronic state is due to the vibrational relaxation [4].

Internal conversion is the quick transition from a higher excited state to a lower excited state of the same spin multiplicity (e.g. from S_2 to S_1) while intersystem crossing is generally a much slower process of transition between two electronic states with different spin multiplicities (e.g. from S_1 to T_1) [4].

In the process of fluorescence, energy loss occurs due to vibrational relaxation and internal conversion and this leads to a longer emission wavelength compared to the excitation wavelength [1].

The difference in the excitation and emission wavenumbers is called the Stokes shift.

The number of photons emitted by a fluorophore, relative to the number of photons absorbed, is called the quantum yield. A fluorophore endowed with a large quantum yield will exhibit a bright emission. The emitted radiation is always of a longer wavelength (and then lower energy) than the excitation radiation (higher energy). For instance, if the incoming light was blue (shorter wavelength), then the appropriate fluorophore will emit green light (longer wavelength). This observation, which was first described by Sir George Gabriel Stokes and

therefore called the Stokes shift, is due to the rapid return of the excited molecule to its ground state [1,3].

Fluorescent molecules or fluorophores are generally characterised by π-conjugated systems and rigid planar structures: the degree of conjugation and rigidity affects their fluorescent properties [8]. Another feature which modifies the fluorescence capacity is the type of substituents in the molecule: the presence of O, N and S functional groups such as phenol, carboxyl, carbonyl, acyl, hydroxyl and thiols contributes to this luminescence phenomenum [8].

2. Fluorescence spectroscopy

Fluorescence spectroscopy (also known as spectrofluorometry or fluorimetry) is a type of electromagnetic spectroscopy to analyse fluorescence in a sample. It uses a beam of light, generally ultraviolet light, that excites the electrons of the investigated molecules and causes them to emit light, which is typically visible light[5].

The advantages of applying these analytical methods are reliability, sensitivity and accuracy of the results. Moreover, molecular fluorescence measurements can be carried on quickly and at low costs.

Two types of instruments can be used for fluorescence spectroscopy: filter fluorometers that use filters to isolate the incident and the fluorescence lights and modern spectrofluorometers that use monochromators. In both instruments, the light generated from an excitation source passes through a filter or monochromator and reaches the sample. A portion of the incident light is absorbed by the sample and its fluorophore molecules emit fluorescent light in all directions. Therefore, a part of this fluorescent light passes through a second filter or monochromator and arrives to a detector, which is generally placed at 90° to the incident beam of light in order to avoid interference of transmitted or reflected incident light reaching the detector.

The conventional fluorescence spectroscopy is called right-angle and it is used for characterizing diluted and transparent samples [9]. When the absorbance of the sample is not greater than 0.05, the emitted fluorescence's intensity is proportional to the concentration of the fluorophore: for this reason, standard practices to dilute the original sample with appropriate solvents until an absorbance smaller than 0.05 are generally applied [9].

However, right angle fluorescence spectroscopy is not effective in analysing original samples characterized by turbidity or opacity. To overcome this problem

and analyse native samples directly, that can be turbid, concentrated or even solid, the front-face fluorescence technique is more appropriate [9,10].

The main difference of front-face fluorescence compared to the classic right-angle methodology is change of the incidence angle: this is the angle formed between the excitation beam and the perpendicular to the illuminated surface of the cell. The incident angle is normally 30° to 60° in the front face technique, while it is always 0° in right-angle technique. The emitted radiation is detected from the same cell-face as excitation beam incidence, and therefore it is called front-face fluorescence. In this way, the passage of the radiation through the bulk solution is avoided and scattered radiation, reflected light and depolarisation phenomena are minimised [1,10]. This means that front-face fluorescence measurement may allow not to employ any sample preparation, resulting perfectly suitable for online, real-time and non-destructive detection and monitoring.

Nowadays, modern spectrofluorometers are widely employed for quantitative detections.

A spectrofluorometer is generally equipped with the following [2]:

- High-pressure xenon arc lamp
- Monochromators
- Sample chamber (called “cuvette” in right-angle fluorescence or “holder” in the front-face mode)
- Fluorescence detection system

The high-pressure xenon arc lamp is the excitation source. It can provide a continuous emission spectrum from the ultraviolet into the infrared, with constant intensity in a range from 300 to 800 nm and with enough irradiance to measure down to approximately 200 nm.

A monochromator is the device that allows to produce individual wavelengths from a broadband light source [11]. It is possible to record both excitation and emission spectra, since monochromators can keep emission fixed at a single wavelength to obtain the excitation spectrum or vice versa, it makes possible to record the fluorescence emission spectrum keeping excitation fixed [1,12].

