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BIOCATALYTIC TRANSFORMATION AND ANALYTIC CHARACTERIZATION OF BIOACTIVE VEGETABLE SUBSTANCES

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"E' una follia odiare tutte le rose perché una spina ti ha punto,

abbandonare tutti i sogni perché uno di loro non si è realizzato,

rinunciare a tutti i tentativi perché uno è fallito.

E' una follia condannare tutte le amicizie perché una ti ha tradito,

non credere in nessun amore solo perché uno di loro è stato infedele,

buttare via tutte le possibilità di essere felici solo perché qualcosa non è andato per il verso giusto.

Ci sarà sempre un'altra opportunità, un'altra amicizia, un altro amore, una nuova forza.

Per ogni fine c'è un nuovo inizio."

Antoine de Saint Exupéry da "Il Piccolo Principe"

A mio padre e mia madre,

punti di riferimento sempre presenti nella mia vita.

A Fulvio,

che mi ha insegnato a credere nei miei sogni.

A me stessa,

perchè ho avuto la forza di superare ogni ostacolo.

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ABSTRACT

The study is organized into two main topics: a) the qualitative/quantitative characterization of natural substances in plant extracts, and b) biocatalytic transformations of natural substances through immobilized enzymes.

Regarding the first topic, the main phenolic components of a large panel of plant extracts, provided by Aboca S.p.a., have been characterized in detail by applying GC-MS, LC-MS and NMR techniques. The knowledge of the structure of bioactive components in plant extracts is an important topic related to cosmetic and pharmaceutical applications, as well as for the certification of quality processes. In fact, the biological activity of vegetable extracts is strictly connected to integrity and complexity of the original chemical composition. In this system, any substances with its specific activity can contribute to total activity in a synergistic way with the others. This work is part of a series of pharmaceutical analysis and controls necessary for the use of a plant extract as phytochemical.

The study of biocatalytic transformation of natural substance is related to the use of enzymatic processes within industries in pharmaceutical, fine chemical, fuel and biosensor applications. Different immobilization procedures have been reported to improve the stability of the enzyme. In this context, novel nano-structured materials are gradually emerging as promising supports for immobilization, mainly due to their high surface area, high loading capacity, poor resistance to mass transfer and favourable physical and chemical properties. The aim of this work is to design and characterize biocatalysts based on carbon nanotubes (CNTs) and to apply such systems in synthetic transformations. The attention was directed to laccase from *Trametes versicolor* and the immobilization techniques included physical methods, covalent bond linkage and the Layer by Layer technique. In the presence of redox mediators, such as TEMPO, the novel biocatalysts were selective and efficient systems for the synthesis of aldehydes in high yield and under mild experimental conditions, with better results than those previously obtained with other immobilized laccases.

INTRODUCTION

CHAPTER 1

NUTRACEUTICALS AND PHYTOCHEMICALS

1.1 Introduction to nutraceutical

Most of the diseases of body organs, such as general vulnerability to infectious diseases and ageing processes, are related to the deficiencies of one or more of the molecules that are required for body's functions but that are not synthesized by the organism.[1] Dietary factors play an important role in premature chronic disease appearance, disease progression, morbidity and mortality. Approximately 40-50% proportion in cardiovascular disorder, 35-50% proportion in cancers, and 20% proportion in osteoporosis is attributable to dietary factors.[2]

About 2500 years ago, Hippocrates, the well recognized father of modern medicine, conceptualized the relationship between the use of appropriate foods for health and their therapeutic benefits.[3] Then Theophrastus, Cato, Pliny the Elder and Galen warned against adulteration of food products.[4]

In the 1989 Dr. Stephen de Felice coined the term nutraceuticals, that derived from the words "nutrition" and "pharmaceutical", as a food or food product that provides health and medical benefits, including the prevention and treatment of disease.[5] Later, Lachance defined nutraceuticals as naturally occurring (usually botanical) bioactive compounds that have health benefits.[6]

A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease. The bioactive ingredients, usually called phytochemicals, sustain or promote health and occur at the intersection of food and pharmaceutical industries. Such substances may range from isolated nutrients, dietary supplements and specific diets to genetically engineered designer foods, herbal products, processed foods and beverages.[7,8]

1.2 Phytochemicals

Plant foods contain many bioactive compounds in addition to those which are traditionally considered as nutrients, such as vitamins and minerals. These physiologically active compounds, referred to simply as phytochemicals, are produced via secondary metabolism in relatively small amounts.

