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A GREEN CHEMISTRY APPROACH FOR THE VALORIZATION OF PHENOLIC COMPOUNDS FOUND IN AGRO-INDUSTRIAL WASTES AND BY-PRODUCTS ACCORDING TO THE CIRCULAR ECONOMY MODEL

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Index

page

1. General introduction

1.1 Green chemistry 4
1.2 Circular economy 6
1.3 Agro-industrial wastes and by-products 8
1.3.1 Recovery of bioactive compounds by traditional and innovative techniques 10
1.4 Phenolic compounds 13
1.4.1 Classification and occurrence in nature 13
1.4.2 Biological activities 24
1.4.3 *Olea europaea* L. 29
1.4.4 *Castanea sativa* Mill 37

2. Aim of the research 43

3. Lipophilization of phenolic compounds found in olive oil and sweet chestnut wastes and by-products under green chemistry conditions

3.1 Introduction: lipophilicity concept and synthetic strategies in polyphenols-chemistry 45
3.2 Results and discussion 51
3.2.1 Lipophilization of tyrosol, homovanillyl alcohol, hydroxytyrosol and an hydroxytyrosol-enriched extract 51
3.2.2 Lipophilization of gallic acid and a gallic acid-enriched extract 59
3.3 Conclusions 73
3.4 Experimental section 74
3.4.1 Chemicals and instruments 74
3.4.2 Synthesis of hydroxytyrosol 74
3.4.3 Synthesis of lipophilic esters of tyrosol, homovanillyl alcohol and hydroxytyrosol: general procedure 75
3.4.4 Preparation and characterization of an hydroxytyrosol-enriched extract 76
3.4.5 Esterification of an hydroxytyrosol-enriched extract: general procedure 77
3.4.6 Synthesis of lipophilic esters of gallic acid: general procedure 77
3.4.7 Recycling of Amberlyst-15 78
3.4.8 Preparation and characterization of a gallic-enriched extract 78
3.4.9 Esterification of a gallic acid-enriched extract: general procedure 79
3.4.10 Characterization of lipophilic esters 80

4. Preparation and characterization of novel poly (vinyl alcohol)-based films using hydroxytyrosol and oleuropein-enriched extracts for active antioxidant food packaging

4.1 Introduction: active antioxidant food packaging 85
4.2 Results and discussion: hydroxytyrosol and oleuropein-enriched extracts obtained from olive oil wastes and by-products as active ingredients for poly (vinyl alcohol)-based films 88
4.3 Conclusions 97
4.4 Experimental section 98
4.4.1 Materials 98
4.4.2 Preparation and characterization of an oleuropein-enriched extract 98
4.4.3 Preparation and characterization of PVA-based formulations 99

4.4.3.1 Morphological and colour analysis 100
4.4.3.2 Determination of the total phenolic content 101
4.4.3.3 Evaluation of the radical scavenging activity of films 101
4.4.3.4 Release studies in a food simulant 101
4.4.4 Statistics 102

5. Synthesis of new phosphodiester dimers from tyrosol, homovanillyl alcohol and hydroxytyrosol as potential anti-Alzheimer's disease agents
5.1 Introduction: polyphenols and neurodegenerative disorders 103
5.2 Results and discussion: synthesis of new phosphodiester dimers of tyrosol, homovanillyl alcohol and hydroxytyrosol and their effect on amyloid β aggregation and metal chelation ability 105
5.3 Conclusions 113
5.4 Experimental section 113
5.4.1 General methods 113
5.4.2 Characterization of dimers 114
5.4.3 Evaluation of the radical scavenging activity 116
5.4.4 Studies on the kinetic of Aβ fibrillation 116

6. General conclusions 118

Index of abbreviations 119

Funding 120

References 121

1. General introduction

1.1 Green chemistry

The concept of green chemistry was first formulated in the late 1990s.[1] According to the definition given by Paul Anastas and John C. Warner, green chemistry is the *"design of chemical products and processes to reduce or eliminate the use and generation of hazardous substance"*.

The ambitious goal of green chemistry is to revolutionize the approach to all chemical processes, and, for this purpose, design criteria, known as "the twelve principles", have been introduced (**Figure 1**).[1]

Figure 1. The twelve principles of green chemistry.

These guidelines do not represent a utopian vision of chemistry, as they are conceived by scientists, people who are aware of the obstacles and problems that can be encountered in chemical synthesis. By putting into practice the indications provided, it will be possible to develop processes that involve the minimum, or even better completely avoid, negative consequences for humans and the environment. The proposed criteria are helpful not only in

the academic field, for chemical processes on a laboratory scale, but also for extending preparation on a large scale, at industrial level.[2]

To make the green chemistry approach even more attractive for the world of industry, the concept of chemical product performance should be revised, consequently resulting in an effect on the business model of the chemical sector. The more performing the product, the greater the profit. But the new notion of performance should include not only maximum efficiency but also less intrinsic hazard. According to green chemistry theory, the concept of "hazard" is extended to all levels of a chemical production process, from raw materials to wastes and by-products generated during the process, up to the final product. This means that tomorrow's chemicals will be evaluated not only for the technical requirements to be met but also for the process through which they were obtained, i.e. considering at each stage of the preparation the possible physical, toxicological and environmental hazards. So, the future chemicals should be non-depleting, non-toxic and non-persistent.[3]

The current chemical production, that relies on petroleum chemistry as a source of starting molecules for the synthesis of more than 100.000 chemicals, will have to be totally redesigned. The new chemical value chain will have to be based on a closed carbon cycles, using feedstock and energy from renewable sources. Moreover, the process should require the least possible energy consumption and the least production of wastes and by-products. Where this is not possible, consideration should be given to how to reuse wastes in other processes. The use of the solvents should be avoided, or at least alternative solvents with low impact on health and the environment, such as water, fluorous and ionic liquids, supercritical media, should be chosen. Catalytic reagents should be preferred to the stoichiometric quantities of materials, so as to avoid subsequent separation processes and therefore the generation of waste substances to be disposed of.[3,4] Lignocellulosic biomass and carbon dioxide are among the most widely available materials on earth and so they constitute a good example of raw material from which to draw to obtain carbon-based building blocks. There are already known cases where the alternative production process of chemicals from renewable sources is highly competitive.[3] The conversion of maleic anhydride into succinic acid through engineered microorganisms is more advantageous than classic petrochemical synthesis both from an economic (0.55-1.1 \$/Kg *vs.* 3-8 \$/Kg) and environmental point of view (in terms of CO_2 that is consumed in the process).[5] In other cases, raw materials from renewable sources have become new build-blocks in already known processes. In the field of polymer chemistry, furanosic monomers, obtained from ligno-cellulosic biomass, are increasingly emerging as potential

candidates to replace fossil-origin building blocks. In particular, 2,5-furandicarboxylic acid has proven to be an ideal substitute for terephthalic acid, widely used to produce polyesters (e.g. polyethylene terephthalate, PET) and polyamides (e.g. polyphthalimides).[6]

The development of a process as safe as possible at all stages of production implies that the substances to be used must guarantee the minimum risk of accidents for operators (explosions, fires, releases). Furthermore, analytical techniques, such as process analytical chemistry, could be exploited for "live" monitoring the chemical transformations, so to evaluate the environmental impact of the process.[2] Since non-toxicity is a requirement also for the chemicals produced, their hazard will have to be assessed. Therefore, it will be necessary to carry out a preliminary study on the chemical-physical properties of a molecule (solubility, cell permeability) and on the biological interaction (how a molecule is absorbed, metabolized and excreted by the body).[3]

Another aspect to take in account is the persistence of the synthetized compounds, which should be designed to be degraded easily and rapidly into innocuous products. However, if a molecule embeds high levels of molecular complexity and has high costs of production in terms of energy, its persistence can become an added value. It will be advisable not to disperse the value of this molecule and think of its reuse rather than a degradation pathway.[3]

1.2 Circular economy

In more recent times, this idea of revolutionizing the chemical sector, moving to a more sustainable production model, overlapped with the broader concept of circular economy, which embraces all production sectors. This concept arises from the need to adopt a development model that can replace the current one, defined as linear. The linear economy model is based on the idea that the sources are inexhaustible, and that the environment can indefinitely withstand pollution. Therefore, the actual production process uses natural sources to obtain materials to be converted into consumer products which will then be thrown away when they are no longer useful. This "resource-to-wastes" economy is not going to last long. The increase in the population, the depletion of non-renewable resources, the increase in consumption with the consequent increase in wastes produced, will induce the current production system to collapse.[7,8] Therefore, it is no longer possible to wait for a transition from a linear economy to a closed-loop economy, so to restore a balance between the environment and humans' need. According to the circular economy approach, the intrinsic

value of the product must be enhanced at each point of its life, with the aim of reducing wastes, not depleting resources, and saving energy.

This new sustainable approach can be summarized with the so-called three rules:[9, 10]

- Reducing the consumption of resources and the generation of wastes in the process of production and consumption processes.
- Reusing wastes as product directly or after repair or remanufacture; or reuse their components as parts of other products.
- Recycling wastes to turn it into a raw material for a new production process.

The transition towards the circular economy model was encouraged by the European Commission, which on December 2015 has presented a plan including the strategies to be realize through 54 actions.[11] A recent report published in 2019 has highlighted that all actions have been delivered or are being implemented.[12]

It can be concluded that the application of the 12 principles would trigger a production mechanism in the chemical sector that would help to overcome the current linear economy model, favouring the implementation of a circular economy model. This approach based on a close synergy between green chemistry and circular economy will allow to achieve many of the 17 sustainable development goals (SDGs) included by the United Nations in the 2030 agenda (**Figure 2**).[13]

Figure 2. The sustainable development goals (SDGs) fixed by the United Nations.[13]

A close relationship between the principles of green chemistry and the SDGs has been highlighted. As far as the environment is concerned, there is a connection between principles 2,3,6,7,8,9,10 and SDGs 9,11,12,13,14,15. Moreover the safeguarding of human health, by minimizing exposure to toxic substances and the use of dangerous products, is another common aspect, since the SDGs have been designed specifically to improve people's well-being (principles 3, 4, 5, 11, 12 and SDGs 3, 8, 12, 2,14, 6, 9, 11). Finally, it must be considered that the economic aspect is not negligible. In fact the innovation associated with the introduction of sustainable alternative chemical processes will require investment in infrastructure (goal 9), leading to sustainable industrialization, which will improve the quality of life (goal 12).[14]

Each member state of the United Nations will be monitored for the implementation of a development model that allows it to achieve these goals. Each state will be called upon to review internal policies, establish new partnerships between various departments and agencies, and plan new government structures. It will also be essential to raise public awareness of sustainability.

For this purpose, there are educational programs concerning science and sustainable chemistry [14,15] currently used only in the academia that could be readjusted to prepare communicational material with the aim of forming more aware citizens.

1.3 Agro-industrial wastes and by-products

The agro-industrial sector is particularly suitable for the implementation of the circular economy model. Only in Europe, of the1.3 billion tons of wastes generated every year, 700 million tons are agricultural wastes and by-products.[16]

They are generated at all levels of the production chain through which agricultural raw materials are transformed in food, from cultivation and collection, up to the product to be marketed. Examples of wastes and by-products deriving from agro-industrial manufacturing processes are leaves, roots, stalks, bark, wood residues, seeds and peel, liquid residues (wastewater) as well as those fruits that are discarded because they are not ripe, damaged or because they do not meet market requirements (such as regular shape and/or colour).

The management of agro-industrial wastes and by-products represents a problem both from an economic and environmental point of view. The high chemical oxygen demand (COD) and

biochemical oxygen demand (BOD) values indicate that agri-food wastes show a high organic load. Therefore, careful management of these harmful environmental wastes is critical to avoid pollution of surface and underground waters, alteration of soil quality, phytotoxicity, coloured natural waters and smells. For this reason, disposal provisions have become increasingly stringent as well as expensive. However, the disadvantage associated with the massive presence of organic material can become an advantage in the perspective of a circular economy model, which considers wastes as a resource.[17,18]

According to this strategy, agro-industrial wastes and by-products constitute a high potential non-fossil raw material that can be reused o recycled in other production processes. The valorisation strategies adopted, mostly based on green technologies, can be summarized in four different approaches.[19]

The first one concerns the use of agro-food wastes as a clean source of biofuels. The anaerobic and aerobic digestion, and the microbial fermentation constitute the most widely used and convenient technologies which have been employed for generation of gaseous and liquid biofuels. Hydrogen which is it-self a clean alternative to fossil energy sources, has been obtained in good yield by fermentation of apple pomace, a by-product of juice extraction.[20] Generation of bioethanol has been broadly investigated and the alternative sustainable procedures has proven to be economically advantageous compared to traditional ones. Citrus peel and grape and sugar beet pomace, due to their high content of carbohydrates, are among the wastes that have been widely used as feedstock for bioethanol production.[21, 22, 23]

The second approach is based on the production of bio-based materials via biotechnological procedures. The growing consumption of plastic makes the use of alternative biodegradable materials, like biopolymers, increasingly urgent. A particular attention has been addressed to polyhydroxyalkanoates, a family of polyesters whose high strength and toughness and moisture resistance make them suitable for the production of water-resistant bottles and films. However currently high production costs made their use still disadvantageous, but this obstacle could be overcome thanks to the possibility of using the agri-food wastes as a low-cost feedstock. A consistent research is devoted to sugarcane and beet molasses, cheese whey effluents, plant oils, swine waste liquor, vegetable and fruit wastes, effluents of palm oil mill, olive oil mill, paper mill, pull mill, and hydrolysates of starch (e.g., corn and tapioca), cellulose, and hemicellulose.[24] Enzymes are a further example of biomaterials that can be produced at low cost using those wastes that have the suitable composition (nutrients and

polysaccharides) to support the growth of microorganisms. Spent grain, grape pomace, banana wastes are examples of substrates used to produce industrially relevant enzymes such as cellulase, xylanase, amylase, lipase by various microorganisms as filamentous fungi, yeasts, and bacteria.[19]

In addition, the demand for cheaper and cleaner technologies for wastewater treatment, to recover clean water while saving water resources, can be met by using bio-adsorbents. In fact, different molecules present in the agro-industrial wastes contain functional groups that allow chelation of polluting ions present in the water. So, the third valorisation strategy consists in the use of these wastes as a green alternative to traditional adsorbent material, i.e. activated carbon. Spent tea leaves have shown a high efficacy in removing lead from aqueous solutions.[25] Furthermore, the high carbon content of agricultural by-products has also been exploited as feedstock to produce activated carbon by chemical method.[26]

Recovery of high value-added and bioactive compounds, like polyphenols, lipids, alkaloids, and terpenoids, is the fourth strategy that has been adopted. In such a way, processing wastes have become a source of products widely used in drugs, nutraceuticals, flavours, fragrances cosmetics and antimicrobics.

1.3.1 Recovery of bioactive compounds by traditional and innovative techniques

The most commonly and traditionally used techniques to recover bioactive compounds from biomass are liquid-liquid (LLE) and solid-liquid extractions (SLE).

LLE is a mass transfer process in which a solute is transferred between two immiscible liquids, typically an aqueous solution and an organic solvent. When the two phases come into contact, the solute, usually in an aqueous solution, is distributed between the immiscible solvents according to an equilibrium which depends on its affinity for one or the other phase.[27]

In the case of SLE, the raw material in adequate size is exposed to different solvents which remove the compounds of interest. The contact between the solid particles and solvent is facilized by conducting the extraction under agitation, which also guarantees an acceleration of the process. Usually, solvent treatment is performed at room temperature. The temperature can be increased to maximize the extraction yield, however the possibility of modifying or destroying the thermolabile compounds must be considered.[18]

The choice of the solvent is fundamental in these techniques, as it allows to recover the compounds of interest as selectively as possible. The solvents commonly employed are volatile organic compounds which can be easily removed, such as chloroform, diethyl ether, dichloromethane but also, ethyl acetate and hexane are used.[27] Indeed it is precisely the use of these solvents, which are highly toxic to operators and the environment, to make these traditional extraction procedures disadvantageous. Moreover, for SLE, in order maximize the transfer of material to the solvent phase, long extraction times are required.[18] In biomass biorefinery, LLE was used in the recovery of hemicelluloses and monomer sugars from aqueous effluents, recovery of biofuels from fermentation broths and extraction of carboxylic acids from biomass hydrolysates.[27] However, it must be said that the use of low-cost and non-toxic ecological solvents is known in SLE. Mixtures of water and ethanol were used for the extraction of polyphenols from vegetable matrices such as olive leaves and black tea leaves.[28]

Greener alternative technologies have become increasingly popular and, in some cases, have shown to be also more efficient. They include microwave-assisted extraction and ultrasound-assisted extraction, through which it is possible to shorten the extraction times, with a consequent energy saving whenever it has been necessary to work at high temperatures; as well as they have allowed to conduct extraction processes in the absence of solvents.[18,19] An example is represented by the valorisation of ginger by-products deriving from juice production.[29] Improved efficiency of the extraction process in terms of yield has been reported for the extraction of D-limonene from orange peel,[30] chlorogenic acid from bark and leaves of *Eucommia ulmoidies* Oliv.,[31] tea polyphenols and tea caffeine from green tea leaves.[32] The reason of best effectiveness of these methodologies lies in the principles on which they are based.

The thermal heating associated with the use of microwaves (frequencies ranging from 300 MHz to 300 GHz) causes the destruction of the non-covalent interactions that have occurred between the plant matrix and the compounds, as well as between the compounds themselves, so that the molecules get rid of the matrix and can spread in the extraction medium.[18,19]

The cavitation phenomenon is responsible for the effects that the ultrasounds have on the medium and on the matrix. When a liquid is subjected to the action of ultrasounds (frequencies ranging from 20 kHz to 10 MHz), voids (cavitation bubbles) are created. When these bubbles reach a critical point explode, releasing energy. In these conditions of high

temperature and pressure the cell wall of the plant is rapidly destroyed, with the subsequent release of the substances of interest in the medium.[18,19]

In recent times, supercritical fluid extraction is a green technique that has achieved great success, especially in the industrial field. A substance near or above its critical point possesses intermediate chemical-physical properties between a liquid and a gas. The combination of solvating power of liquids with low viscosity, high diffusivity low surface tension of the gases allows supercritical fluids to penetrate more easily and rapidly into the solid matrix, improving time and efficiency of extraction. Furthermore, by suitably tuning the critical conditions of pressure and temperature and/or by adding appropriate co-solvents (such as low molecular mass alcohols) it is possible to affect the affinity towards specific molecules to be extracted.[18] The most used fluid for extractions is supercritical carbon dioxide (sCO_2) because its mild critical parameters (T= 31 °C, P=73.8 MPa) allow to work at room temperature, avoiding the degradation of temperature sensitive molecules. Furthermore, CO_2 is cheap, non-toxic, can be easily recycled at the end of the process; with its use no solvent residues are found in the final products, since CO_2 after depressurization returns to the gaseous state.[33] Employment of sCO_2 for the isolation of high added values compounds from agri-food processing wastes is well-known. Lycopene, β-carotene, tocopherols, fatty acids and sitosterols have been extracted from tomato pomace, a by-product of tomato industrial processing;[34] vitamin E and provitamin A have been isolated from red-pepper by-products.[35] Also a lipid fraction, which has proven to be a promising cosmetic product for sunscreen formulations, has been recovered using this approach on coffee grounds and green coffee defective beans.[36] In addition to sCO_2, subcritical water (sH_2O) has proved to be an interesting alternative to traditional organic solvents. The subcritical conditions are achieved by raising the water temperature (between 100 and 374 °C) and, at the same time, applying appropriate pressures so that the water remains in the liquid state. In such conditions, dielectric constant decreases and this superheated water has shown to be able to extract also slightly non-polar compounds. A successful extraction of resveratrol from wastes of wine production by using sH_2O extraction has been reported in literature.[37]

A suitable separation of different components of a complex mixture can be carried out by membrane technologies. These technologies are based on the use of selective filters (membranes) that allow the selective passage of molecules with specific molecular weights and dimensions. The driving force of the process is a transmembrane pressure, which is applied in a tangential direction with respect to the flow of material to be separated facilitating

the recovery of solutes from the membrane surface. Membranes techniques generally used for recovering high-added value compounds from aqueous extracts and food aqueous by-products are microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). MF membrane prevents the passage of solutes with a size greater than 10000 nm; UF membrane repels molecules with a molecular weight between 350 and 1000 Da as macromolecules, proteins, and colloids; the small pore size of the NF membrane allows the passage of small molecules, with a molecular weight lower than 120 Da; RO membrane is able to concentrate sugars, monovalent ions and salts in aqueous solution, so only water remains into the permeate. Considering the complexity of the composition, the approach that has been applied to process agro-industrial wastes and by-products is the integrated membrane system. According to their permeability, different membranes are suitably used in succession to obtain increasingly refined fractions at each step.[38, 39] Processes for valorisation of agro-industrial wastes through an integrated membrane system have been widely reported. Although the most studied samples are olive mill wastewater,[40, 41, 42, 43] other applications include artichoke wastewater, [44, 45] orange press liquor, [46] and coconut by-products.[47] Membrane technologies fit perfectly in the perspective of green processes as they are not energetically expensive, do not require the use of chemicals and high temperatures. Furthermore, the ultimate permeate of a membrane integrated system is water that can be recycled and/or reused, thus forming a closed-cycle process. However, a problem associated with the use of the membrane system is a decreasing in permeate flow over time. This is due to two phenomena: polarization concentration and fouling. The first one is caused by rejected molecules that accumulate on the membrane surface and it can be overcome by modulating the operating conditions. The fouling problem is due to unwanted material clogging the pores of the membrane and one of the approaches that has been attempted to avoid it is the chemical modification of membrane surface.[48]

1.4 Phenolic compounds

1.4.1 Classification and occurrence in nature

Phenolic compounds (also named polyphenols) represent the most abundant and widespread class of plant secondary metabolites, deriving from the shikimate/phenylpropanoid and/or the polyketide pathway, featuring more than one phenolic unit and deprived of nitrogen-based functions.[49]

They occur in nature primarily in glycosylated forms, linked to one or more sugar residues (glucose is the most common one, although galactose, rhamnose, xylose, and arabinose are also found) as well as esters, in particular methyl esters.[50]

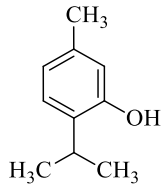
Polyphenols are present in almost all food of plant origin (vegetables, cereals, legumes, fruits, nuts) and beverages (wine, cider, beer, tea). In each matrix, their content is influenced by several parameters such as environmental conditions and genetic factors, germination, degree of ripeness, variety, processing, and storage.[51]

To date, in the plant kingdom were identified more than 8000 polyphenols [52] with a wide range of structural variety. According to the common classification based on the basic carbon skeleton, they can be distinguished in the following classes (**Table 1**): simple phenols (C6), phenolic acids (C6-C1 and C6-C3), phenethyl alcohols (C6-C2), coumarins and chromones (C6-C3), xanthones (C6-C1-C6), stilbenes (C6-C2-C6), flavonoids (C6-C3-C6), lignans $(C6\text{-}C3)_2$, lignins $(C6\text{-}C3)_n$, condensed tannins (or proanthocyanidins) $(C6–C3–C6)_n$.[51]

Table 1. Main classes of natural polyphenols.

Class	***Basic skeleton***
Simple phenols	C6
Phenolic acids	C6-C1
Phenethyl alcohols	C6-C2
Phenolic acids	C6-C3
Coumarins and chromones	C6-C3
Xanthones	C6-C1-C6
Stilbenes	C6-C2-C6
Flavonoids	C6-C3-C6
Lignans	$(C6\text{-}C3)_2$
Lignins	$(C6\text{-}C3)_n$
Condensed tannins	$(C6–C3–C6)_n$

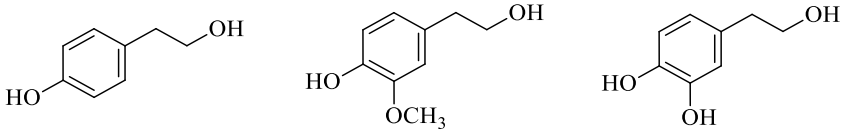
A simple phenol C6 is thymol (**Figure 3**), the main compound found in the essential oils obtained from plants belonging to the Lamiaceae (*Thymus, Ocimum, Origanum, Monardagenera*), Verbenaceae, Scrophulariaceae, Ranunculaceae and Apiaceae family. The extracts from these plants have been used in the food industry since ancient times as flavouring and preservative agents.[53]



Thymol

Figure 3

The C6-C2 class includes phenethyl alcohols. The most representative compounds are tyrosol, homovanillyl alcohol and hydroxytyrosol (**Figure 4**), bioactive compounds found in *Olea europaea* L. that will be described in the paragraph 1.4.3. [54]



Tyrosol **Homovanillyl alcohol** **Hydroxytyrosol**

Figure 4

Hydroxybenzoic and hydroxycinnamic acids are two subgroups of phenolic acids. Hydroxybenzoic acids include gallic acid (which will be discussed in detail in the paragraph 1.4.4.), *p*-hydroxybenzoic acid, protocatechuic acid, vanillic acid and syringic acid, which in common have the C6–C1 structure (**Figure 5**). The hydroxybenzoic acid content of edible plants is generally very low, except for certain red fruits (such as strawberries, cranberries), black radish, and onions, which can have amounts of several tens of milligrams per kilogram fresh weight.[55]

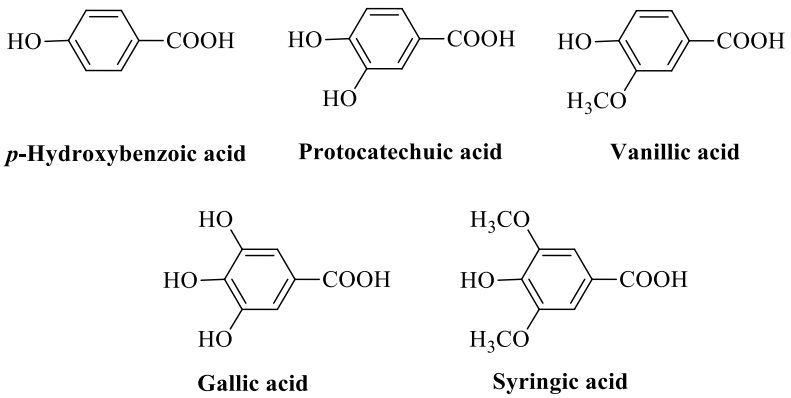


Figure 5

The chemical structure of hydroxycinnamic acids is characterized by a C6-C3 carbon skeleton, with an aromatic ring linked to an unsaturated three carbon atoms side chain. The most important compounds are *p*-coumaric acid, caffeic acid, ferulic acid, and sinapic acid (**Figure 6**), which are present in red colour fruits, wine, tea and, in the case of caffeic acid, also in coffee as chlorogenic acid.[56]

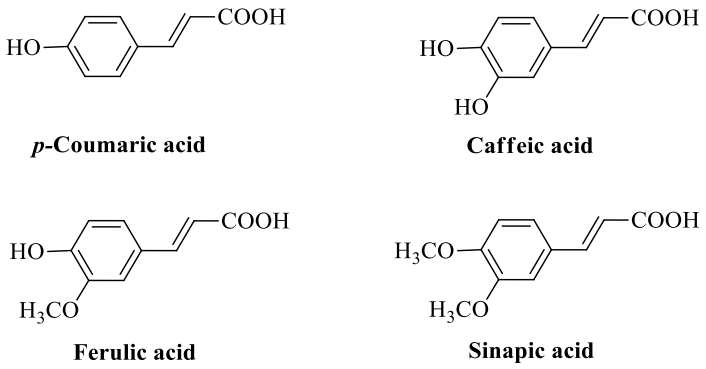


Figure 6

Coumarins are characterized by a 1,2-benzopyranic ring system as basic skeleton. In plants, they are overproduced in response to attacks of pathogens or abiotic stress, so they are considered phytoalexins.[57] The simplest compound is coumarin (**Figure 7**), which is a constituent of many plants having a pleasant spicy smell of fresh hay, woodruff, or vanilla. It was isolated for the first time in 1882 from tonka bean and used as a food flavouring until

1954, when it was discovered that coumarin caused hepatic damages. In 1988 the use of coumarin was again allowed, setting a limit concentration of 2 mg per kg of food.[58]

Chromones are a class of natural products widely distributed in several botanical species such as Aloaceae, Caesalpinaceae, Chaetomiaceae, Moraceae, Piperaceae, Rubiaceae, Staphyleaceae and Thymelaeaceae.[59] In recent years, medical chemistry has shown a growing interest towards these compounds. In fact, the chromone scaffold is an excellent template to perform structural modifications to obtain a wide number of compounds with potential pharmacological properties.[60] Since ancient times, khellin (**Figure 7**) has been used as an agent for the treatment of respiratory problems such as asthma.[59]

Xanthones are a backbone C6-C1-C6, with a dibenzo-γ-pyrone scaffold. Naturally occurring compounds include simple xanthones, xanthone glycosides, prenylated xanthones, xanthonolignoids, bisxanthones, and miscellaneous xanthones. α–Mangostin, well-known for the anti-inflammatory activity,[61] is the major compound found in the pericarp of mangosteen fruit. (**Figure 7**).

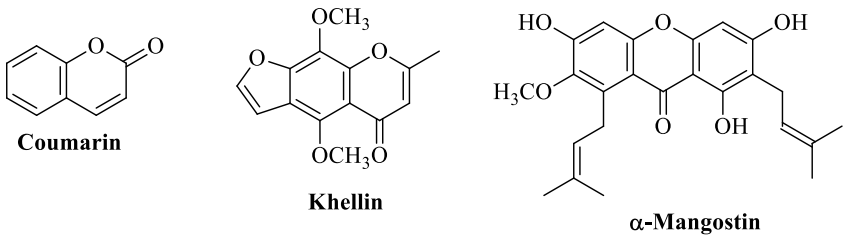


Figure 7

Stilbenes are found in almonds, beans, blueberries, bilberries, peanuts, grapevine, cranberries, mulberries, plum, and wine. It consists of two aromatic rings linked by a double bond, generally in (*E)* configuration. One of the most interesting and widely studied stilbenes is resveratrol (**Figure 8**), a phytoalexin present in grapes.[57]

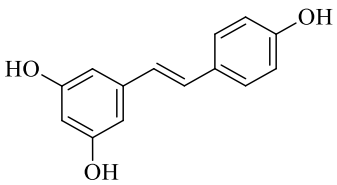


Figure 8

Flavonoids constitute the largest group of plant phenolics, accounting for over half of the 8.000 naturally occurring phenolic compounds.[50] The basic structure consists of 15 carbon atoms arranged in 2 benzene rings (A e B) joined by a benzopyranic ring C (**Figure 9**). The hydroxyl groups are free or present as methyl esters, acetyl esters, linked to sugars. Generally, glycosylation occurs at C-3, C5and C-7 position and sugars are generally rhamnose, glucose, galactose, and arabinose.[62]

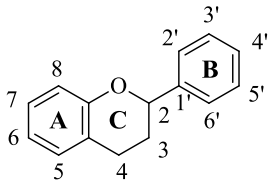
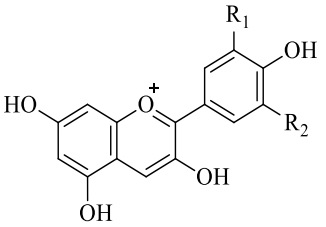


Figure 9

Depending on the level of oxidation and pattern of substitution of the C ring, flavonoids can be divided into different subclasses: anthocyanins, flavanols, flavanones, flavonols, flavones, and isoflavones. Compounds within the same subclass differ for the position of the substituents on the ring A and B.[62]

Anthocyanins take their name from the Greek words *anthos* (=flower) and *kyáneos* (=blue), being responsible for the blue, purple, red and orange colour of many fruits and vegetables. The structure backbone of the aglycon, the anthocyanidin, is the flavilium ion or 2-phenylbenzopyrilium. Six compounds are abundant in plants: cyanidin, delphinidin, pelargonidin, peonidin, petunidin and malvidin (**Figure 10**). The main sources of anthocyanins are red fruits, mainly berries and red grapes, cereals, principally purple maize, vegetables and red wine.[63]



$R_1 = R_2 = H$	**Pelargonidin**
$R_1 = OH, R_2 = H$	**Cyanidin**
$R_1 = R_2 = OH$	**Delphinidin**
$R_1 = OCH_3, R_2 = H$	**Peonidin**
$R_1 = OH, R_2 = OCH_3$	**Petunidin**
$R_1 = R_2 = OCH_3$	**Malvidin**

Figure 10

The subclass of flavanols, generally indicated as "*catechins*" to refer to juice or boiled extract of *Mimosa catechu*, include catechin, epicatechin, epigallocatechin, gallocatechin, epicatechin gallate, epigallocatechin gallate and epicatechin gallate (**Figure 11**). In the basic structure, A ring is a resorcinol moiety, while B ring is similar to catechol moiety and carbon in position 3 on C ring brings a hydroxyl group. Green tea is a principal source of catechins, and the major component is epigallocatechin gallate. Other sources of catechins are grapes and blackberries, apples, or nuts like beans, almond, pistachios, red wine, and cocoa-based products.[64]

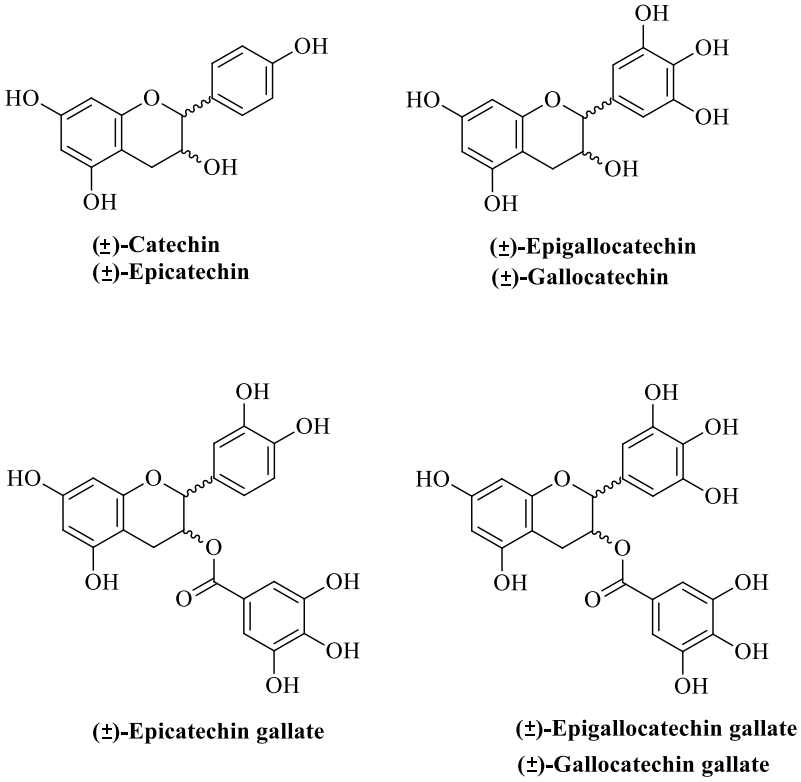


Figure 11

Flavanones occur in tomato, potato, rosemary, peppermint and fruits such as lemon, orange, strawberry, raspberry, and plum. Examples of molecules belonging to this category are naringenin and hesperidin (**Figure 12**).[57]

The subclass of flavonols is well represented by quercetin and kaempferol, which are the most ubiquitous polyphenols in fruit and vegetables. Their structures differ only for the presence of hydroxyl group in C-3' position. (**Figure 12**). Quercetin is mainly found in onion, asparagus,

and berries while kaempferol-rich foods are green leafy vegetables, including spinach and kale, and herbs such as dill, chives, and tarragon.[65]

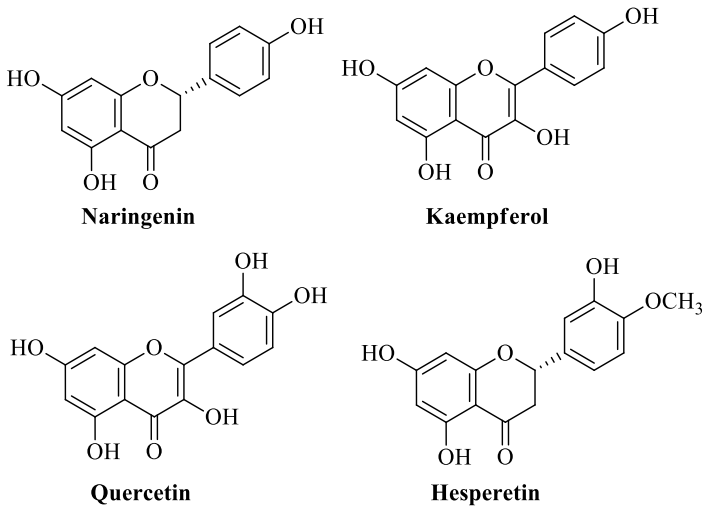


Figure 12

In the flavone basic structure, there is a double bond between carbon atoms in positions 2 e 3. Flavones are white and cream pigments of some flowers and co-pigments together with anthocyanins in blue flowers. As regards beverages, they are abundant in citrus juices; apigenin and diosmetin 6,8-di-C-glucosides can be found in bergamot, mandarin orange, orange, and citron juices. Examples of food sources are apricot, cashew, bean, cabbage, cardon, apple, artichoke, mango, papaya, onion, olive oil and honey.[57]

Isoflavones are the most common form of phytoestrogens, phytochemicals having a structure similarity to mammalian oestrogens and so they mimic the same action on target organs.[66] The main source of isoflavones are plants of leguminous family, especially soy which contains daidzein, genistein, and glycitein (**Figure 13**).[66]

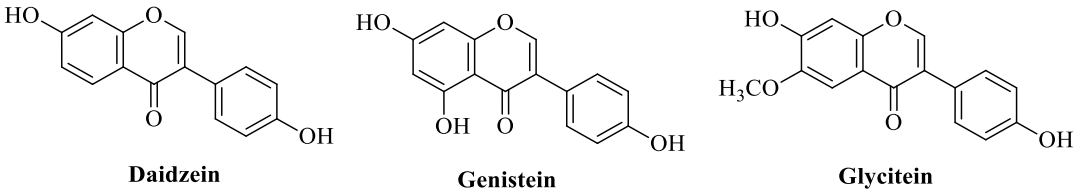


Figure 13

Lignans are high molecular weight polyphenols showing a wide structural range. The main commonly studied show two phenylpropanoid units C6-C3 linked through the β and β' carbon atoms.[67] Sources of dietary lignans are oilseeds (i.e., flax, soy, rapeseed, and sesame), whole-grain cereals (i.e., wheat, oats, rye, and barley), legumes, various vegetables and fruit (particularly berries) as well as beverages (coffee, tea, and wine). Recently, lignans are also found in dairy products, meat, and fish.[57] Examples of dietary lignans are secoisolariciresinol, pinoresinol, and matairesinol (**Figure 14**).