The most common type of monochromator works with a diffraction grating: the collimated light illuminates a grating and exits with a different angle depending on the wavelength. Thus, the monochromator can be adjusted to select the wavelengths to transmit.

The fluorescence detection system is constituted by photomultiplier tubes for emission amplification. Detectors can be single-channeled or multichanneled. The single-channeled can only detect the intensity of one single wavelength at a

time, while the multichanneled detects the intensity of all wavelengths simultaneously, making the emission monochromator unnecessary.

Electronic devices are then employed to quantify the signal and display it electronically [1].

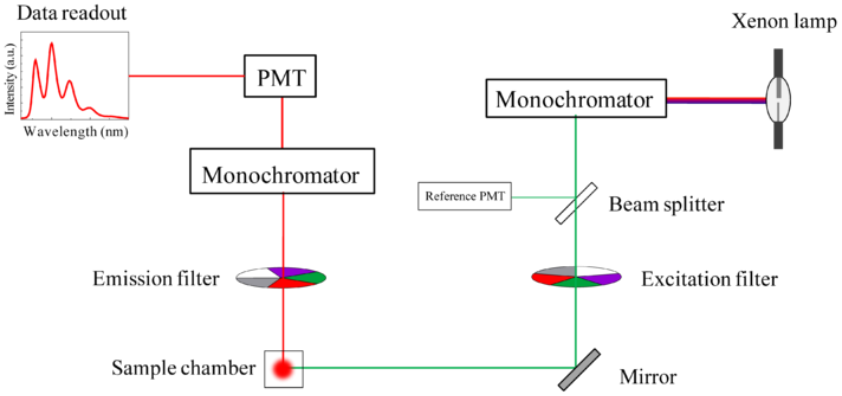

Figure 29. Schematic diagram of the component of a spectrofluorometer [13].

2.1. Excitation Emission Matrix (EEM)

An analytical technique becoming widely used in the field of fluorescence spectroscopy is the excitation emission matrix, or EEM, also called three-dimensional fluorescence spectrum [14].

EEM is a three-dimensional scan, recorded by scanning the fluorescence intensity across a range of excitation and emission wavelengths to generate a 3D contour plot of excitation (Ex) vs. emission (Em) vs. intensity. Compared with traditional fluorescence spectra, EEM allows to detect changes of fluorescence intensity with simultaneous variations of excitation and emission wavelengths resulting in a more comprehensive multidimensional determination of fluorescence.

Three-dimensional excitation–emission matrix (EEM) fluorescence spectroscopy is a fast, sensitive and selective analytical tool [15]. EEM could be applied to identify and quantify many different types of analytes with remarkable resolution even in concentrations in the ppb range, also providing a molecular fingerprint of each analyte [16].

EEMs are then used for a wide variety of applications where multi-component analysis is required, such as detection of environmental pollutants [17,18] and food contaminants [15,19], biochemical analysis [20] and quantitative analysis of multi-component fluorescent solutions [20,21].

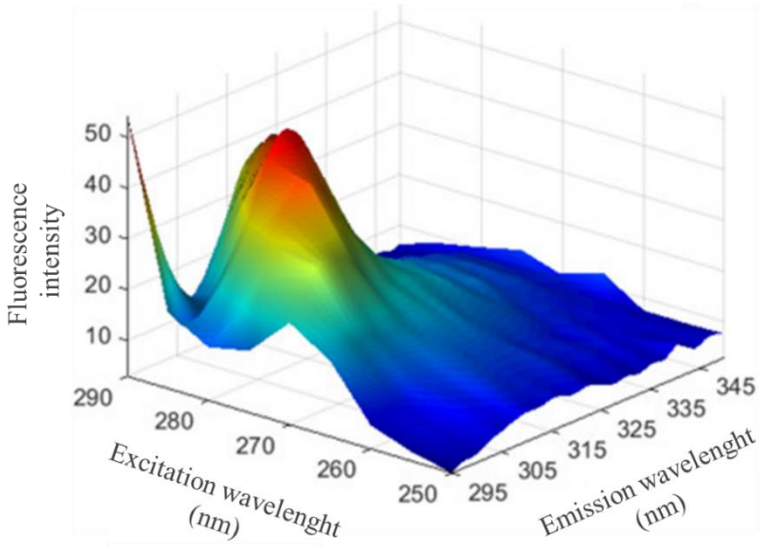

Figure 30. Example of a typical Excitation Emission Matrix (EEM) [17].

The operational Ex and Em wavelengths data are usually≥200 nm and≥250 nm, respectively. Some researchers even suggest to record the fluorescence intensity at Ex≥220 nm due to the large deviation in measurement at lower Ex [22].