Phytochemicals (from the Greek word phyto, meaning plant) are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients.[9] They protect plants from disease and damage and contribute to the plant's colour, aroma and flavour.[10,11]

Dietary intake of phytochemicals may promote health benefits, protecting against chronic degenerative disorders. Majority of foods, such as whole grains, beans, fruits, vegetables and herbs contain phytonutrients/ phytochemicals. These phytochemicals, either alone and/or in combination, have tremendous therapeutic potential. Phytochemicals in food are of enormous significance due to their beneficial effects on human health since they offer protection against numerous diseases or disorders such as cancers, coronary heart disease, diabetes, high blood pressure, inflammation, microbial, viral and parasitic infections, psychotic diseases, spasmodic conditions, ulcers, osteoporosis and associated disorders.[12]

More than 4,000 phytochemicals have been catalogued and are classified by protective function, physical characteristics and chemical characteristics and about 150 phytochemicals have been studied in detail.[13]

Phytochemicals accumulate in different parts of the plants, such as in the roots, stems, leaves, flowers, fruits or seeds.[14] Many phytochemicals, particularly the pigmented molecules, are often concentrated in the outer layers of the various plant tissues. Concentrations vary from plant to plant depending upon the variety, processing, cooking and growing conditions.[15] Phytochemicals are also available in supplementary forms, but evidence is lacking that they provide the same health benefits as dietary phytochemicals.[16] Phytochemicals are not essential nutrients and are not required by the human body for sustaining life, but have important properties to prevent or to fight some common diseases.

1.2.1 Classification and characteristics

The exact classification of phytochemicals could have not been performed so far, because of the wide variety of them. In recent year phytochemicals are classified as primary or secondary constituents, depending on their role in plant metabolism. Primary constituents include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll's etc. Secondary constituents are the remaining plant chemicals such as alkaloids, terpenes, saponins, phenolic compounds (flavonoids, phytoestrogens, phenolic acids, lignans, tannin, curcumines), glucosides, carotenoids, phytosterols and phytostanols, tocotrienols, organosulfur compounds (allium compounds and glucosinolates), and non-digestible carbohydrates (dietary fiber and prebiotics).[17,18]

Carotenoids

Carotenoids are tetraterpenoids responsible for the yellow, orange and red color of many fruits, vegetables, a few roots, egg yolk, fish like salmon and trout, crustaceans. They are synthesized by plants, algae, fungi, yeasts and bacteria, but are merely accumulated from the diet, unchanged or slightly modified, in some animals. Typically a food would have one to five major carotenoids with a series of minor carotenoids in trace or very small amounts. The principal carotenoids encountered in human blood are: β-carotene, α-carotene, β-cryptoxanthin, lutein and lycopene. These are also the carotenoids most commonly found in foods.[19,20] The provitamin A activity of some carotenoids (e.g. β-carotene, α-carotene, β-cryptoxanthin) has been known for a long time. In more recent years, carotenoids, provitamins A or not, have been studied for other health-promoting effects, including immuno-enhancement and reduction of the risk of developing degenerative diseases such as cancer, cardiovascular diseases (CVD), cataract and macular degeneration.[21,22,23,24,25] These physiological activities have been attributed to antioxidant property, specifically to the ability to quench singlet oxygen and interact with free radicals.[26,27] However, other mechanisms of action against chronic diseases have been increasingly cited: modulation of carcinogen metabolism, regulation of cell growth, inhibition of cell proliferation, enhancement of cell differentiation, stimulation of cell-to-cell gap junctional communication, retinoid-dependent signalling and filtering of blue light.[28,29,30,24,25]

Phenolic compounds

Phenolic compounds comprise one of the most numerous and widely distributed groups of substances in the plant kingdom, with more than 8000 phenolic structures currently known.[237] Natural phenolics can range from simple molecules, such as phenolic acids, to highly polymerized compounds, such as tannins, and their occurrence in foods is extremely variable. The health-related properties of phenolic compounds, particularly flavonoids, are believed to be based on their antioxidant activity as scavengers of free radical.[31,32] The primary target of radicals are proteins (including enzymes), lipids (relevant to the induction of heart disease), DNA (relevant to the induction of cancer) and RNA. Many researchers have shown that lipid peroxides and reactive oxygen species are involved in the development of a variety of diseases, including cancer, atherosclerosis, heart disease, kidney damage, and even accelerated aging.[33,34] Flavonoids are also metal chelators and have been found to bind metals, such as copper and iron, that can catalyze lipid oxidation.