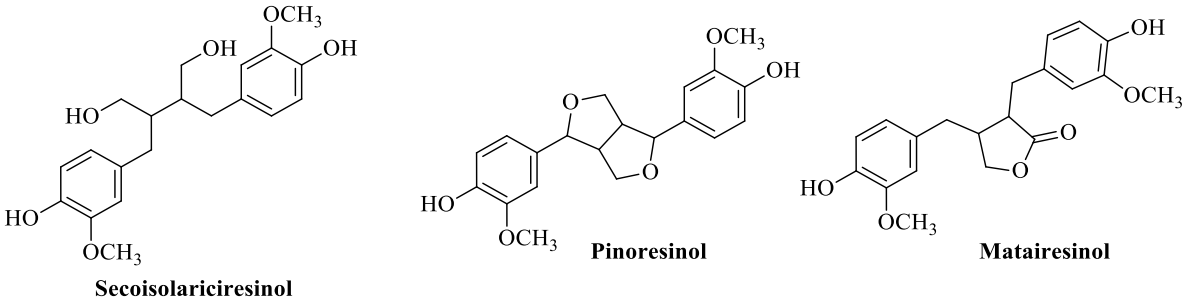


Figure 14

Lignins are found in the cell walls, where they perform structural and protective functions. As regards chemical structure, they are three-dimensional heteropolymers resulting from the oxidative coupling of phenylpropanoids units (C6-C3). The monolignol composition of the lignan varies considerably among taxa, tissues, and cell types. However, the three predominant monomers are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (**Figure 15**), which are connected through different types of chemical bonds including non-condensed ether-O-4 bonds and condensed carbon-carbon bond.[68] Lignins are considered a high potential raw material from which it is possible to obtain biofuels and fine chemicals by pyrolysis and solvothermal treatment.[69]

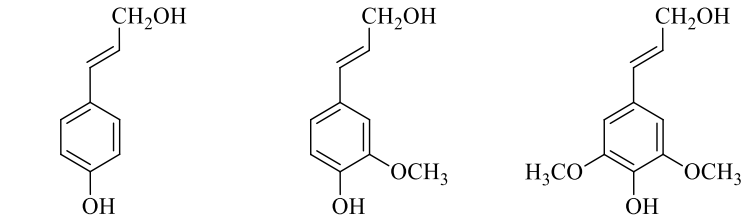


Figure 15

Tannins have been used in tanneries since ancient time being able to react with animal proteins and protect animal skin from parasites attack during the leather manufacturing process. On the basis of their chemical structure, they can be distinguished in hydrolysable and non-hydrolysable. The formers are further subdivided in gallotannins and ellagitannins, esters of gallic acid and hexahydroxydiphenic acid with a polyol, generally glucose. The hydrolysis of gallotannins yields gallic acid while from ellagitannins, hexahydroxydiphenic acid is isolated as its stable dilactone, ellagic acid (**Figure 16**).[70]

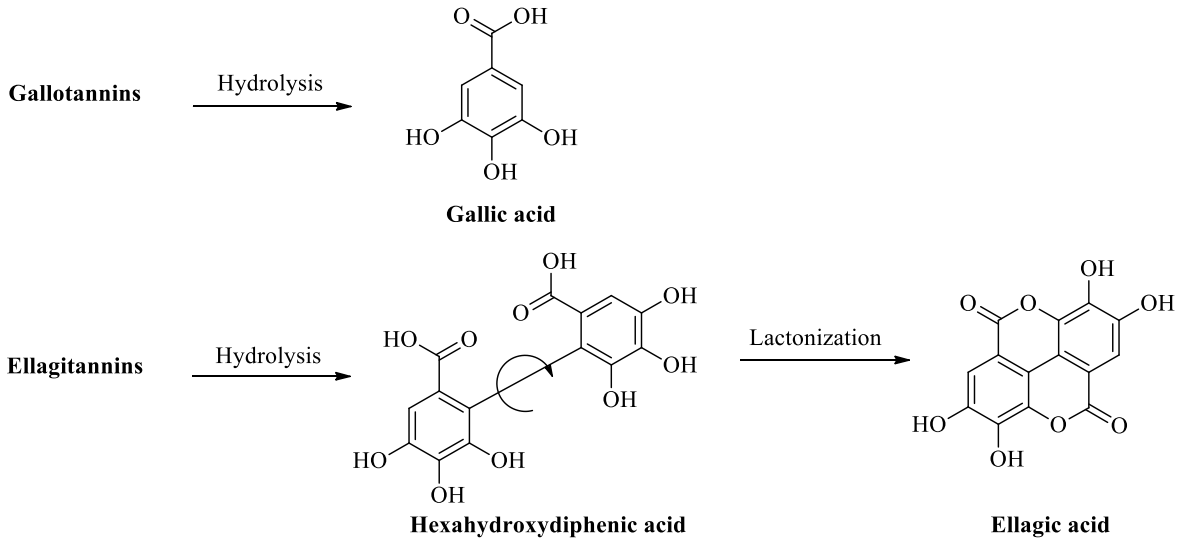


Figure 16

Examples of hydrolizable tannins are pentagalloylglucose, vescalagin and castalagin (**Figure 17**).[71]

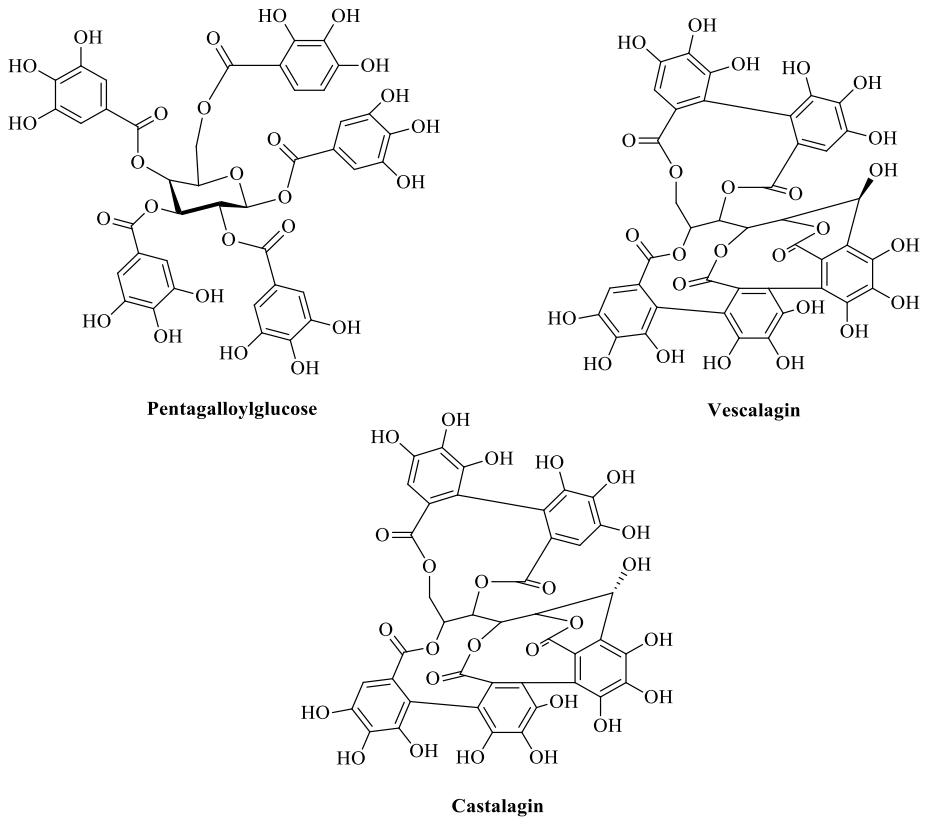


Figure 17

Condensed tannins or proanthocyanidins are oligomers or polymers whose the monomer unit is flavan-3-ol. Depending on the numbers of the bonds between the monomeric units, there are B-type and A-type proanthocyanidins. The B-type are characterized by only one C-C bond between C4 and C6 or C4 and C8 positions of the 2 units; in the A-type additional linkage between C2-C7 positions occurs (**Figure 18**).[71]

Legumes, nuts, grape seeds, pine bark extracts and other minority cereals such as sorghum and barley are sources of proanthocyanidins. Ellagitannins can be found in all varieties of berries and their derivatives (such as juices, jams, and jellies), pecans, walnuts, brazil nuts, peanuts, pomegranate (fruit and juice), red apples, pink and white grapefruit, white and red grapes, oak-aged wines.[57] Among gallotannins, can be mentioned tannic acid, a not yet clearly

identified mixture of different gallic acid esters of glucose, generally obtained from sweet chestnut aqueous extract.[72]

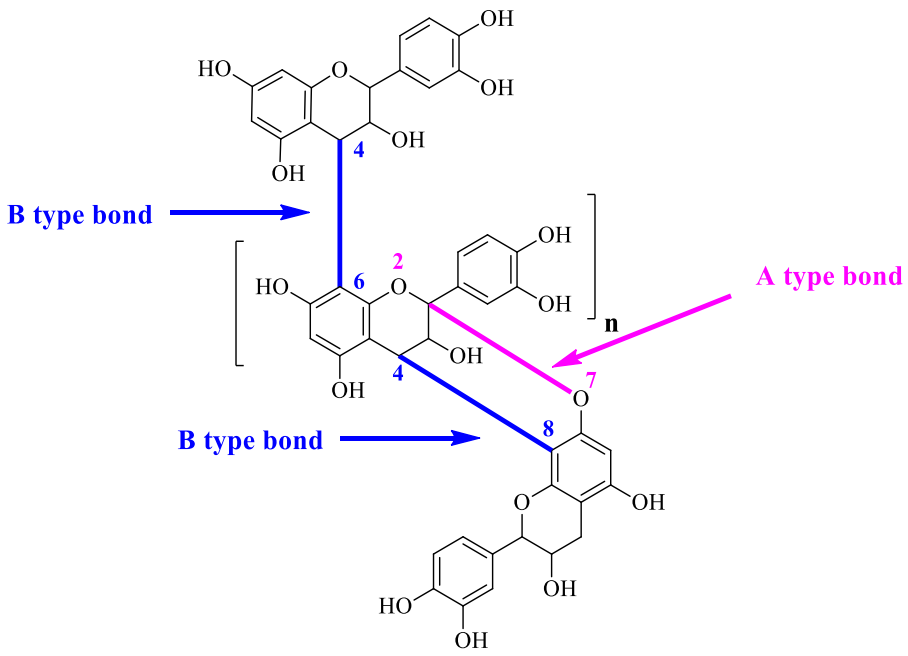


Figure 18

1.4.2 Biological activities

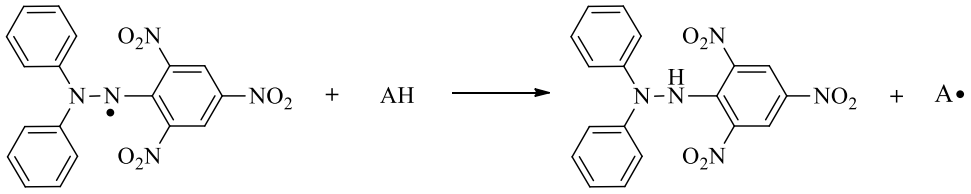
Polyphenols are essential and non-essential bioactive compounds that occur in nature, they are part of the food chain and have proven effects on human health.[73] Their numerous biological activities justify the increasing interest toward these natural substances.

The most relevant is the antioxidant activity. The function of an antioxidant is to protect the human body from oxidative stress, a peculiar state resulting from an imbalance between generation and consumption of reactive oxygen species (ROS), such as hydroxyl radical ($HO^{\cdot}$), superoxide radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) or reactive nitrogen species (RNS) such as nitric oxide radical ($NO^{\cdot}$), nitrogen dioxide radical ($NO_2^{\cdot}$), nitrous acid (HNO_2). In condition of oxidative stress, the endogenous antioxidant defence system (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase) is unable to counteract the overproduction and accumulation of ROS and/or RNS which can damage biological macromolecules as DNA, proteins and lipids.[74] Oxidative

damage is therefore responsible for developing important disorders such as degenerative diseases,[75] atherosclerosis, cardiovascular diseases,[76] inflammatory diseases [77] and cancer.[78]

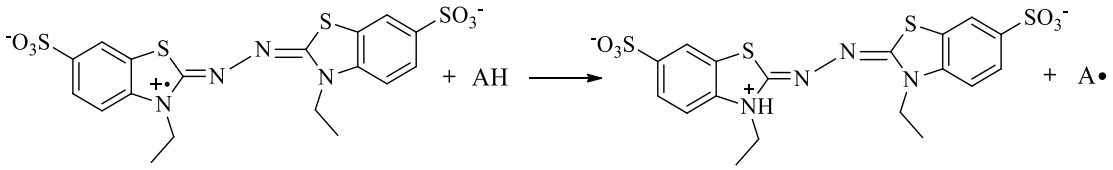
Different methods have been developed to test *in vitro* antioxidant capacity of phenolic compounds. The most common in laboratory practice are the 2,2-diphenyil-picrylhydrazyl (DPPH) radical assay and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) radical cation assay.

The DPPH assay is based on the ability of an antioxidant (AH) to reduce the stable organic radical DPPH•, generally in a methanol solution (**Scheme 1**). The solution changes colour from purple to yellow, with a consequent decreasing in absorbance which is measured by UV-Vis spectrophotometry at wavelength 517 nm. The antioxidant activity of AH can be expressed using different parameters, such as IC50, which represents the concentration of antioxidant able to inhibit 50% of the DPPH radical.[79]



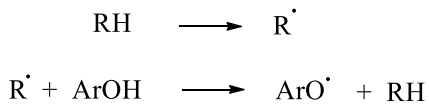
Scheme 1. Reduction of DPPH• radical by an antioxidant (AH).

Also ABTS assay is a spectrophotometric method. In this case it is necessary to generate the ABTS•+ radical cation, which is a blue/green chromophore with a maximum absorption at wavelength of 734 nm, and it is obtained by reacting ABTS with potassium persulfate for 16-24 h. The addition of an antioxidant to the radical cation reduces it to ABTS, with a consequent decrease in absorbance (**Scheme 2**). The antioxidant activity is generally expressed as TEAC (Trolox Equivalent Antioxidant Capacity), which represents the concentration of antioxidant that determines an absorbance inhibition of the ABTS•+ radical cation equal to that of a 1 mM solution of trolox (acid-6-hydroxy-2,5,7,8-tetramethylchromane), the reference standard.[80]



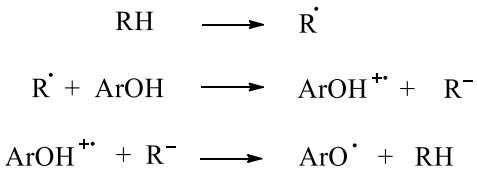
Scheme 2. Reduction of ABTS•+ radical cation by an antioxidant (AH).

Phenolic compounds can act as antioxidants in several ways: scavenging free-radicals, chelating transition metals ions and inhibiting enzymes. As regards their capacity to break chain radical reactions, a mechanistic dichotomy exists between hydrogen atom transfer (HAT) and single electron transfer (SET).[81] In HAT mechanism (**Scheme 3**), the phenolic compound, ArOH inactivates free radical by donating a hydrogen atom. The radical form of antioxidant produced by reaction, $ArO^{\cdot}$, is much more chemical stable than $R^{\cdot}$, thanks to the capacity of the aromatic ring to stabilize the electron by delocalization.



Scheme 3. HAT mechanism.

Polyphenols can interrupt the chain radical reaction by donating an electron to a radical substrate; also in this case, the net result is the production of stable radical $ArO^{\cdot}$ (**Scheme 4**).



Scheme 4. SET mechanism.

These two mechanisms must occur in parallel but with differ rates. Two parameters, bond dissociation enthalpy (BDE) and ionization potential (IP), play an important role; in particular, BDE is relevant for HAT and IP for SET. A computational study suggests that most of substances act according to the HAT mechanism.[81]

Phenolic compounds, thanks to the presence of adjacent OH groups, also have tendency to chelate transition metals ions (especially iron and copper) preventing involvement of such ions in free radical formation reactions. For example, during catabolism of dopamine is produced H_2O_2 that in presence of a metal at low oxidation state releases hydroxyl radicals (Fenton reaction, Scheme 5). The capture of metal ions by chelating agent inhibit accumulation of hydroxyl radicals in neurons, an event associated with the onset of Parkinson disease.[82]

Scheme 5. Fenton reaction.

Furthermore, the presence on phenolic structures of the π-electrons of the aromatic ring and hydrogen-bonding property of OH group, allows them to interact with amino acids within active sites of enzymes involved in the production of radicals such as various cytochrome P450 isoforms, lipoxygenases, cyclooxygenase, and xanthine oxidase.[56]

The cardioprotective effect of polyphenols is related to their capacity of act as inhibitor of the mammalian target of rapamycin (mTOR) a protein belonging to phosphatidylinositol kinase-related kinase (PIKK) family. Signaling mechanism mediated by mTOR is considered a target for therapy of cardiovascular diseases: reduction of signals along this pathway involves beneficial effects against atherosclerosis, cardiac hypertrophy, and heart failure. Resveratrol inhibits mTOR pathway suppressing oxidized low-density lipoprotein (oxLDL) proliferation of smooth muscle cells, one of the major contributing factors for atherosclerosis. Epigallocatechin-3-gallate proved to be a good candidate as a cardioprotective drug to treat cardiac hypertrophy. In fact, it is capable to block transactivation of epidermal growth factor receptor (EGFR), through inhibition of its downstream target mTOR.[83] Another study showed how intake of *p*-hydroxycinnamic acids contributes to protect against endothelial dysfunction, which is associated with the onset of cardiovascular risks. For example, chlorogenic acid through activation of endothelial nitric oxide synthase increases endothelial level of NO, a factor responsible of vasal relaxation. [84]

Polyphenols showed protective and therapeutic effect against development of cancer. As reported in a study, anthocyanidins inhibit cell proliferation in human vulva carcinoma cells

(A431) and human colon carcinoma cells (HT29) interfering in signalling pathway involved in the regulation of cell growth.[63]

Catechins can inhibit tumour cell proliferation by affecting molecular pathways associated with apoptosis, cell cycle, angiogenesis, invasion, and growth factor-related proliferation. It was observed that consumption of green tea, a catechins-rich beverage, reduces risk of breast cancer.

Polyphenolic compounds act as anticancer agent also modulating epigenetic mechanisms involved in tumorigenesis. Examples are resveratrol and genistein, whose anticancer effect has been reported in renal carcinoma cell line, prostate cancer cells, esophageal cell carcinoma and colon cancer cell lines.[85] Isoflavones are phytoestrogens and are therefore able to bind to the estrogen receptor inducing estrogen-like effects *in vivo* in animals and humans. For this reason, preparations based on genistein and daidzein are used therapeutically to relieve menopausal symptoms.[57]

There are also evidences concerning the anti-diabetic activity of phenolic compounds. It was reported that polyphenol-rich extracts from berries inhibit two enzymes, α-amylase and α-glucosidase, involved in starch digestion, one of the mechanisms that control blood glucose levels. Black currant and rowanberry extracts enriched, respectively, in anthocyanins and chlorogenic acid, potently inhibit α-glucosidase. Extracts enriched in tannins, such as raspberry and rowanberry, are more effective in amylase inhibition.[86]

Antimicrobial activity of polyphenols makes them promising candidates to fulfil the growing consumer demand for natural food preservatives, as well as to face emergence and spread of antibiotic resistance among human pathogenic microorganisms. Polyphenols can act as antimicrobial agent according to various mechanisms: the presence of hydrogen bond donors on their structure and the difference in the HOMO and LUMO energy levels between phenolics and lipids of bacterial membrane are responsible for their ability to damage cell membranes; in the intracellular environment, they can bind to enzyme, varying their normal functions; they can also alter fungal morphology, disrupting cell wall. As example, quercetin and phenolic acids modify permeability of cell membrane, while tannins affect activity of intracellular enzymes. However, in the case of some flavonoids and resveratrol was also observed effects on DNA synthesis and replication and on protein synthesis. [87]

Polyphenols also play a role in contrasting inflammation, a defensive process of natural immunity induced by internal (tissue injury or trauma) or external (diet, microbial infection)

factors. The onset of inflammatory process triggers several responses, such as production of pro-inflammatory cytokines and chemokines, hydrolysis of cyclic adenosine monophosphate (cAMP), through phosphodiesterase, release of arachidonic acid (a mediator), production of ROS and RNS. Flavonoids, such as genistein, quercetin, kaempferol, are found to have in impact on these mechanisms, blocking the inflammatory process, that would otherwise become chronic.[88] Many xanthones with potent anti-inflammatory properties are also known. In a recent study, these identified molecules were valuated according to series of drug-likeness analysis parameters, such as physicochemical properties and pharmacokinetics parameters (absorption, distribution, metabolism and excretion, so called ADME). Since xanthones have proven to possess drug-likeness chemical properties are receiving increasing interest from the pharmaceutical industries.[89]

1.4.3 *Olea europaea* L.

Olea europaea L. is a tree native to Asia Minor and Syria. Nowadays, olive cultivation is widespread throughout the Mediterranean basin, with Spain, Italy and Greece being the major producers of olives and olive oil.

From agricultural practices and olives' processing two types of wastes and by-products are generated: leaves, olive pomace (solid) and olive mill wastewaters (liquid). Leaves are wastes resulting from pruning of olive trees, a process carried out in the period between the end of winter and flowering (in the norther hemisphere March and May respectively) which, only in Europe, involves the generation of 11.8 million tons of biomass.[90] The processing of olives to produce extra virgin oil is related to the production of olive pomace and olive mill wastewaters. In particular, for the production of 100 kg of extra virgin olive oils 400 kg of pomaces and 100 kg of wastewaters are obtained.[91] These wastes are characterized by high values of chemical oxygen demand (COD). Notably, for olive pomace, a COD value of 444.068 g/Kg was found.[92] Instead, for olive mill wastewater COD is 45–170 g L^{-1}.[93]

Olive leaves are generally used for direct combustion or as animal feed while pomace to produce biogas. However, the disposal and management of these wastes represent a serious problem from an environmental point of view due both to their high amount produced and the related toxicity.[94] These wastes are indeed an interesting source to apply the *circular economy* model, considering that only 0.5% of the total polyphenols present in olives are found in the extracted oil. In fact these valuable compounds can be recovered from wastes and reused as

food supplements, functional foods, nutraceuticals and pharmaceutical products. To give some examples, olive leaf extracts have been sold as dried leaves, powders, extracts, or tablets used as herbal teas or food supplements.[54] In a recent study, polyphenols deriving from olive mill wastewater are used as potential UV booster in cosmetic formulations.[95]

The main phenolic compounds found in olive oil wastes and by-products belong to different classes and are showed in **Figure 19**. The phenolic alcohols present are tyrosol, homovanillyl alcohol and hydroxytyrosol. Also, oleuropein and oleocanthal, secoiridoid precursors of hydroxytyrosol and tyrosol, respectively, were identified. The flavonoids class is represented mainly by luteolin and apigenin. Phenolic acids, such as caffeic acid, ferulic acid and protocatechuic acid, were also found.[54, 89, 96]

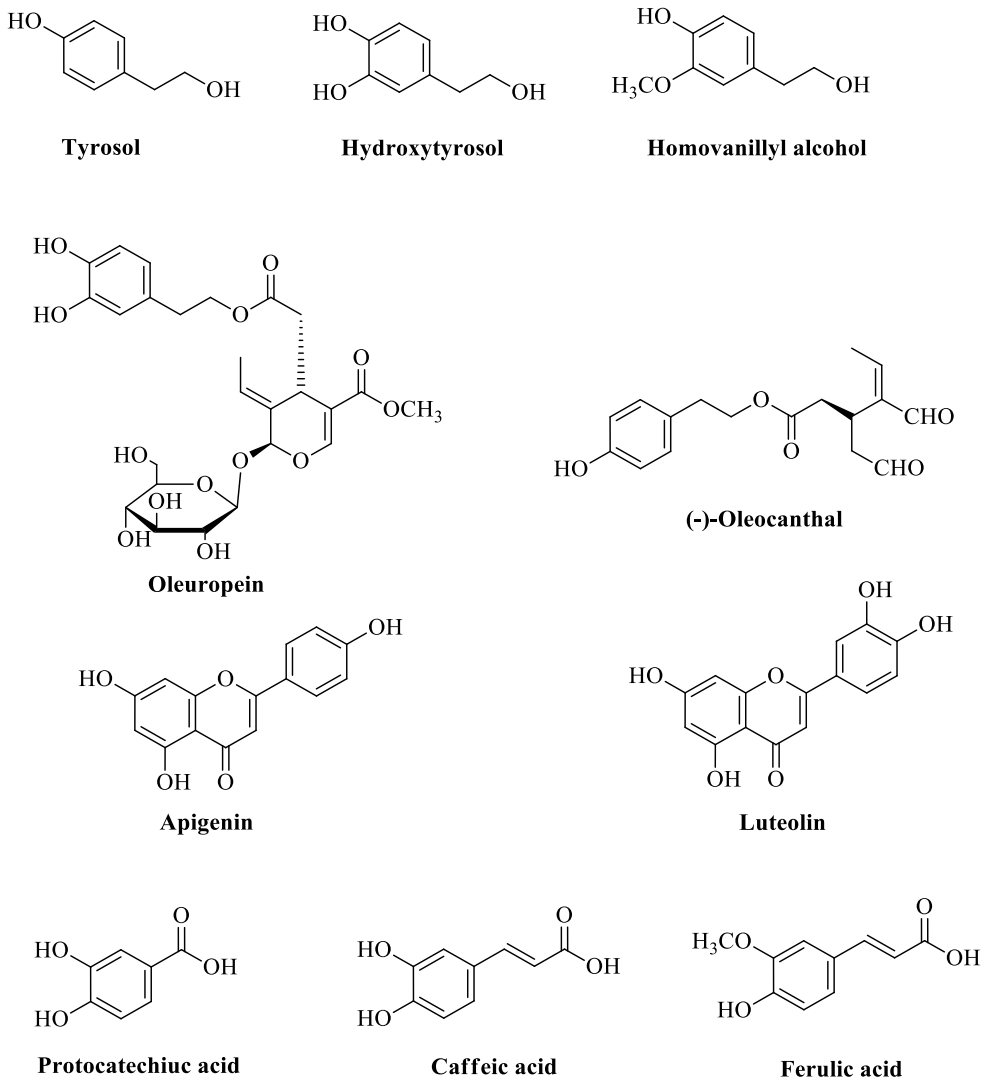


Figure 19

Tyrosol, homovanillyl alcohol, hydroxytyrosol and oleuropein will be discussed in detail for the purposes of this work.

Tyrosol [2-(4-hydroxyphenyl)-ethanol, Tyr] is found in olive fruit (8.1 mg/Kg),[91] pomaces (11.5 mg/Kg)[91] and olive mill wastewater (2.9-4.9 mg/100 ml wastewater).[94] Tyr exerts a long-term protective effect towards ROS production and decrease in GSH induced by oxidized LDL for its ability to accumulate in an intracellular environment.[97] The effectiveness as cellular antioxidant appeared relevant also in the prevention of UVB induced cell death. Treatment of human keratinocytes with Tyr, before exposure to UVB rays, prevents apoptotic cell death. Tyr counteracts damages at mitochondrial level by decreasing poly (ADP-ribose) polymerase, an enzyme which can be activated by ROS. These results are encouraging for developing of functional cosmetics, based on the use of phytochemicals.[98]

Considering the growing number of scientific evidences concerning the need to treat hypertension not only by acting on the level of blood pressure but also by preventing risk factors that lead to the development of cardiovascular complications, Tyr seems promising for new therapeutic strategies. In fact, both *in vitro* and *in vivo* studies, have demonstrated the effectiveness of Tyr to counteract microcirculatory disturbances by affecting blood rheological properties.[99]

Studies performed using animal models and mast cells have highlighted the effect of Tyr on allergic inflammations. Although further studies must be found to establish the exact molecular targets, Tyr likely acts on the signalling pathway that regulates the intracellular level of calcium. A rapid increase in intracellular calcium causes the release of allergic mediators and Tyr has proven to be able to block calcium influx, attenuating anaphylactic shock.[100]

To explore more deeply the molecular mechanisms by which olive oil can influence longevity, going beyond the obvious antioxidant properties of its phenolic components, it was studied the effect of Tyr on longevity in the nematode *Caenorhabditis elegans,* a multicellular model organism. The beneficial effects of Tyr were associated with regulation of heat-shock transcription factor-1 (HSF-1), involved in expression of genes able to extend lifespan in *C. elegans*.[101]

Homovanillyl alcohol [3-hydroxy-4-methoxyphenylethanol, HVa] is found in olive mill wastewater (46 μg/g).[102] It is a good antioxidant and antiradical agent. However, regarding the comparison of the i*n vitro* antioxidant action between HTyr and HVa, conflicting data are

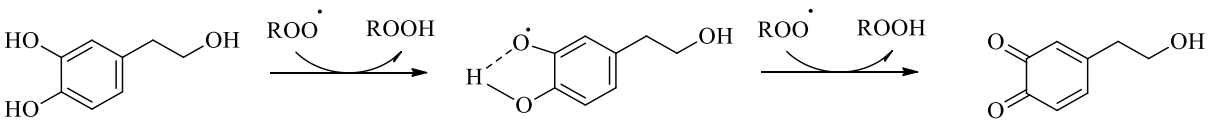
present in literature.[103] In fact, according to the DPPH assay, HTyr resulted more efficient than HVa while, using the ABTS assay, HVa showed a greater antioxidant capacity.[104,105] Moreover, *in vivo* studies demonstrated the ability of HVa to protect against ROS due to the metal chelating properties involved in reducing iron levels in erythrocytes of patients with down syndrome. Altered iron homeostasis results in an increase in ROS, which is responsible for the early onset of Alzheimer's disease-type dementia in patients with down syndrome. Although HVa was less active than HTyr, experimental data evidenced that olive oil polyphenols are promising for dietary and/or nutraceutical approaches in the prevention of dementia.[106]

The effect of HVa on NF-κB driven transcription (a target of several anti-inflammatory agents) has been evaluated in human gastric adenocarcinoma cells. The results demonstrated that metabolic transformation does not inactivate the active principles of olive oil phenols: the anti-inflammatory activity is retained by the metabolite HVa.[107] Its role in preventing inflammation process was confirmed in a study regarding the ability of olive oil phenols to inhibit accumulation of oxysterols, a phenomenon responsible of onset of cardiovascular and degenerative diseases, by regulating release of interleukin-1β, a proinflammatory cytokine.[108]

From a study conducted to investigate the absorption, metabolism, distribution, and elimination of olive oil phenols, resulted that HVa is an HTyr metabolite. In fact, HTyr in the small intestine is subjected to an O-methylation reaction, regulated by catechol-O-methyl transferase, from which HVa is formed.[109]

Hydroxytyrosol [2-(3,4-dihydroxyphenyl)ethanol, HTyr] is a low-molecular weight catecholic compound. It is found in *Olea europaea* fruits (16.6 mg/Kg),[91] in olive pomace (19.9 mg/Kg)[91] and olive mill wastewaters (214 mg/Kg).[91]

Among olive phenols, HTyr is one of the most studied for its biological properties. It exhibits a strong antioxidant activity which can be attributed to the electron donating ability of hydroxyl groups in the *ortho* position and subsequent formation of stable intramolecular hydrogen bonds with the phenoxylic radical (**Scheme 6**).[110]



Scheme 6. Mechanism of antiradical action of HTyr.

HTyr acts as antioxidant scavenging free-radicals, breaking peroxidative chain reactions, preventing lipid peroxidation and inhibiting hypochlorous acid derived radicals.[110] The free-radical scavenging activity was widely evaluated by ABTS and DPPH assays. [111,]

HTyr shows an *in vitro* protective effect against low-density lipoprotein (LDL) oxidation,[111, 112] which plays a primary role in the onset and progression of atherosclerosis. Based on results obtained *in vitro*, several studies were carried out to investigate the effect of olive oil with different phenolic content on *in vivo* capacity to reduce the LDL susceptibility to oxidation. Trial studies showed that the daily intake of a phenol-rich olive oil reduces oxidized LDL in plasma without compromising endogenous antioxidant enzymes (such as reduced glutathione). Therefore, these studies provide evidences that antioxidant phenolic components, although with the contribution of monosaturated fatty acids content, are responsible of cardioprotective effect commonly attributed to olive oil.[113,114] Recently, the European Food Safety Authority (EFSA) claimed that the consumption of olive oil polyphenols (standardized by the content of hydroxytyrosol and its derivatives) prevents oxidative damage from LDL suggesting a daily consumption of 5 mg of HTyr and its derivatives.[110]

HTyr exhibits anticancer effect through an anti-proliferative activity in different tumour cell lines such as colon,[115] breast,[116] prostate,[117] leukaemia,[118] and thyroid [119] cancer cells. It shows inhibitory effect on epidermal growth factor receptor (EGFR), one of the main drivers of colon carcinogenesis. HTyr increased the degradation of EGFR and this effect was selectively directed at colon tumour cells. The same results were observed also *in vivo*, when HTyr was administrated to HT-29 tumour bearing mice.[115] HTyr can inhibit MCF-7 breast cancer cells proliferation through a mechanism that does not involve a classic ER-mediated gene regulation but instead ERK1/2 activation. This mechanism of action makes HTyr promising for its potential application as therapeutics for ER-negative breast cancer. In fact,

the synthetic drug tamoxifen, chemically similar to oestrogens, commonly used for the therapy of ER-positive breast cancer patients, acts as agonist of a G-protein-coupled receptor (GPER) stimulating growth of ER-negative breast cancer cell lines. HTyr, instead, acts by non-mediated gene regulation mechanism and its interaction with GPR30 gives rise to a sustained ERK1/2 activation/phosphorylation. This sustained activation is read in the mitochondrial environment as an antiproliferative signal and the final effect is apoptosis in ER-negative breast cell lines.[116]

HTyr is also as anti-inflammatory agent. At low concentrations (1-10 μmol/L), as achievable in human biological fluids after daily intake of 25-50 mL of extra virgin olive oil, HTyr is able to regulate expression of macrophages cyclooxygenase-2 (COX-2) and related metalloproteins (matrix metallopeptidase 9, MMP-9) involved in the inflammatory process that leads to the onset of atherosclerotic plaques. The significant antioxidant properties of HTyr underlies its capacity to inhibit MMP-9 and COX-2, in fact the ability to scavenge ROS causes the non-activation of the redox-sensitive transcription factor NF-κB, involved in both COX-2 and MMP-9 gene expression.[120]

Some studies demonstrated the neuroprotective effect of HTyr in rat brain slices subjected to an experimental model of hypoxia–reoxygenation *in vitro*, introducing HTyr in incubation medium and measuring the neuronal damage as lactate dehydrogenase (LDH) efflux. The same result is observed repeating experiment also *ex-vivo*, after oral administration of HTyr to rats.[121] Moreover, HTyr has proven to be promising for the development of more effective therapeutic treatment for Parkinson's disease. The strategy adopted to slow the progression of the Parkinson's disease is decreasing production of dopamine metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL), by inhibition of monoamine oxidase (MAO). However, MAO inhibition determines as side effect an increased spontaneous oxidation of dopamine to toxic compounds. HTyr has proven to be able to decrease DOPAL and its oxidized derivatives levels, although the best results have been obtained for a combined action of HTyr with MAO inhibitors, suggesting a possible formulation of HTyr and traditional inhibitors.[122]

An interesting result for immunopharmacology is the ability to modulate the allergy response by inhibiting of mast cells or by binding allergenic molecules and making them less recognizable by the immune system.[123]

Moreover, HTyr shows also antimicrobial activity and as a matter of fact *Olea europaea* has been traditionally used for the treatment of several infectious disorders of bacterial, fungal, and viral origin [124] and, studies on antimicrobial and bacteriostatic activity of olive extracts have been reported since 1970.[125] HTyr and oleuropein have shown a certain degree of effectiveness against fusion of the viral envelope of HIV and the integration of the viral genome into the host cell. Interaction of these molecules with hydrophobic pocket on the surface gp41 fusion complex and catalytic core domain of HIV-1 integrase prevent propagation of viral infection. Results obtained suggested that HTyr may be useful against other viruses with type I transmembrane envelope glycoprotein, including severe acute respiratory syndrome associated coronavirus, Ebola virus, measles virus and avian flu virus.[126] Antifungal activity of HTyr was tested *in vitro* against medically important yeasts and dermatophyte. A powerful activity was observed against *Candida albicans*: HTyr caused a significant cell wall damage, changes in cell surface hydrophobicity and inhibition of germ-tube formation.[127] In 1999 was published the first study regarding the antibacterial activity of HTyr and data reported indicated it as a promising antimicrobial agent for treatment of human intestinal or respiratory tract infections. In particular, HTyr is able to inhibit significantly the growth of bacterial strains of *Salmonella*, *Vibrio* species and *Staphylococcus aureus*.[128] It was also determined the *in vitro* susceptibilities of *Mycoplasma pneumoniae*, *Mycoplasma hominis*, and *Mycoplasma fermentans* (bacteria responsible for respiratory, urinary and genital tract infections) to HTyr and also in this case the results have been encouraging.[129] A recent study was carried out to investigate the bactericidal effects of dietary olive phenolics including Tyr, HTyr and Ole at the molecular level. An *Escherichia coli* model system was used to evaluate the capacity to bind ATP synthase, a molecular target widely used in studies to find drugs against bacterial infections. Olive polyphenols were indeed able to induce inhibition of ATP synthase activity and *E. coli* cell growth, and, among the four olive phenolics tested, only Tyr caused a full inhibition.[124]

Previous studies on the inhibitory activity of raw olive mill wastewater and their total phenolic extracts against plant pathogenic fungi [130] have encouraged a more recent study involving pure HTyr and an extract enriched in HTyr obtained from oil mill wastewater. They were tested on two olive pathogens, *Pseudomonas savastanoi pv. savastanoi* and *Agrobacterium tumefaciens*, responsible for the formation of hyperplastic growths such as galls, tumors or knots in case of infection through wounds due to pruning, frost lesions or hail damage, with consequent serious losses in terms of production. The extract demonstrated a

better antimicrobial action against both pathogens than HTyr alone and this was explained considering the role of the minor phenolic components in the extract. The result obtained makes this approach interesting in view of applying a closed-cycle production process to obtain a natural alternative against phytopathogens.[131]

A final consideration can be made regarding the excellent safety profile of HTyr. No side effects were detected, even at very high concentrations (up to 500 mg/kg/d);[133] in addition, HTyr is not mutagenic, and not genotoxic compound with No Observed Adverse Effects Level (NOAEL) classification. However, it should be noted that toxicity was evaluated mostly *in vitro* and in animal models, so it is necessary to extend the studies on untoward effects in humans, considering the increasing appeal that HTyr has in the field of nutraceuticals and functional foods.[111, 132]

Oleuropein (Ole) is a secoiridoid compound mainly found in olives (19.2 mg/Kg)[91] and leaves (26.47 mg/Kg),[94] but present only at low concentrations in olive by-products (7.6 mg/Kg and 16.5 mg/Kg in pomaces and in olive mill wastewater, respectively).[91] This is due to the action of enzymatic degradation (such as β-glucosidase) during olive oil production. These enzymes can hydrolyse Ole producing glucose, elenolic acid and HTyr (**Figure 20**).