The wavelength ranges of excitation and emission must not be overlapped, in order to avoid Rayleigh and Raman scatterings that interfere with the fluorescent signal complicating the analysis [23].

A large amount of fluorescence information can be obtained from EEM data, including the peak intensity, location and distribution, information extracted from spectral decomposition, and information related to photon energy in the fluorescence process [14].

Moreover, since EEM is an analytical method able to produce second-order data, it can be associated with chemometric tools that exhibit the second-order property, in order to allow the identification and quantification of the analytes of interest also in the presence of uncalibrated interferences. This property is known as the “second-order advantage” [24,25].

3. Chemometric

According to the definition of the International Chemometrics Society (ICS), chemometrics is [26]:

“the chemical discipline that uses mathematical and statistical methods to: design or select optimal procedures and experiments, provide maximum chemical information by analysing chemical data, give a graphical representation of this information, in other words information aspects of chemistry”.

Chemometrics allows to quickly obtain real-time information from multivariate data, providing high quality information to be extracted from less resolved data. It allows to improve measurements and enhance knowledge of existing processes. Moreover, it requires very low capital, providing a cheap and convenient tool [27].

As the other ‘metrics’, chemometrics strongly depends on the use of different types of mathematical models. Though knowledge of statistics, numerical and operational analysis and applied mathematics is required for this task, the real difficult and interesting problems are defined by the applications, as happens in all the applied branches of science [28].

The main issue of chemometrics is to rationalize the chemical problem to a form that can be indicated as a mathematical relation. The related mathematical and statistical problems are quite simple.

Thus, chemometrics must not be separated from chemistry or become even a separate branch of chemistry, but on the contrary it must be an integral part of all chemistry’s areas [28].

A chemical model (M) which experimentally relates determined variables X to each other presents in addition a statistical model (E) associated with it. The

model E describes the noise and the variability of the data around the chemical model M.

The separation of ‘chemistry’ and ‘noise’ made by statistics greatly helps to evaluate the analytical data, allowing to conclude anything from measured data, that are very complex and difficult to resolve or interpret.

$$X = M + E \rightarrow \text{Data} = \text{Chemical Model} + \text{Noise}$$ [28]

Chemometrics, using calibration tools, pattern recognition (modelling and classification) tools and multivariate optimizations, supports the basis of the modern analytical chemistry.

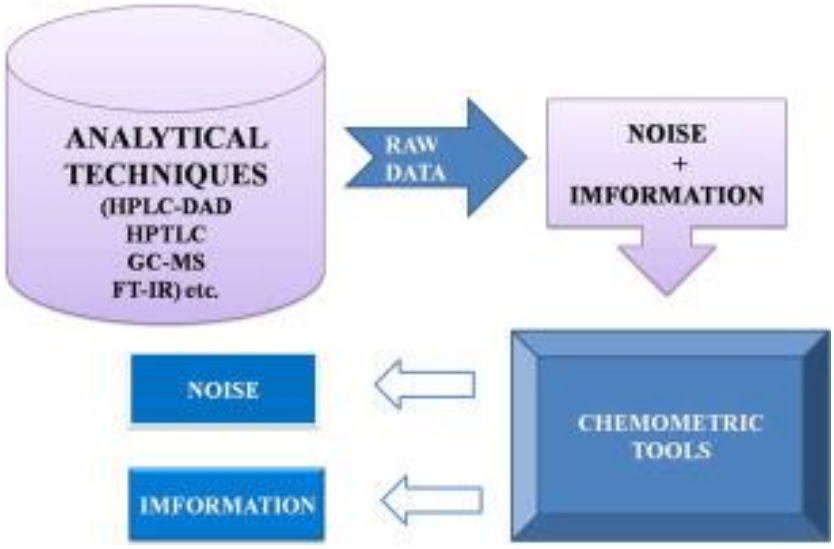

Figure 31. Chemometric tools applied in analytical chemistry [29]

J. Workman identified chemometrics as a process that can solve routine chemical problems at different stages [27]:

1. rationalize a process or phenomenon using chemical instrumentation which generates data inexpensively;
2. analyse the multivariate data;
3. repeat if necessary;
4. set up and test the model;
5. develop multivariate understanding of the process.

The chemometric approach to achieve chemical knowledge derives from the multivariate nature of most chemical systems, in which univariate methods are not able to provide optimum knowledge, but only supply a narrow and limited approach to the problem under study, disregarding variables intercorrelated [30].

Multivariate data analysis implies the analysis of data composed by numerous variables measured in a large/huge number of samples, namely the data matrix under study. The purpose of multivariate data analysis is to determine all the variations in the investigated data matrix; thus chemometric tools try to find the relationships between samples and variables in the data set and often to convert them into a smaller number of new and more informative latent variables[29]. In this sense, mathematics employed in chemometrics are not used to intrinsically model a processes or phenomenon but rather to establish hidden relationships between the data and the state of the investigated system [31].