Phytosterols

Plant sterols or phytosterols are structurally similar and functionally analogous to the animal sterol, like cholesterol. Phytosterols are triterpenoids occurring in both free and esterified form. Of more than 40 phytosterols identified, β-sitosterol, stigmasterol and campesterol are the most abundant and are predominantly supplied by vegetable oils,[35,36] which are rich sources of sterol esters. A less abundant class of related compounds are the plant stanols or phytostanols, which are completely saturated forms of phytosterols. Phytostanols are derived primarily from corn, wheat, rye and rice. Phytosterols and phytostanols inhibit intestinal absorption of cholesterol.[36]

Tocotrienols

Tocotrienols are unsaturated analogs of tocopherol (vitamin E). There are at least four known forms of tocotrienol, with γ-tocotrienol as the main and most potent cholesterol-lowering form.[37] It has been suggested that α-tocopherol attenuates the inhibitory effect of γ-tocotrienol.[38] Tocotrienols have also been demonstrated to possess vitamin E activity,[39] antioxidant activity[40] and antitumor properties[41].

Glucosinolates

The glucosinolates are sulfur-containing glucosides and over a hundred different glucosinolates have been identified in the plant kingdom.[42] Although the glucosinolates are structurally diverse, there are only three principal groups, based on the side-chain structure: aliphatic, aromatic and indolyl (heteroaromatic) glucosinolates.[43] All the glucosinolates show the β-D-thioglucose group, a sulphonated oxime moiety and a variable side-chain derived from methionine, tryptophan, phenylalanine or some branched-chain amino acids.[44] Glucosinolate breakdown products exert a variety of antinutritional and toxic effects in higher animals, the most thoroughly studied of which is the goitrogenic effect of some products.[45]

Dietary fibers

Dietary fibers are oligosaccharides, polysaccharides and their hydrophilic derivatives, which cannot be digested by the human digestive enzymes to absorbable components in the upper alimentary tract; this definition includes lignin.[46] They have several recognized physiological effects: modulation of glucose absorption, regulation of gastrointestinal transit time, fecal bulking, acidification of colonic content and control of cholesterol bioavailability.[47,48]

Alkaloids

Alkaloids are natural product that contains heterocyclic nitrogen atoms, and are basic in character. The name of alkaloids derives from “alkaline” and it was used to describe any nitrogen-containing base.[49] Alkaloids are so numerous and involve such a variety of molecular structure that their rational classification is difficult. However, the best approach to the problem is to group them into families, depending on the type of heterocyclic ring system present in the molecule.[50] Alkaloids are significant for the protecting and survival of plant because they ensure their survival against micro-organisms (antibacterial and antifungal activities), insects and herbivores (feeding deterrens) and also against other plants by means of allelopathically active chemicals.[51] Alkaloids have many pharmacological activities including antihypertensive effects (many indole alkaloids), antiarrhythmic effect (quinidine,

spareien), antimalarial activity (quinine), andanticancer actions (dimeric indoles, vincristine, vinblastine).[52]

Terpenoids

The terpenoids are a class of natural products which have been derived from five-carbon isoprene units. Most of the terpenoids have multi cyclic structures that differ from one another by their functional groups and basic carbon skeletons.[53] Among the plant secondary metabolites, terpenoids function as phytoalexins in plant direct defense, or as signals in indirect defense responses which involves herbivores and their natural enemies.[54] Many plants produce volatile terpenes in order to attract specific insects for pollination or otherwise to expel certain animals using these plants as food. Less volatile but strongly bitter-tasting or toxic terpenes also protect some plants from being eaten by animals (antifeedants).[55] Last, but not least, terpenes play an important role as signal compounds and growth regulators (phytohormones) of plants, as shown by preliminary investigations. In addition, terpenoids can have medicinal properties such as anticarcinogenic (e.g. perilla alcohol), antimalarial (e.g. artemisinin), anti-ulcer, hepaticidal, antimicrobial or diuretic (e.g. glycyrrhizin) activity and the sesquiterpenoid antimalarial drug artimisinin and the diterpenoid anticancer drug taxol.[56,57]