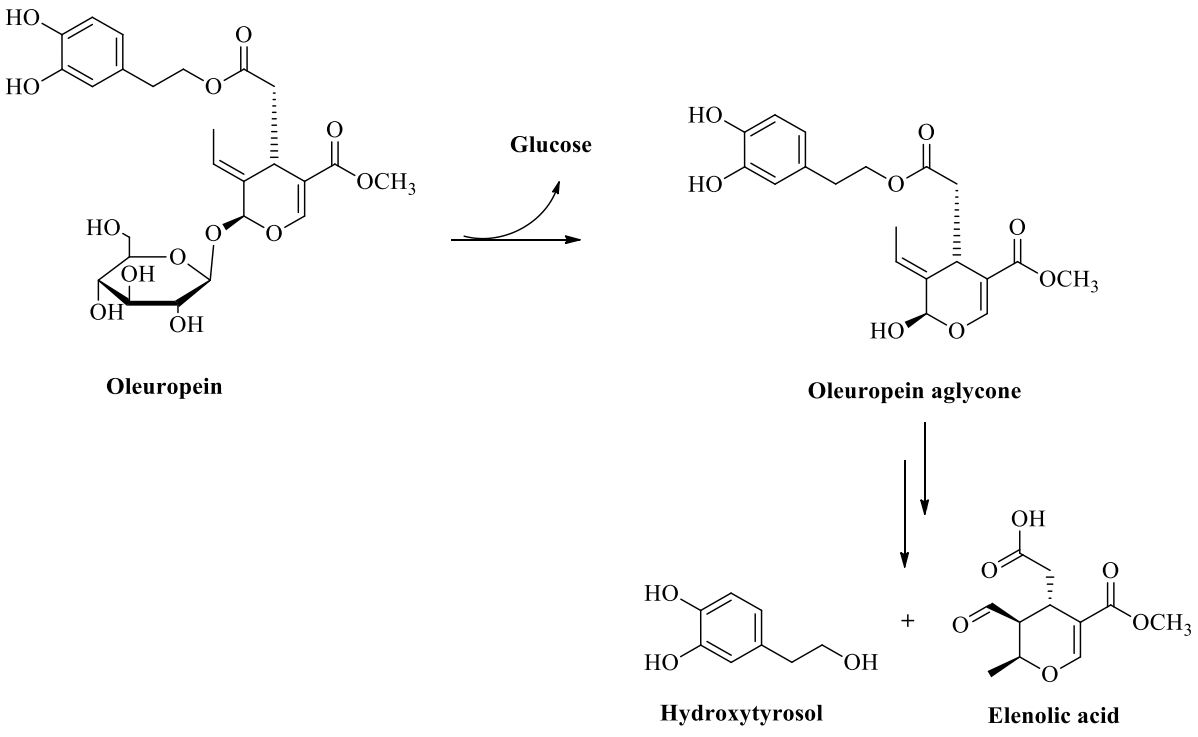


Figure 20. Enzymatic conversion of oleuropein into hydroxytyrosol.

In addition to the antioxidant property,[133] Ole showed cardioprotective, anti-cancer, antimicrobial, anti-inflammatory, and neuroprotective effects. From *in vivo* studies it appears that the administration of Ole to rabbits leads to a reduction in the levels of total cholesterol and triglycerides, which are very well known risk factors of heart attack.[134] Moreover, Ole has decreased cell viability, inhibited cell proliferation and induced apoptosis in breast, colon, liver, thyroid cancer cell lines.[135] A significant decrease in the concentration of interleukin-1β, a mediator of inflammation, was observed in human mononuclear cells as consequence to the addition of Ole.[136]

Olive leaf extract has proven to be promising agent to regulate the composition of the gastric flora, thanks to their antimicrobial activity against *H. pylori* and *C. jejuni*.[137] Ole aglycone has showed to play a role in preventing aggregation of α-synuclein, a protein involved in the pathogenesis of Parkinson's disease.[138]

1.4.4 *Castanea sativa* Mill.

The sweet chestnut (*Castanea sativa* Mill.) is a medium-large deciduous tree belonging to the Fagaceae family. In Europe, it is spread over an area of more than 2.5 million hectares, most of which is concentrated in the Mediterranean area.[139] The main producing country is Italy (34.5%), followed by Greece (23.7%) and Portugal (19.7%).[140] The production is generally meant for the food industry, which uses seeds to obtain products such as gluten-free flours, marron-glacés, chestnut puree. Since the edible part is minimal, tons of wastes, such as pericarp (outer shell), integument (inner shell) leaves and burs, are generated after the industrial process. In addition, forestry processes also involve the formation of wastes and by-products (i.e. pruning leaves and bark), as well as industrial woodworking for furniture production.[140,141] The approach for the disposal of these residues consisted in the incineration of the inedible material, with the aim to produce energy while the wastes deriving from the harvesting process was generally left on the ground, favouring the growth of larvae that could damage the plants.[140]

In recent years, these treatments have been re-evaluated. Sweet chestnut is a source of precious bioactive compounds, as polyphenols, and the recovering of these molecules from wastes and by-products has become very attractive. Phenolic acids (ellagic and gallic acid, **Figure 16**), flavonoids as rutin and its aglycone quercetin (**Figure 12**), apigenin (**Figure 19**)]

and tannins are the main phenolic compounds found in chestnut by-products.[142] Leaves have the highest polyphenol content, followed by the outer shells (28.5 and 9.4 mg/g extract, respectively), while inner shell is the part containing lowest phenolic compounds (1.88 mg/g extract).[140]

Quercetin, rutin, ellagic acid were found in extracts from *C. sativa* leaves.[143,144] Thanks to this phenolic content, *C. sativa* leaf ethanolic extracts have been demonstrated to be effective in the prevention and treatment of oxidative stress-mediated diseases and photoaging and for this reason are excellent candidates for topical formulations.[145] Extracts from chestnut shells were obtained by using alkaline aqueous solution and, due to their high content in condensed tannins, they have been proposed as a suitable alternative to chromium in leather tanning, a process that poses a serious pollution problem. In addition, tannins have a structure and reactivity that make them suitable to replace phenol in adhesive formulations.[142]

Among the products that can be obtained from sweet chestnut aqueous extract there is the so-called tannic acid, which contains hydrolysable tannins. Although its chemical composition has not been fully understood, it can be said that it consists in a mixture of gallic acid esters of glucose. The beneficial effects of tannic acid on human health are well-known: it possesses antimutagenic, anticancer, antioxidant properties, as well as the ability to reduce serum cholesterol.[142] Recently, sweet chestnut wood has been used as raw material to obtain two standardized fractions in hydrolysable tannins content.[146] These fractions are commercially available and are promising to find applications in several fields.

The antimicrobial properties of hydrolysable tannins are well known,[147] therefore formulations of phytosanitary products based on sweet chestnut extracts could replace traditional copper-based pesticides. An application in the zootechnical industry could also be very interesting. Satisfactory results were obtained when the commercial sweet chestnut tannin extract was integrated in the common diet of two laying hens in order to study the effects on the cholesterol content and nutritional quality of the eggs.[148] The analytical data obtained from HPLC-DAD-ESI-MS analysis of the commercial fractions, showed that the main compounds were vescalagin and castalagin (**Figure 17**), ellagic acid and gallic acid. For the purposes of this work, gallic acid will be discuss in detail below.

Gallic acid [3,4,5-trihydroxybenzoic acid, GA] is a low molecular weight phenolic compound widely spread in the plant world. It can be found, in free form or as a derivative, in gallnuts, grapes, sumac, witch hazel, tea leaves, hops, oak bark, different berries, mango, rhubarb and

soy.[149,150] The most common commercial source of GA are tannins, fourth most abundant class of constituents of plants, from which GA is obtained by chemical or enzymatic hydrolysis; it can be also produced by microbial fermentation, by taking advantage of fungal or bacterial tannases.[151]

Many applications of GA in industry are known. It is widely used in the production of paper, for the stabilization of collagen in the leather manufacturing process and as a base material for inks, paints, dyes.[150] Moreover, in analytical research it is used as a standard antioxidant in total phenolic content assay, where results are commonly expressed as gallic acid equivalents (GAE).[152] However, given its numerous and powerful biological activities, it is plausible to find employment in several other industrial sectors.

GA is a strong antioxidant compound and this activity is due to the number and position of the hydroxyl groups on the aromatic ring.[153] Compared to catechol analogues, the presence of an additional *ortho*-hydroxylic group tends to further stabilize the radical formed and therefore to increase the antioxidant effect (**Figure 21**). The removal of the hydrogen atom is favoured in the *para*-position, where bond dissociation enthalpy is the lowest due the possibility of stabilizing radical by two intramolecular hydrogen bonds on either sides. A further explanation of the strong antioxidant efficiency of GA can be given by considering the concept of *captodative* or *push-pull* effect,[154] a synergetic stabilizing action exerted by an electron-donor and an electron-withdrawing substituent simultaneously present on a molecule. In GA, it is thought that the presence of the electron-donating OH group in the *ortho*-position and the electron-withdrawing -COOH group in the *para*-position can have a stabilizing effect on radical center.[149]

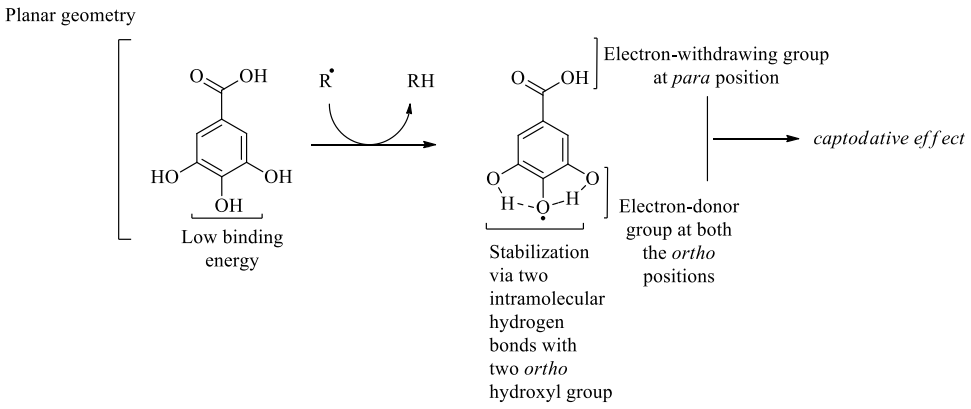


Figure 21. Structural factors responsible for the strong antioxidant activity of gallic acid.

DPPH assay and ABTS assays confirmed a stronger antioxidant activity of GA compared to ascorbic acid, trolox, uric acid and vitamin E.[149] GA is able to act as antioxidant also by restoring the depleting levels of endogenous antioxidant defence systems. In senescence-accelerated mice, after administration of GA an increased activity of enzymes catalase and glutathione peroxidase and a reduction of the extent of lipid peroxidation in the liver, brain and kidney were observed.[155]

The powerful chelating ability of GA towards transition metals which is due to the two adjacent hydroxyl groups on the aromatic ring, led to think about possible effects of GA on the bioavailability of metals. However, in a study regarding the effect of polyphenols on absorption of Fe (II), the most abundant transition metal in the body, it was found that GA is able to promote the absorption of iron in Caco-2 cells. Anyway, Fe (II) is chelated by GA and the degree positively correlates to increase in pH. The complex GA/Fe (II) has an intense blue colour, and it was used to produce writing inks (iron gall inks). Moreover, a series of GA and azo complexes with different metal ions (Co, Ni, Cu) were also prepared and some of them showed activity against bacteria and fungi.[149]

GA shows a pro-oxidant action in a Fenton-type system. The latter is a system in which the simultaneous presence of H_2O_2 and a transition metal in the reduced form results in production of the strong oxidant hydroxyl radical (**Scheme 7**). It is widely studied due to its importance in biochemistry, medicine, food, environmental chemistry, and materials research.

Scheme 7. Fenton-type system.

An investigation of the behaviour of GA in a such system revealed that, as a function of the molar ratio Fe (III)/GA, GA can act as antioxidant or pro-oxidant. In particular, if molar ratio GA:Fe < 2:1 prevails the ability of GA to reduce Fe (III) leading to the formation of Fe (II) which accelerates the production of the $OH^{\cdot}$ radical species. If molar ratio GA:Fe > 2:1, the overall effect of GA is antioxidant, showing its potent radical scavenger activity against the $OH^{\cdot}$ radical.[156]

The pro-oxidant action of GA is thought to be responsible for its potent anti-cancer activity. The activity against different cancer cells (such as leukaemia,[157] prostate,[158] lung,[159] colon[160], breast [161] cancer) involves several oncogenic pathways due to its capacity of regulating cell cycle, metastasis, angiogenesis, and apoptosis.[149] GA was found to induce apoptosis selectively in tumour cells, which resulted more susceptible to GA action than healthy cells. To clarify reasons beyond this selective cytotoxicity, was conducted a study using rat hepatoma cells. As reported, cancer cells, unlike healthy cells, do not have the appropriate enzymatic defence system that would allow them to counteract the damages of H_2O_2 produced by the pro-oxidant action of GA.[162]

The same ROS-dependent apoptosis was observed also in HCT-15 colon cancer cell lines after treatment with GA, which caused a time-dependent cell cycle arrest at the sub-G1 phase.[160] For what concerns its effect on the cell cycle, in another study GA showed to be able to inhibit proliferation of prostate cancer cells through activation of Chk1 and Chk2 kinases, resulting in activation of cyclin B1, Cdc2 and Cdc25C, which are involved in G2/M phase of cell cycle.[158] It was also investigated the effect of GA on glioma, the most common primary malignancies in the central nervous system. GA can inhibit glioma cell viability, proliferation, and invasiveness by downregulating signalling pathways of PI3K/Akt and Ras/MAPK, kinase proteins involved in control of cell survival, proliferation, motility, and metabolism.[163]

GA demonstrated to be a good anti-inflammatory agent. It induces endothelium-dependent contraction and inhibited endothelium-dependent relaxation of rat thoracic aorta. This vascular effect is probably associated with capacity of GA to inhibit endothelium production of nitric oxide, a molecule which plays an important role in inflammatory disorders.[164]

The antimicrobial properties of GA are also well known. It shows a significant inhibitory effect on the growth of *Helicobacter pylori*,[165] a Gram-negative spiral-shaped bacterium that colonizes the human stomach causing a serious transmissible infectious disease, as well as onset of gastric atrophy, peptic ulcer disease, gastric adenocarcinoma. It was also reported the antimicrobial activity of GA against four human pathogenic bacteria (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, and Listeria monocytogenes*) of biomedical prominence. Investigation regarding the mechanism of action demonstrated that GA interacted with the surface of bacteria, promoting changes in physicochemical surface properties of membrane, with consequent release of intracellular content leading to cell death. The phenolic acid-lipid interaction is stronger in Gram-negative bacteria, due to the highest

lipid content of cell walls and this explains the greater susceptibility of the two gram-negative bacteria (i.e. *E. coli*, *P. aeruginosa*) than Gram-positive ones.[166] GA, tested against *Candida albicans*, demonstrated to reduce the activity of the enzyme of the membrane CYP51 and of squalene epoxidase, targets for azole antifungals, with an efficacy comparable to the standard drug fluconazole. The results were confirmed also in an *in vivo* study, carried out using immunosuppressed mice susceptible to fungal infection.[167] These evidences support a recent study that has been led to develop innovative multi active-substance loaded liposomal formulations containing quercetin and GA for treatment of the symptoms of vulvovaginal candidiasis.[168] In addition, GA affects Herpes Simplex Virus-type 1 (HSV-1) infectivity by detaching viruses that have already bound to cells. Probably, the expression of ICP27, gC, gD, and VP5 proteins was inhibited in infected cells treated with GA, resulting in a viricidal effect on the new progenies of virus and a non-spread of cell-to-cell infection.[169]

2. Aim of the research

Phenolic compounds are secondary metabolites exhibiting interesting biological properties such as antioxidant, anticancer, anti-inflammatory and antimicrobial activities. Their large occurrence in plants, dietary vegetables and fruits implies that agro-industrial wastes and by-products are rich of these valuable compounds. At the same time, the disposal of these materials represents an environmental and economic problem for the high toxicity and cost. However, according to the green chemistry principles and the circular economy model, they are a source of bioactive compounds to valorize and this topic was studied from more than ten years in the Laboratory of Organic and Natural Products of the Department of Agriculture and Forest Sciences of the University of Tuscia.

The olive and the sweet chestnut are very common tree species in Italy and this implies a significant spread throughout the national territory of companies dealing with the processing of such matrices. In this light, the experimental activity carried out during the PhD program was focused on the valorization of phenolic compounds found in wastes and by-products deriving from olive oil and sweet chestnut processing. In particular, the attention was devoted to selected small phenols as tyrosol, homovanillyl alcohol, hydroxytyrosol, oleuropein, gallic acid and the research activity carried out can be divided into three parts:

1. Lipophilization of phenolic compounds found in olive oil and sweet chestnut wastes and by-products under green chemistry conditions. Despite their important biological activities, the hydrophilic nature of some phenolic compounds represents a drawback when the solubility in fatty and oily matrices or a better bioavailability in cellular environmental is required as for some food, cosmetic and pharmaceutical applications. Tyrosol, homovanillyl alcohol, hydroxytyrosol and gallic acid were esterified under green chemistry conditions in order to obtain the corresponding derivatives showing alkyl chains of variable length. The optimized procedures on pure compounds were then applied to an hydroxytyrosol-enriched extract and a gallic acid-enriched extract obtained from olive oil and sweet chestnut wastes and by-products, respectively, through eco-friendly and economically sustainable technologies.

2. Preparation and characterization of novel poly (vinyl alcohol)-based films using hydroxytyrosol and oleuropein-enriched extracts for active antioxidant food packaging. Active food packaging is an innovative strategy strongly supported by both academia and industry, which aims to increase food shelf life and preserve food quality. It consists in the

incorporation of active ingredients such as antioxidants into polymeric materials to achieve their controlled release over time into food, in order to avoid or delay oxidative deterioration. An hydroxytyrosol-enriched extract and an oleuropein-enriched extract, obtained from olive oil wastes and by-products, were used as active ingredients for poly(vinyl) alcohol-based films. The novel formulations were characterized about their morphological, optical and antioxidant properties to test their effectiveness as active packages.

3. Synthesis of phosphodiester dimers of tyrosol, homovanillyl alcohol and hydroxytyrosol as potential anti-Alzheimer's disease. Phenolic compounds showing radical scavenging activity are promising for both prevention and therapy of Alzheimer's disease. In this light, tyrosol, homovanillyl alcohol, and hydroxytyrosol were used as scaffolds for the design and synthesis of a mini-library of derivatives containing two phenylethanoid fragments linked through a phosphodiester bond. All dimers were evaluated relatively to different aspects involved in Alzheimer's disease pathology evidencing the best results for hydroxytyrosol-derived compounds.

The research activity was carried out in collaboration with the research group of Prof. Annalisa Romani (University of Florence), Prof. Luigi Torre and Debora Puglia (University of Perugia), Prof. Armando Zarrelli and Di Fabio Giovanni (University Federico II of Naples).

The results obtained led to the following publications on international journal peer reviewed:

Bernini, R.; Carastro, I.; Santoni, F.; **Clemente, M**. Synthesis of lipophilic esters of tyrosol, homovanillyl alcohol and hydroxytyrosol. *Antioxidants* **2019**, 8, 174; doi: 10.3390/antiox8060174.

Luzi, F.; Pannucci, E.; **Clemente, M.**; Grande, E.; Urciuoli, S.; Romani, A.; Torre, L.; Puglia, D.; Bernini, R.; Santi, L. Hydroxytyrosol and oleuropein-enriched extracts obtained from olive oil wastes and by-products as active antioxidant ingredients for poly-(vinyl alcohol)-based films. *Molecules* **2021**, *26*, 2104. doi: 10.3390/molecules26072104.

Romanucci, V.; Giordano, M.; De Tommaso, G.; Iuliano, M.; Bernini, R.; **Clemente, M.**; Garcia-Viñuales, S.; Zarrelli, A.; Di Fabio, G. Synthesis of new tyrosol-based phosphodiester derivatives: effect on amyloid β aggregation and metal chelation ability. *ChemMedChem*. **2021**, *16*, 1172-1183. doi: 10.1002/cmdc.202000807.

3. Lipophilization of phenolic compounds found in olive oil and sweet chestnut wastes and by-products under green chemistry conditions

3.1 Introduction: lipophilicity concept and synthetic strategies in polyphenols-chemistry

Lipophilicity is one of the most studied physicochemical properties in drug discovery and development affecting effectiveness, distribution and clearance. In addition, it plays an important role in cosmetic, pharmaceutical and food applications when the solubility in non-aqueous media is required. Then, lipophilicity is often an effective guide for modifying the structure of molecules to improve their biological and/or pharmacological properties.[170] Generally, it is quantified through a parameter, the Log P, defined as the decimal logarithm of P, where P is the partition-coefficient of an un-ionized compound in two immiscible phases as 1-octanol and water/buffer at the equilibrium. Depending on this value, all compounds, including phenols can be divided into lipophilic or hydrophobic or apolar (Log P=0-3) and hydrophilic or polar (Log P=3-10).[171]

Hydrophilic polyphenols include tyrosol (Tyr), homovanillyl alcohol (HVa), hydroxytyrosol (HTyr) and gallic acid (GA). In some cases, the hydrophilicity of these compounds can represent a drawback for using them in pharmaceutical as well as in food and cosmetic industry. Then, different strategies of lipophilization have been developed to modulate their physicochemical properties. Lipophilization consists of covalently introducing a hydrophobic portion into a compound without altering the functional moieties responsible for the specific biological activities. One of the strategies used in drug discovery is the halogenation.[172,173] The introduction of a halogen or a trifluoromethyl residue into a molecule leads to a better capacity to membrane binding and permeation. Moreover, this chemical modification results in stabilizing protein-ligand complexes, due to capacity of halogen atoms to form intermolecular bonds similarly to H-bonds, with a possible improvement of drug metabolism. For example, the replacement of a CH_3 group with a CF_3 increases the stability in human liver of an analgesic drug. This strategy has been used also in polyphenols-chemistry with the synthesis of halogenated flavonoids [174] and phenethyl alcohols trifluoroacetate.[104] Another strategy adopted for increasing the lipophicility of polyphenols is based on the introduction of an alkyl chain of variable length, without altering the phenolic moiety responsible for the biological activities. These chemical modifications can be carried out by etherification and esterification reactions using chemical or enzymatic methods.[175, 176 177, 178] The resulting compounds, generally named lipophenols, have proved to be particularly interesting because

the modification of the chemical structure, in addition to making an improvement in the pharmacokinetic profile, also allows to combine the beneficial effects of polyphenols and fatty acids.[179] Lipophenols showed antioxidant action with a behaviour in agreement with the so-called Porter's polar parodox.[180] This theory is so defined because, contrary to what one might expect, polar compounds are more efficient in their antioxidant action in bulk oils and fats than their corresponding apolar homologues. Instead, the latter best performs their action in emulsified systems (emulsions, micelles, liposomes). The explanation of this phenomenon lies in the concept of interfacial oxidation. In fact, in a biphasic system, the oxidation occurs at the interface. In oily solution at the oil-air interface, where there will be a higher concentration of polar antioxidant and so they will carry out the antioxidant action. In an emulsified system, oxidation takes place in the oil-water interface where the lipophilic antioxidants are able to concentrate more than the polar homologues, which remain mostly in the aqueous phase.[181] However, in many cases the relationship between the antioxidant capacity and the hydrophobicity of a molecule in emulsion is not as linear as expected but has a parabolic trend. This is the so-called cut-off effect,[182] a phenomenon observed also for other biological activities, consisting in an increase of the activity as the alkyl chain is lengthened, with a threshold for generally the middle-length chain, after which further chain extension leads to a drastic decrease in antioxidant capacity. A study has reported the evaluation of the antioxidant activity in emulsified medium for a complete homologous series of C1-C18 chlorogenic acid esters, and the resulting cut-off effect was explained assuming that the longer chains compounds tend to form aggregates greater than the tendency to move towards the interface and that thus the concentration of antioxidant at the site of oxidation decreased.[181] The high hydrophilic nature of HTyr (Log P=0.09)[105] has led to the synthesis of lipophilic alkyl ethers using alkyl iodides and potassium hydroxide in dimethyl sulfoxide (**Figure 22**).[183] In agreement with the Porter's polar paradox, the hydrophobic derivatives have shown in the lipid matrix a slightly lower antioxidant power than HTyr. Instead, they were more efficient in hydrophilic media, although a chain length effect was observed and only derivatives with a number of carbon atoms less than or equal to 4 on the side chain showed a better antioxidant action than HTyr.[183] The anti-platelet effects of some of these derivatives have been evaluated both *in vitro* and *in vivo* experiments, demonstrating a higher cardioprotective effects compared to HTyr.[184] Alkyl ethers of Tyr and HVa have also been synthesized (**Figure 22**).[185]

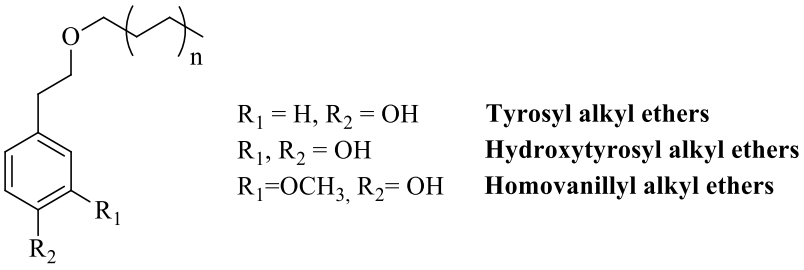


Figure 22

Acylation of phenolic compounds with saturated and unsaturated fatty acids, through an esterification reaction, has been a widely attempted approach. The most common method to prepare these compounds is the reaction between alcohols and carboxylic acids or acyl chlorides and anhydrides, in presence of a catalyst. Fisher reaction is an example of acid-catalysed esterification. Both strong Bronsted acids (hydrochloric acid, sulfuric acid, camphorsulfonic acid, *p*-toluensulphonic acid) and milder Lewis acids (boron trifluoride etherate, scandium trifluoromethanesulfonate) are commonly employed as activators of the carboxylic group.[186] Acylation of alcohols with acyl chlorides and anhydrides is generally carried out using pyridine or 4-substituted derivatives, such as 4-dimethylaminopyridine (DMAP), as nucleophilic catalyst.[187] Derivatization of vanillin, carvacrol and quercetin with linoleic acid has been performed by a Fisher esterification reaction using concentrated sulfuric acid as a catalyst.[175] Lipophilic phenols have been prepared also by enzymatic reactions through *Candida antarctica* lipase-catalysed acylation and methylation. The use of a biocatalyst allowed to modulate the regioselective substitution of the -OH groups to obtain the corresponding monoester derivatives. Moreover, it has been as well reported the enzymatic synthesis of malvidin 3-glucoside derivatives using saturated fatty acids with variable length chain.[176] Tyr and HTyr alkyl esters were prepared by direct esterification [188,189,190,191] or transesterification with vinyl,[192] ethyl [177] or methyl ester [193] of fatty acid, using lipase as biocatalyst. Several studies have been carried out to verify that lipophilization does not affect the antioxidant efficacy of the starting compounds. Results obtained by application of Rancimat method, the most common method used to evaluate antioxidant efficacy in lipophilic food matrices, has showed that the antioxidant behaviour of HTyr and Tyr lipophilic derivatives in oils agrees with the polar paradox, in fact the most polar compound in each series is the most effective antioxidant.[193, 194] Instead, in the case of ABTS assay, generally carried out in a hydrophilic medium, only Tyr esters disregard what is expected

according to polar paradox and are less efficient than Tyr.[193] In a fish oil-in-water emulsion system, it has been observed a parabolic trend of antioxidant activity of esters as function of chain length, the "the cut-off effect" previously described. The antioxidant capacity increases until it reaches a maximum (for C8-C10 derivatives) and then decreases due to the surfactant properties of the lipophilic derivatives. Plotting the surfactant effectiveness against the alkyl chain length, a parabolic shape with a maximum around 8-10 carbon atoms can be observed. Then, the best surfactants are also the best antioxidants, because they can reside at oil-water interface, creating a protecting barrier for the underlying oily phase.[192] For HTyr esters a similar behaviour has been reported also in hydrophilic environment. According to the results obtained applying the ABTS assay, an increased antioxidant efficacy has been observed up to the medium-sized (C4-C9) lipophilic derivatives esters, whereas the elongation of the acyl chain no longer has any effect.[195]

Gallic acid (GA) represents another bioactive phenolic compound characterized by a high hydrophilic nature (Log P=0.42).[196] Lipophilization of GA via a direct esterification reaction with alkyl alcohols to obtain the corresponding esters (**Figure 23**) is well known in literature.

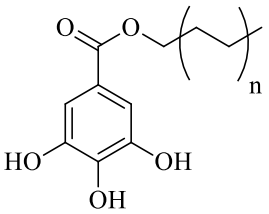


Figure 23

Common chemical methods to obtain alkyl gallates are based on the use of both acid and basic activators of the carboxylic moiety. The conventional Fisher esterification, involving the use of strong acid catalysts, such as sulphuric acid [197,198] and *p*-toluensulphonic acid [199,200] has been employed. Use of carbodiimides as activators was another strategy adopted.[201,202] Dicyclohexylcarbodiimide was rarely applied in the formation of ester bonds due some drawbacks, i.e. yields not always high, and tendency to form dicyclohexylurea as a by-product which makes more laborious the chromatographic purification.[203] New alternative carbodiimide reagents as N,N-diisopropylcarbodimide were used according to Neises and Steglich conditions, [204] i.e. in presence of 4-dimethylaminopyridine as nucleophilic

catalyst.[202] However, greener alternative methods to obtain alkyl gallates were applied. Tannase [205] and lipase [206,207] were used as biocatalysts to carry out a chemoselective direct esterification of gallic acid with alkyl alcohols. The biological activities of these lipophilic derivatives were widely investigated and, in some cases, a correlation between the increase in lipophilia and the activity studied has been observed. Due the presence of the pyrogallol moiety, alkyl gallates are strong antioxidant agents. The antioxidant activity agrees with the Porter's polar paradox*:* in an oil system, GA has proven to be clearly more powerful than its derivatives, while among alkyl gallates the best result has been obtained for methyl gallate.[208] Like already observed for HTyr fatty acid esters, a so-called cut-off effect due to their surfactant properties was detected in a fish oil-water emulsion. By plotting the percentage of oxidation inhibition with respect to the length of the alkyl chain, a parabolic trend has been observed. The maximum corresponding to the medium alkyl chain derivatives, which have proved to be the best surfactants.[209] The radical scavenging ability of alkyl gallates was evaluated by DPPH and ABTS assays. The tested compounds have shown a comparable antioxidant capacity or, in some cases, only slightly lower than GA.[210] Furthermore, the effect of the chain length on the antioxidant efficiency is not very significant as the gallates have a very similar scavenging activity.[197,198,211,212] The antioxidant action of alkyl gallates is not limited to their capacity of quenching radical species; for this reason, these compounds have been defined multifunctional antioxidants.[213] In fact, they have shown an inhibitory activity against pro-oxidant enzymes, such as lipoxygenase and xanthine oxidase; in addition, a relation between lipophilicity and enzymatic inhibition has been observed. In particular, as lipophilia increases, the inhibitory action increases according to a parabolic trend, reaching a maximum for esters with a number of carbon atoms on the chain between 12 and 16. The presence of a hydrophobic portion on the molecule has proved essential to exert this inhibitory action, since GA as such is not able to inhibit the peroxidation of linoleic acid catalyzed by lipoxygenase, while it has been able to inhibit xanthine oxidase only at very high concentrations. Although the presence of the pyrogallol moiety is important for the interaction with the aromatic rings of the phenylalanine residues, the flexibility and hydrophobicity of the long alkyl chain constitute essential characteristics for anchoring to the active site and preventing the enzyme substrate from entering.[213] Moreover, both GA and alkyl gallates have been able to bind the superoxide generation site of xanthine oxidase, modifying the enzyme by reduction. However, lipophilic derivatives have been shown to be significantly less efficient than GA and their effect on superoxide anion generation has not been affected by chain length.[213] A further aspect of their antioxidant action is the capacity to chelate the

transition metal ions, preventing them from taking part in reactions such as the Fenton's , involved in the production of ROS.[213] Alkyl gallates showed also antimicrobial activity. The best antifungal action against *Saccharomyces cerevisiae* was observed for compounds with a middle length alkyl chain; instead, for those with a shorter or longer chain, efficacy decreases considerably.[202] The cut-off effect is not so evident when alkyl gallates were tested for their antibacterial activity against *Staphylococcus aureus* and *Salmonella choleraesuis*. In fact, starting from the most active compounds, no variation of the bactericidal action was observed as consequence of the addition of the methylene group on the alkyl chain.[214,215] The poor fungicidal action of highly hydrophobic derivatives has been explained considering the different mechanism by which alkyl gallates act against microorganisms, depending on whether they are prokaryotes or eukaryotes. The antibacterial action of alkyl esters was shown to be based on the inhibition of the cellular respiration system. The electron transport chain in prokaryotic microorganisms is located inside the cytoplasmic membrane, therefore the high activity observed up to dodecyl gallate, can be explained by the increasing ability to cross the membrane. In eukaryotic organisms, such as fungi, the electron transport chain is found in the mitochondria and alkyl gallates with increasing hydrophobicity would have difficulty to cross the cytosol.[215] Indeed, it has been discovered that in fungi they behave mainly as surfactants and the highly hydrophobic molecules do not adsorb on the cellular surface but remain in the surrounding aqueous medium probably in the form of insoluble monolayer or diffused film. Then, compounds with more than 10 carbon atoms on alkyl chain are inactive against the tested fungi.[214] However, in other studies concerning the activity of alkyl gallates against a series of human [216] and plant [196] pathogenic fungi, a good fungicidal action was observed up to dodecyl gallate. These results disagreed with what has been observed previously and suggested that alkyl gallates could act not only as surfactants. In particular, in the case of plant pathogenic fungi, it has been thought that alkyl gallates can inhibit the mitochondrial complex II.[196] Alkyl gallates showed also anticancer activity and this effect seems to be linked to their ability to induce apoptosis on cancer cells.[217]

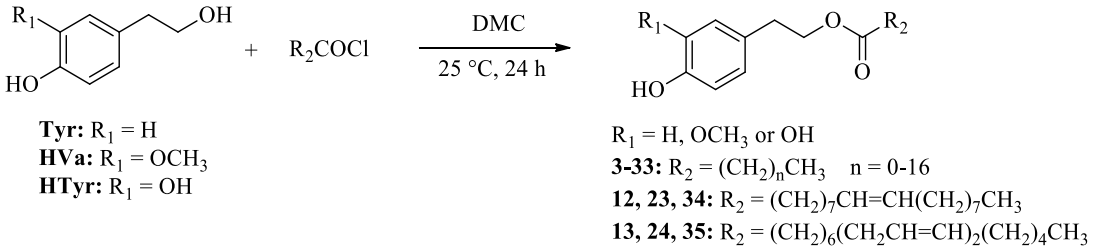
Based on the relevant biological properties of lipophilic phenols above described, a first topic of the research carried out during the PhD program, has been devoted to design and develop eco-friendly synthetic procedures for preparing Tyr, HVa, HTyr e GA esters. After the optimization of the procedure, pure compounds were isolated. Finally, the best experimental conditions were applied to HTyr e GA-enriched extracts obtained, respectively, from *Olea*

europaea L. and *Castanea sativa* Mill. wastes and by-products, as valuable examples of recovery and reuse of these materials according to the circular economy model.