The main fields of application of chemometric are [26]:

- Quality control
- Process Analytical Technology
- Process monitoring and control
- Food traceability
- QSAR / QSPR and REACH
- Genomics, proteomics and metabolomics
- Experimental Design and Optimization
- Drug and material design
- Image analysis
- Industrial and environmental applications

4. Chemometric tools applied to EEMs

Since fluorescence spectroscopy covers a wide range of excitation and emission wavelengths, it may happen that the signals of the analytes under study resulted overlapped with each other, generating a complex mixture of the fluorescent matrix constituents and even presenting quenching effect [17]. This issue makes the determinations difficult, decreases the selectivity of the analytical method and requires the use of a separation technique before using spectroscopy to obtain a specific fluorescent signal.

To solve the problem, the use of excitation-emission fluorescence matrices (EEMs) coupled with chemometric methods with second-order property is often employed. In this way, it is possible to identify and quantify the analytes of interest

even in the presence of unknown interferences that are absent in the calibration samples[24].

Several chemometric methods exhibiting the second-order property can be applied to EEM matrices.

In the present thesis a research topic is presented involving the use of EEMs fluorescence spectroscopy coupled with the multivariate decomposition method PARAFAC.

The multivariate decomposition methods (PCA and PARAFAC) allow to resolve multivariate data through the reduction of their dimensionality, that are called latent variables or principal components (PCs). For this purpose, it is necessary to decompose the data into lesser dimensions, by unfolding the three-way data, which means to slice up the three-dimensional data cube into two-dimensional tables. And then, by placing these tables side by side large two-dimensional data matrix can be created [29].

Principal Components Analysis (PCA) reduces the dimensionality of a data set containing several correlated variables, retaining at the same time as much as possible of the variation (i.e. the information) present in the data set: PCA is based on the assumption that a high variability (i.e. a high variance value) is synonymous with a high amount of information. In other words, PCA finds the sub-space in the space of the original variables where data mostly vary [30].Thus, the original correlated variables are linearly transformed into a lower number of uncorrelated variables (the already mentioned PCs) [29,32,33]. The new coordinates that describe each sample in the new space (PCA space) graph are called “scores”. Since PCs are expressible as linear combinations of the original variables, the coefficients that multiply each variable are called “loadings” [34].

Parallel factor analysis (PARAFAC) can be considered a generalization of bilinear PCA to higher order arrays. It is a decomposition method for three-way arrays and the most advanced tool employed for the resolution of three-dimensional data obtained from different hyphenated techniques [35,36]. Moreover, PARAFAC algorithm calculates simultaneously all the components while PCA requires different steps.

The decomposition of data is made into tri-linear components: each component includes one score vector and two loading vectors instead of one score and one loading as in PCA. By using the right number of components, PARAFAC allows to find the true spectra with the proper signal-to-noise ratio. In this way, it is possible to directly interpret the loadings obtained with PARAFAC [37].

The fluorescence spectra coming from the EEMs can be arranged into a three-way data tensor and a PARAFAC decomposition can be applied to these data [34].

The decomposition of the experimental data array $\underline{X}=(x_{ijk})$ gives a unique estimation of the:

1) Sample profile, $a_f=(a_{1f}, a_{2f}, \ldots a_{If})$
2) Emission profile or emission spectrum, $b_f=(b_{1f}, b_{2f}, \ldots, b_{Jf})$
3) Excitation profile or excitation spectrum, $c_f=(c_{1f}, c_{2f}, \ldots, c_{Kf})$

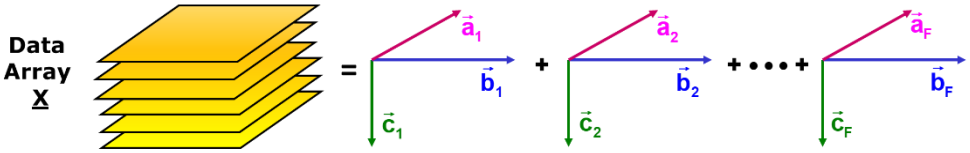

Figure 33. PARAFAC decomposition of three-dimensional data.

Under trilinearity, PARAFAC decomposition provides unique profiles estimations when an appropriate number of factors is chosen to fit the model, so that it is possible to unequivocally identify each factor with each analyte [34]. This guarantees the second order property, thanks to which it is possible to measure also in the presence of uncalibrated interferers [22].