Saponins

Saponins are a group of secondary metabolites widely distributed in the plant kingdom Chemically, saponins as group include compounds that are glycosylated steroids, triterpenoids, and steroid alkaloids. Many saponins are known to be antimicrobial, to inhibit mould, and to protect plants from insect attack.[58] Extensive research has been carried out into the membrane-permeabilising, immunostimulant, hypocholesterolaemic and anticarcinogenic properties and they have also been found to significantly affect growth, feed intake and reproduction in animals. These structurally diverse compounds have also been observed to kill protozoans and molluscs, to be antioxidants, to impair the digestion of protein and the uptake of vitamins and minerals in the gut, to cause hypoglycaemia, and to act as antifungal and antiviral compounds.[59,60,61]

CHAPTER 2

PHENOLIC COMPOUNDS

2.1 Phenolic compounds and plants: physiological and ecological role

Phenolic compounds are plant secondary metabolites that constitute one of the most common and widespread groups of substances in plants. As stated by Harborne[84], the term "phenolic" or "polyphenol" can be precisely defined chemically as a substance which possesses an aromatic ring bearing one (phenol) or more (polyphenol) hydroxyl substituents, including functional derivatives (esters, methyl ethers, glycosides, etc.). As a general rule, the terms phenolics and polyphenols refer to all secondary natural metabolites arising biogenetically from the shikimate-phenylpropanoids-flavonoids pathways, producing monomeric and polymeric phenols and polyphenols. Phenol itself is a natural product but most phenolics have two or more hydroxyl groups. All phenolic compounds exhibit intense absorption in the UV region of the spectrum and those that are coloured absorb strongly in the visible region as well. Each class of phenolic compounds has distinctive absorption characteristics.[62,63] Plants need phenolic compounds for pigmentation, growth, reproduction, resistance to pathogens and for many other functions. These compounds form one of the main classes of secondary metabolites and several thousand different compounds have been identified with a large range of structures: monomeric, dimeric and polymeric phenolics.[64,65,66,67,68,69]

In contrast with basic metabolism that refers to the anabolic and catabolic processes required for cell maintenance and proliferation, secondary metabolism refers to compounds present in specialized cells that are not directly essential for basic photosynthetic or respiratory metabolism but are thought to be required for plants survival in the environment.[68,70,71,72] Secondary metabolites apparently act as defence (against herbivores, microbes, viruses or competing plants) and signal compounds (to attract pollinating or seed dispersing animals), as well as protecting the plant from ultraviolet radiation and oxidants.[73,74]

The pattern of secondary metabolites in a given plant is complex. Differences can be seen between different developmental stages (e.g., organs important for survival and reproduction have the highest and most potent secondary metabolites), between individuals and populations.[75,76,77,78,79,80,81] Phenolic compounds are found throughout the plant kingdom but

the type of compound present varies considerably according to the phylum under consideration. Generally, the role of phenolic compounds in defence is related to their antibiotic, antinutritional or unpalatable properties. Besides their involvement in plant-animal and/or plant-microorganism relationships, plant phenolics have also key roles as the major red, blue and purple pigments, as antioxidants and metal chelators, as signalling agents both above and below ground between plant and other organisms, and as UV light screens. Thus the primary established roles of plant phenolics are clearly ecological in nature, some having dual or even multiple functions.[82]

2.2 Classification of phenolic compounds

Phenolics are characterized by having at least one aromatic ring with one or more hydroxyl groups attached. In excess of 8000 phenolic structures have been reported and they are widely dispersed throughout the plant kingdom.[83] Phenolics range from simple, low molecular-weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols. They can be classified based on the number and arrangement of their carbon atoms (**Table 1**).[84]