3.2 Results and discussion

3.2.1 Lipophilization of tyrosol, homovanillyl alcohol, hydroxytyrosol and an hydroxytyrosol-enriched extract

Based on the preliminary results previously obtained in our Laboratory,[218] a large panel of Tyr, HVa and HTyr lipophilic esters has been synthetized under green chemistry conditions, as depicted in **Scheme 8**.



Scheme 8. Lipophilization of Tyr, HVa and HTyr.

The solvent of choice to carry out these reactions was dimethyl carbonate (DMC), a chemical produced through a green catalytic process by ENICHEM (Italy). The low toxicity, the absence of irritating or mutagenic effects and the high biodegradability qualify DMC as an environmentally friendly chemical useful in organic synthesis (**Table 2**).[219]

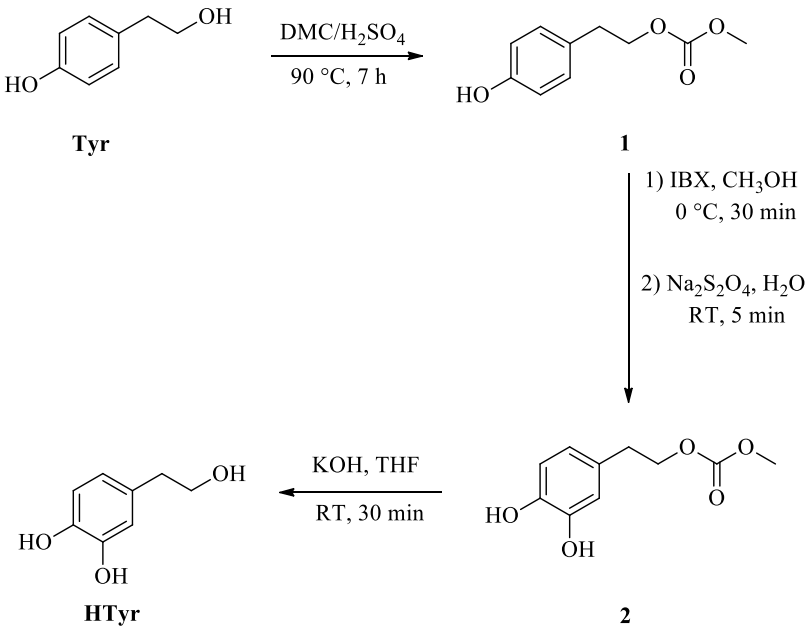
In addition, DMC can be used also as reagent. In fact, it can react with a nucleophile through two electrophilic centers, the carbonyl and the methyl groups, which are classified according to Hard-Soft Acid-Base theory as the hard and soft centre, respectively. The presence of an acid or a base as catalyst in the reaction environment emphasizes the hard or soft character of the centers on the reagents involved in the reaction. As a consequence, DMC can react with nucleophiles as a carboxymethylating or methylating agent and represents an eco-friendly alternative to more hazardous and toxic reagents such as methyl halides and methyl sulphate.

Table 2. Properties of DMC.[219]

Property	***Value***
Oral acute toxicity (rats)	LD_{50} 13.8 g/Kg
Acute toxicity per contact (cavy)	LD_{50} > 2.5 g/Kg
Acute toxicity per inhalation (rats)	LC_{50} 140 mg/L (4 h)
Mutagenic properties	None
Irritating properties (rabbits, eyes, skin)	None
Biodegradability	>90% (28 days)
Acute toxicity (fish)	NOEC [a] 1000 mg/L
Acute toxicity on aerobic bacteria of wastewaters	EC_{50} >1000 mg/L

[a] NOEC=concentration which does not produce any effect

Tyr, HVa and HTyr are commercially available compounds; however, HTyr is very expensive. Thanks to a patented synthetic procedure, in our Laboratory HTyr is largely available at the high degree of purity (>98%).[220,221] The synthesis, depicted in **Scheme 9**, was based on the selective oxidation of Tyr by 2-iodoxybenzoic acid (IBX), a reagent able to selectively produce catecholic compounds via *ortho*-quinones.[222, 223]



Scheme 9. Synthetic procedure to obtain HTyr by oxidation of Tyr.

Briefly, Tyr was selectively protected on the alcoholic moiety using DMC used both as reagent and solvent. The carboxymethylated derivative **1** was obtained via an interaction

between the hard-electrophilic center of the DMC, i.e. the carboxyl group, and the hard-nucleophilic center of the phenolic compound, i.e. alkyl hydroxyl group. By using a catalytic amount of sulfuric acid, an increase in the electrophilic character of the carbonyl group was achieved and a selective carbomethoxylation of the alcoholic chain of tyrosol was obtained in quantitative yield. Then, compound **1** was subjected to an oxidation reaction using IBX in methanol at room temperature. The oxidation occurred selectively in the *ortho*-position. After the reduction reaction, carried out using sodium dithionite ($Na_2S_2O_4$), HTyr carboxymethylated **2** was the only oxidation product (86% yield). The following deprotection of alcoholic group via basic hydrolysis led to the final product, HTyr, obtained in 85% yield. The chemical structure and purity were confirmed by ^{1}H NMR, ^{13}C NMR and HPLC analysis.[57]

The synthesis of lipophilic Tyr, HVa and HTyr derivatives was carried out as follow. Each substrate was solubilized in DMC and then a little excess of the appropriate acyl chloride was added. With the aim to obtain derivatives of different lipophilicity, acyl chlorides with C2-C18 chain length were used. The mixtures were stirred at room temperature and monitored by thin-layer chromatography. After 24 h, the reactions were stopped; the final esters were purified on column chromatography and then characterized by ^{1}H and ^{13}C NMR spectroscopy. As reported in **Table 3**, Tyr esters **3-13** and HVa esters **14-24** were obtained in excellent yields (90-98%); HTyr esters **25-35** were isolated in satisfactory yields (60-66%).

Table 3. Esterification reactions of Tyr, HVa and HTyr.

Substrate	***Product***	***Yield (%)[a]***	***Substrate***	***Product***	***Yield (%)[a]***	***Substrate***	***Product***	***Yield (%)[a]***
Tyr	**3**	94	HVa	**14**	92	HTyr	**25**	66
Tyr	**4**	98	HVa	**15**	92	HTyr	**26**	62
Tyr	**5**	96	HVa	**16**	95	HTyr	**27**	68
Tyr	**6**	98	HVa	**17**	94	HTyr	**28**	62
Tyr	**7**	96	HVa	**18**	92	HTyr	**29**	62
Tyr	**8**	96	HVa	**19**	98	HTyr	**30**	64
Tyr	**9**	97	HVa	**20**	95	HTyr	**31**	60
Tyr	**10**	95	HVa	**21**	98	HTyr	**32**	62
Tyr	**11**	94	HVa	**22**	92	HTyr	**33**	60
Tyr	**12**	96	HVa	**23**	90	HTyr	**34**	64
Tyr	**13**	90	HVa	**24**	90	HTyr	**35**	60

[a] Yields calculated after chromatographic purification

The experimental results confirmed that all substrates were selectively esterified on the alcoholic functional group. Probably, this is due to the higher nucleophilicity of the alkyl

hydroxyl group compared to the phenolic moiety, emphasized by DMC, as already observed.[220] Even if HTyr esters were isolated in lower yields, the simplicity of the operations and low cost of the reagents make the above described procedure attractive. In fact, the reactions were performed in DMC, at room temperature, avoiding both protection/deprotection procedures and employment of strong acid catalysts: these conditions fit with the green chemistry approach, which encourages the development of processes involving the use of less hazardous substances and the minimization of energy consumption.[2]

The alkyl ester derivatives obtained are depicted in **Figure 24.** Within each series, a gradual increase in the number of carbon atoms on alkyl chain resulted in a parallel increase of the LogP values, varying between 1 and 10.[105]

These compounds are relevant for biological activities. Tyr esters with different alkyl chain lengths (from C2 to C18:1) showed antimicrobial and anti-leishmanial activities. Tyr caprylate **6**, Tyr capriate **7**, and Tyr laurate **8** showed a remarkable antimicrobial activity against *Leishmania major*, *Leishmania infantum*, *Staphylococcus aureus*, *Staphylococcus xylosus*, *Bacillus cereus* and *Brevibacterium flavum.*[189] Another study carried out on an *in vitro* simulated gastrointestinal tract model has demonstrated that the absorption from the intestinal lumen of Tyr esters are better than Tyr.[224] HTyr acetate **25**, found also in olive oil,[225] exhibits antioxidant effect in oil and emulsions.[226] It showed antibacterial activity against Vibrio spp., and a better anti-angiogenic activity than HTyr; moreover, it was better absorbed than free HTyr in small intestine, enhancing the delivery of HTyr to the enterocytes. Among a series of esters tested for anti-inflammatory activity, the best results have been obtained for HTyr oleate **34**, recently found in extra virgin olive oil and olive oil (5 and 1µg/g, respectively).[227] Both derivatives **25** and **34** show antiproliferative activity on human cervical cells (HeLa).[228]

A recent study carried out in our Laboratory has evidenced that HTyr butanoate **26**, octanoate **28** and oleate **34** showed antiproliferative activity on the human colon cancer cell line HCT8-β8 engineered to overexpress estrogen receptor β.[218] The experimental data showed not only that all lipophilic compounds significantly reduced the proliferation of tumour cells, but also HTyr oleate **34** was the most effective confirming the role of a long carbon chain on the bioavailability of the compound. HTyr butanoate **26**, decanoate **29**, palmitate **31**, stearate **32**, oleate **34** and linoleate **35** are promising therapeutic agents for the treatment of cutaneous diseases in consideration to their permeability.[229] The introduction of the alkyl chain has

proven to be strategic for considerably improving the permeation profile of HTyr, which would otherwise be able to pass only in a low percentage through the human stratum corneum and viable epidermis (SCE) membranes.[229]

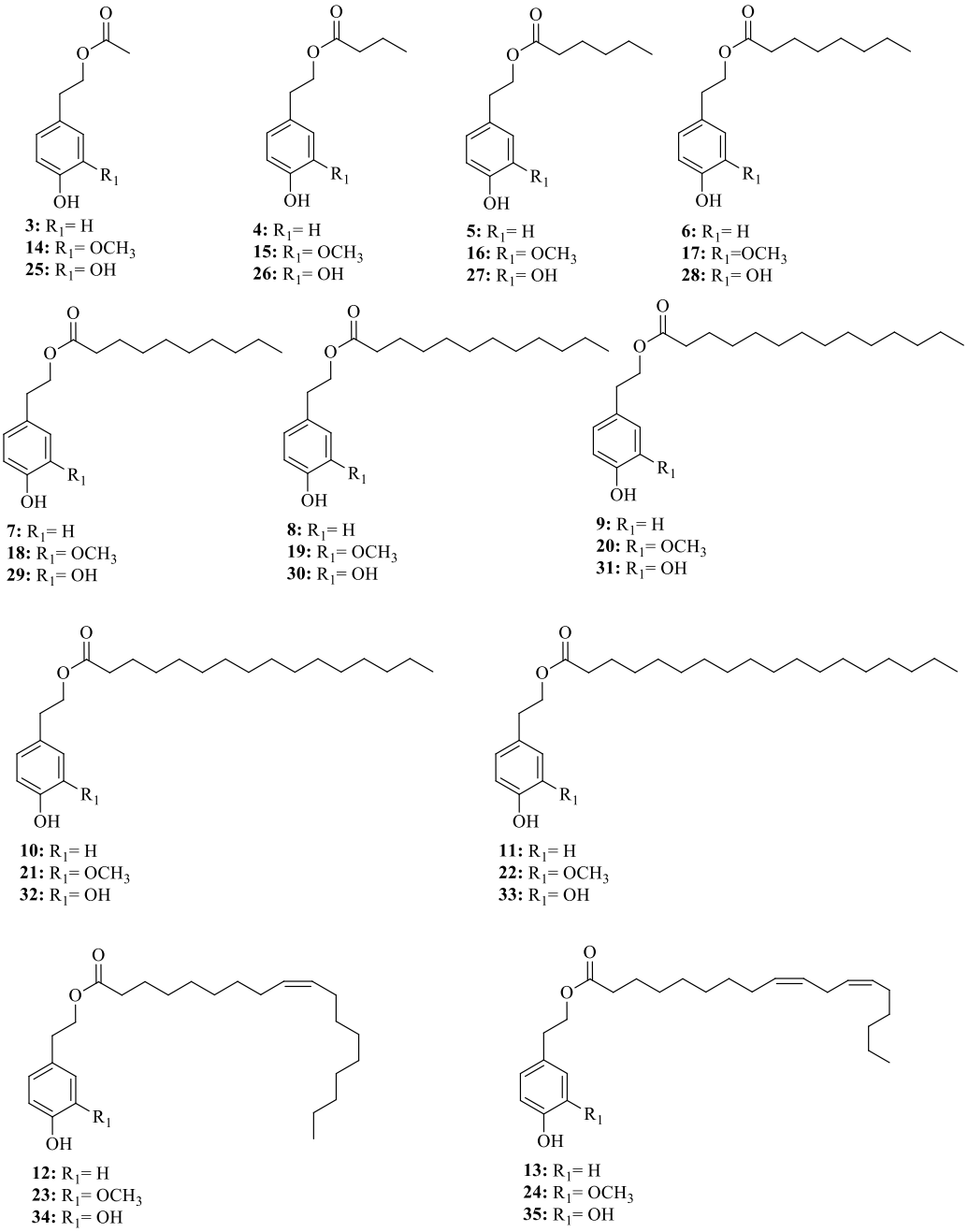


Figure 24. Chemical structures Tyr, HVa and HTyr lipophilic derivatives.

In consideration of the biological relevance of HTyr esters, and according to the circular economy model, the lipophilization reaction optimized using pure HTyr was applied to an HTyr-enriched extract (HTyrE) obtained from *Olea europaea* wastes.

The extract was prepared by the research group of Prof. Annalisa Romani (University of Florence) using an eco-friendly and patented procedure.[44] The raw material (free-oil olive pump) was pre-treated with concentrated HCl acidified with citric acid (1% v/v) and then subjected to a solid-liquid extraction in water using a pneumatic extractor (**Figure 25**). Then, the extract was fractionated by an innovative separation process based on membrane technology, consisting of microfiltration (MF), nanofiltration (NF), and reverse osmosis (RO) stages. The final fraction collected was concentrated using a heat pump evaporator to increase the concentration of HTyr. Fraction was then purified by chromatography to obtain the final product, HTyrE, which was characterized by HPLC-DAD and ^{1}H and ^{13}C NMR analysis. Flash and preparative chromatography of the fractions was carried out using the Pure C-850 FlashPrep chromatography system (Büchi Italia Srl, Milan, Italy). A C18 column was used as stationary phase, while water and ethanol as eluents.

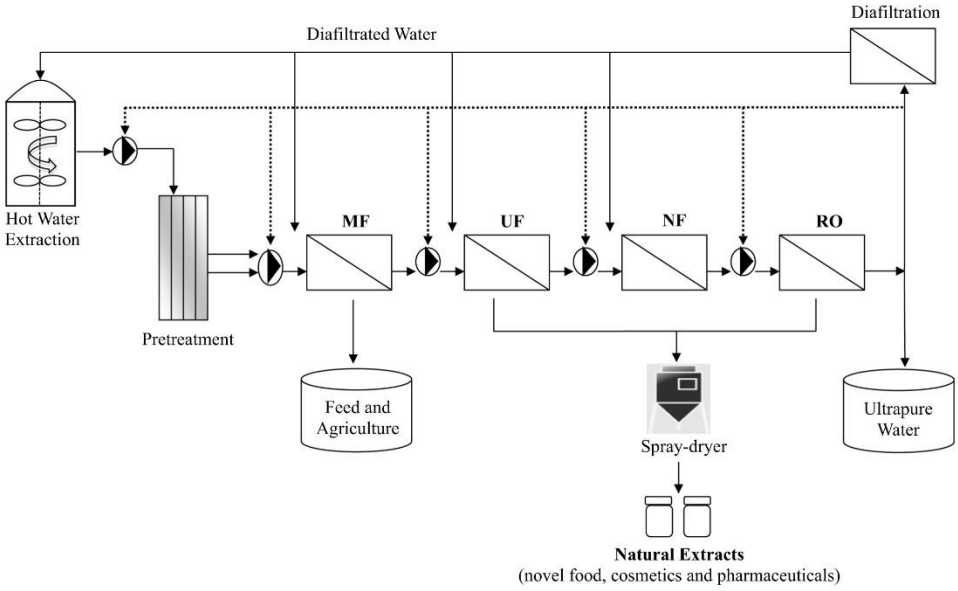


Figure 25. HTyrE obtained by membrane technologies.

As showed in the chromatographic profile recorded at 280 nm and reported in **Figure 26**, HTyr proved to be the main component of HTyrE with 115.24 mg/g, (77%, w/w) and tyrosol was the secondary component with 34.50 ± 1.04 mg/g (23% w/w).

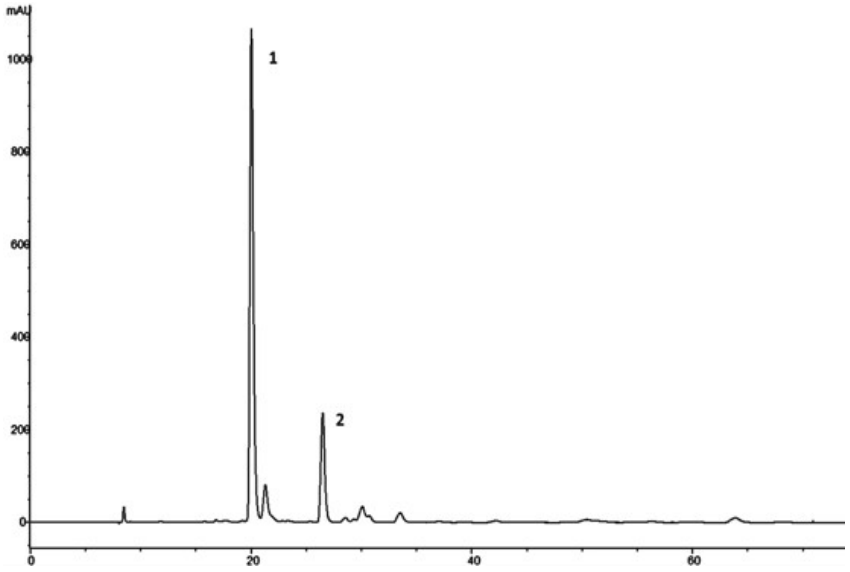


Figure 26. HPLC profile of HTyrE at 280 nm. 1. HTyr; 2. Tyrosol.

The ^{1}H NMR analysis of extract confirmed the chemical compositions determined by HPLC. In fact, the characteristic absorption signals of pure HTyr [220] found by ^{1}H NMR spectrum analysis (**Figure 27a**) were predominantly present in the HTyrE spectrum (**Figure 27b**) clearly proving that HTyr is indeed the main components of HTyrE. Focusing the attention on the aromatic area, HTyrE spectrum revealed the presence of the signals of the three aromatic protons of HTyr (H-4, H-7 and H-8). The doublets at δ=6.70-6.68 and 6.67-6.66 were assigned to H-7 and H-4 protons, respectively. The multiplicity of the H-7 signal was due to the coupling with the H-8 proton in the *ortho* position (J_{ortho}= 8.0 Hz). Meta-coupling of protons H-4 and H-8 (J_{meta}= 4.0 Hz) resulted in splitting of the H-4 signal into a doublet. The signal of the third aromatic proton of HTyr (H-8) resulted as a double doublet (δ=6.56-6.53) whose largest constant was relative to the coupling in *ortho* with H-7 (J_{ortho}= 8.0 Hz), while the smallest constant resulted from the *meta* coupling with H-4 (J_{meta}= 4.0 Hz). The doublets at δ=7.06-7.03 and δ=6.73-6.70 was consistent with the aromatic proton of Tyr, the secondary phenolic compound present in the extract.

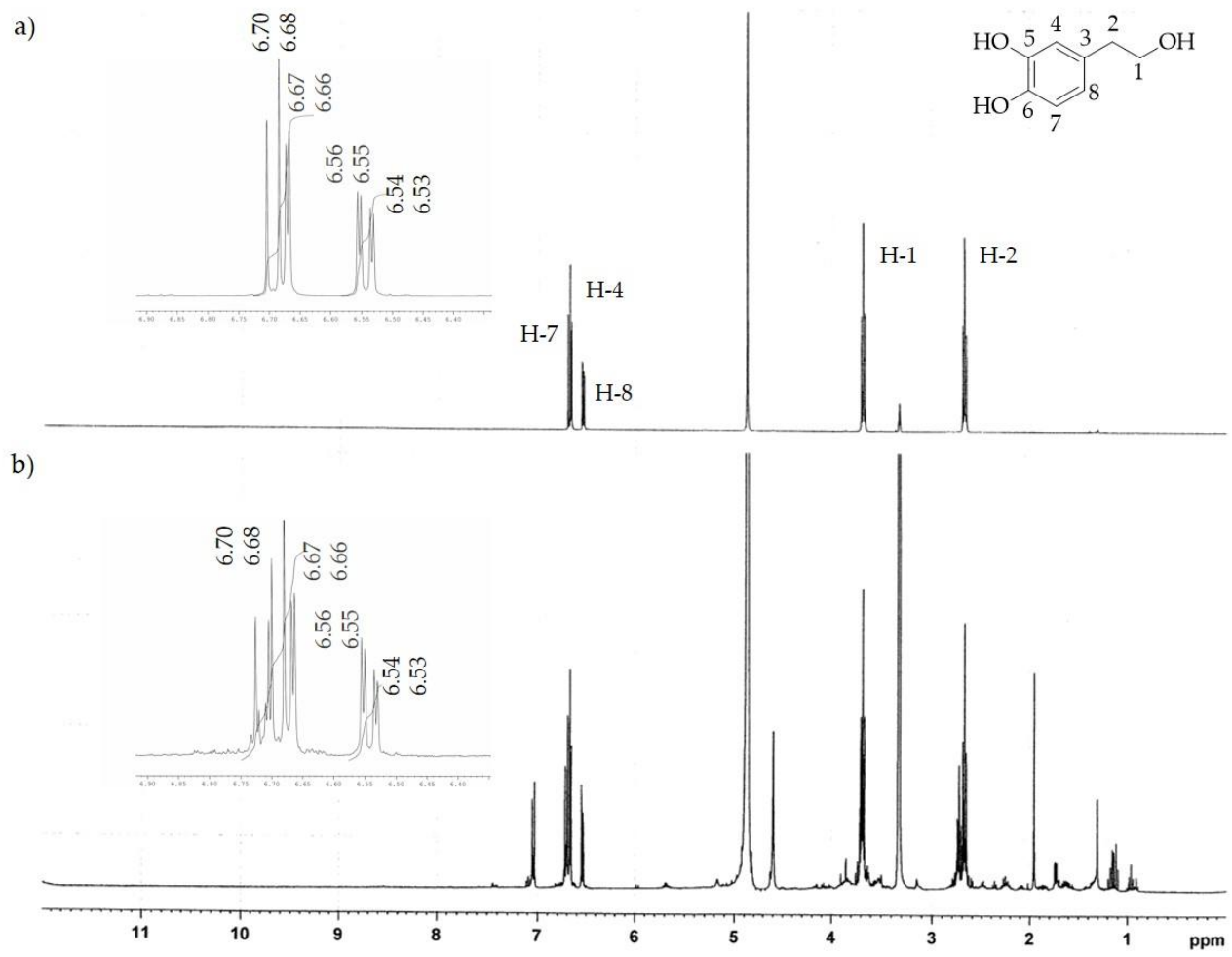


Figure 27. ^{1}H-NMR spectrum of (a) pure HTyr; (b) Ole enriched extract (OleE).

The mild esterification procedure optimized for pure HTyr was applied to HTyrE. A sample of the extract corresponding to a known amount of HTyr was dissolved in DMC and the appropriate C2-C18 acyl chloride was introduced. Each reaction was kept at room temperature under magnetic stirring for 24 h and monitored by TLC on silica gel plates using a mixture of dichloromethane and methanol (9.8/0.2 or 9.5/0.5) as eluent. Each final product was identified using the pure compound previously synthetized as reference. After the work-up, the resulting lipophilic fraction was purified through chromatographic column obtaining the corresponding HTyr esters **25**-**35**. The yields of the final products ranging from 60 to 66%, according to previously observed for pure HTyr.

The described results demonstrated that bioactive lipophilic compounds as HTyr esters can be prepared also starting from extracts obtained by olive oil wastes and by-products representing a valuable example of their recovery and reuse of bioactive.

3.2.2 Lipophilization of gallic acid and a gallic acid-enriched extract

During the PhD program, a new green procedure for the synthesis of alkyl gallates has been optimized. Unfortunately, the procedure previously used to prepare Tyr, HVa and HTyr alkyl esters failed for the low solubility of gallic acid (GA) in DMC. So that, an alternative way based on the reaction between GA and alkyl alcohols in acidic medium has been designed and developed.

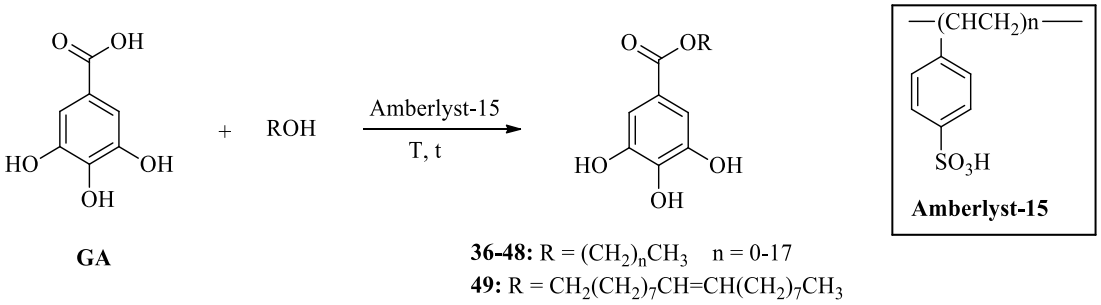
In last years, the employment of ion-exchange resins to carry out process in heterogeneous phase has attracted increasing interest as green alternative to conventional reactions performed in homogeneous phase for practical and environmental advantages. After an extensive bibliographic research, Amberlyst-15 was chosen. It consists of grey-brown spheres; chemically, it is a polystyrenic resin with sulfonic groups. The physical properties are reported in **Table 4**.[230]

Table 4. Properties of Amberlyst-15.

Properties	***Value***
Concentration of active sites	≥ 4.7 eq/Kg
Moisture holding capacity	52-57%
Particle size	0.600-0.850mm
Average pore diameter	300 Å
Total pore volume	0.40 mL/g
Maximum operating temperature	120°C

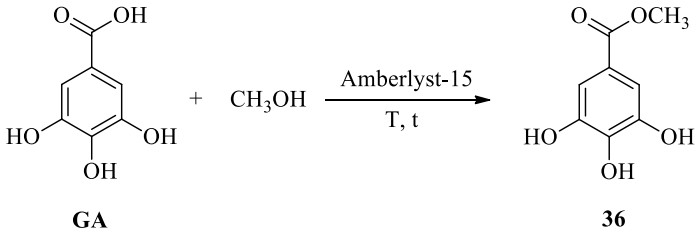
The use of Amberlyst-15 in organic synthesis is very attractive. It is commercially available at low cost, easy and safe to use, and it can be regenerated and reused several times.[230] Its use avoids the formation of by-products and subsequent purification steps according to the green chemistry approach. The versatility of application allowed the use of Ambelyst-15 in organic synthesis. It was used in Michael addition, Friedel-Crafts, halogenation and protection and deprotection reactions.[230] It was widely used for the preparation of esters starting from carboxylic acids and alcohols. For example, perfumery and flavour grade esters as phenethyl acetate and cyclohexylphenyl acetate were prepared by using Amberlyst-15 as acidic catalyst.[231,232] Propyl nonanoate was prepared in 90% yield by reaction of nonanoic acid with 1-propanol in 1,4-dioxane.[233] Esterifications of fatty acids with ethanol were investigated and yields of esters up to 87% were obtained.[234] Esters of levulinic acid with a C1-C4 alkyl chain

were prepared in 55 to 82% yields.[235,236] These literature data, together with the remarkable properties, encouraged us to choose Amberlyst-15 as acidic ion exchange resin to activate the carboxyl group of GA in esterification reaction. The general reaction was depicted in **Scheme 10**.



Scheme 10. Esterification reaction of GA.

At first, the experimental conditions were optimized, and the reaction model was the reaction between GA and methanol in the presence of Amberlyst-15 **(Scheme 11)**.



Scheme 11. Esterification of GA with methanol in the presence of Amberlyst-15.

Methyl gallate **36**, used as reference compound, was synthesized by a Fisher reaction in the presence of sulfuric acid, as reported in the literature.[199] After the chromatographic purification of the crude reaction mixture, compound **36** was characterized by Nuclear Magnetic Resonance spectra (^{1}H and ^{13}C NMR).

The experimental results of the reactions carried out in the presence of Amberlyst-15 are reported in **Table 5**. Each reaction was carried out adding the appropriate amount of Amberlyst-15 to a solution of GA in methanol in large excess (GA/methanol: 1/247).[199]

A blank reaction was carried out to verify that no product was formed in the absence of the resin (*entry 1*). The following reactions were performed varying reaction time, temperature and molar ratio GA/Amberlyst-15.

As depicted in **Figure 28**, at the end of each reaction, Amberlyst-15 was removed from the reaction medium by simple filtration to recover the solution; after distillation at reduced pressure of methanol, the crude reaction mixture was obtained.

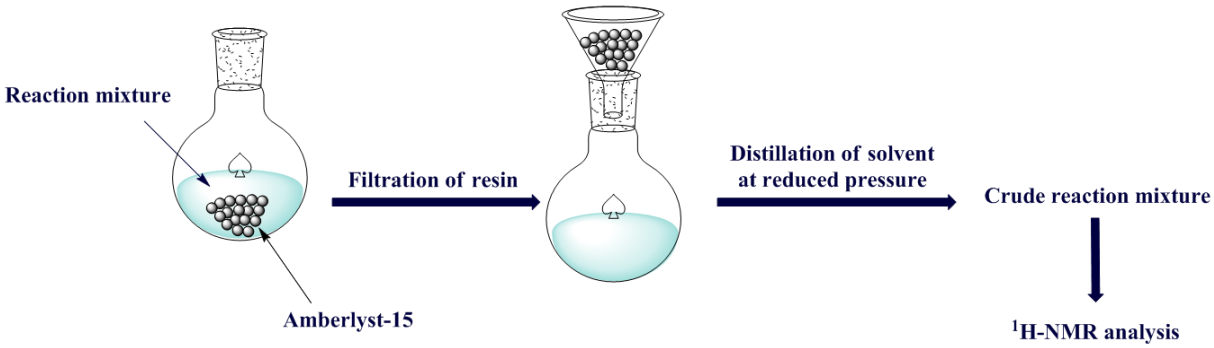


Figure 28. Schematization of the different phases of the esterification reaction of GA with Amberlyst-15.

The yield of methyl gallate **36** was calculated using the crude reaction products by ^{1}H NMR analysis thanks to the different chemical shift of the singlet of the aromatic protons of GA (δ= 7.09 ppm) and that of the aromatic protons of the ester (δ= 7.07 ppm), as showed in **Figure 29**.

As reported in **Table 5**, using a molar ratio GA/Amberlyst-15=5.3/1, 2.7/1, or 1/1, GA methyl ester 36 was obtained in very low yields (11-32%) also at 60 °C and after 48 h (entries 2-7).

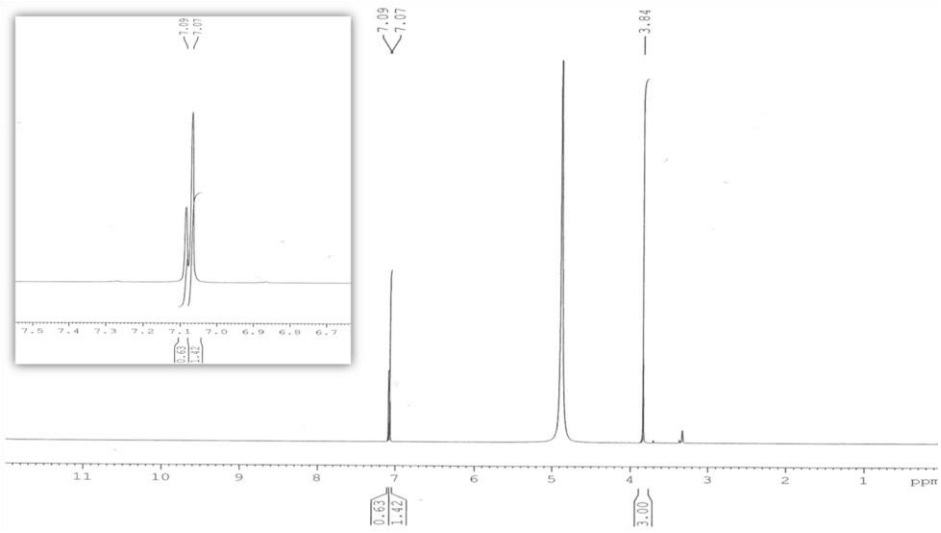


Figure 29.

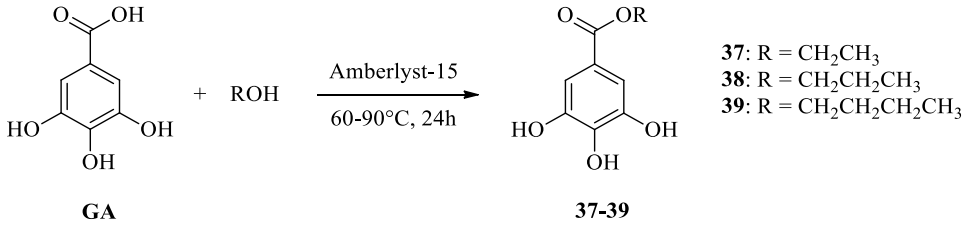
A significant improvement in the yield was observed only when the molar ratio GA/resin was unbalanced in favour of the latter. According to the literature data,[232,233,236] the amount of resin used affects the yield; in fact, increasing the amount of Amberlyst-15, the ester yield increases to 50% (*entry 8*) and 100 % (*entry 9*).

Table 5. Esterification reactions of GA with methanol.

	Reaction conditions [a]	*Yield (%)* [b]
1	**GA**/--, 25°C, 24 h	--
2	**GA**/Amberlyst-15=5.3/1, 25°C, 24 h	--
3	**GA**/Amberlyst-15=5.3/1, 60°C, 24 h	**36**: 11
4	**GA**/Amberlyst-15=5.3/1, 60°C, 48 h	**36**: 12
5	**GA**/Amberlyst-15=2.7/1, 60°C, 24 h	**36**: 18
6	**GA**/Amberlyst-15=2.7/1, 60°C, 48 h	**36**: 32
7	**GA**/Amberlyst-15=1/1, 60°C, 24 h	**36**: 23
8	**GA**/Amberlyst-15=1/4, 60°C, 24 h	**36**: 50
9	**GA**/Amberlyst-15=1/4, 60°C, 24 h	**36**: 100

[a] GA: 0.3 mmol; GA/Methanol=1/247; V=3.0 ml.
[b] Calculated by ^{1}H NMR

Based on these results, the best experimental conditions were extended to reaction performed in the presence of C2-C4 alcohol to obtain ethyl **37**, propyl **38** and butyl gallate **39** (**Scheme 12** and **Table 6**). Considering that ethanol, 1-propanol and 1-butanol show higher boiling points (78 °C, 90 °C, 118 °C, respectively) the reactions were carried out also at T>60 °C, obtaining excellent yields (*entries 3, 5 and 7*).



GA **37-39**

Scheme 12 Esterification of GA with ethanol, 1-propanol and 1-butanol in the presence of Amberlyst-15.

As reported above, the alcohol was used in large excess, playing the dual role of solvent and reagent. This was also useful in terms of reaction yields, as the acid-catalyzed esterification reaction is an equilibrium and excess amount of one of the reactants represents one of the commonly adopted strategies to promote product formation.[186] However, as the alkyl chain of the alcohol increases, so does its boiling point, and so the elimination at reduced pressure becomes increasingly difficult for the higher homologs of 1-butanol.

Therefore, it was necessary to introduce a solvent. Since 1-butanol is the limiting case, the synthesis of butyl gallate **39** was chosen as a reaction model to optimize the new experimental conditions. Considering the temperature at which the reactions were carried out, 1,4-dioxane, which has a boiling point of 101 °C, seemed to us an adequate solvent for the investigation (**Table 6**). 1,4-Dioxane is widely used not only in organic synthesis but also in other fields of application.[237] It shows high water solubility, low volatility, low susceptibility to chemical and biological reactions, and bio-degradability.[238] With the introduction of a solvent, a lower molar ratio GA/1-butanol was used. In fact, with a molar ratio 1/55, butyl gallate **39** was obtained in quantitative yield (*entry 8*). However, the amount of alcohol was still too high. In order to investigate also the effect of the amount of Amberlyst-15 and reaction volume on the yields, the molar ratios GA/1-butanol and the reaction volume were simultaneously lowered (*entries 9-11*).

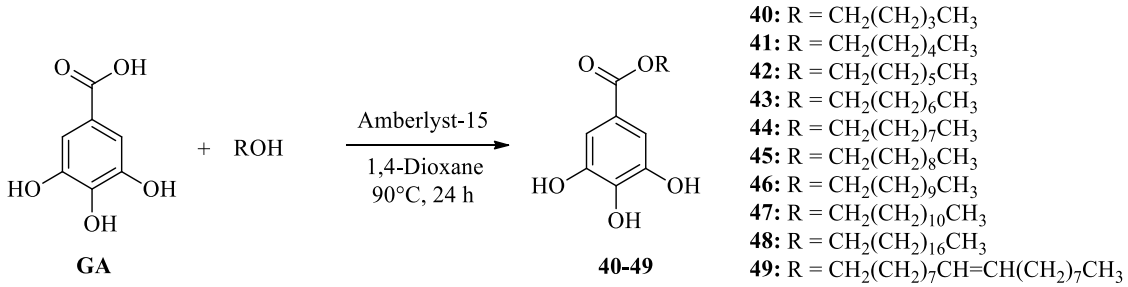
As shown in the Table 6, the best result was obtained using a molar ratio GA/Amberlyst-15=1/5 and GA/1-butanol=1/5, with a total reaction volume of 1 ml (entry 10).