The research work presented in this thesis (Chapter 3-Part A) employed PARAFAC decomposition associated with EEMs to unequivocally identify and quantify three carcinogenic polycyclic aromatic hydrocarbons in a sample of smoked tuna.

A more detailed discussion about PARAFAC can be found in the related scientific paper.

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CHAPTER 3

PART A

"Determination of carcinogenic polycyclic aromatic hydrocarbons in food"

1. Introduction

1.1. Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) consist of a wide class of natural occurring organic compounds, that are among the most ubiquitous pollutants in the natural environment, existing in more than hundreds different combinations in mixtures [1].

For what concern their chemical structure, PAHs are compounds consisting of only carbon and hydrogen atoms (hydrocarbons), composed of multiple aromatic rings bonded in linear, cluster, or angular arrangements [2]. Naphthalene, which is the simplest PAH compound, consists of two coplanar fused benzene rings. The distinctive properties of these molecules are in part caused by the delocalization of the electrons in their aromatic rings.

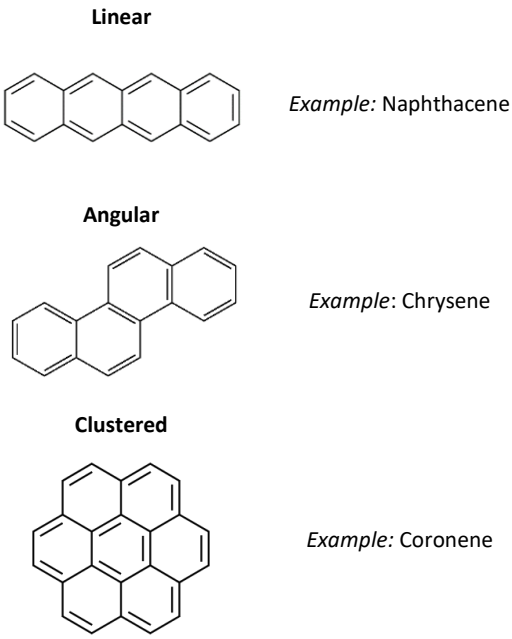


Figure 34. Molecular arrangement of the polycyclic aromatic hydrocarbons: linear, angular and clustered.

The most commonly PAHs encountered in nature are composed by two (naphthalene) to seven (coronene) fused aromatic rings, even if PAHs with greater number of rings are also found.

They are widespread in the environment as products of incomplete combustion or pyrolysis of organic material, such as coal, petroleum, wood and natural gas [6,7].

PAHs do not exist alone, but they are generally present as complex mixtures of many related compounds characterised by a wide range of physico-chemical properties and toxicity. In fact polycyclic aromatic hydrocarbons are an important class of toxic compounds, with possible mutagenic, carcinogenic, teratogenic and genotoxic activities [8].

1.2. Toxicity of PAHs in humans

Based on long-term and high-dose animal studies and in vitro and in vivo genotoxicity tests, several PAHs have been classified by the International Agency for Research on Cancer (IARC) as probably carcinogenic to humans (Group 2A) or possibly carcinogenic to humans (Group 2B) and one in particular, i.e. benzo[a]pyrene (BaP), directly as carcinogenic to humans (Group 1) [9].

16 PAHs in particular have been categorized as priority environmental pollutants by the European Union (EU) and the US Environmental Protection Agency (EPA) based on their toxicity, potential for human exposure, frequency of occurrence at dangerous waste sites and level of information available [10]. Among these PAHs, i.e. benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, chrysene, dibenz(a,h)anthracene, indeno(1,2,3-cd)pyrene and benzo(a)pyrene (Group 1) are considered by EPA as probable human carcinogens [10].

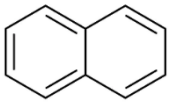
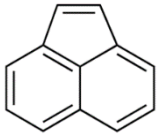
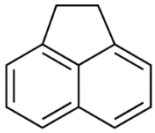
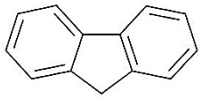
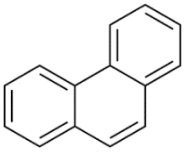
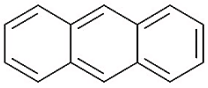
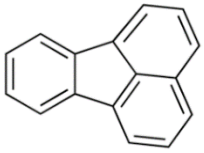
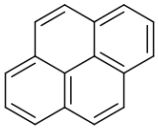
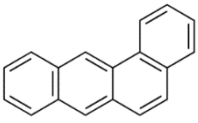
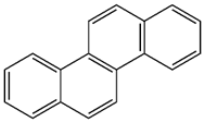
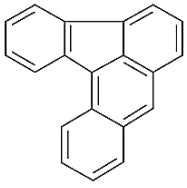
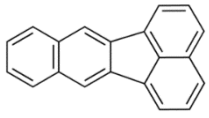
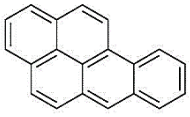
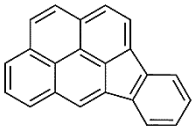
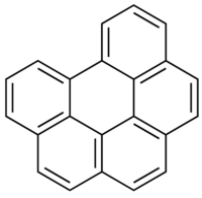
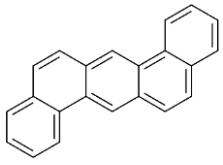
 Naphthalene	 Acenaphthylene	 Acenaphthene	 Fluorene
 Phenanthrene	 Anthracene	 Fluoranthene	 Pyrene
 Benzo[a]anthracene	 Chrysene	 Benzo[b]fluoranthene	 Benzo[k]fluoranthene
 Benzo[a]pyrene	 Indeno[1,2,3-cd]pyrene	 Benzo[ghi]perylene	 Dibenzo[ah]anthracene