Table 6. Esterification reactions of GA with ethanol, 1-propanol and 1-butanol.

	Reaction conditions	*Yield (%)* [c]
1	**GA/Ethanol**=1/170, **GA**/Amberlyst-15=1/10, 60°C, 24 h [a]	**37**: 45
2	**GA/Ethanol**=1/170, **GA**/Amberlyst-15=1/10, 70°C, 24 h [a]	**37**: 72
3	**GA/Ethanol**=1/170, **GA**/Amberlyst-15=1/10, 80 °C, 24 h [a]	**37**: 96
4	**GA/1-Propanol**=1/133, **GA**/Amberlyst-15=1/10, 70°C, 24 h [a]	**38**: 55
5	**GA/1-Propanol**=1/133, **GA**/Amberlyst-15=1/10, 90°C, 24 h [a]	**38**: 100
6	**GA/1-Butanol**=1/110, **GA**/Amberlyst-15=1/10, 60°C, 24 h [a]	**39**: 78
7	**GA/1-Butanol**=1/110, **GA**/Amberlyst-15=1/10, 90°C, 24 h [a]	**39**: 100
8	**GA/1-Butanol**=1/55, **GA**/Amberlyst-15=1/10, 90°C, 24 h, 1,4-dioxane [a]	**39**: 100
9	**GA/1-Butanol**=1/10, **GA**/Amberlyst-15=1/5, 90°C, 24 h, 1,4-dioxane [b]	**39**: 100
10	**GA/1-Butanol**=1/5, **GA**/Amberlyst-15=1/5, 90 °C, 24 h, 1,4-dioxane [b]	**39**: 80
11	**GA/1-Butanol**=1/3, **GA**/Amberlyst-15=1/5, 90°C, 24 h, 1,4-dioxane [b]	**39**: 67

[a] GA: 0.3 mmol, V=3 ml.
[b] GA: 0.3 mmol, V=1 ml.
[c] Calculated by ^{1}H NMR.

Based on these results, the optimized conditions were applied to GA and long chain alcohols such as 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, 1-nonanol, 1-decanol, 1-undecanol, 1-dodecanol, 1-octadecanol and oleyl alcohol (**Scheme 13**). As reported in **Table 7**, the alkyl esters **40**-**49** were obtained with excellent yields, ranging from 84% to 100%.



Scheme 13. Esterification of GA with long chain alcohols (C5-C18) in the presence of Amberlyst-15.

Table 7. Esterification reactions of GA with long chain alcohols (C5-C18).

Reaction conditions [a]	*Yield (%)*[b]
GA/1-Pentanol=1:5, 90°C, 24 h,1,4-dioxane	**40**: 95
GA/1-Hexanol=1:5, 90°C, 24 h, 1,4-dioxane	**41**: 84
GA/1-Heptanol=1:5 90 °C, 24 h, 1,4-dioxane	**42**: 89
GA/1-Octanol=1:5, 90°C, 24 h, 1,4-dioxane	**43**: 93
GA/1-Nonanol=1:5, 90°C, 24 h, 1,4-dioxane	**44**: 91
GA/1-Decanol=1:5, 90°C, 24 h, 1,4-dioxane	**45**: 96
GA/1-Undecanol=1:5, 90°C, 24 h, 1,4-dioxane	**46**: 100
GA/1-Dodecanol=1:5, 90°C, 24 h, 1,4-dioxane	**47**: 94
GA/1-Octadecanol=1:5, 90°C, 24 h, 1,4-dioxane	**48**: 94
GA/Oleyl alcohol=1:5, 90°C, 24 h, 1,4-dioxane	**49**: 92

[a] GA: 0.3 mmol, GA/Amberlyst-15=1/5, V= 1 ml,
[b] Calculated by ^{1}H NMR.

The advantage of performing reactions in heterogeneous phase lies in the ease of the operations involved in the process, schematized in **Figure 30**. As above described, at the end of the reaction, Amberlyst-15 was easily removed from the reaction medium by filtration and, subsequently, the solvent was removed from the remaining solution by distillation at reduced pressure, obtaining a crude reaction mixture containing the expected ester to be purified by chromatography. At this point, a key step comes into play for the heterogeneous phase processes, namely the possibility of reusing resin, after regeneration.

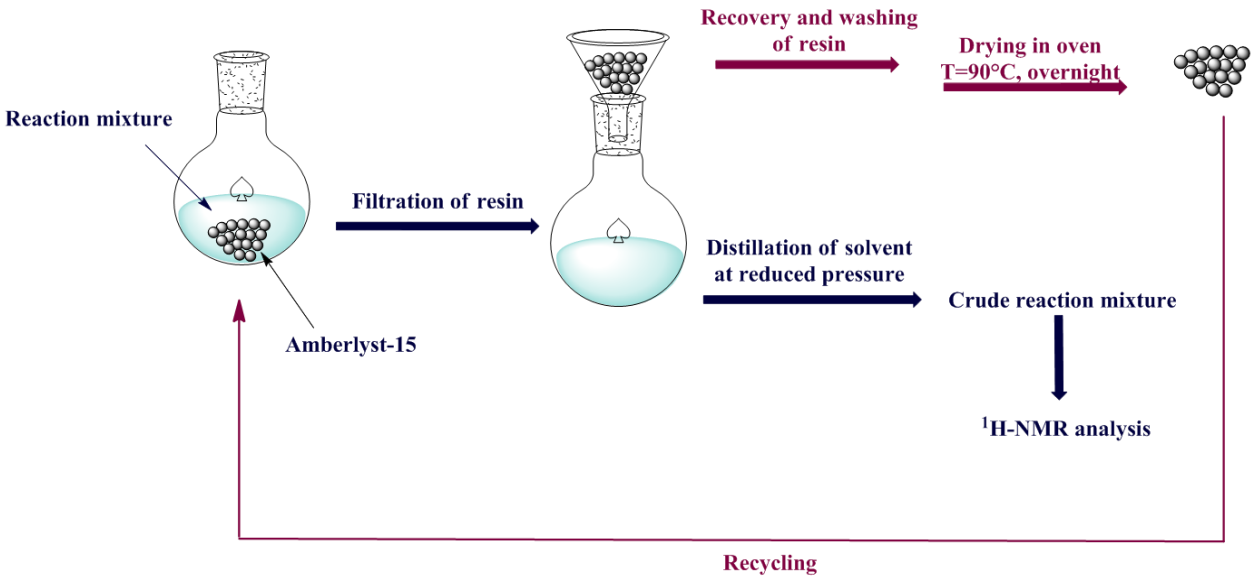


Figure 30. Schematization of different phases of using and recycling of Amberlyst-15.

Then, according to the green chemistry approach, the recycling of Amberlyst-15 was investigated. The performances of the recovered resin were evaluated in the two model reactions: the first, in the presence of methanol and the second in the presence of 1-butanol under the optimized experimental conditions.

As depicted in **Figure 30**, the recovered Amberlyst-15 was washed with diethyl ether [237] and methanol; then, it was dried in oven at T=90 °C overnight.[232, 236] As showed in **Figure 31**, the resin proved to be efficient in the esterification reactions for at least five runs. The following runs were characterized by a progressive light reduction of yields probably for the loss of resin during the filtration and washing phases but also for the deposition of residual reagents and product on the active sites of the resin, whose removal by washing becomes more and more difficult as recycling proceeds, as already reported in the literature.[235, 236]

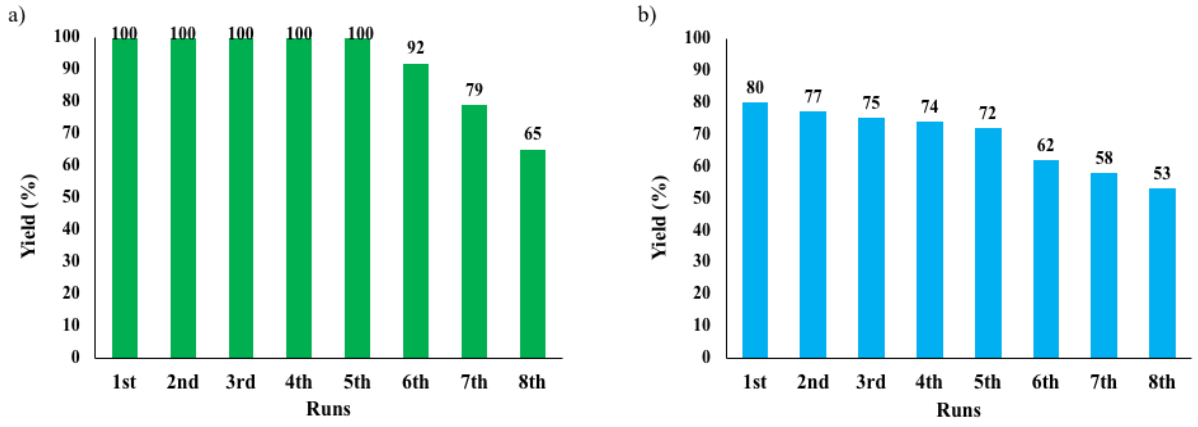


Figure 31. **(a)** Recycling of Amberlyst-15 on esterification of GA with methanol (GA/methanol=1/247, GA/Amberlyst-15=1/10, T=60°C, t=24 h); **(b)** Recycling of Amberlyst-15 on esterification of GA with 1-butanol (GA/1-butanol=1/5, GA/resin=1/5, 1,4-dioxane, T=90°C, t=24 h).

The procedure described here satisfies some of the criteria proposed by green chemistry theory to achieve more safe and sustainable processes. The optimized procedure is a valid green alternative for the synthesis of lipophilic derivatives of GA, if we consider the higher yields obtained in comparison with the classic chemical esterification methods in homogeneous phase (80-100% vs 50-89%).[197,199,201,202]

Finally, the scalability of the process was evaluated using the reaction of GA with methanol. Starting from 1 g of GA, methyl gallate **36** was obtained in quantitative yield, demonstrating that the method is promising for a large-scale production of alkyl gallates.

All alkyl gallates prepared are reported in **Figure 32.** They are more lipophilic than the starting gallic acid (Log P = 0.42);[196] in addition, the gradual increase in the number of carbon atoms on alkyl chain resulted in a parallel increase of the LogP value, varying from 0.69 for methyl gallate **36** up to 7.68 for octadecyl gallate **43**.[196, 239]

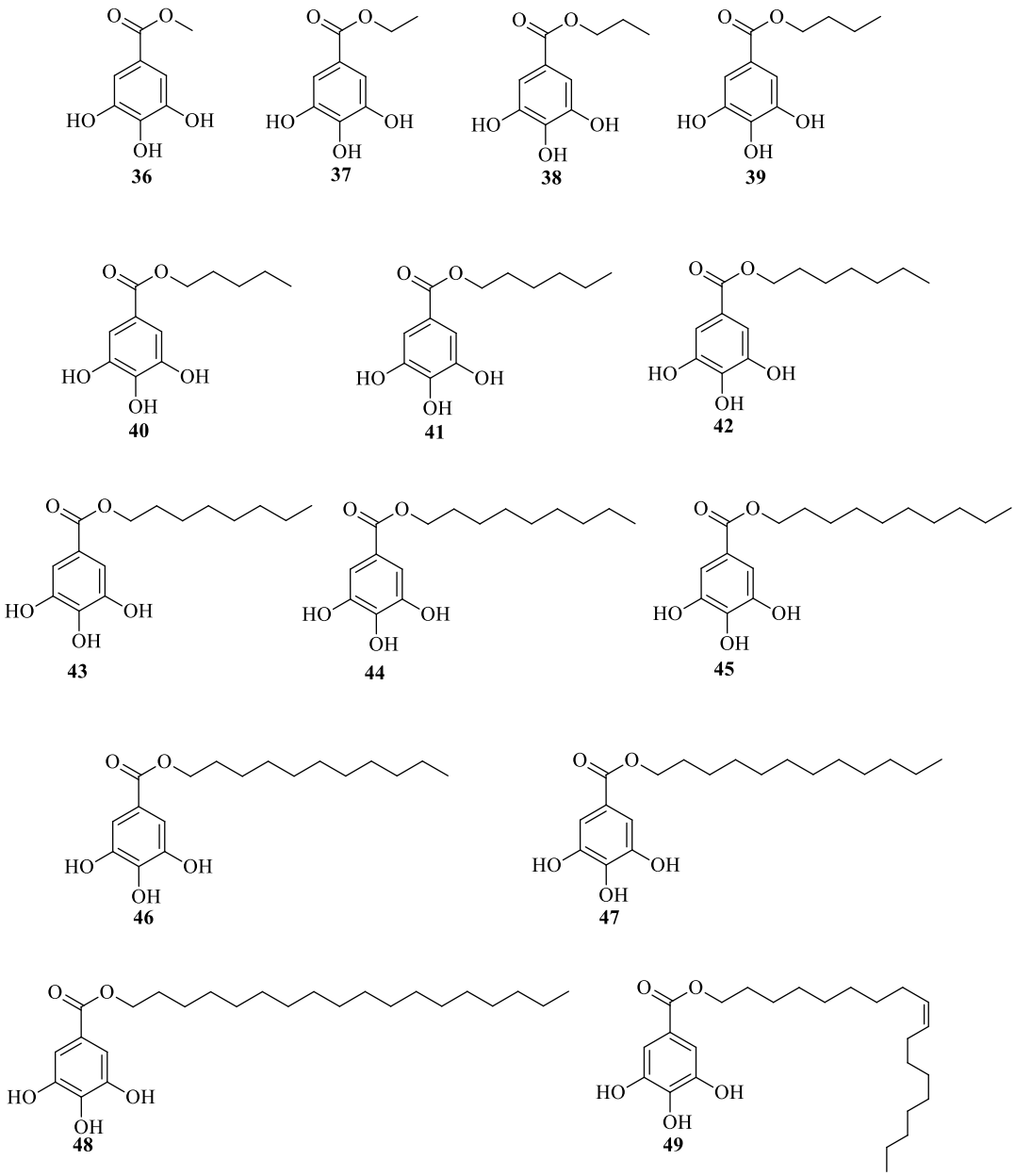


Figure 32. Chemical structures of GA lipophilic derivatives.

Some of them are naturally occurring compounds. As an example, methyl gallate **36** is present in the coats of red and black sword beans,[240] in extracts of baru nuts,[241] in pods of jiringa [242] and in wastes from wine production.[243] In addition, octyl gallates **43** has been isolated from *Terminalia bellarica*, a potent medicinal herb.[244]

These compounds are interesting for their relevant biological activities, and in many cases these properties result emphasized for their lipophilicity. Propyl gallate **38**, octyl gallate **43** and dodecyl gallate **47** are currently used as additives for food. They preserve fats and oils-based food from their deterioration delaying or interrupting the propagation steps of lipid peroxidation process.[245] Propyl gallate **38** is the most used also in cosmetic and personal care products (bath products, skin cleansing and skin care products, makeup products, sunscreen and suntan products) as well as in pressure-sensitive adhesives, lubricating oil additives and transforming oils.[246]

The lipophilicity of alkyl gallate has been highlighted in a study concerning the effects of these compounds on the proliferation of two mouse tumour cell lines, the Wehi 231 B cell lymphoma and the L929 fibroblast. The strongest activity has been observed for octyl gallate **43** and dodecyl gallate **47**; in particular, the latter has proven to be 40 times more efficient than GA.[247] Again, evaluating the cytotoxic effect of alkyl gallates against four human tumour cell lines (HepG2, DU-145, A549 and MDA-MB-231), octyl gallate **43** showed the most powerful cytotoxic action.[248] These results really reflect the best efficacy of hydrophobic compounds in interaction with biological membranes, as they are perfectly in agreement with the literature data concerning the transport of these lipophilic derivatives of GA across the intestinal barrier. Using Caco-2 cell monolayers as model, it has been observed that only when there is a substantial increase in chain length (i.e. from methyl to octyl gallate), a better absorption of the compound was observed.[249] By studying the mechanism through which alkyl esters cause cell death in melanoma cells, it has been reported that they are able to act through different pathways and the different hydrophobicity affect the type of mechanism that each compound can trigger. Octyl gallate **43** has been the only compound able to cause an imbalance of intracellular redox species, by depleting glutathione and increasing production of ROS, simultaneously. Decyl gallate **45** and dodecyl gallate **47**, instead, have regulated the transcription factor NF-κB which also causes a reduction in glutathione levels as final effect. Finally, tetradecyl gallate has showed the best ability to inhibit cell adhesion, a phenomenon related to treatment resistance that melanoma patients often show.[250] Hexyl gallate **41** and dodecyl gallate **47** inhibited the growth of different human breast cancer cell lines and a

strong cytotoxic effect of **47** against two particular cell lines (MCF7 ADR and MDA-MB-231), which are respectively resistant to multidrug and highly proliferative and invasive, was found.[251, 252] Esters with short alkyl chain (no more of five carbon atoms) showed to be the most efficient antiviral agents. In particular, methyl gallate **36** has been 50-fold more active than GA against Herpes Simplex Virus type 2 (HSV-2),[253] while both GA and pentyl gallate **40** have showed the best effectiveness against Herpes Simplex Virus type 1 (HSV-1) and Human Immunodeficiency Virus (HIV-1).[169] Investigating the mechanism of action of these compounds, it has been found that they have an effect on the attachment and penetration of the virus into the cells but are also able to affect the virus when it has already infected the cells by inhibiting the expression of viral proteins with a consequent virucidal effect on progenies.[169]

In consideration of the biological relevance of alkyl gallates, and according to the circular economy model, the lipophilization reaction optimized on pure GA was applied to a GA-enriched extract (GAE) obtained from *Castanea sativa* Mill. wastes and, in particular, from hydrolysable tannins deriving from wood processing.

The extract was produced in the industrial plant of Gruppo Mauro Saviola Srl operating in Radicofani (SI). The company produces ecological chipboard panels made with recycled wood. This manufacturing process requires that the chipboards used contain the least possible amount of tannins. The presence of these polymers, in fact, causes a rapid hardening of the glues used to make the panel itself, making it impossible to model. Then, these tannins represent wastes that have been valorised. Saviola is the leading post-consumption wood processor in the world and the largest producer of tannins in Europe. Its philosophy is based on the notions of recycling and upcycling, that is to say the salvaging and re-using of waste to give it a second life by way of an environmentally sustainable economic cycle.

The extractive process (**Figure 33**) is based on an eco-friendly and patented procedure starting from tannins broths; all process is solvent-free according to the circular economy model. It involves an extraction with hot-water and a sequence of membrane filtration steps with different molecular cut-offs for fractionation to obtain different powders.[72] The liquid permeate resulting from the last filtration step is a brown solution (GAE).

This activity is related to an Industrial PhD program carried out in collaboration between Saviola and the University of Florence.

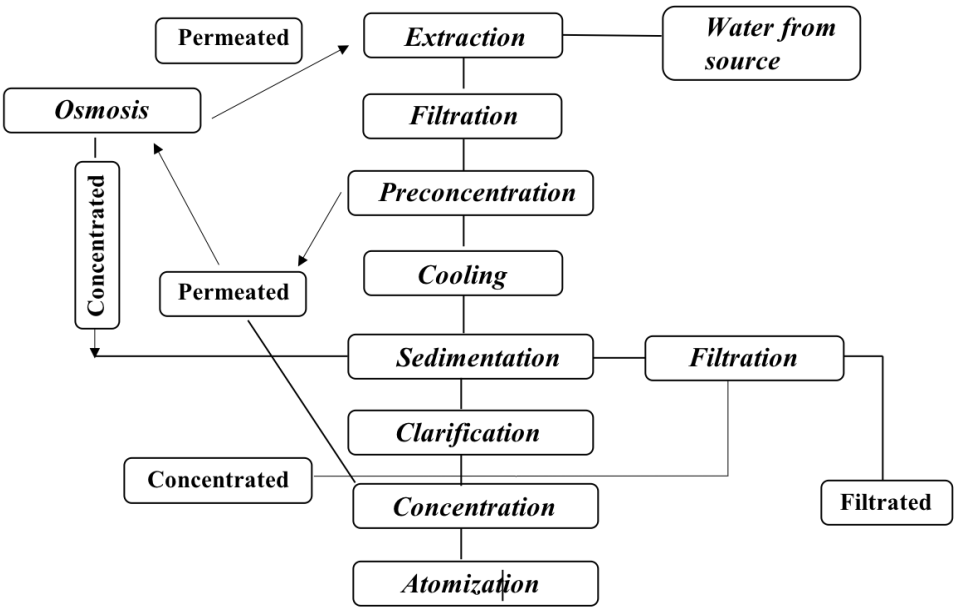


Figure 33. Extractive process to obtain GAE.

The HPLC-DAD analysis confirmed the presence of GA as the only phenolic compound (11.47 mg/ml, **Figure 34**).

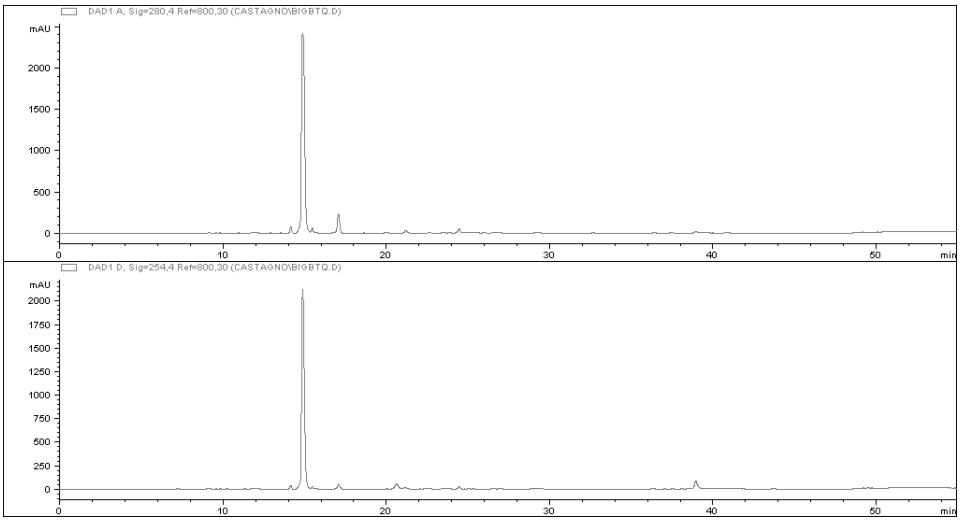


Figure 34. HPLC profile of GAE at 280 and 254 nm.

The NMR analysis confirmed the qualitative profile evidenced by HPLC-DAD analysis. **Figure 35** reported the comparison between the ^{1}H NMR spectrum of pure GA and GAE. Focusing the attention on the aromatic area, GAE spectrum revealed the presence of the signals of the two aromatic protons of GA (singlet at δ=7.08).

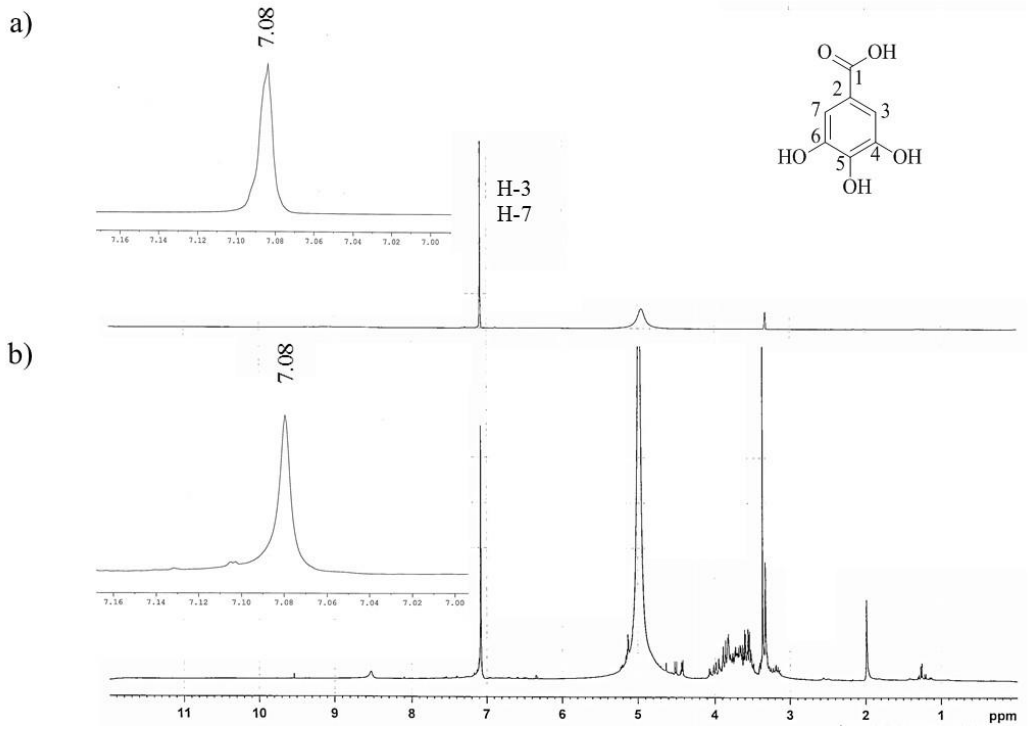


Figure 35. ^{1}H-NMR spectrum of (a) pure GA; (b) GA-enriched extract (GAE).

After removing the solvent by evaporation under reduced pressure, a brown solid was obtained, which was used as starting material for the esterification reactions under the best conditions optimized for pure GA. Then, the reactions with methanol, ethanol, 1-propanol and 1-butanol were performed using the alcohol as both solvent and reagent. For alcohols having a longer alkyl chain, 1,4-dioxane was introduced. After addition of the Amberlyst-15, the mixture was kept under magnetic stirring for 24 or 48 h, at 60 or 90 °C. At the end of reaction, the resin was removed by filtration and washed as already described. The solvent was evaporated under reduced pressure. Each crude reaction mixture was analysed by ^{1}H-NMR in order to calculate the yields using pure alkyl gallates as reference (**Table 8**).

Table 8. Esterification of GA in GAE

Reaction conditions	*Yield (%)* [c]
GA/Metanol=1:247, 60°C, 24 h [a]	**36**: 100
GA/Ethanol=1:170, 80°C, 24 h [a]	**37:** 72
GA/Ethanol=1:170, 80°C, 48 h [a]	**37:** 89
GA/Ethanol=1:170, 80°C, 72 h [a]	**37**: 100
GA/1-Propanol=1:133, 90 °C, 24 h [a]	**38**: 100
GA/1-Butanol=1:110, 90°C, 24 h [a]	**39:** 100
GA/1-Pentanol=1:5, 90°C, 24 h,1,4-dioxane [b]	**40:** 55
GA/1-Pentanol=1:5, 90°C, 48 h,1,4-dioxane [b]	**40**: 43
GA/1-Hexanol=1:5, 90°C, 24 h, 1,4-dioxane [b]	**41**: 32
GA/1-Hexanol=1:5, 90°C, 48 h, 1,4-dioxane [b]	**41**: 62
GA/1-Heptanol=1:5 90 °C, 24 h, 1,4-dioxane [b]	**42**: 75
GA/1-Heptanol=1:5 90 °C, 48 h, 1,4-dioxane [b]	**42**: 81
GA/1-Octanol=1:5, 90°C, 24 h, 1,4-dioxane [b]	**43**: 54
GA/1-Octanol=1:5, 90°C, 48 h, 1,4-dioxane [b]	**43**: 85
GA/1-Nonanol=1:5, 90°C, 24 h, 1,4-dioxane [b]	**44**: 89
GA/1-Nonanol=1:5, 90°C, 48 h, 1,4-dioxane [b]	**44**: 88
GA/1-Decanol=1:5, 90°C, 24 h, 1,4-dioxane [b]	**45**: 56
GA/1-Decanol=1:5, 90°C, 48 h, 1,4-dioxane [b]	**45**: 78
GA/1-Undecanol=1:5, 90°C, 24 h, 1,4-dioxane [b]	**46**: 70
GA/1-Undecanol=1:5, 90°C, 48 h**,** 1,4-dioxane [b]	**46**: 86
GA/1-Dodecanol=1:5, 90°C, 24 h, 1,4-dioxane [b]	**47**: 21
GA/1-Dodecanol=1:5, 90°C, 48 h, 1,4-dioxane [b]	**47**: 67
GA/1-Octadecanol=1:5, 90°C, 24 h, 1,4-dioxane [b]	**48**: 60
GA/1-Octadecanol=1:5, 90°C, 48 h**,** 1,4-dioxane [b]	**48**: 70
GA/Oleyl alcohol=1:5, 90°C, 24 h**,** 1,4-dioxane [b]	**49**: 33
GA/Oleyl alcohol=1:5, 90°C, 48 h, 1,4-dioxane [b]	**49**: 64

[a] GA:0.32 mmol (55 mg) in 155 mg of enriched fraction, GA/Amberlyst-15=1/10, V=3 ml.
[b] GA:0.32 mmol (55 mg) in 155 mg of enriched fraction, GA/Amberlyst-15=1/10, V=1 ml.
[c] Yield calculated by ^{1}H NMR.

Experimental results proved that in the extracts GA was esterified with C1-C4 alcohols obtaining the corresponding esters in quantitative yields *(entries 1 and 4-6*), according to what has already been observed using pure GA. Only in the case of ethyl gallate **37**, it was necessary to increase the reaction times up to a maximum of 72 h in order to increase the yield from 72 to 100%. The effect of increase in the reaction times (from 24 to 48 h) on the yield trend was instead shown to be a constant in the esterification of GA in GAE with C5-C18 alcohols *(entries 7-26*).

The results obtained using as a model the reaction between GA in the GAE and 1-butanol in 1,4-dioxane, indicated an improvement in yield even for a greater amount of resin. Then, the molar ratio GA/resin was increased from 1/5 to 1/10.

Furthermore, it should be noted that unlike what was observed with commercial GA, the new conditions imposed by the use of the solvent affect yields quite significantly. This is true both

in comparison to the yields of the lipophilic enriched fractions in solvent-free conditions (62-89% *vs* 100%) and in comparison, with the yields of esters prepared starting from commercial GA (62-89% *vs* 89-100%). However, given the complexity of the starting material, the results obtained after esterification reactions are satisfactory.

3.3 Conclusions

Tyr, HVa, HTyr and GA are low-molecular weight phenols characterized by a wide number of interesting biological activities. However, their hydrophilicity represents a drawback when the solubility in fatty and oily matrices or a better bioavailability in cellular environmental is required such as in some food, cosmetic and pharmaceutical formulations.

In this light, the first topic of the PhD program was the optimization of green chemistry procedures to obtain lipophilic ester derivatives of Tyr, HVa, HTyr and GA. Tyrosyl, homovanillyl and hydroxytyrosyl esters were isolated in good yields (60-98%), solubilizing phenolic compound in dimethyl carbonate, an eco-friendly solvent, and adding a little excess of the appropriate C2-C18 acyl chloride. The ion-exchange resin Amberlyst-15 showed to be a valid green alternative to strong acids, such as sulfuric acid and hydrochloric acid, for the synthesis of lipophilic derivatives of GA. A panel of alkyl esters of GA with an alkyl chain up to 18 carbon atoms was prepared in satisfactory yields (from 84 up to 100%), operating in heterogeneous phase. The reactions were initially carried out using methanol, ethanol, propanol and 1-butanol both as reagent and as solvent. However, as the alkyl chain of the alcohol increases, so does its boiling point, and so the elimination at reduced pressure became increasingly difficult. Therefore, it was necessary to introduce an organic solvent and to lower the quantity of alcohol to be used. 1,4-Dioxane, widely used in the organic synthesis, was chosen as reaction medium. In addition, the recycling of Amberlyst-15 was investigated and the resin proved to be efficient in the esterification reactions for at least five runs.

According to the circular economy model, the lipophilization reaction optimized on pure HTyr and GA was applied to HTyr-enriched extract and GA-enriched extract obtained, respectively, by *Olea europaea* L. and *Castanea sativa* Mill. wastes and by-products using sustainable processes based on membrane technologies. The yields of the final products are consistent with what was previously observed for pure HTyr and GA, although a slight decrease in yield was observed for alkyl gallates with carbon numbers greater than 4.

Considering the complexity of the starting material, the results obtained are satisfactory and lipophilic fractions containing alkyl esters of HTyr and GA will find use for applications where the solubility in lipid media is required.

3.4 Experimental section

3.4.1 Chemicals and instruments

Reagents, standards, silica gel 60 F254 plates, and silica gel Lichroprep® Si 60 (40-63 μm) were purchased from Merck Life Science. Solvents were purchased from VWR international. All chemicals used were of analytical grade. IBX was freshly prepared as described in literature. [254] All weighing were carried out using the Shimadzu ATX 124 analytical scale. Solvents were distilled using the rotary evaporator Rotavapor® R-100 (Buchi company). NMR spectra (^{1}H NMR, ^{13}C NMR) were recorded using a 400-MHz nuclear resonance spectrometer Advance-III Bruker. The chemical shifts were expressed in parts per million (δ scale) and referenced to either the residual protons or carbon of the solvent. HPLC analysis were carried out using a HP 1200 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA). The detector was a diode array and the column was a LiChrosorb RP-18 (250 × 4.60 mm, 5 μm i.d.). Melting points were determined with a Büchi B-545 apparatus. Mass spectra were determined with a Shimadzu QP2010-Plus Gas Chromatograph Mass spectrometer (EI ion source, DI).

3.4.2 Synthesis of hydroxytyrosol

Hydroxytyrosol was synthesized using a patented procedure optimized in our laboratories,[220,221] based on three steps: *(a) selective derivatization of the alcoholic group of tyrosol (2-hydroxyphenethyl alcohol) with dimethyl carbonate (DMC)*. Tyrosol (138 mg, 1.0 mmol) was dissolved in DMC (3 mL), and then H_2SO_4 96% (10.7 μL, 0.2 mmol) was added. The solution was stirred at reflux (T=90 °C) for 7 h until disappearance of the substrate. At the end, it was cooled to room temperature, and DMC was evaporated under vacuum as an azeotropic mixture with methanol (DMC/CH3OH=1:3) boiling at 64 °C. The residue was extracted with ethyl acetate, washed with a saturated solution of NaCl, and dried over Na_2SO_4. After filtration and evaporation under vacuum, carboxymethylated tyrosol [2-(4′-hydroxyphenyl)ethyl methyl carbonate] was obtained as colorless oil in quantitative yield. *(b)*

Oxidation of carboxymethylated tyrosol with 2-iodobenzoic acid and in situ reduction with sodium dithionite to obtain carboxymethylated hydroxytyrosol. Carboxymethylated tyrosol (1.0 mmol) was dissolved in CH_3OH (4 mL), and then IBX (336 mg, 1.2 mmol) was added. The solution was stirred at 0 °C until disappearance of the substrate (30 min). At the end, water (4 mL) and $Na_2S_2O_4$ (348 mg, 2.0 mmol) were added, and the solution was stirred for 5 min at room temperature. After evaporation of the solvent under vacuum, the residue was solubilized with ethyl acetate and treated with a saturated solution of $NaHCO_3$. The aqueous phase was extracted with ethyl acetate. The organic phases were washed with a saturated solution of NaCl and dried over Na_2SO_4. After evaporation of the solvent, carboxymethylated hydroxytyrosol [2-(3′,4′-dihydroxyphenyl)ethyl methyl carbonate] was obtained as a colorless oil in 86% yield. *(c) Hydrolysis of carboxymethylated hydroxytyrosol under basic conditions.* To a solution of carboxymethylated hydroxytyrosol (226 mg, 1.0 mmol) in THF was added KOH 1 M (3 mL, 3.0 mmol). The mixture was stirred at room temperature for 30 min. After evaporation of the solvent, the residue was solubilized with ethyl acetate and treated with 1 M HCl. The aqueous phase was extracted with ethyl acetate. Organic phases were washed with a saturated solution of NaCl and dried over Na_2SO_4. After evaporation of solvent, colorless oil was obtained (85% yield) and characterized by ^{1}H-NMR, ^{13}C-NMR and HPLC to verify both the chemical structure and purity.

3.4.3 Synthesis of lipophilic esters of tyrosol, homovanillyl alcohol and hydroxytyrosol: general procedure

The appropriate phenethyl alcohol (0.5 mmol) was solubilized in dimethyl carbonate (1.5 mL) at 25 °C. Then the acyl chloride was added (0.6 mmol) and the mixture was kept under stirring for 24 h. The reaction was monitored by thin-layer chromatography on silica gel plates using mixtures of dichloromethane and methanol (9.8/0.2, 9.5/0.5 or 9.0/1.0) as eluents. At the end, the solvent was distilled under reduced pressure and the residue was solubilized with ethyl acetate (10 mL); then a saturated solution of NaCl was added (5.0 mL). After the extraction with ethyl acetate (3 × 10 mL), the combined organic phases were washed with NaCl s.s. (10 mL), dried over Na_2SO_4, and filtered. The solvent was distilled under reduced pressure and the residue was purified by a silica gel chromatographic column using mixtures of dichloromethane and methanol (9.8:0.2, 9.5:0.5 or 9.0/1.0) as eluents. All compounds were characterized by NMR analysis, dissolving 20 mg of each sample in chloroform-d3 or

methanol-d4 (0.5 mL). Tyrosyl **3-13** and homovanillyl esters **14-24** were obtained in yields ranging from 90 to 98%, and hydroxytyrosyl esters **25-35** from 60 to 68% (**Table 3**, in Results and discussion section).