Table 5. US EPA's 16 priority-pollutant PAHs and their chemical structure.

1.2.1. Sources of exposure

Typically, humans are not exposed to an individual PAH, but to mixtures of PAHs. They may be exposed to these substances at home, outside or at the workplace, where environmental PAHs are present as vapours, attached to dust and other particles in the air [11]. Cigarette smoking, asphalt roads, vehicle exhausts, coal and coal tar, forest fires, agricultural and residential wood burning, municipal and industrial waste incineration and hazardous waste sites are important sources of exposition [11]. Anyway, the major source of PAHs exposure, in non-cigarette smokers and non-occupationally exposed workers, is diet, responsible for more than 90% of the total exposure to PAHs of population in many countries of the world [12,13]. Several studies have shown that consumption of PAH-tainted dairy items, red meat (barbecued/grilled) and fatty foods (lard and pork fat) contribute to substantial intake of PAHs [14–16].

Therefore, PAHs can enter body through lungs, by breathing air that contains them, by ingestion of contaminated food, water and beverages or by contact through skin [11].

1.2.2. Short-term and long-term health effects

The impact of PAHs on human health greatly varies depending on several factors, such as the duration and source of exposition, the amount of PAHs to which one is exposed and their relative toxicity, but also subjective factors that can affect health impact such as age and pre-existing health status [8].

An assessment of occupational exposure to high levels of pollutant mixtures containing PAHs in the United Kingdom revealed the appearance of symptoms such as nausea, vomiting, diarrhoea, eye irritation and confusion [17], although it is not clear which component of the mixture was responsible for these effects, since also other compounds commonly found with PAHs may cause the symptoms.

PAHs mixtures are also known to cause inflammation and skin irritation. Benzo(a)pyrene and anthracene, in addition to be direct skin irritants, like naphthalene too, have been reported to be skin sensitizers, since they lead to allergic reactions in skin of animals and humans [17].

Long-term or chronic exposure to PAHs can provoke harmful health effects including suppress immune reaction, kidney and liver damage and jaundice, cataracts, breathing problems, asthma-like symptoms and lung function abnormalities. Repeated contact with skin, especially to naphthalene, may cause redness and skin inflammation. Naphthalene, if inhaled or ingested in large amounts, can also induce the breakdown of red blood cells [17].

Chronic exposure to PAHs ultimately leads to gene mutation and cell damaging, becoming a severe cause of cancers.

1.2.3. Carcinogenicity and teratogenicity

Although unmetabolized PAHs can provoke toxic effects, the main responsible for the harmful health effects in body are their reactive metabolites, deriving from multiple metabolic transformations of PAHs, such as epoxides and dihydrodiols, that are electrophilic derivatives able to link to cellular proteins and DNA [18]. The occurrence of cell damage and biochemical disruptions lead to mutations, malformations and cancer.

Geno-toxicity effects of PAHs have been demonstrated in several in vitro studies, using mammalian cell lines, including human ones [19], and in vivo using wild rodents model [20], playing an important role in the carcinogenicity process.

Many findings about the carcinogenic effects of PAHs primarily derive from occupational studies of workers exposed to PAHs mixture. These long-term studies showed an increased risk of mainly lung and skin as well as gastrointestinal and bladder cancers. However, since workers were exposed to other cancerogenic compounds such as aromatic amines, it is not clear from these studies if PAHs were the main cause of cancer [8].

Moreover, laboratory studies on animals exposed to PAHs for long periods showed the formation of lung cancer after inhalation, stomach cancer by ingesting PAHs through food and skin cancer from skin contact [8].

PAHs are also responsible for embryotoxic effects as described by in vivo studies on animals exposed to PAH such as benzo(a)pyrene, benzo(a)anthracene and naphthalene [21]. The assumption of high levels of benzo(a)pyrene during pregnancy resulted in birth defects and decrease of the offspring body weight in mice [22].

For what concern humans, it has been demonstrated that exposure to PAH pollution during pregnancy is associated to adverse birth outcomes including premature delivery, low birth weight and malformations of heart [23]. Prenatal

exposure to high levels of PAHs is also related to lower IQ at age three, increased behavior problems at ages six and eight, affecting children's cognitive development by five years of age. Cord blood of exposed babies also showed DNA damage that has been linked to cancer [23,24].

1.3. Food contamination by PAHs

Food can be contaminated by PAHs that are present in water, air, soil or packaging materials, as well as those that are formed during food processing or certain home cooking practices, e.g. smoking, barbecuing, roasting, grilling, toasting, frying, heating, baking, drying and ohmic-infrared cooking [25].

In particular, smoking techniques, in which the smoke produced by the incomplete combustion of the wood, by painted wood or by corrugated and smooth cardboards comes into direct contact with the product, can lead to a high contamination by PAHs [25,26]. Levels as high as 200 μg/kg of PAH have been found in smoked fish and meat [8].

In addition, thermal degradation of some food components as fatty acids, triglycerides, steroids and amino acids [6,27], free radical reactions, intramolecular addition processes or polymerization of small molecules [28] contribute to food pollution by PAHs. Concerning the pathway of PAHs formation, even if work are available studying model lipids and food lipids [6] or speculating formation mechanisms [25], further studies are necessary for a more accurate comprehension.

In the past decade the Scientific Committee on Food (SCF), the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the International Programme on Chemical Safety (IPCS) evaluated the PAHs food pollutants.

In 2002 SCF concluded that the 16 already mentioned in Section 1.2., Chapter 3-Part A, may be considered as potentially genotoxic and carcinogenic to humans thus representing a priority group in the assessment of the risk of long-term adverse health effects following dietary intake of PAHs [29].

In addition, SCF suggested to use BaP as a marker of occurrence and effect of the carcinogenic PAHs in food [29].

Later in 2007 and 2008, following a recommendation on the further investigation about PAHs levels in certain foods (2005/108/EC) [30] and according to results provided by eighteen Member States, EFSA demonstrated that BaP could not be considered a reliable marker since resulted to be not always detectable, providing in about 30% of all the tested samples a negative

response, even if others PAHs, above all chrysene, were found [29]. In view of these findings, the Commission requested a full review of the 2002 SCF opinion on PAHs and as a consequence the Panel on Contaminants in the Food Chain from EFSA (CONTAM Panel) suggested that benzo[a]pyrene, chrysene, benzo[a]anthracene, benzo[b]fluoranthene (PAH4) and four additionally ones, i.e. benzo[k]fluoranthene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3-cd]pyrene (PAH8), have to be considered, either individually or in a combination, as the only possible indicators for the carcinogenic potency of PAHs in food [29]. This decision was then implemented in the Regulation EC 1881/2006 in 2011 and revised in the new Regulation UE 835/2011 [31] that set the maximum level of benzo[a]pyrene in smoked meat and fish to 5 μg Kg^{-1} and included an additional limit of 30 μg Kg^{-1} for the sum of PAH4.

1.4. Determination of PAHs in foods

Determination of PAHs in foods matrices, where they usually occur in concentrations of ppb or ppt, requires an extraction procedure followed by multistep clean-up works in order to isolate these compounds from all the interferers present in a complex fat and protein containing food matrix.

Standard methods for PAHs detection in food have been published by IARC [32] and by the Association of Official Analytical Chemists (AOAC) [33].

Grimmer and Böhnke's procedure [34], involving saponification, extraction with cyclohexane, liquid-liquid partition of cyclohexane extract with N, N-dimethylformamide (DMF), followed by silica gel column chromatography is still widely used.

Among the most common modifications to improve the outcomes, the change of extraction and partition solvents is usually performed. In brief, dimethylsulfoxide (DMSO), that is a specific solvent for PAHs which allows their separation from triglycerides, is used in place of DMF [35]. In addition, the use of pre-packed cartridges for solid phase extraction (SPE) [36], which guarantee time and solvent savings as well as better reproducibility compared to chromatographic columns, is also often adopted. The liquid–liquid extraction with organic solvents followed by column chromatography and SPE are frequently replaced by other techniques like supercritical fluid extraction (SFE) [37], accelerated solvent extraction (ASE) [38], microwave assisted extraction (MAE) [39], solid-phase microextraction (SPME), gel permeation chromatography (GPC) [40] and preparative size-exclusion chromatography (SEC) [41].