3.4.4 Preparation and characterization of an hydroxytyrosol-enriched extract

Olea europaea L. wastes (free-oil olive pulp) were obtained from Leccino cultivar (Vinci, Florence, Italy). Free-oil olive pulp was collected in October 2019 after oil extraction. A patented procedure based on membrane technology was applied to obtain hydroxytyrosol-enriched extracts from Olea europaea wastes.[255] The raw material was extracted with water and then subject to microfiltration (MF), nanofiltration (NF), and reverse osmosis (RO). Microfiltration was carried out with tubular ceramic membranes in titanium oxide; nanofiltration and reverse osmosis were carried out using spiral wound module membranes of poly (ether sulfone). By applying a spray drying process at the end of the extraction, a powder was obtained. Flash and preparative chromatography were carried out by a HPLC-PURE C-850 (Büchi Italia Srl, Cornaredo, Milan, Italy) equipped with a Sepacore pump C-605/control module C-615- T-valve, backpressure regulators from Pure sampling pump-column FlashPure Ecoflex C18 cartridges. For each run, 600 mg of the sample were introduced in the chromatography system with a syringe and purified. The eluents were water (eluent A) and ethanol (eluent B). A five-step linear solvent gradient was used starting from 20% of solvent B for two minutes, for 3 minutes at 70% of solvent B and for 14 minutes at 100% of solvent B at a flow rate of 30mL/min. The UV wavelengths were 240 nm, 285 nm, 330 nm and 310 nm (UV threshold: 0.05 AU). The fractions obtained after the chromatographic process were concentrated under vacuum, at 25 ° C using a Rotavapor B-490 (Büchi Italia Srl, Cornaredo, Milan, Italy); the resulting samples were then concentrated in water and freeze-dried using the Lyovac GT2 freeze dryer (Leybold - Heraeus, Cologno Monzese, Milan, Italy)" at T< - 30°C and P= 1 bar.

Qualitative and quantitative content of HTyrE was determined using a HP 1260 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an analytical column Lichrosorb RP18 250 x 4.60 mm i.d, 5 μm (Merck Darmstadt, Germany) was used. The eluents were water adjusted to pH=3.2 with formic acid (solvent A) and acetonitrile (solvent B). A four-step linear solvent gradient was used starting from 100% of solvent A up to 100% of solvent B, for 88 min at a flow rate of 0.8 mLxmin-1. Polyphenols found in the

extracts were identified by comparing retention times and UV/Vis spectra with those of the authentic standards. Each compound was quantified at the selected wavelength (240, 280, 330, and 350 nm) using a five-point regression curve. The ^{1}H spectrum was recorded dissolving 30 mg of sample in methanol-d4 (0.5 ml).

3.4.5 Esterification of an hydroxytyrosol-enriched extract: general procedure

A total of 25 mg (0.16 mmol) of hydroxytyrosol-enriched extracts were dissolved in dimethyl carbonate (5.0 mL), and 0.19 mmol (40–200 μL) of the appropriate acyl chloride was introduced. The reaction was kept at 25°C under magnetic stirring for 24 h; then, dimethyl carbonate was distillated under reduced pressure by using a rotary evaporator. The mixture was solubilized with ethyl acetate and washed with a saturated solution of NaCl; then, the combined organic phases were dried over Na_2SO_4. After filtration, the solution was recovered, and the solvent was evaporated under reduced pressure. The content of hydroxytyrosol and the corresponding alkyl ester present in each sample was determined by HPLC–DAD analysis at λ = 280 nm. The yields of hydroxytyrosyl esters range from 60 to 66%.

3.4.6 Synthesis of lipophilic esters of gallic acid: general procedure

(a) Synthesis with methanol, ethanol, propanol, 1-butanol

Gallic acid (50 mg, 0.3 mmol) was dissolved in a fixed volume (3 ml)[199] of the appropriate alcohol, and finally Amberlyst-15 (640 mg, 3 mmol) was introduced. The mixture was kept at 60-90 °C under magnetic stirring for 24 h. The reaction was monitored by thin-layer chromatography on silica gel plates using a mixture of dichloromethane:methanol=9:1 as mobile phase. After 24 h the resin was filtered and washed with diethyl ether [237] (3 ml x 2, 10 minutes under magnetic stirring) and methanol (3 ml x 1, 10 minutes under magnetic stirring). Solvents recovered were combined with reaction mixture and evaporated under reduced pressure. An aliquot (10 mg) of the crude reaction mixture was dissolved in methanol-d4 and used for the ^{1}H-NMR analysis by which the yield was calculated (**Table 5** and **6** in Results and discussion section). The residue was purified by a silica gel chromatographic column using mixtures of dichloromethane and methanol (from 10:0 to 9.9:0.1) as eluents. The final

compounds **36-39** were characterized by NMR analysis, dissolving 20 mg of each sample in methanol-d4 (0.5 mL).

(b) Synthesis with long chain alcohols (C5-C18)

To 50 mg of gallic acid (0.3 mmol) was added 1,4-dioxane in a complementary amount to the appropriate alcohol (1.5 mmol) in order to obtain a final volume of 1 ml. Finally, Ambelyst-15 (331 mg, 1.5 mmol) was introduced into the mixture. The reaction was kept at 90 °C under magnetic stirring for 24 h. The reaction was monitored by thin-layer chromatography on silica gel plates (using a mixture of dichloromethane:methanol=9:1 as mobile phase). After 24 h the resin was filtered and washed with diethyl ether [237] (1 ml x 2, 10 minutes under magnetic stirring) and methanol (1 ml x 1, 10 minutes under magnetic stirring). Solvents recovered were combined with reaction mixture and evaporated under reduced pressure. An aliquot (10 mg) of the crude mixture was dissolved in methanol-d4 and used for the ^{1}H-NMR analysis through which the yield was calculated (**Table 7** in Results and discussion section). The residue was purified by a silica gel chromatographic column using mixtures of dichloromethane and methanol (from 10:0 to 9.9:0.1) as eluents. The final compounds **40-49** were characterized by NMR analysis, dissolving 20 mg of each sample in methanol-d4 (0.5 mL).

3.4.7 Recycling of Amberlyst-15

Gallic acid (50 mg, 0.3 mmol) was dissolved in methanol (3 ml, 74.1 mmol), or, alternatively, in 1,4-dioxane (863 µl) by subsequently adding 1-butanol (1.5 mmol, 137 µl). After addition of the resin (640 mg, 3 mmol or 331 mg, 1.5 mmol), the mixture was left under magnetic stirring for 24 h, at T=60 or 90 ° C. At the end of reaction, the resin was filtered and washed with diethyl ether [237] (3 ml or 1 ml x 2, 10 minutes under magnetic stirring) and methanol (3 ml or 1 ml x 1, 10 minutes under magnetic stirring). The solvents recovered from washing were combined with reaction mixture and evaporated under reduced pressure. An aliquot (10 mg) of the crude reaction mixture was dissolved in methanol-d4 and used for the ^{1}H-NMR analysis by which the yield was calculated. The resin was dried in oven at T=90 °C overnight [232,235] and was employed for subsequent runs.

3.4.8 Preparation and characterization of a gallic acid-enriched extract

The extract was a commercial fraction furnished by GRUPPO MAURO SAVIOLA Srl (Viadana, MN, Italy). The patented procedure includes several solvent-free process streams and was carried out in the industrial tannin extraction and concentration/purification plant operating in Radicofani (SI, Italy).[72] The extract was analyzed by using a HP-1200 liquid chromatograph equipped with a DAD detector and a HP 1100 MSD API-electrospray (Agilent Technologies, Santa Clara, CA) operating in negative and positive ionization mode. A Luna, C18 250×4.60 mm, 5 μm column (Phenomenex, Torrance, CA), operating at 25 °C was used. The eluents were H_2O (adjusted to pH 3.2 with HCOOH) and CH_3CN. A four-step linear solvent gradient starting from 100% H_2O up to 100% CH_3CN was performed with a flow rate of 0.8 mL/min over a 55 minutes period, as previously described. Mass spectrometer operating conditions were: gas temperature 350 °C at a flow rate of 10.0 L/min, nebulizer pressure 30 psi, quadrupole temperature 30 °C and capillary voltage 3500 V. The fragmentor was set at 120 eV.

3.4.9 Esterification of a gallic acid-enriched extract: general procedure

(a) Synthesis with methanol, ethanol, propanol, 1-butanol

155 mg of extract were dissolved in 3 ml of the appropriate alcohol and then Amberlyst-15 (680 mg, 3.2 mmol) was introduced. The mixture was kept at 60-90 °C under magnetic stirring for 24-72 h. Then the resin was filtered and washed with diethyl ether (3 ml x 2, 10 minutes under magnetic stirring) and methanol (3 ml x 1, 10 minutes under magnetic stirring). Solvents recovered were combined with reaction mixture and evaporated under reduced pressure. An aliquot (20 mg) of the crude reaction mixture was dissolved in methanol-d4 and used for the ^{1}H-NMR analysis by which the yield was calculated. Compounds **36-39** were obtained in 100% yield. (**Table 8**, in Results and discussion section).

(b) Synthesis with long chain alcohols (C5-C18)

To 155 mg of extract was added 1,4-dioxane in a complementary amount to the appropriate alcohol (1.5 mmol respect to gallic acid) in order to obtain a final volume of 1 ml. Finally, Ambelyst-15 (680 mg, 3.2 mmol) was introduced into the mixture. The reaction was kept at 90 °C under magnetic stirring for 24-48 h. Then the resin was filtered and washed with diethyl ether (3 ml x 2, 10 minutes under magnetic stirring) and methanol (3 ml x 1, 10

minutes under magnetic stirring). Solvents recovered were combined with reaction mixture and evaporated under reduced pressure. An aliquot (20 mg) of the crude mixture was dissolved in methanol-d4 and used for the ^{1}H-NMR analysis through which the yield was calculated. Compounds **40-49** were obtained in yields ranging from 43 to 89% (**Table 10**, in Results and discussion section).

3.4.10 Characterization of lipophilic esters

4-Hydroxyphenethyl acetate (Tyrosyl acetate) **3**. Colorless oil. NMR spectra are in accordance with those reported in the literature.[188,194,220]

4-Hydroxyphenethyl butanoate (Tyrosyl butyrate) **4**. Colorless oil. NMR spectra are in accordance with those reported in the literature.[194]

4-Hydroxyphenethyl hexanoate (Tyrosyl hexanoate) **5**. Colorless oil. NMR spectra are in accordance with those reported in the literature.[192,220]

4-Hydroxyphenethyl octanoate (Tyrosyl caprylate) **6**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[188,192]

4-Hydroxyphenethyl decanoate (Tyrosyl capriate) **7**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[188,192]

4-Hydroxyphenethyl decanoate (Tyrosyl laurate) **8**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[192,198]

4-Hydroxyphenethyl tetradecanoate (Tyrosyl myristate) **9**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[188,194]

4-Hydroxyphenethyl palmitate (Tyrosyl palmitate) **10**. Colorless oil. NMR spectra are in accordance with those reported in the literature.[188,194,220]

4-Hydroxyphenethyl stearate (Tyrosol stearate) **11**. Colorless oil. NMR spectra are in accordance with those reported in the literature.[188,194]

4-Hydroxyphenethyl oleate (Tyrosol oleate) **12**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[188,194]

4-Hydroxyphenethyl linoleate (Tyrosol linoleate) **13**. Yellow oil. NMR spectra are in accordance with those reported in the literature. [188,194,220]

4-Hydroxy-3-methoxyphenethyl acetate (Homovanillyl acetate) **14**. Colorless oil. NMR spectra are in accordance with those reported in the literature.[105,220]

4-Hydroxy-3-methoxyphenethyl butanoate (Homovanillyl butyrate) **15**. Colorless oil. NMR spectra are in accordance with those reported in the literature.[105]

4-Hydroxy-3-methoxyphenethyl hexanoate (Homovanillyl hexanoate) **16**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[220]

4-Hydroxy-3-methoxyphenethyl octanoate (Homovanillyl caprylate) **17**. Yellow oil. ^{1}H-NMR (400 MHz,$CDCl_3$) δ: 6.88-6.86 (1H, m, Ph-*H*), 6.74-6.72 (2H, m, Ph-*H*), 5.57 (1H, bs, O*H*), 4.27 (2H, t, J = 6.0 Hz, OC*H*$_2$), 3.90 (3H, s, OC*H*$_3$), 2.88 (2H, t, J = 6.0 Hz, Ph-C*H*$_2$), 2.31 (2H, t, J = 8.0 Hz, COC*H*$_2$), 1.60 (2H, m, C*H*$_2$), 1.28 (8H, m, $CH_2(CH_2)_4CH3$), 0.90 (3H, m, C*H*$_3$). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 173.4, 145.9, 143.8, 129.2, 121.1, 113.8, 110.9, 64.5, 55.4, 34.3, 33.9, 31.1, 28.6, 28.5, 24.5, 22.1, 13.5.

4-Hydroxy-3-methoxyphenethyl decanoate (Homovanillyl capriate) **18**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[105]

4-Hydroxy-3-methoxyphenethyl dodecanoate (Homovanillyl laurate) **19**. Yellow oil. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 6.97-6.95 (1H, m, Ph-*H*), 6.87-6.72 (2H, m, Ph-*H)*, 5.40 (1H, bs, O*H*), 4.27 (2H, t, J = 8.0 Hz, OC*H*$_2$), 3.89 (3H, s, OC*H*$_3$), 2.88 (2H, t, J = 8.0 Hz, Ph-C*H*$_2$), 2.31 (2H, t, J = 8.0 Hz, COC*H*$_2$), 1.64-1.60 (2H, m, C*H*$_2$), 1.28 (16H, m, $CH_2(CH_2)_8CH3$), 0.92-0.89 (3H, m, C*H*$_3$). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 173.4, 145.9, 143.8, 129.1, 121.1, 113.9, 110.9, 64.5, 55.4, 34.3, 33.9, 31.4, 29.1, 29.0, 28.9, 28.8, 28.7, 28.5, 24.4, 22.1, 13.6.

4-Hydroxy-3-methoxyphenethyl tetradecanoate (Homovanillyl myristate) **20**. Yellow oil. ^{1}H-NMR (400 MHz, CDCl3) δ: 6.87-6.85 (1H, m, Ph-*H*), 6.74-6.72 (2H, m, Ph-*H*), 5.60 (1H, bs, O*H*), 4.27 (2H, t, J = 8.0 Hz, OC*H*$_2$), 3.89 (3H, s, OC*H*$_3$), 2.88 (2H, t, J = 8.0 Hz, Ph-C*H*$_2$), 2.31 (2H, t, J = 8.0 Hz, COC*H*$_2$), 1.64–1.60 (2H, m, C*H*$_2$), 1.28 (20H, m, $CH_2(CH_2)_{10}CH3$), 0.92-0.89 (3H, m, C*H*$_3$). ^{13}C-NMR (100 MHz, CDCl3) δ: 173.4, 145.9, 143.8, 129.1, 121.1, 113.9, 110.9, 64.5, 55.4, 34.3, 33.9, 31.4, 29.1, 29.0, 28.9, 28.8, 28.7, 28.6, 28.5, 28.4, 24.5, 22.2, 13.6.

4-Hydroxy-3-methoxyphenethyl palmitate (Homovanillyl palmitate) **21**. Colorless oil. NMR spectra are in accordance with those reported in the literature.[220,256]

4-Hydroxy-3-methoxyphenethyl stearate (Homovanillyl stearate) **22**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[105]

4-Hydroxy-3-methoxyphenethyl oleate (Homovanillyl oleate) **23**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[220]

4-Hydroxy-3-methoxyphenethyl linoleate (Homovanillyl linoleate) **24**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[220]

3,4-Dihydroxyphenethyl acetate (Hydroxytyrosyl acetate) **25**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[105,220,225]

3,4-Dihydroxyphenethyl butanoate (Hydroxytyrosyl butyrate) **26**. Colorless oil. NMR spectra are in accordance with those reported in the literature.[105,218,229]

3,4-Dihydroxyphenethyl hexanoate (Hydroxytyrosyl hexanoate) **27**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[220]

3,4-Dihydroxyphenethyl octanoate (Hydroxytyrosyl caprylate) **28**. Colorless oil. NMR spectra are in accordance with those reported in the literature.[195]

3,4-Dihydroxyphenethyl decanoate (Hydroxytyrosyl capriate) **29**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[105,229]

3,4-Dihydroxyphenethyl dodecanoate (Hydroxytyrosyl laurate) **30**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[193]

3,4-Dihydroxyphenethyl tetradecanoate (Hydroxytyrosyl myristate) **31**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[192]

3,4-Dihydroxyphenethyl palmitate (Hydroxytyrosyl palmitate) **32**. Yellow oil. NMR spectra are in accordance with the literature.[193,220,229]

3,4-Dihydroxyphenethyl stearate (Hydroxytyrosyl stearate) **33**. Yellow oil. NMR spectra are in accordance with those reported in the literature.[105,229,196]

3,4-Dihydroxyphenethyl oleate (Hydroxytyrosyl oleate) **34**. Colorless oil. NMR spectra are in accordance with those reported in the literature.[193,220,229]

3,4-Dihydroxyphenethyl linoleate (Hydroxytyrosyl linoleate) **35**. Colorless oil. NMR spectra are in accordance with those reported in the literature.[193,220,229]

Methyl 3,4,5-trihydroxybenzoate (Methyl gallate) **36**. White solid. m.p. = 200-202 °C,[197] found 195-197 °C. NMR spectra are in accordance with those reported in the literature.[197,199,201] MS (EI ion source): *m/z* (%) = 184 (21 [M^+]), 153 (100).[197,199,201]

Ethyl 3,4,5-trihydroxybenzoate (Ethyl gallate) **37**. Grey solid. m.p. = 148-150 °C,[197] found 147-148 °C. NMR spectra are in accordance with those reported in the literature.[197,199,201] MS (EI ion source): *m/z* (%) = 198 (51 [M^+]), 153 (100).[197,199, 201]

Propyl 3,4,5-trihydroxybenzoate (Propyl gallate) **38**. Grey solid. m.p.= 145-146 °C, 147-148 °C,[197,200] found 146-147 °C. NMR spectra are in accordance with those reported in the literature.[197,199,200,201,257] MS (EI ion source): *m/z* (%) = 212 (46 [M^+]), 170 (85), 153 (100).[197,199, 201]

Butyl 3,4,5-trihydroxybenzoate (Butyl gallate) **39**. Yellow solid. m.p.= 126-127 °C,[197] found 131-132 °C. NMR spectra are in accordance with those reported in the literature.[197,199,201] MS (EI ion source): m/z (%) = 226 (33 [M+]), 170 (100), 153 (69).[197,199,201]

Pentyl 3,4,5-trihydroxybenzoate (Pentyl gallate) **40**. White solid. m.p. = 93-94 °C,[197] found 94-95 °C. NMR spectra are in accordance with those reported in the literature.[197,199,201] MS (EI ion source): m/z (%) = 240 (27 [M+]), 170 (100), 153 (55). [197,199,201]

Hexyl 3,4,5-trihydroxybenzoate (Hexyl gallate) **41**. Yellow solid. m.p.=92-93 °C, [197,200] found 94-95 °C. NMR spectra are in accordance with those reported in the literature.[201,204,205,206,257] MS (EI ion source): m/z (%) = 254 (23 [M+]), 170 (100), 153 (48).[197,201,252]

Heptyl 3,4,5-trihydroxybenzoate (Heptyl gallate) **42**. Grey solid. m.p.=90-91 °C, 95-96 °C,[197,258] found 89-90 °C. NMR spectra are in accordance with those reported in the literature.[197,201,202,258] MS (EI ion source): m/z (%) = 268 (18 [M+]), 170 (100), 153 (43).[197,201,258]

Octyl 3,4,5-trihydroxybenzoate (Octyl gallate) **43**. Grey solid. m.p. =91-92 °C, 96-97 °C,[197,200] found 93-94 °C. NMR spectra are in accordance with those reported in the literature.[197,201,202,258] MS (EI ion source): m/z (%) = 282 (15 [M+]), 170 (100), 153 (35).[197,200,201]

Nonyl 3,4,5-trihydroxybenzoate (Nonyl gallate) **44** Yellow solid. m.p.= 92-93 °C,[258] found 90-92 °C. ^{1}H-NMR spectrum is in accordance with that reported in the literature.[202,258] ^{13}C NMR (100 MHz, CD_3OD) δ: 167.24; 145.09 (2C); 138.30; 120.33; 108.60 (2C); 64.40; 31.4; 29.26; 29.03; 29.01; 28.49; 25.81; 22.34; 13.06. MS (EI ion source): m/z (%) = 296 (15 [M+]), 170 (100), 153 (31).[258]

Decyl 3,4,5-trihydroxybenzoate (Decyl gallate) **45**. White solid. m.p.=94-95 °C, 96-97 °C,[197,200] found 94-95 °C. ^{1}H-NMR spectrum is in accordance with that reported in the

literature.[197,202,258] ^{13}C NMR (100 MHz, CD_3OD) δ: 167.24; 145.09 (2C); 138.31; 120.37; 108.64 (2C); 64.40; 31.65; 29.28; 29.26; 29.03; 29.00; 28.48; 25.79; 22.32; 13.04. MS (EI ion source): m/z (%) = 324 (15 [M+]), 170 (100), 153 (29).[197,200,258]

Undecyl 3,4,5-trihydroxybenzoate (Undecyl gallate) **46**. White solid. m.p.= 89-91 °C, 94-95°C,[197,258] found 91-92 °C. ^{1}H-NMR spectrum is in accordance with that reported in the literature.[197,202] ^{13}C NMR (100 MHz, CD3OD) δ: 167.24; 145.10 (2C); 138.31; 120.42; 108.67 (2C); 64.39; 31.64; 29.29; 29.28; 29.25; 29.03; 28.97; 28.48; 25.77; 22.30; 13.00. MS (EI ion source): m/z (%) = 324 (15 [M+]), 170 (100), 153 (29).[197,258]

Dodecyl 3,4,5-trihydroxybenzoate (Dodecyl gallate) **47**. White solid. m.p.= 94-96 °C, 97-98 °C,[197,200] found 96-97 °C. NMR spectra are in accordance with those reported in the literature.[197,200,202,257] MS (EI ion source): m/z (%) = 338 (12 [M+]), 170 (100), 153 (23).[197,200]

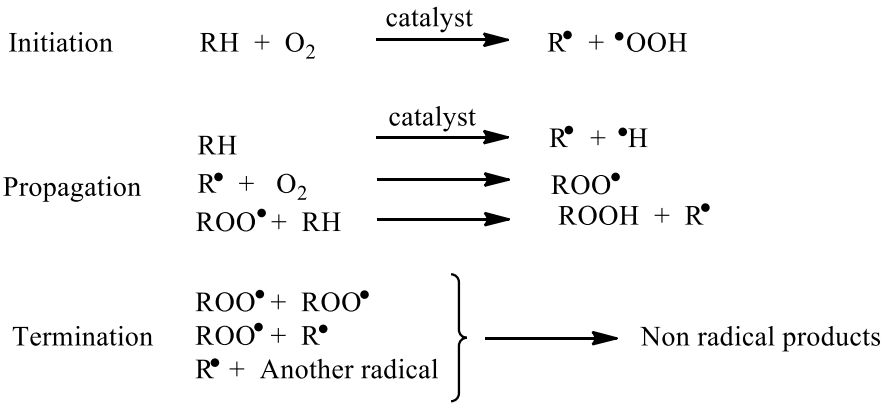
Octadedecyl 3,4,5-trihydroxybenzoate (Octadecyl gallate) **48**. White solid. m.p.= 102-104 °C, 96-97 °C,[197-200] found 95-96 °C. ^{1}H-NMR spectrum is in accordance with that reported in the literature.[197,200] ^{13}C NMR (100 MHz, CD_3OD) δ: 167.23; 145.09 (2C); 138.30; 120.4; 108.66 (2C); 64.36; 31.66; 29.36 (7C); 29.29; 29.27; 29.06; 29.00; 28.50; 25.79; 22.32; 13.03. MS (EI ion source): m/z (%) = 422 (15 [M+]), 170 (100), 153 (21).[197,200]

Oleyl 3,4,5-trihydroxybenzoate (Oleyl gallate) **49**. Yellow solid. ^{1}H NMR (400 MHz, CD_3OD) δ: 7.07 (2H, s, Ph-*H*), 5.44-5.34 (2H, m, C*H*=C*H*), 4.86 (1H, bs, O*H*); 4.24 (2H, t, J=6.54 Hz, OCH_2), 2.05-1.98 (4H, m, CH_2CH=CHCH_2), 1.78-1.71 (2H, m, OCH_2CH_2), 1.29 (22H, m, CH_2 (CH_2)$_{11}CH_3$), 0.91 (3H, t, J=6.64 MHz, 3H, CH_3). ^{13}C NMR (100 MHz, CD_3OD) δ: 167.22; 145.09 (2C); 138.30; 130.10; 120.34; 108.61 (2C); 64.38; 32.21; 31.68; 29.35 (7C); 29.29; 29.27; 29.07; 29.02; 28.50; 25.80; 22.34; 13.06. MS (EI ion source): m/z (%) = 420 (12 [M+]), 170 (100), 153 (49).

4. Preparation and characterization of novel poly (vinyl alcohol)-based films using hydroxytyrosol and oleuropein-enriched extracts for active antioxidant food packaging

4.1 Introduction: active antioxidant food packaging

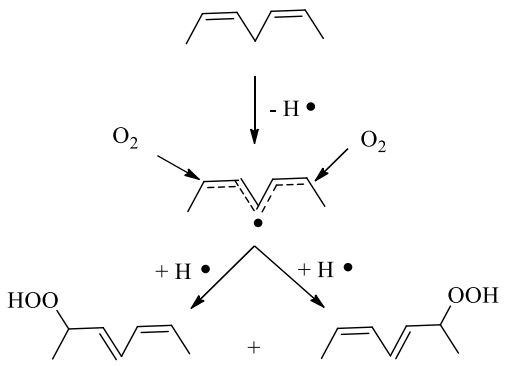
Food deterioration is a phenomenon occurring in all the different stages of the food chain (production, transport, processing, storage and marketing) and according to a recent FAO report, it is a relevant problem to solve to both guarantee food quality and dispose of food for world population.[259] Oxidation is one of the main external factors responsible for the chemical-physical alteration of food. High levels of oxygen in contact with food lead to oxidation and deterioration of nutrients. As example, in fats and oils containing unsaturated fatty acids, the most frequently occurring reaction is lipid oxidation.[260] It can be carried out by triplet oxygen ($^3O_2$) and singlet oxygen ($^1O_2$) by different mechanisms.[261] Autoxidation and photosensitized oxidation are radical mechanisms, which proceed according to the classic three steps: initiation, propagation, and termination (**Scheme 14**).[260, 261]



Scheme 14. Three stages of lipid oxidation.

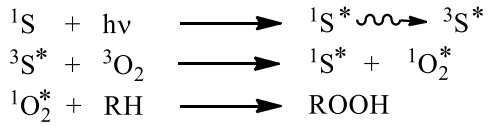
In the autoxidation process, the reactive specie is $^3O_2$, having a radical character. This means that fatty acids must be in a radical form to react; heat, metals or light act as catalysts to form allylic radical. In fact, hydrogen bond to carbon in the double bond is easily removed due to its low bond dissociation energy. The reaction of lipid radical with triplet oxygen is very quick at normal oxygen pressure. The final product is a conjugate hydroperoxide; in the

presence of polyunsaturated fatty acids such as linoleic acid, linolenic acid and arachidonic acid, positional isomerization occurs (**Scheme 15**).[260,261]



Scheme 15. Autoxidation process in polyunsaturated fatty acids.

In a photosensitized process, singlet oxygen acts as the oxidant, a non-radical specie, which directly reacts with high-electron-density double bonds on unsaturated fatty acids. However, in this case the presence of a photosensitizer, as chlorophyll, is required. Following the absorption of light energy, the singlet state chlorophyll changes to the excited state and reacts with atmospheric triplet oxygen. Therefore, the photosensitizer loses the acquired energy returning to ground state and, at the same time, singlet oxygen is the final product together to conjugated and non-conjugated hydroperoxides.[260,261] **Scheme 16** shows the general mechanism of this process, where S is the photosensitizer.



Scheme 16. General mechanism of a photosensitized oxidation process.

Hydroperoxides are unstable primary products that easily undergo another process, the thermal oxidation. Secondary products deriving from heating of oils can be volatile or non-volatile compounds. The first ones are aldehydes, ketones, short-chain hydrocarbons, alcohols, esters and are responsible for the onset of rancid flavour.[261] Dimerization and

polymerization are the main reactions involved in the thermal oxidation of fats and oils. The resulting products are triacylglycerol dimers and polymers, rich in oxygen and producing a residue similar to brown resin.[261] Furthermore, the oxidation of lipids can be catalysed by lipoxygenase by a non-radical mechanism. Oils containing linoleic, linolenic, and arachidonic acids are favoured substrates; conjugated hydroperoxides are the resulting products of oxidation.[261]

Reduction or destruction of essential nutrients, as well as formation of brown pigments and toxic aldehydes, changes in flavour and colour, are all aspects having a negative impact on quality and safety of food and influence the consumers choices. To delay or inhibit oxidation processes, antioxidants are commonly added to packages food. Antioxidants are able to prevent and delay the damages caused by free radicals as ROS and RNS; then, their incorporation into food packaging results also beneficial for health consumers. Synthetic phenolic compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ) are the most widely used food additives due their high efficacy, low cost and easy availability. However, the potential toxic and carcinogenic effects of some synthetic antioxidants have encouraged the study of natural alternatives for safer foods.[261]

In this light, an innovative solution strongly supported both by academia and industry, which aims to increase food shelf-life and preserve food quality is active food packaging.[262] It consists in the incorporation of active ingredients (as example, antimicrobial or antioxidant agents) into the polymeric material of a film to achieve a controlled release over time into food. In recent times, natural phenolic compounds as ferulic acid, caffeic acid,[263] gallic acid, quercetin,[264, 265] tocopherol, quercetin,[266] hydroxytyrosol [267,268,269] or complex plant-derived extracts [270, 271] have been added to polymeric films to produce active food packages. The need to implement a transition towards a *circular economy* model has led to investigate novel sustainable packaging solutions based on renewable resources. With the aim to satisfy the "zero waste concept", wastes and by-products of the olive oil industry represent a very attractive plant material for obtaining polyphenols-based antioxidants useful for a variety of industrial applications, including design and production of active food packages.[272]

Even in the choice of polymeric materials, a shift towards more sustainable alternatives is necessary. Due to their ease modelling, low prices and resistance to environmental degradation, plastics are the most popular polymers known. As example, polypropylene (PP)

and polyethylene (PE) are commonly used in packaging applications.[273] Finding environmentally friendly alternatives for food packaging materials is not an easy task, since they must possess specific properties (such as barrier and mechanical properties) and finally their employment must be approved by the food safety regulatory agencies.[261] Poly (vinyl alcohol) (PVA) is an example of eco-friendly material employed in the food packaging industry. It is a semi-crystalline polymer produced by hydrolysis of poly (vinyl acetate); is a water soluble, biocompatible, cheap and non-toxic film which indeed offers good thermal stability, thermomechanical characteristics, flexibility and strength, physical and optical properties relevant for packaging applications. Furthermore, Food and Drug Administration (FDA) officially approved this material for producing stretchable and flexible food packaging systems.[274,275]

On the basis of these considerations, and thanks to the scientific collaboration of our research groups with Prof. Luigi Torre and Debora Puglia (University of Perugia),[266,268-270] novel antioxidant PVA-based films were obtained incorporating hydroxytyrosol or oleuropein-enriched extracts (HTyrE and OleE, respectively) obtained by olive oil wastes and by-products into PVA.

4.2 Results and discussion: hydroxytyrosol and oleuropein enriched extracts obtained from olive oil wastes and by-products as active ingredients for poly (vinyl alcohol)-based films

HTyrE and OleE were used as active ingredients for the preparation of novel PVA-based formulations useful for antioxidant food packaging. They were obtained from free-oil olive pulp and leaves by membrane technologies.[276,277] As already reported for HTyrE (see paragraph 3.2.1), to prepare OleE, raw materials were first subjected to a solid-liquid extraction in water using a pneumatic extractor and then filtered in sequence using different membranes (microfiltration, ultrafiltration, nanofiltration) and then reverse osmosis.

The final fractions were then purified by chromatography to obtain OleE, which was characterized by HPLC-DAD and NMR spectroscopy (^{1}H and ^{13}C NMR) to define the qualitative and quantitative phenolic profile. Flash and preparative chromatography of the fractions was carried out using the Pure C-850 FlashPrep chromatography system (Büchi Italia Srl, Milan, Italy). A C18 column was used as stationary phase, while water and ethanol as eluents. As showed in the chromatographic profile recorded at 280 nm and reported in

Figure 36, Ole was the main component of OleE with 341.47 ± 13.66 mg/g (89%, w/w); HTyr, verbascoside and a secoiridoid derivative were the secondary components with 1.2 ± 0.04 mg/g, 2.53 ± 0.08 mg/g and 38.12 ± 1.22 mg/g, respectively, for a total of 11% w/w.

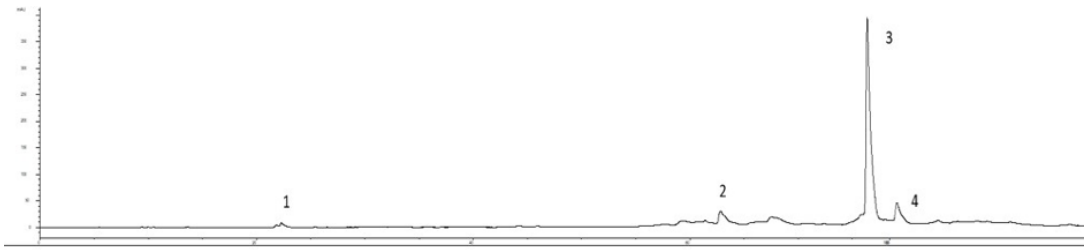


Figure 36. Chromatographic profile of OleE at 280 nm. 1. HTyr; 2. Verbascoside; 3. Oleuropein; 4. Secoiridoid derivative.

NMR analysis of the extract confirmed the chemical compositions determined by HPLC-DAD. In fact, the characteristic absorption signals of pure Ole [278] found by ^{1}H NMR spectrum analysis (**Figure 37a**) were predominantly present in the OleE spectra (**Figure 37b**) clearly proving that Ole is indeed the main components of OleE. The focus was turned on the area including signals in the range δ=7.8-5.6 ppm. The peculiar signal of Ole at δ=7.53 was assigned to the proton H-16. This singlet was strongly deshielded as was affected by both the proximity (in terms of σ bonds) of ethereal oxygen and the resonance effects due to the unsaturated ester. The signals at δ=6.71-6.55 were assigned to the aromatic protons (H-2, H-1, H-5) of the HTyr moiety; the signal of the vinyl proton H-13 (δ=6.12-6.07) was a quartet as consequence of the vicinal coupling with the methyl group (H-14). The singlet at δ=5.93 was assigned to the proton H-15 not having protons in such a close proximity able to give coupling.

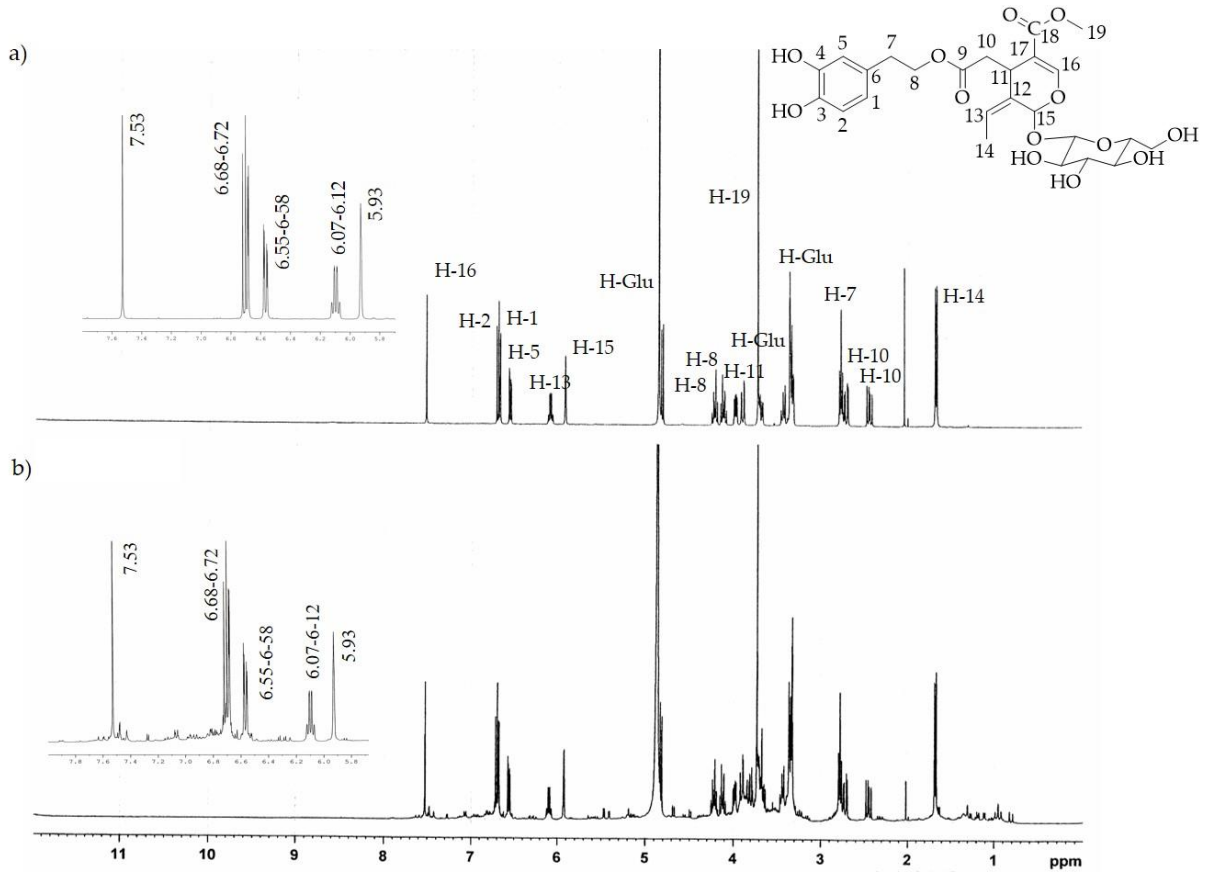


Figure 37. [1]H-NMR spectrum of (a) pure Ole; (b) Ole enriched extract (OleE).

Visual appearance and the morphological analysis of HTyrE and OleE in powder and in water solution are showed in **Figure 38.** HTyrE powder showed a light beige color. FESEM image shows that HTyrE pristine powder was characterized by spherical shape in accordance with literature (**Figure 38a**).[279] HTyrE solution resulted totally transparent (**Figure 38b**) maintaining the same colour previously detected for the powder, the transparency and solubility were guaranteed by the use of water as solvent. OleE powder denoted a brown-yellowish colour (**Figure 38c**) characterized by microparticles with circular/spherical shape. FESEM image of OleE in water dispersion (**Figure 38d)** showed that solution was partially transparent and maintained the same colour observed for the powder. Morphological investigations of both extract solutions are reported in **Figure 38b** and **38d**. In water solution, they lost the initial shape and during the evaporation process the different drops of HTyrE or OleE solution created a film.

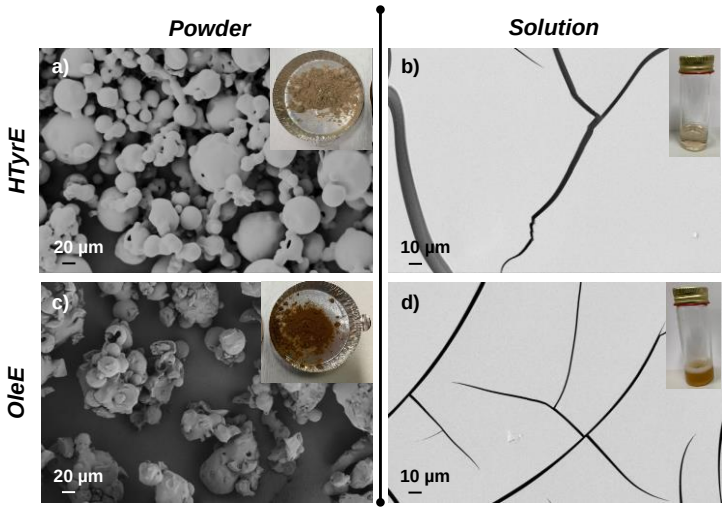


Figure 38. Visual aspect and FESEM images of **a)** HTyrE and **c)** OleE powder; **b)** and **d)** after dispersion in water.

After characterization of HTyrE and OleE, novel PVA-based films were prepared incorporating them into PVA polymeric films by solvent casting method. The use of water as solvent represents another eco-friendly aspect of this research avoiding thermal process and toxic organic solvents.

Firstly, the effect of different percentages of each enriched extract (5, 10 and 20% wt.) on morphological behaviour, optical, and antioxidant properties of the corresponding films was investigated. Then, PVA/HTyrE-5, PVA/HTyrE-10, PVA/HTyrE-20 and PVA/OleE-5, PVA/OleE-10, PVA/OleE-20 were prepared.

The morphological properties were investigated by FESEM (**Figure 39**). Experimental data clearly show that the incoroporation of HTyrE and OleE into PVA did not negatively affected the homogeneous and smooth internal fractured surface dected for neat PVA film (**Figure 39**). Therefore, no evident alterations were observed in the corresponding PVA-based films in terms of homogeneity and uniformity for both extracts at the different concentrations.This positive behavior was ascribable to the good dispersion of each extract in the PVA matrix processed by solvent casting, which was obtained also thanks to their high solubility in water.[269, 280]

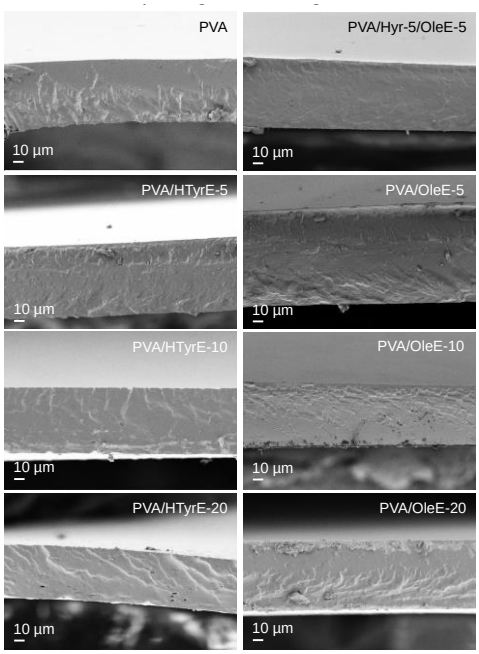


Figure 39. FESEM images of fractured surfaces of PVA, PVA/HTyrE and PVA/OleE films.

Transparency of films was analyzed by UV-Vis spectroscopy (**Figure 40**). The transparent nature of PVA film (transmittance of 94% at a wavelength of 600 nm) was slightly affected by the addition of HTyrE at different concentrations (transmittance at 600 nm: PVA/HTyrE-5 = 93%, PVA/HTyrE-10 = 92% and PVA/HTyrE-20 = 91%).[268,269] On the contrary, the incoroporation of OleE affected the transparency of the resulting films. The transmittance trend was kept stable up to the wavelength value of 450 and 550 nm for PVA/HTyrE and PVA/OleE films, respectively. This behavior highlights the possibility to realize transparent systems in the wavelength range of 600-900 nm. PVA/HTyrE films showed a visible absorption band centered at 280 nm as a consequence of the presence of the extract.[269] Furthermore, the transparency behavior for PVA/HTyrE films rapidly decreased from 450 nm to lower wavelength values. Precisely, the addition of OleE reduced drastically the transmittance of UVA (315-400 nm) and UVB (280-315 nm), while HTyrE limited the transparency in the UVA region and increased the protection in the UVB region (lower transmittance in UVB region respect to UVA wavelengths). In this respect, the development of PVA/HTyrE-5/OleE-5 combined the dual and positive behavior of each enriched extract. This behavior can positively act guaranteeing an increase in the shelf life in the presence of food sensitive to light, concluding that the extracts hindered the passage of light radiation, with specific wavelengths, which could damage the packaged products.[269,281]

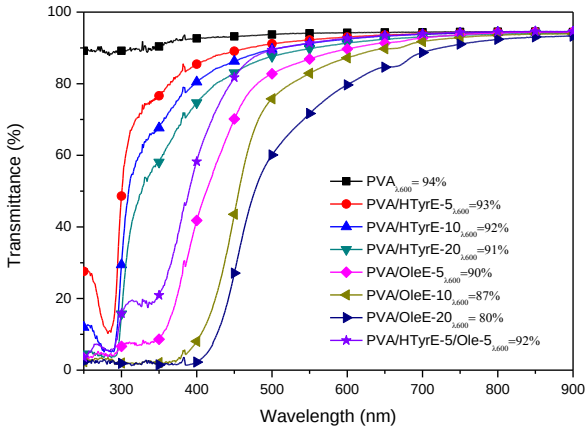


Figure 40. UV-Vis analysis of PVA, PVA/HTyrE and PVA/OleE films.

The colorimetric and gloss analysis were performed to evaluate the effect of HTyrE and OleE on the optical appearance of the films. The results are reported in **Table 9**. Neat PVA is characterized by high lightness value (L*=99.16 ± 0.04) respect to the other films. The measured value indicated high transparency of the matrix and a slight reduction of transparency when HTyrE or OleE was added into PVA, in accordance with the results of UV-Vis characterization. The addition of the extracts determined a colorimetric deviation from lighter yellow (desert sand) to darker yellow/brown using HTyrE or OleE, respectively. These variations were magnified by increasing the concentration of both extracts (**Table 9**) and using OleE (b* PVA=0.13±0.02, b* PVA/20HTyrE=9.17±0.06 and b* PVA/20OleE=55.76±0.13). The increase in concentration significantly contributed in modifying the ΔE* values. The highest ΔE* value for PVA films has been registered for PVA/OleE-20 film (ΔE* = 60.14±0.37). The PVA/HTyrE-5/OleE-5 showed similar color parameters of PVA/Ole-5 evidencing that OleE greatly influenced the final color of polymeric system. The presence of the extracts influenced the aesthetic quality of PVA-based films; this characteristic was expressed through the Gloss value that was reduced as the concentration of the extracts increased.[264,281]

Table 9. Color coordinates of PVA/HTyrE and PVA/OleE films.

Formulations	L*	a*	b*	ΔE*	Gloss (°)
Control	99.47±0.00	-0.08±0.01	-0.08±0.01	-	121±0
PVA	99.16±0.04	-0.10±0.01	0.13±0.02	0.37±0.05	254±4
PVA/HTyrE-5	97.39±0.15	-0.11±0.03	3.71±0.22	4.33±0.25	228±9
PVA/HTyrE-10	95.93±0.02	-0.08±0.00	6.52±0.11	7.49±0.10	215±2
PVA/HTyrE-20	94.45±0.10	-0.25±0.03	9.17±0.06	10.53±0.10	171±5
PVA/OleE-5	92.41±0.58	-2.98±0.10	24.57±1.23	25.81±1.34	225±3
PVA/OleE-10	87.62±0.47	-2.97±0.61	32.73±0.12	32.73±0.12	185±2
PVA/OleE-20	77.50±0.68	3.97±0.50	55.76±0.13	60.14±0.37	122±9
PVA/HTyrE-5/Ole-5	92.70±0.89	-3.35±0.12	23.59±2.33	24.84±2.46	222±5

Finally, total phenolic content (TPC) and antioxidant properties of each formulation (PVA/HTyrE-5, PVA/HTyrE-10, PVA/HTyrE-20, PVA/OleE-5, PVA/OleE-10 and PVA/OleE-20) were evaluated through the Folin-Ciocâlteau and the DPPH assays respectively. Total phenolic content (TPC) analysis and antioxidant activity were measured as an indirect way to evaluate the release properties and the antiradical activity of the produced films, and therefore to select the better concentration of HTyrE and OleE to be incorporated in the PVA films. Indeed, an important property of the developed formulations is the antioxidant activity induced by the active ingredients, which is directly linked with the phenolic content.

The results, depicted in **Figure 41a**, showed a corresponding increase in TPC value in relation to the phenolic concentration of the formulations. A similar trend was observed for the antioxidant activity determined by DPPH assay on the solutions obtained from each formulation.[281] Indeed, as showed in **Figure 41b**, all formulations proved an antioxidant activity expressed as RSA% value in direct relation to the phenolic concentration present in the formulations.

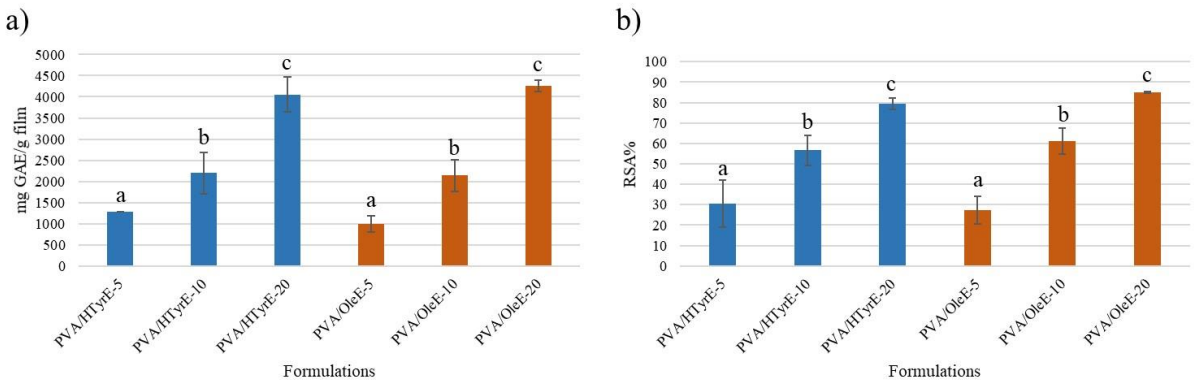


Figure 41. a) Total phenolic content (TPC) and b) antioxidant activity of PVA/HTyrE-5, PVA/HTyrE-10, PVA/HTyrE-20, PVA/OleE-5, PVA/OleE-10 and PVA/OleE-20 formulations determined by Folin-Ciocalteu method and DPPH assay respectively. Results are expressed as mg of gallic acid equivalent (GAE)/g of film for TPC and as RSA% for DPPH assay.

On the basis of the transparency behavior as well as balancing morphological features, optical properties and antioxidant performances of the resulting materials, the 10% w/w concentration of HTyrE and OleE used for casting the PVA films was chosen. In fact, the presence of a higher content of the extract (20%wt, PVA/HTyrE-20 and PVA/OleE-20) radically reduced the transparency in the visible spectrum around wavelengths (λ) of 400-700 nm (**Figure 40**).

In addition, a blend of HTyrE (5% w/w) and OleE (5% w/w) was selected to verify a possible synergic effect of both extracts on the properties of the corresponding formulations. After the preparation of PVA/HTyrE-10, PVA/OleE-10 and PVA/HTyr-5/OleE-5, the antioxidant activity was evaluated in a food simulant to test effectiveness as active packages. Release tests were performed according to the European Standard EN 13130-200522 [282] and European Commission Regulation 10/2011.[283] Each formulation was analysed in the proper water-based foods simulant solution (ethanol 10% v/v) for 21 days. All samples were analyzed at 1, 3, 7, 10 and 21 days quantifying HTyr and Ole released via UV-Vis spectrophotometry. As shown in **Figure 42**, in all the tested formulations, concentration of HTyr and Ole did not sensibly increase during the 21 days of the test.

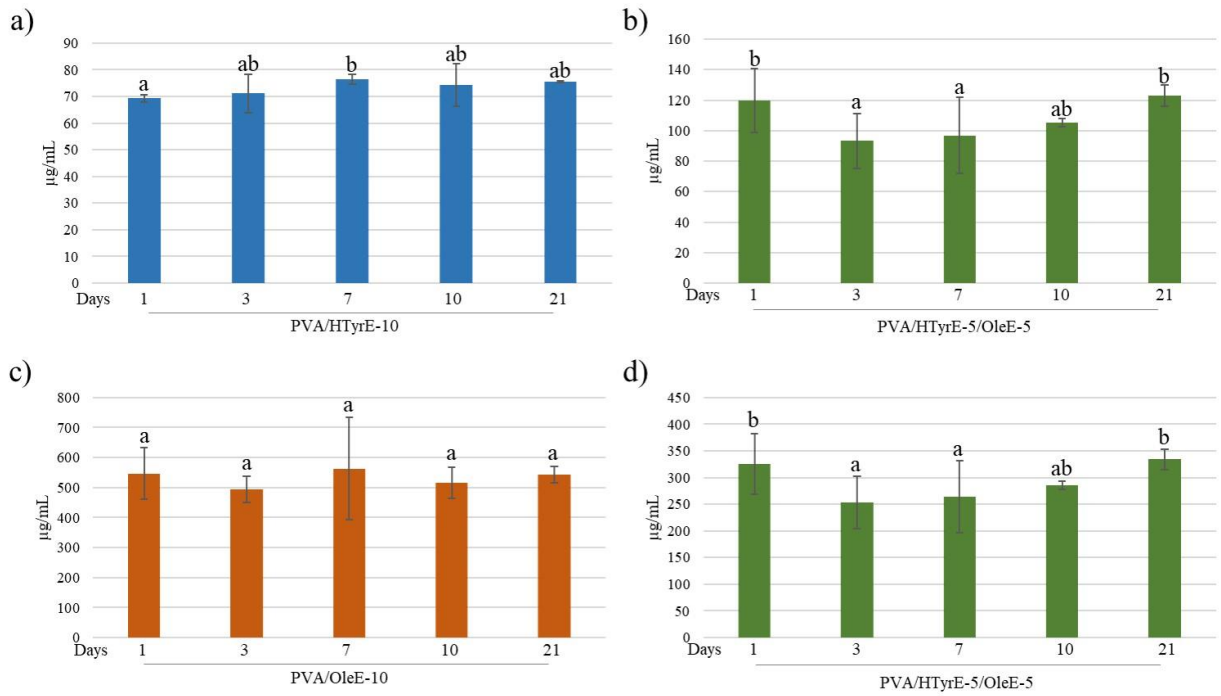


Figure 42. a), c) HTyr and b), d) Ole released (µg/ml) from each formulation (PVA/HTyrE-10, PVA/OleE-10 and PVA/HTyrE-5/OleE-5) into the simulant solution at different contact times. Analysis have been carried out in a food simulant (ethanol 10% v/v) for 21 days.

The antioxidant activity was evaluated through the DPPH assay as well performed on the food simulant solutions at different days (1, 3, 7, 10 and 21 day). As shown in **Figure 43,** a remarkable antioxidant activity was observed for all formulations tested over time. In line with the experimental data concerning the specific migration, antioxidant activity did not sensibly increase during the 21 days of the test. These results prove an uncontrolled quick release and are indeed in total accordance with what has been observed for PVA films incorporating pure active ingredients such as HTyr, gallic acid and quercetin into PVA.[271,273] As depicted, all formulations showed antioxidant activity in the following order: PVA/OleE-10, PVA/HTyrE-5/OleE-5, and PVA/HTyrE-10. These results were consistent with the greater concentration of Ole into OleE compared to that of HTyr into HTyrE (90% and 77%, respectively).

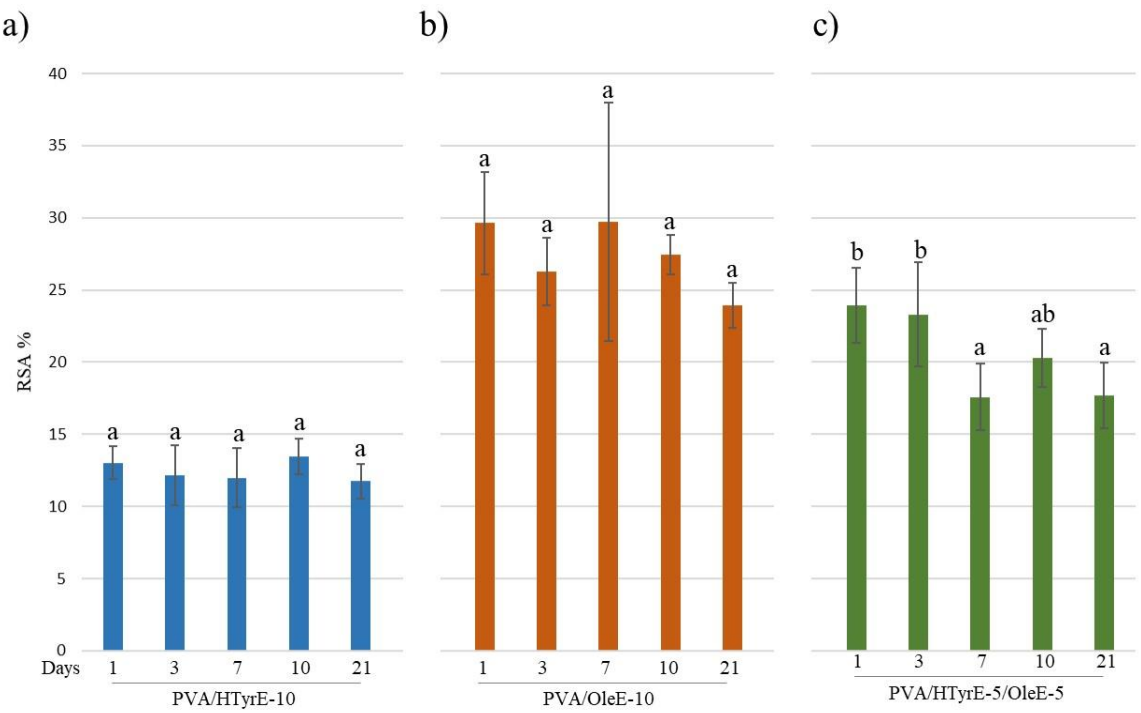


Figure 43. Radical scavenging activity (RSA%) of food simulant solutions after migration tests from PVA/HTyrE-10 (a), PVA/OleE-10 (b) and PVA/HTyrE-5/OleE-5 (c) formulations.

4.3 Conclusions

Novel films were designed and produced incorporating HTyrE and OleE as active ingredients into PVA by solvent casting method. Both extracts were obtained from olive oil wastes and by-products (free-oil olive pulp and leaves, respectively) through sustainable processes based on membrane technologies and finely characterized by analytical advanced techniques as HPLC-DAD and NMR. The resulting films (PVA/HTyrE-10, PVA/OleE-10, and PVA/HTyrE-5/OleE-5) were characterized investigating the morphological behaviour, optical and antioxidant properties. All formulations showed good performances and the best antioxidant effect was observed for PVA/OleE-10, followed by PVA/HTyrE-5/OleE-5 and then PVA/HTyr-10. Based on their properties, these films seem promising as active packages for the preservation of oxidative-sensitive food products. A relevant environmental aspect of this work is the reuse and valorization of olive oil industry wastes and by- products according to the circular economy model.

4.4 Experimental section

4.4.1 Materials

Olea europaea L. wastes and by-product (free-oil olive pulp and leaves) were obtained from Leccino cultivar (Vinci, Florence, Italy) and were collected in April during the pruning of olive trees. Poly(vinyl alcohol) (average MW 124-146 kg mol^{-1}, 99% hydrolyzed) was purchased from Sigma-Aldrich® (Milan, Italy). Chemicals and HPLC grade solvents were purchased from Sigma-Aldrich (Milan, Italy). Tyrosol and oleuropein used as standards were supplied by Extrasynthèse (Genay, France). Hydroxytyrosol was synthesized according to an optimized and patented procedure.[220,221]

4.4.2 Preparation and characterization of oleuropein-enriched extract (OleE)

HtyrE was prepared and characterized as previously described in paragraph 3.4.4. OleE were prepared from leaves using an industrial process of recovery and concentration, as already described.[277] By applying a spray drying process at the end of the extraction, a powder for each sample was obtained. Flash chromatography and preparative were carried out by a HPLC-PURE C-850 (Büchi Italia Srl, Cornaredo, Milan, Italy) equipped with a Sepacore pump C-605/control module C-615- T-valve, backpressure regulators from Pure sampling pump-column FlashPure Ecoflex C18 cartridges. For each run, 600 mg of the sample were introduced in the chromatography system with a syringe and purified. The eluents were water (eluent A) and ethanol (eluent B). A five-step linear solvent gradient was used starting from 20% of solvent B for two minutes, for 3 minutes at 70% of solvent B and for 14 minutes at 100% of solvent B at a flow rate of 30mL/min. The UV wavelengths were 240 nm, 285 nm, 330 nm and 310 nm (UV threshold: 0.05 AU). The fraction obtained after the chromatographic process were concentrated under vacuum, at 25 ° C using a Rotavapor B-490 (Büchi Italia Srl, Cornaredo, Milan, Italy); the resulting sample were then concentrated in water and freeze-dried using the Lyovac GT2 freeze dryer (Leybold - Heraeus, Cologno Monzese, Milan, Italy)" at T< - 30°C and P= 1 bar.

Qualitative and quantitative content of OleE were determined using a HP 1260 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an analytical column Lichrosorb RP18 250 x 4.60 mm i.d, 5 μm (Merck Darmstadt, Germany) was used. The eluents were water adjusted to pH=3.2 with formic acid (solvent A) and acetonitrile

(solvent B). A four-step linear solvent gradient was used starting from 100% of solvent A up to 100% of solvent B, for 88 min at a flow rate of 0.8 mLxmin-1. Polyphenols found in the extracts were identified by comparing retention times and UV/Vis spectra with those of the authentic standards. Each compound was quantified at the selected wavelength (240, 280, 330, and 350 nm) using a five-point regression curve.[284]

Ole (20 mg) or OleaE (30 mg) were solubilized in methanol-d4 (0.5 mL). ^{1}H NMR spectra were recorded using the 400 MHz Nuclear Magnetic Resonance Spectrometer Avance III (Bruker, Germany). Chemical shifts were expressed in parts per million (δ scale) and referred to the residual protons of the solvent.

4.4.3 Preparation and characterization of PVA-based formulations

PVA-based films were prepared by solvent casting technique. The content of HTyrE and OleE were 5, 10 and 20 % wt. in accordance with available literature [265,278] and then fixed at 10% wt for each formulation.[265,278] Initially, PVA (0.5 g) was dissolved in distilled water (10 mL) under magnetic stirring for 2 h at 80 °C. The polymeric solution was kept under magnetic stirring to reach room temperature (RT) and then cast. PVA-based films listed in **Table 10** were obtained mixing, under magnetic stirring, the polymeric solution with a specific amount of HTyrE and/or OleE. Each extract was previously dispersed in distilled water (0.1 g of extract in 10 mL of distilled water) for 1 h at RT.

Table 10. Material formulations of PVA and PVA based films.

Formulations	PVA	HTyrE (% wt)	OleE (% wt)
PVA	100	-	-
PVA/HTyrE-5	95	5	-
PVA/HTyrE-10	90	10	-
PVA/HTyrE-20	80	20	-
PVA/OleE-5	95	-	5
PVA/OleE-10	90	-	10
PVA/OleE-20	80	-	20
PVA/HTyrE-5/OleE-5	90	5	5

Homogeneous HTyrE or OleE dispersions in aqueous solution were obtained applying a magnetic stirring and a sonication bath, both at RT for 1 h. The polymeric solutions were cast in a Teflon® substrate and the evaporated at RT. PVA films with thicknesses between 50 and 80 μm were equilibrated for 1 week at 53% RH in desiccators before the characterizations. The RH conditions during the storage time were guaranteed using magnesium nitrate-6-hydrate oversaturated solution.[267,285]

4.4.3.1 Morphological and colour analysis

The morphological investigation was performed by Field emission scanning electron microscopy (FESEM, Supra 25-Zeiss). The analysis was firstly carried out on HTyrE and OleE in powder state and after dissolution in deionized water. The powders were deposited on conductive adhesive, gold sputtered and analyzed, a similar procedure was also adopted for the AI in water solution. Few drops of the AI suspensions were deposited onto a substrate of silicon, dried at room temperature and visualized after gold sputtering. A magnetic stirring at room temperature for 1 h was applied in order to obtain a uniform dispersion of active ingredients in the solvent. Subsequently, the effect of both HTyrE and/or OleE on the fracture surface of PVA based films was investigated. The surfaces were gold sputtered in order to offer electric conductivity and the samples were observed using an accelerating voltage of 2.5 kV.

The colour parameters of PVA films were determined by means of a spectrophotometer (CM-2300d Konica Minolta, Japan). Data were acquired by using the SCI 10/D65 method whereas CIELAB colour variables, as defined by the Commission Internationale de l′Éclairage (CIE 1995), were utilized. The different produced formulations were positioned on a white standard plate and L*, a*, and b* parameters were analyzed. L* value ranges from 0 (black) to 100 (white); a* value ranges from -60 (green) to 60 (red); and b* value ranges from -60 (blue) to 60 (yellow). Samples were evaluated in triplicate, and three measurements were taken at random locations on each of the studied films. The total colour difference ΔE* and gloss parameters were calculated as indicated in Equation:

$$\Delta E^{*} \quad * = \sqrt{(\Delta L *)^2 + (\Delta a *)^2 + (\Delta b)^2}$$

4.4.3.2 Determination of the total phenolic content

Total phenolic content (TPC) of PVA/HTyrE-5, PVA/HTyrE-10, PVA/HTyrE-20, PVA/OleE-5, PVA/OleE-10 and PVA/OleE-20 formulations was measured through the Folin-Ciocâlteu method.[286] Briefly, each PVA system (0.1 g) was placed in methanol (2 mL) for 24 h at room temperature.[285] Then, 100 µL of samples or standard, 2 ml of H_2O and 200 µL of Folin-Ciocâlteau reagent were mixed and incubated at room temperature for 3 min. Next, 1 mL of Na_2CO_3 (20/80 p/v) was added and the solutions were placed in the dark for 1 h. Finally, absorbance was measured at 765 nm using a UV/VIS spectrophotometer (UV-2600-Shimadzu, Kyoto, Japan). TPC values were quantified using gallic acid (GA) as standard (300-590 mg/L) and expressed as mg of Gallic Acid Equivalent (GAE)/g of extract.

4.4.3.3 Evaluation of the radical scavenging activity of films

Radical scavenging activity was determined using the DPPH assay.[265] Analyses were conducted on PVA/HTyrE-5, PVA/HTyrE-10, PVA/HTyrE-20, PVA/OleE-5, PVA/OleE-10 and PVA/OleE-20 methanolic solutions and on food simulant solutions from PVA/HTyrE-10, PVA/OleE-10 and PVA/HTy-5/OleE-5 at every contact time (1, 3, 7, 10, and 21 days). All solutions (1 mL) were mixed with 1 mL of 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) in methanol (50 mg/L) and incubated at room temperature in the dark for 60 min. The absorbance was measured at 517 nm using a UV/VIS spectrophotometer (UV-2600-Shimadzu, Kyoto, Japan). DPPH radical scavenging activity (RSA) was calculated according to the Equation:

$$RSA\% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where A_{sample} is the absorbance of sample and $A_{control}$ is the absorbance of the control.

4.4.3.4 Release studies in a food simulant

Specific migration tests were performed into ethanol/water 10% (v/v) solution as food simulant, according to European Standard EN 13130-2005 [282] and European Commission Regulation 10/2011.[283] Double-sided, total immersion migration tests were carried out with 12 cm^2 of films and 20 mL of simulant (area-to-volume ratio around 6 dm^2 L^{-1}) in triplicate at 40 ºC in an oven. Samples were taken at 1, 3, 7, 10, 21 days in triplicate; a blank test for the

simulant was also included. After the migration tests, each solution was recovered and stored at -4 ºC before analysis.

HTyrE and OleE released by each PVA-based formulation in food simulant (10% (v/v) ethanol water solution) for every contact time (1, 3, 7, 10, and 21 days) were analysed using a UV/VIS spectrophotometer (UV-2600-Shimadzu, Kyoto, Japan). Absorbance values were measured at 280 nm. Standard curves for HTyr (8-60 µg/ml) and Ole (25-110 µg/ml) were used. Samples were analysed in triplicate and results are reported as the average ± SD and expressed as µg of antioxidant per mL of food simulant.

4.4.4 Statistics

Data were expressed as averages ± SD of three replicate determinations. Statistical differences were calculated using one-way analysis of variance (ANOVA), followed by Tukey's honest significant difference (HSD) post hoc test.

5. Synthesis of new phosphodiester dimers of tyrosol, homovanillyl alcohol and hydroxytyrosol as potential anti-Alzheimer's disease agents

5.1 Introduction: polyphenols and neurodegenerative disorders

Neurodegenerative disorders include a variety of diseases related to the nervous system. The most common cause both of dementia and death is Alzheimer's disease (AD), identified more than 100 years ago. Dementia is a progressive decline in neurocognitive skills that affects the ability of a person to perform daily activities. Therefore, neurodegenerative diseases have a significant medical and financial impact on society and the aging of the world population as well as the lack of effective treatments is worrying.[287] Neurodegenerative diseases are responsible for the damage of the brain components related to cognitive functions, namely neurons. Extracellular accumulation of amyloid-β (Aβ) protein (called β-amyloid plaques) and oxidative stress are supposed to be major contributors to neuronal cell damage and therefore play a significant role in causing the AD onset.[288] Massive deposition of amyloid fibers is believed to interfere with the neuron-to-neuron communication at synapses and to contribute to cell death. The causes responsible for this abnormal accumulation of proteinaceous deposits are still unclear, "*the amyloid hypothesis*" tries to explain the mechanism.[289] According to this hypothesis, mutations in the genes encoding the precursor protein β-amyloid (APP) are the events at the origin of the neurotoxic cascade leading to the pathogenesis of AD. Genetic mutations promote the proteolytic cleavage of APP by β- or γ-secretase, causing an overproduction of fragments with neurotoxic Aβ domain which form the amyloid plaques.

At the same way, the oxidative stress, a consequence of an imbalance between ROS production and antioxidant defences, contributes to pathogenesis of AD. Accumulation of ROS in mitochondria, organelle-producing semi reduced oxygen species during respiration, led to harmful radical reactions, such as lipid and DNA oxidations, responsible of the ageing processes and neurodegenerative disorders.[290] Moreover, aggregation of Aβ and oxidative stress are strictly interconnected. The simultaneous presence of high levels of redox-active metals (e.g. copper, iron) and Aβ aggregations favours the overproduction of intracellular ROS. The peptide itself is believed to cause a reduction in the oxidation state of the metal ion probably via the Aβ methionine 35 sulphur atom or biological reducing agents such as dopamine and ascorbate. The metal ion is thus involved in a Fenton-like chemistry, causing oxidative stress and neuronal damage.[289] Furthermore, many studies have shown that some

divalent metal cations (e.g. Cu^{2+} and Zn^{2+}) play a role in Aβ aggregation, insomuch to propose the *metal hypothesis*. Specifically, the presence of these ions influences the aggregation rate of Aβ, leading to a faster generation of more amorphous aggregates and/or potentially neurotoxic oligomers.[291]

Compounds that show both radical scavenging activity and capacity for bio-metal modulation in the brain may be useful for both prevention and therapy of AD. Natural polyphenols appear to be ideal candidates because in addition to their well-known antioxidant action, they also show anti-amyloid effects. In addition, the daily intake of polyphenols through the diet makes their potential use in the treatment of AD more interesting and advantageous compared to other compounds. Specifically, dietary polyphenols containing a catecholic or a pyrogallol moiety showed a disrupting effect on Aβ aggregation.[292] Epigallocatechin gallate (EGCG), found mainly in green tea, interferes directly with amyloid fibril formation from several peptides and proteins and remodels preformed fibrils, thus generating non-toxic species. The interaction with amyloidogenic peptides/proteins can take place in two ways. EGCG is able to bind to the unfolded or misfolded peptide/protein with non-covalent random interactions involving the peptide backbone, leading to the formation of small off-pathway oligomers or amorphous material. On the other hand, EGCG is able to interact with the side-chains of specific residues so to maintain peptide/protein native configuration, thus preventing its aggregation. Resveratrol, found mainly in red wines, besides acting upstream of one (or more) signalling cascades, possibly activated by binding to a membrane receptor, is also capable of directly interfering with the amyloid aggregation of various peptides. Specifically, resveratrol binds to His-18 human pancreatic islet amyloid polypeptide (hIAPP), preventing it to promote the oligomerization of Aβ.[292]

Recently, *Olea europaea* polyphenols, i.e. HTyr, Tyr, HVa and Ole, have attracted particular interest in this field. Ole was found to prevent the growth of toxic $Aβ_{1–42}$ oligomers through its interaction with the peptide N-terminus. The π-π interaction between the Tyrosine-10 aromatic residue in amyloidogenic proteins and the aromatic ring in the phenolic compound would disrupt the π stacking between protein units by blocking self-assembly in amyloid fibrils.[293] Tyr and HTyr are identified as neuroprotective agent against Aβ-induced toxicity. Results demonstrated that the number of cell death decreased when cells were co-treated with Aβ and Tyr or HTyr.[294]

The key role of the catechol moiety in the inhibition of amyloid plaque formation has been recently confirmed.[295,296] The ability of the three slightly different polyphenols, Tyr, HTyr and HVa, to interfere with $A\beta_{1-40}$ aggregation was investigated by Thioflavin T (ThT) assay. Experimental data showed that HTyr was a potent inhibitor of amyloid growth, while Tyr and HVa catalyse fibril formation. The different behaviour was explained using molecular dynamics simulations. Due to the additional hydroxyl group at the C3 position, HTyr was able to establish stronger interactions via hydrogen bonds with the amino acid residues strictly involved in the aggregation process[297]

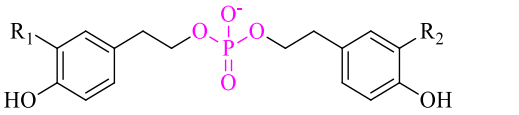
Finally, considering that the potential role of HTyr in neurogenesis processes *in vivo* has only been partially investigated, a recent study carried out in collaboration with the research group of Dr. Felice Tirone (Institute of Biochemistry and Cell Biology, National Research Council, Monterotondo) evidenced the effects of the oral administration of HTyr on hippocampal neurogenesis.[298] Different stages of production of new neurons in aged and adult mice and in a mouse model which shows impaired neurogenesis (i.e. the knockout of the antiproliferative B-cell translocation 1 gene (Btg1)) were investigated. The experimental results revealed that HTyr treatment leads to an increase in survival of new neurons and a decrease in apoptosis. In addition, in the aged dentate gyrus and Btg1-null, HTyr treatment stimulates the proliferation of stem and progenitor cells.

Considering that HTyr is one of the low-weight molecular phenols present in extra virgin olive oil, this study once again highlights the health benefits of Mediterranean style nutrition. Although equivalent for humans of the dose tested in the study is above the consumption of HTyr within the Mediterranean diet, it could be increased by the administration of dietary supplements formulations as preventing agent to improve age-related brain dysfunctions.

5.2 Results and discussion: synthesis of new phosphodiester dimers of tyrosol, homovanillyl alcohol and hydroxytyrosol and their effect on amyloid β aggregation and metal chelation ability

The interesting anti-amyloid activity previously discussed relatively to catechol-based polyphenols, encouraged the design of new potential anti-AD agents. During the PhD program, in collaboration with the research group of Prof. Armando Zarrelli and Giovanni Di Fabio (University Federico II, Naples) Tyr, HVa and HTyr have been used as scaffolds for the

design and synthesis of a mini-library of derivatives containing two phenylethanoid fragments linked through a phosphodiester bond (**Figure 44**).