Furthermore, as regard the analytical methods, Gas Chromatography–Mass Spectrometry (GC–MS) [42], high resolution GC-MS (HRGC–MS) [43] and high performance liquid chromatography (HPLC) assisted by fluorescence detection (FLD) [44–46] are nowadays the most applied techniques for qualitative–quantitative analysis of PAHs in food.

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2. Unequivocal identification and quantification of PAHs content in ternary synthetic mixtures and in smoked tuna by means of excitation-emission fluorescence spectroscopy coupled with PARAFAC

2.1. The present work

The present work is the result of my three-month research period at University of Burgos, in the Chemometric and Qualimetry Group of the department of Analytical Chemistry, made possible by a collaboration with the research Group of Chemistry of Food and Dietary Products of Genoa University.

The aim of the present study was to determine and quantify three of the main carcinogenic PAHs occurring in food, i.e. benzo[a]pyrene (BaP), benzo[a]anthracene (BaA) and chrysene (Chry), in a matrix of commercial smoked tuna. The analytical method exploited to achieve the goal was EEMs fluorescence spectroscopy using classical right-angle technique, coupled with the chemometric tool PARAFAC.

For this purpose, a preliminary study on artificial ternary mixtures of PAHs was performed to tune the selected analytical method. BaP, BaA and Chry were arranged in mixtures following a five-level experimental design, which is an orthogonal design consisting of 25 experiments: each individual compound is measured at each of the five selected different concentration five times, providing a well balanced mixture design.

Then, the presence of the three PAHs was investigated and quantified in smoked tuna by exploiting the previously tuned analytical approach. After lyophilization, the dried samples of smoked tuna were extracted with *n*-hexane through an ultrasonic bath followed by a multi-step clean-up procedure involving solid phase extractions with pre-packed cartridges and liquid-liquid extractions, using *n*-hexane and dimethylsulfoxide as solvents.

In both cases, the fluorescent signals coming from the ternary mixtures and from the smoked tuna extract were arranged into three-way data tensor and decomposed by PARAFAC, in order to identify the investigated compounds and quantify them based on their calibration models.

Thanks to the ‘second order property’ of PARAFAC, PAHs were unequivocally identified and quantified with very good decision limit (CCα) and capability of detection (CCβ) both in the ternary mixtures and smoked tuna.

The relative errors in quantifying BaP, BaA and Chry in the artificial ternary mixtures (“predicted concentration” versus “true concentration”) resulted to be only of 6.8%, 3.6% and 2.5%, respectively.

As regard smoked tuna, BaP was detected in a concentration of 5.42 μg kg^{-1}, while BaA and Chry resulted to be absent.

2.2. Scientific paper

Please find the article at the following link:

https://doi.org/10.1016/j.microc.2019.104561

S. Catena, S. Sanllorente, L.A. Sarabia, R. Boggia, F. Turrini and M.C. Ortiz, Unequivocal identification and quantification of PAHs content in ternary synthetic mixtures and in smoked tuna by means of excitation-emission fluorescence spectroscopy coupled with PARAFAC, Microchem. J. 154 (2019) 10456.

Conclusions

Chapter 3 is the final part of the present doctoral thesis dedicated to the field of analytical chemistry.

In particular, a specific technique of fluorescence spectroscopy has been studied and applied, analysing the achieved chemical data through chemometrics, i.e. that branch of chemistry that uses mathematics and statistics.

The studies described in this chapter have been conducted at the University of Burgos, where I spent a research period with the Chemometric and Qualimetry Group of the department of Analytical Chemistry.

The topic of the work was chosen based on the desire to combine the excellent knowledge of chemometrics of the Spanish group with my doctoral experience in food chemistry.

The emission-excitation matrix (EEM) fluorescence spectroscopy coupled with the chemometric tool PARAFAC has been employed, in fact, to analyse three polycyclic aromatic hydrocarbons, carcinogenic bioactive compounds well known as food contaminants.

Benzo[a]pyrene, benzo[a]anthracene and chrysene, have been investigated in a matrix of commercial smoked tuna, after an appropriate multi-step extraction, and benzo[a]pyrene have been detected and quantified in a concentration just within the limit allowed by legislation.

OTHER PUBLICATIONS

Please find the articles at the following related links:

1. https://doi.org/10.3390/app10082795

   F. Turrini, P. Malaspina, P. Giordani, S. Catena, P. Zunin, R. Boggia, Traditional Decoction and PUAE Aqueous Extracts of Pomegranate Peels as Potential Low-Cost Anti-Tyrosinase Ingredients, Applied Sci. 10 (2020) 2795.

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