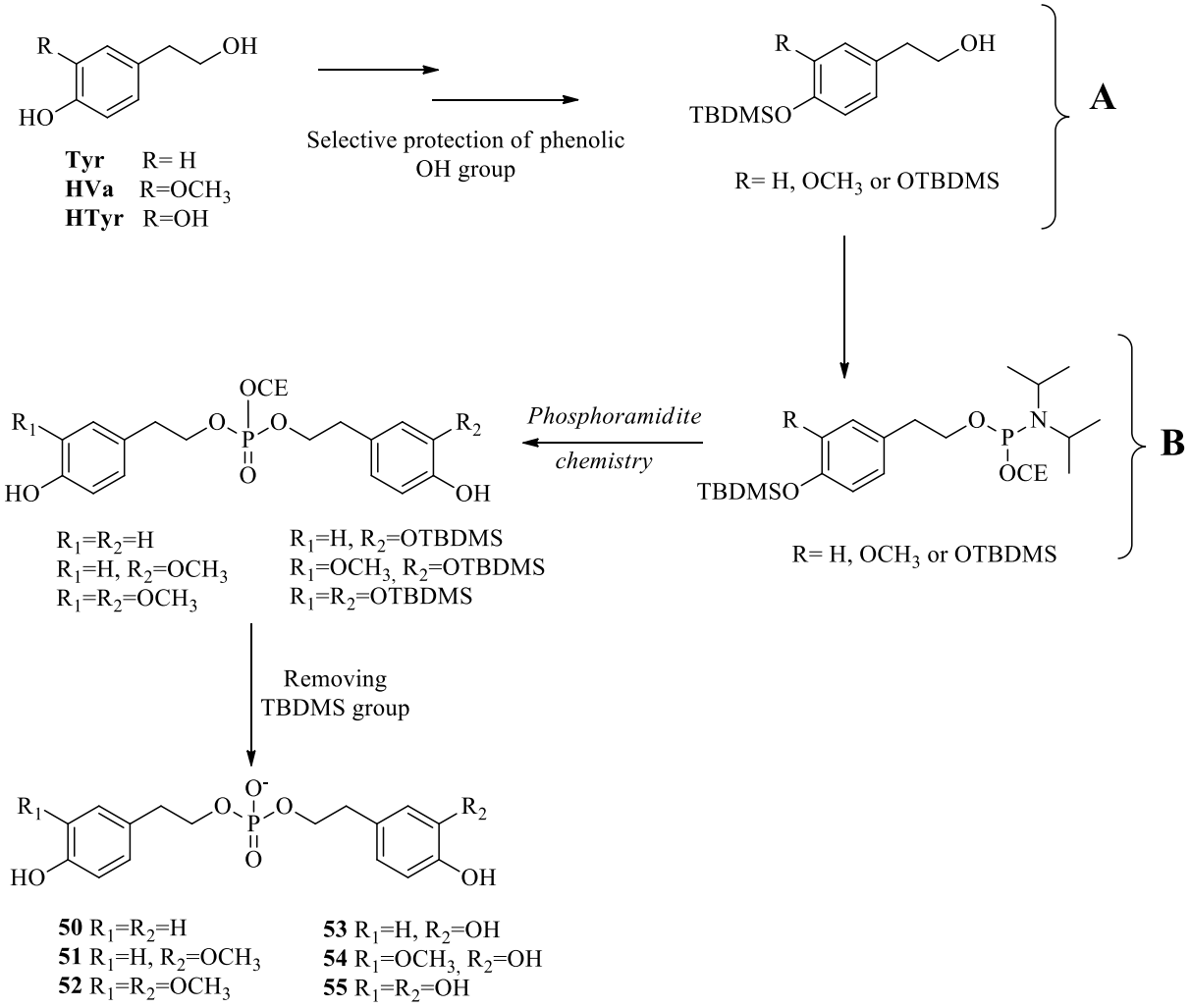


50 R_1=R_2=H
51 R_1=H, R_2=OCH_3
52 R_1=R_2=OCH_3
53 R_1=H, R_2=OH
54 R_1=OCH_3, R_2=OH
55 R_1=R_2=OH

Figure 44.

After the synthesis, the behaviour of these dimers was evaluated relatively to different aspects involved in AD pathology: oxidative stress, Aβ aggregation and role of biometals on Aβ aggregation, in order to identify new potential molecules against AD. For this purpose, the radical scavenging activity, $A\beta_{40}$ aggregation inhibitory activity and the chelating ability of bio-metals as Cu (II) and Zn (II) were carried out.

The mini library of phosphodiester dimers was prepared as shown in **Scheme 17.** The *tert*-butyldimethylsilyl (TBDMS) group was chosen to protect the phenolic functionalities due to the easy introduction procedure and above all the mild regio-selective removal conditions to obtain the building blocks **A**. Starting from the latter intermediates, the other building blocks containing phosphoramidite function (**B**) were obtained. The two building blocks **A** e **B** were coupled and after removal of TBDMS groups, the final compounds **50-55** were obtained. The HPLC analysis showed that the dimers are obtained with a high degree of purity (> 97%).



Scheme 17

The novel dimers **50-55** were tested for their radical scavenging capacity using the *in vitro* radical cation 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS•+) assay and compared to that of the corresponding monomers Tyr, HVa and HTyr. The experimental data of the assay, expressed as Trolox Equivalent Antioxidant Capacity (TEAC in mM equivalent), are summarized in **Table 11**.

Among monomers, Tyr resulted less effective than both HVa and HTyr, showing TEAC values of 0.45 ± 0.02, 0.81 ± 0.04 and 0.68 ± 0.02, respectively, according to what has been previously reported.[104] When HVa and HTyr were linked together the activity increased; in particular, Tyr-p-HVa **51** showed a TEAC value of 1.21 ± 0.07. A similar behaviour was observed for HVa-p-HVa and Tyr-p-HVa with TEAC values of 1.43 ± 0.03 and 1.21 ± 0.07

for **52** and **51**, respectively. Unexpectedly, HTyr-p-Tyr **53**, HTyr-p-HVa **54** and HTyr-p-HTyr **55** was as effective as the respective monomers.

Table 11. Radical scavenging activity of Tyr, HVa, HTyr and dimers **50-55.**

Compounds	**$ABTS^{\bullet+}$ (TE mM)[a]**
Tyr	0.45 ± 0.02
HVa	0.81 ± 0.04
HTyr	0.68 ± 0.02
Tyr–*p*–Tyr **50**	0.97 ± 0.23
Tyr–*p*–HVa **51**	1.21 ± 0.07
HVa–*p*–HVa **52**	1.43 ± 0.03
HTyr–*p*–Tyr **53**	0.66 ± 0.05
HTyr–*p*–HVa **54**	0.65 ± 0.03
HTyr–*p*–HTyr **55**	0.69 ± 0.03

[a] TE: Trolox Equivalent

To evaluate the ability of dimers (ligands) to modulate Aβ40 aggregation, ThT (Thioflavin T) experiments have been carried out in the absence and presence of increasing concentrations of ligands (peptide/ligand molar ratios 1:0.25, 1:0.5 and 1:1). The experimental results were in accordance with literature data.[298]

In the same way of parental monomers, the dimers **50-52** containing two Tyr or HVa moieties or a combination of both, catalysed the aggregation phenomenon. These compounds reduced the lag phase and increased the total amount of the formed fibers at all tested peptide/ligand ratios. The effect was more accentuated at 1:0.25 peptide/ligand molar ratio and the compounds presented a progressive loss of activity by a further increase (**Figure 45a**, **b** and **c**).

Interestingly, dimers containing HTyr **53-55** showed a bimodal behavior, producing an acceleration or an inhibition of the aggregation depending on the concentration of dimer ligand. As shown in **Figure 45**, the efficacy of inhibiting aggregation is only present at the higher tested peptide ligand molar ratios (1:1 and in some cases 1:0.5) while the ability to favor the aggregation of HVa and Tyr counterbalances the catechol properties at the smaller tested ratio (1:0.25) (**Figure 45d**, **e** and **f**). Analyzing all data, it appears clear that the catechol moiety is responsible for the inhibitory effect on Aβ aggregation of the compounds that contain it.

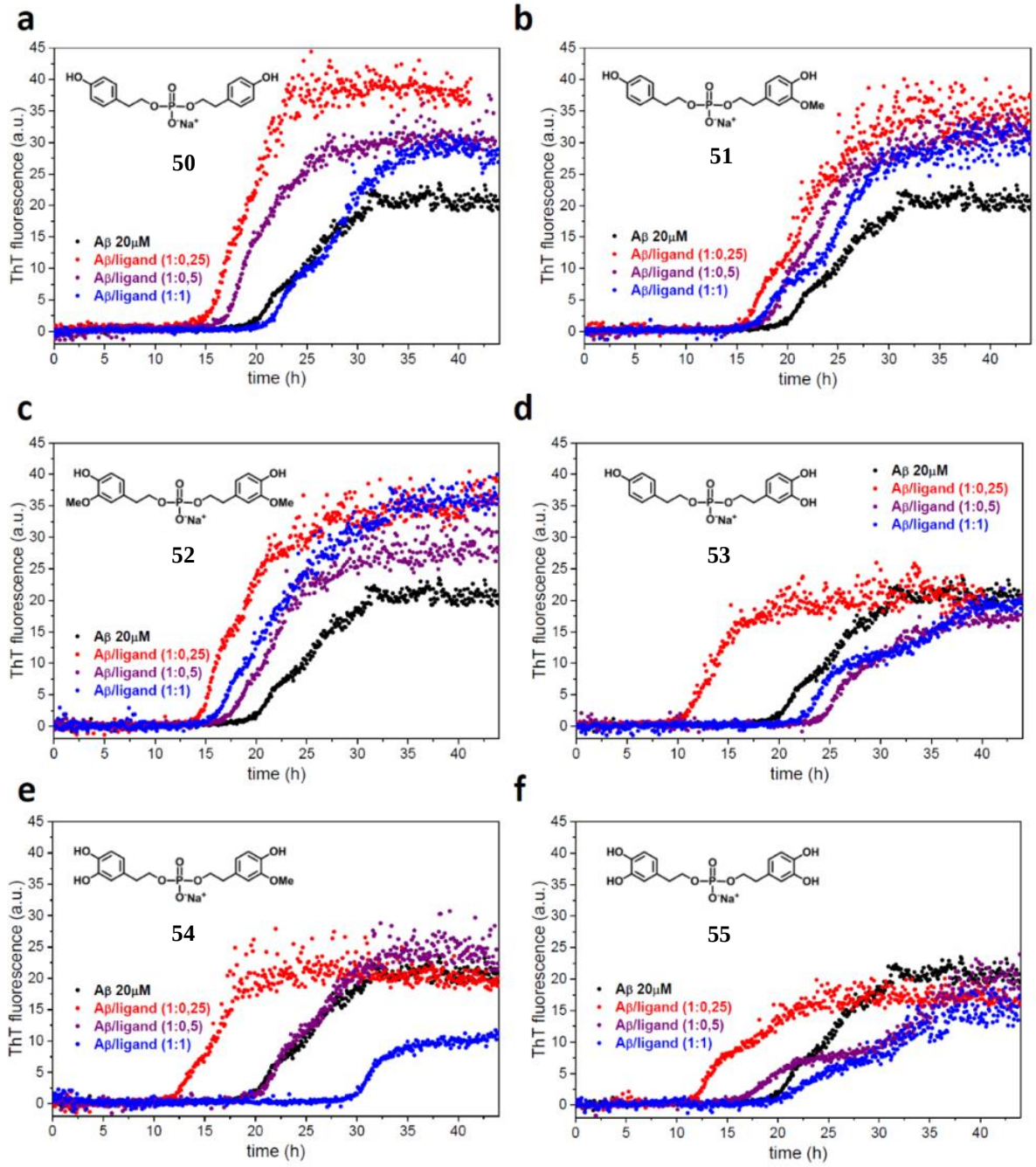


Figure 45. a) Amyloid fibrillation kinetics of Aβ in absence (black curve) and in presence of Tyr-*p*-Tyr **50** in peptide/ligand molar ratios 1:0.25 (red curve), 1:0.5 (purple curve) and 1:1 (blue curve). Dots represent experimental data; each condition has been tested in triplicate. Experiments were carried out using Aβ 20 μM at 37°C in phosphate buffer 10 mM NaCl 100mM pH 7.4. b) As a) except for Tyr-*p*-HVa **51**. c) As a) except for HVa-*p*-HVa **52**; d) As a) except for HTyr-*p*-Tyr **53**. e) As a) except for HTyr-*p*-HVa **54**; f) As a) except for HTyr-*p*-HTyr **55**.

A further investigation concerned the bio-metal chelating capacity of dimers towards Cu^{+2} and Zn^{+2} ions. The study of complexation was conducted both by UV-Vis, fluorescence spectroscopy and a potentiometric method.

The data were collected by titration where the pH measurement was conducted with a glass electrode in 0.1 M $NaClO_4$ as ionic medium. In **Table 12** are showed the protolithic constants of dimers **50-55** obtained by elaboration of data deriving from potentiometric measurements carried out on the dimers in the absence of metals.

Table 12. Acidity constants for dimers

Compound	**50**	**51**	**52**	**53**	**54**	**55**
pKa_1	9.8 ± 0.1	9.2 ± 0.1	9.0 ± 0.1	8.5 ± 0.1	9.5 ± 0.1	8.8 ± 0.1
pKa_2	10.1 ± 0.2	10.6 ± 0.2	10.0 ± 0.2	10.5 ± 0.2	11.3 ± 0.2	9.4 ± 0.2
pKa_3	–	–	–	13.1 ± 0.5	13.0 ± 0.5	10.5 ± 0.3
pKa_4	–	–	–	–	–	13.1 ± 0.5

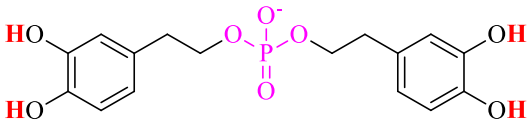
Potentiometric measurements were carried out also in presence of metals; the experimental data revealed that all derivatives show a strong chelating ability for both metal ions. However, the role of the catecholic moiety is evident from the excellent Cu^{+2} and Zn^{+2} chelating capacity of the derivatives **53-55** containing HTyr. Potentiometric measurements showed the formation of complexes with very high stability constants in the range of $10^{6.5}$ – $10^{20.2}$ (**Table 13**).

Table 13. Stability constants for HTyr dimers.

Me^{2+}	***Compound***	***Equilibrium***	***log (Keq)***
Cu^{2+}	**53**	$Cu^{2+} + HL^{2-} \leftrightarrows CuHL$ $Cu^{2+} + 2\ HL^{2-} \leftrightarrows Cu(HL)_2^{2-}$	11.0 20.2
	54	$Cu^{2+} + HL^{2-} \leftrightarrows CuHL$	11.5
	55	$Cu^{2+} + H_2L^{2-} \leftrightarrows CuH_2L$ $Cu^{2+} + 2\ H_2L^{2-} \leftrightarrows Cu(H_2L)_2^{2-}$	9.5 16.9
Zn^{2+}	**53**	$Zn^{2+} + HL^{2-} \leftrightarrows ZnHL$	6.5
	54	$Zn^{2+} + HL^{2-} \leftrightarrows ZnHL$	7.1
	55	$Zn^{2+} + H_2L^{2-} \leftrightarrows ZnH_2L$	8.1

For both metals the formation of complexes with stoichiometry 1:1 were observed. While, for compounds **53** and **55**, Cu^{2+} complexes with stoichiometry 2:1 were also observed. This last result could be due to the participation of two catecholic moieties to chelation with Cu^{2+} ion.

The distribution diagram of the Cu^{+2}**- 55** system is reported in **Figure 46a**. A prevalence of free metal is observed at pH up to 5, while there is a prevalence of the CuH_2L complex at pH range 5 - 7 (which reaches a maximum of about 80%). For the Zn^{+2} **- 55** system (**Figure 46b**), the free metal represents the prevalent specie up to pH 7, while the ZnHL complex species becomes considerable at pH values greater than 8.



55 (H_4L)

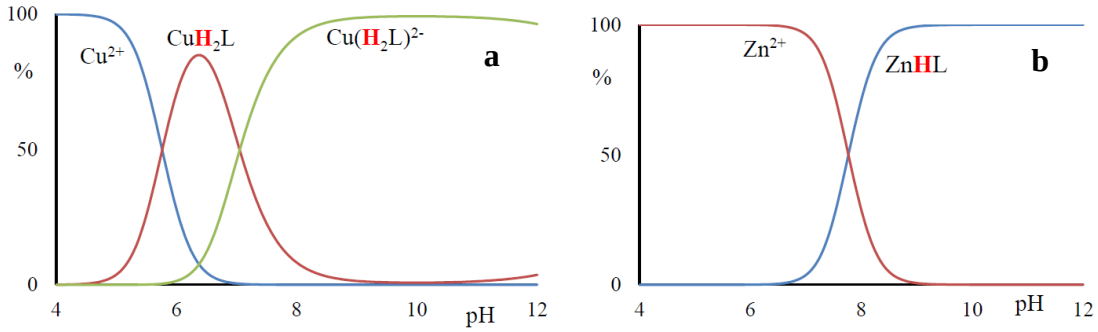


Figure 46. a) Distribution diagram of solutions containing 1.0 x 10^{-4} M Cu (II) and 2.0 x 10^{-4} M of compound **55** in 0.1 M $NaClO_4$, and b) Zn (II) and 2.0 x 10^{-4} M of compound **55** in 0.1 M $NaClO_4$.

UV spectra showed a similar profile. In particular, UV spectra of dimer **55,** in absence of complexation (**Figure 47**), presents two typical phenolic group absorptions at 220 nm (ε = 485cm-$1M^{-1}$) and 280 nm (ε = 860$cm^{-1}M^{-1}$), which show minimal variations with an increase of pH.[299] On the other hand, at pH greater than 7, the Cu $(H_2L)_2^{2-}$ species is the only one present (greater than 95%).

The presence of the metal generates in both cases an increase of absorbance together with a bathochromic shift of the absorption band at 280 nm as a consequence of the participation of

aromatic moieties in the formation of the complex. However, this effect is more evident with Cu^{2+} ions, probably thanks to the greater stability of the complex present in both compounds.

Spectrofluorimetric measurements performed on the same solutions showed a much more marked decrease in intensity in the presence of metal cations with respect to free compound **55** (depending on the pH). Also, in these measurements a greater variation was observed in the presence of Cu^{2+} ions (**Figure 47**).

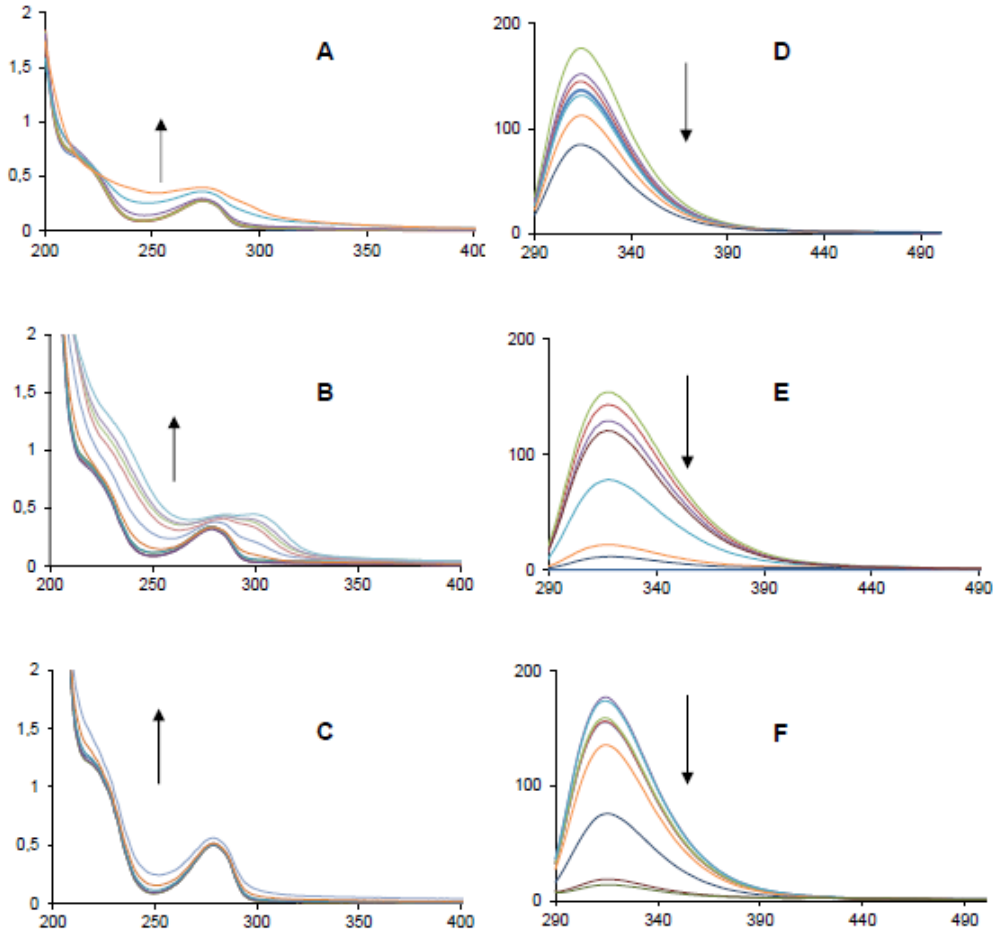


Figure 47. UV spectra (on the left) and fluorescence spectra (on the right) of solution with composition: A, D) 0.90 mM of compound **55** (pH: from 6.59 to 10.84); B, E) 0.90·mM of compound **55** and 0.45·mM Cu (II) (pH: from 2.68 to 8.29); C, F) 0.90·mM of compound **55** and 0.45·mM Zn (II) (pH: from 3.36 to 7.57).

5.3 Conclusions

In conclusion, by using small phenolic compounds as building blocks and by exploiting the well-established phosphoramidite chemistry, an efficient synthetic strategy was developed to obtain a mini library of phosphodiester dimers of these natural metabolites. The antioxidant activity ($ABTS^{\bullet+}$ test) of phosphodiester dimers resulted generally greater than that of the precursors. Initially, dimers were tested by thioflavin-T fluorescence assay to evaluate their $A\beta_{40}$ aggregation inhibitory activity. Interestingly, dimers that contain the HTyr moieties **53-55**, showed a bimodal behaviour, producing an acceleration or an inhibition of the aggregation depending on the concentration of dimer ligands. Instead, dimers containing Tyr or HVa moieties **53-55**, catalysed the aggregation phenomenon as the monomeric precursors. Based on these interesting antiaggregant activities, the ability of dimers **53-55** to complex Cu (II) and Zn (II) ions has been also investigated by potentiometric and UV spectrophotometric analyses. Although with different behaviour, all dimers showed a strong chelating ability for both metal ions forming complexes with high stability constants in the range of $10^{6.5} – 10^{20.2}$.

Therefore, the new synthetized phosphodiester dimers are promising agent in prevention and treatment of AD.

5.4 Experimental section

5.4.1 General methods

All chemicals were purchased from Sigma-Aldrich (Italy) except for hydroxytyrosol that was synthetized according to reported in the literature.[220] HPLC−grade CH_3CN and CH_3OH were purchased from Carlo Erba Reagents and Sigma-Aldrich, respectively. The $A\beta_{1-40}$ peptide was purchased from GensScript.

Reactions were monitored by TLC (precoated silica gel plates F254, Merck) and column chromatography (Merck Kieselgel 60, 70-230 mesh). HPLC analysis was performed with a Shimadzu LC−8A PLC system equipped with a Shimadzu SCL-10A VP System control and a Shimadzu SPD−10A VP UV−Vis detector. HPLC analysis were carried out on Phenomenex Luna RP18 column (5 μm particle size, 4.6 mm × 150 mm i.d.) using a gradient that started at 5% of CH_3CN in 0.1 M Ammonium Acetate in H_2O (pH 7.0) for 5 min and increased to 100% of ACN over 30 min at a flow rate of 0.8 mL/min with detection at 260 nm. For

ESI–MS analysis, a Waters Micromass ZQ Instrument equipped with an electrospray source was used.

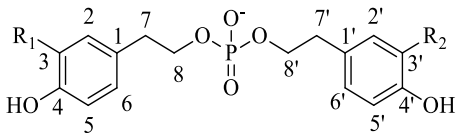
For Aβ aggregation experiments, samples were prepared by adding 1-2 µL of the studied molecules stock solutions in water or DMSO to 50 µL of ThT 20 µM in PBS (phosphate 10 mM, pH 7.4, 100 mM NaCl). Experiments were carried out in 384-well plates. Immediately after addition of $A\beta_{1\text{-}40}$ (20 µM, final concentration) time traces were recorded using a Varioskan plate reader (ThermoFisher, Waltham, MA) with λ_{ecc} 440 nm and λ_{em} 480 nm at 37 °C. Each experiment was performed in triplicate.

The potentiometric titrations were conducted in an air–bath thermostat kept at 25.00 ± 0.05 °C. A programmable computer–controlled data acquisition unit 3421A, supplied by Hewlett and Packard, was used to perform the potentiometric measurements. The glass electrodes were Metrohm (Herisau, Switzerland) of 60102-100 type and Ag/AgCl electrode was utilized as reference. The EMF values were measured with a precision of ± 0.01 mV using a Keithley 642 type Digital Electrometer.

UV–Vis spectra were recorded by model Cary 5000 Spectrophotometer by Varian C., from 200 to 600 nm (optical path 0.2 cm) at 25.0 °C, under a constant flow of nitrogen.

Fluorescence spectra were obtained using a JASCO spectrofluorimeter model FP–750, from 200 to 600 nm (optical path 0.2 cm).

5.4.2 Characterization of dimers



50 $R_1=R_2=H$ **53** $R_1=H$, $R_2=OH$
51 $R_1=H$, $R_2=OCH_3$ **54** $R_1=OCH_3$, $R_2=OH$
52 $R_1=R_2=OCH_3$ **55** $R_1=R_2=OH$

Bis(4-hydroxyphenethyl) phosphate **50.** ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.03 (4H, d, J=8.4 Hz, H–2, H–2', H–6 and H–6'); 6.72 (4H, d, J=8.4 Hz, H–3, H–3', H–5 and H–5'); 3.90 (4H, q, J=6.9 Hz, H–8 and H–8'); 2.77 (4H, t, J =7.1 Hz, H–7 and H–7'). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 155.4; 129.6; 129.2; 114.7; 66.2; 66.1; 36.1; 36.0. ^{31}P NMR (161.98 MHz, $CDCl_3$) δ: 0.56. MS (ESI^+): calcd. for $C_{16}H_{20}O_6P$ $[M+H]^+$ 339.10, found 339.22 $[M+H]^+$.

4-hydroxy-3-methoxyphenethyl 4-hydroxyphenethyl phosphate **51.** ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.01 (2H, d, J=8.5 Hz, H–2' and H–6'); 6.82 (1H, d, J=1.8 Hz, H–2); 6.73 (1H, d, J=8.5 Hz, H–5); 6.71 (2H, d, *J* 8.5 Hz, H–3' and H–5'); 6.66 (1H, dd, *J* 8.1, 1.8 Hz, H–6); 3.93 (4H, m, H–8 and H–8'); 3.83 (3H, s, OC*H*$_3$); 2.76 (4H, q, *J* 7.1 Hz, H–7 and H–7'). ^{13}C NMR (100 MHz, $CDCl_3$): δ 155.5; 147.3; 144.5; 130.1; 129.6; 129.1; 121.2; 114.8; 114.7; 112.4; 66.3; 66.2; 55.0; 36.4; 36.0. ^{31}P NMR (161.98 MHz, $CDCl_3$) δ: 0.30. MS (ESI^+): calcd. for $C_{17}H_{22}O_7P$ $[M+H]^+$ 369.11, found 369.55 $[M+H]^+$.

Bis(4-hydroxy-3-methoxyphenethyl) phosphate **52.** ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.83 (2H, d, J=1.9 Hz, H–2 and H–2'), 6.72 (2H, d, J= 8.0 Hz, H–5 and H–5'); 6.64 (2H, dd, J=8.0, 1.9 Hz, H–6 and H–6'); 3.95 (4H, q, J=6.9 Hz, H–8 and H–8'); 3.80 (6H, s, OC*H*$_3$); 2.78 (4H, t, J=7.0 Hz, H–7 and H–7'). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 147.3; 144.5; 130.0; 121.2; 114.7; 112.4; 66.3; 66.2; 55.0; 36.4; 36.3. ^{31}P NMR (161.98 MHz, $CDCl_3$) δ: 0.36. MS (ESI^+): calcd. for $C_{18}H_{24}O_8P$ $[M+H]^+$ 399.12, found 399.23 $[M+H]^+$.

3,4-dihydroxyphenethyl 4-hydroxyphenethyl phosphate **53.** ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.03 (2H, d, J=8.3 Hz, H–2' and H–6'); 6.71 (2H, d, J=8.3 Hz, H–3' and H–5'); 6.69 (2H, d, J=8.2 Hz, H–5); 6.67 (1H, m, H–2); 6.53 (1H, dd, J=7.8, 1.8 Hz, H–6); 3.93 (4H, q, J=6.3 Hz, H–8 and H–8'); 2.78 (2H, t, J=6.8 Hz, H–7); 2.73 (2H, t, J=6.7 Hz, H–7'). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 155.4; 144.7; 143.3; 129.9; 129.6; 129.1; 119.9; 115.8; 114.8; 114.7; 66.3; 66.2; 36.0; 35.9. ^{31}P NMR (161.98 MHz, $CDCl_3$) δ: –2.3. MS (ESI^+): calcd. for $C_{16}H_{20}O_7P$ $[M+H]^+$ 355.09, found 355.38 $[M+H]^+$.

3,4-dihydroxyphenethyl 4-hydroxy-3-methoxyphenethyl phosphate **54.** ^{1}H NMR (400 MHz, CD_3OD) δ: 6.79 (1H, d, J=1.9 Hz, H–2'); 6.72 (1H, d, J=7.8 Hz, H–5); 6.69 (1H, d, J=7.6 Hz, H–5'); 6.68 (1H, br s, H–2); 6.65 (1H, dd, J=7.8, 2.0 Hz H–6); 6.53 (1H, dd, J=7.6, 1.9 Hz H–6'); 3.94 (4H, m, H–8 and H–8'); 3.82 (3H, s, OC*H*$_3$); 2.80 (2H, t, J=6.8 Hz, H–7); 2.73 (2H, t, J=6.9 Hz, H–7'). ^{13}C NMR (100 MHz, CD_3OD) δ: 147.4; 144.6; 144.5; 143.3; 130.0; 129.9; 121.1; 120.0; 115.8; 114.9; 114.7; 112.3; 66.4; 66.3; 55.0; 36.2; 36.0. ^{31}P NMR (161.98 MHz, CD_3OD) δ: -3.0. MS (ESI^+): calcd. for $C_{17}H_{22}O_8P$ $[M+H]^+$ 385.10, found 385.38 $[M+H]^+$.

Bis(3,4-dihydroxyphenethyl) phosphate **55**. ^{1}H NMR (400 MHz, CD_3OD) δ: 6.69 (2H, d, J=8.3 Hz, H–5 and H–5'); 6.68 (2H, d, J=2.2 Hz, H–2 and H–2'); 6.54 (2H, dd, J=7.9, 1.8 Hz, H–6 and H–6'); 3.96 (4H, q, J=6.6 Hz, H–8 and H–8'); 2.74 (4H, t, J=6.8 Hz, H–7 and

H–7'). ^{13}C NMR (100 MHz, CD_3OD) δ: 144.7; 143.4; 129.7; 120.0; 115.8; 114.9; 66.8; 66,7; 36.0; 35.9. ^{31}P NMR (161.98 MHz, CD_3OD) δ: –2.1. MS (ESI^+): calcd. for $C_{16}H_{20}O_8P$ $[M+H]^+$ 371.09, found 371.38 $[M+H]^+$.

5.4.3 Evaluation of the radical scavenging activity

The assays were carried out as already described by Bernini, R. et al.[104] Firstly, an ABTS radical cation ($ABTS^{\bullet+}$) stock solution was prepared. ABTS (30.7 mg) was dissolved in water (8.0 mL) to obtain a 7.0 mM solution. Then, 5.3 mg of $K_2S_2O_8$ were added and the mixture was kept in the dark at room temperature for 16 h to produce ABTS radical cation ($ABTS^{\bullet+}$). After this time, the $ABTS^{\bullet+}$ solution was diluted with ethanol until to reach an absorbance value of 0.7±0.02 at λ=734 nm. Phenolic compounds to test were solubilized in ethanol to afford 0.04-0.4 mM solutions. For the radical scavenging assay, 950 μL of the $ABTS^{\bullet+}$ solution and 50 μL of ethanolic solutions of phenolic compounds were mixed at room temperature. After 10 min, the absorbance values at λ=734 nm were registered. Three measurements were recorded for each concentration; each time blanks of pure solvent were run. Trolox was used as the antioxidant control; the results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC), that is the concentration of antioxidant giving the same percentage inhibition of absorbance (I%) of the radical cation at λ=734 nm as 1.0 mM Trolox. A dose-response curve for was obtained plotting I% of the $ABTS^{\bullet+}$ as function of range of concentrations of antioxidant compounds or Trolox. TEAC value is calculated dividing the gradient of the plot of the percentage inhibition of absorbance vs. concentration plot for the antioxidant in question by the gradient of the plot for Trolox. Results are expressed as means ± standard deviations.

5.4.4 Studies on the kinetic of Aβ fibrillation

$Aβ_{1–40}$ was monomerized by solubilizing it in HFIP (1 mg/mL), aliquoted (100 μL), frozen at −80 °C and lyophilized. To quantify Aβ, a lyophilized aliquot was solved in 20 μL of NaOH 1 mM and added to 180 μL of buffer (phosphate 10 mM, pH 7.4) and the absorbance was measured at 280 nm ($ε_{280}$ = 1450 $M^{-1}·cm^{-1}$). Samples for ThT assays were prepared by adding 1–2 μL of the corresponding TPD stock solutions (final ligand concentrations 5 μM, 10 μM or 20 μM) to 50 μL of ThT 20 μM in buffer (phosphate 10 mM, pH 7.4, NaCl 100

mM). Immediately after the addition of $A\beta_{1-40}$ (20 μM, final concentration), time traces were recorded (λ_{exc}= 440 nm and λ_{em} =480 nm) using a 384-well plate in a Varioskan plate reader (ThermoFisher, Waltham, MA) at 37 °C. A glass bead (diam. 1 cm) was added to each well, and the plate was shaken for 10 s before each read (600 shakes per minute with a diameter of the orbital movement of 1 mm). To exclude false ThT positive signals, controls in the absence of $A\beta_{1-40}$ were also carried out for each tested condition. Each condition was tested in triplicate and kinetic curves were fitted with the following equation:

$$I = \frac{Imax}{1 + e^{-k(t - t_{1/2})}}$$

where I is the fluorescence intensity as function of time t, I_{max} corresponds with the maximum intensity of fluorescence, *k* is the apparent rate constant, $t_{1/2}$ and lag time (t_{lag}) represent the point in time where the signal reaches 50% and 10% of the amplitude of the transition, respectively. Data are expressed as the average of the kinetic parameters obtained by fitting each of the replicas and indicated as mean (standard error).

6. General conclusions

The research activity carried out during the PhD course was devoted to the valorization of phenolic compounds found in agro-industrial wastes and by-products according to the principles of green chemistry and circular economy model.

In the first part of the work, lipophilic esters of tyrosol, homovanillyl alcohol, hydroxytyrosol and gallic acid were successfully prepared using eco-friendly synthetic procedures. The reactions were chemoselective and no by-products were obtained. The low cost, and safety of the reagents make them attractive for the preparation of compounds characterized by relevant biological activities. Satisfactory results were obtained applying the optimized reactions on hydroxytyrosol-enriched extract and gallic acid-enriched extract obtained, respectively, from *Olea europaea* L. and *Castanea sativa* Mill. wastes and by-products. They represent raw material from which these interesting bioactive lipophilic esters can be prepared under green chemistry conditions in order to implement a closed loop production cycle. Further investigations will be devoted to evaluate new biological activities of the lipophilic compounds and extracts for future applications.

The second part of the research was focused on the preparation of novel PVA-based films incorporating an hydroxytyrosol-enriched extract and an oleuropein-enriched extract obtained by olive oil wastes and by-products. The good performances suggested that these films are promising active packages of hydrophilic foods to avoid food deterioration and increasing their shelf life. A future prospect could concern the up-scaling of the production of such formulations with antioxidant activity using wastes from the agro-food industry to produce increasingly sustainable, economic and eco-friendly packaging solutions as an alternative to the materials currently used.

In the third part, the research was still focused on using tyrosol, homovanillyl alcohol, hydroxytyrosol as starting materials for the synthesis of a mini-library of new phosphodiester dimers as potential anti-Alzheimer's disease agents. Antioxidant activity, Aβ anti-aggregation behaviour and bio-metal chelating capacity of these dimers were evaluated. The experimental results confirmed that the catechol group is crucial for the inhibition of Aβ aggregation as well as for the formation of high stable complexes with Cu (II) and Zn (II). Therefore, the synthesized dimers are promising as anti-Alzheimer's disease agents and, as future perspectives, their effect could be evaluated through *in vivo* assays.

Index of abbreviations

Tyr	Tyrosol
HVa	Homovanillyl alcohol
HTyr	Hydroxytyrosol
GA	Gallic acid
DMC	Dimethyl carbonate
HTyrE	HTyr-enriched extract
GAE	GA-enriched extract
PVA	Poly (vinyl alcohol)
OleE	Oleuropein-enriched extract
AD	Alzheimer's disease
Aβ	Amyloid-β

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