Number of carbon atoms	**Basic skeleton**	**Class**
6	C_6	Simple phenols
		Benzoquinones
7	C_6-C_1	Phenolic acids
8	C_6-C_2	Acetophenones
		Phenylacetic acids
9	C_6-C_3	Hydroxycinnamic acids
		Phenylpropenes
		Coumarins
		Isocoumarins
		Chromones

10	C_6-C_4	Naphthoquinones
13	C_6-C_1- C_6	Xanthones
14	C_6-C_2- C_6	Stilbenes
		Anthraquinones
15	C_6-C_3- C_6	Flavonoids
		Isoflavonoids
18	$(C_6$-$C_3)_2$	Lignas
		Neolignans
30	$(C_6$-C_3-$C_6)_2$	Biflavonoids
	$(C_6$-$C_3)_n$	Lignins
N	$(C_6)_6$	Catechol melanins
	$(C_6$-C_3-$C_6)_n$	Condensed tannins

Table 1. Classification of phenolic compounds

2.2.1 Phenolic acids

The name "phenolic acids" describes phenols that possess one carboxylic acid functionality. Examples of phenolic acids are: Benzoic acid (**1)**, Salicylic acid (**2)**, *p*-hydroxybenzoic acid (**3)**, Vanillic acid (**4)**, Syringic acid (**5)**, Protocatechuic acid (**6)**, Gentisic acid (**7)**, Gallic acid (**8**) (**Figure 2**). Although a great deal is still unknown regarding the roles of phenolic acids in plants, they have been connected with diverse functions, including nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components, and allelopathy.[85,86,87] Benzoic acid derivatives exist in virtually all plant foods (e.g., fruits, vegetables, and grains) and are physically dispersed throughout the plant in seeds, leaves, roots, and stems.[88],[89] Only a minor fraction exists as "free acids". Instead, the majority are linked through ester, ether, or acetal bonds either to structural components of the plant (cellulose, proteins, lignin),[90,91,92,93,94] or to larger polyphenols (flavonoids), or smaller organic molecules (e.g., glucose, quinic, maleic, or tartaric acids) or other natural products (e.g., terpenes)[95,96].

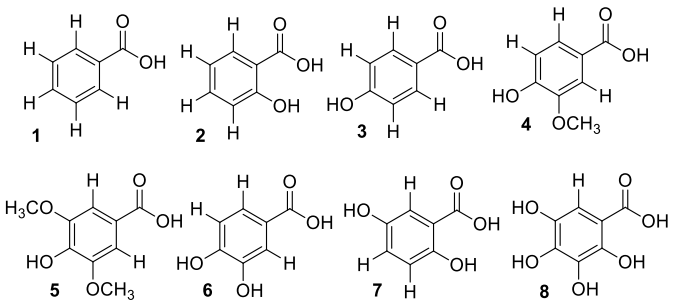


Figure 2. Phenolic acids: Benzoic acid (1), Salicylic acid (2), p-hydroxybenzoic acid (3), Vanillic acid (4), Syringic acid (5), Protocatechuic acid (6), Gentisic acid (7), Gallic acid (8)

2.2.2 Hydroxycinnamic acids

Hydroxycinnamic acids and their derivatives are widely distributed in the plant kingdom and play a very important role in the secondary metabolism in plants.[97,98,99,100] **Figure 3** show some examples of hydroxycinnamic acid as: Cinnamic acid (**1**), *p*-coumaric acid (**2**), Ferulic acid (**3**), Sinapic acid (**4**), Caffeic acid (**5**). Hydroxycinnamic acids are present in fruits and in different parts of plants, either esterified with other hydroxyacids or sugars, or in glycosylated form. Free forms only accumulate in exceptional circumstances, and only in very small quantities.[101,102] This is because, in their free form, hydroxycinnamic acids act as phytohormone regulators[103] or phytotoxic agents, while in the linked form they are involved in toxin elimination mechanisms[101,102,104] and/or storage of defensive agents (phytoalexins) under conditions of stress[100,105]. Furthermore, these compounds have a significant effect on the organoleptic characteristics of plant-based foods and beverages and contribute to bitterness and astringency, and particularly to browning in fruits and beverages.[106,107,108]

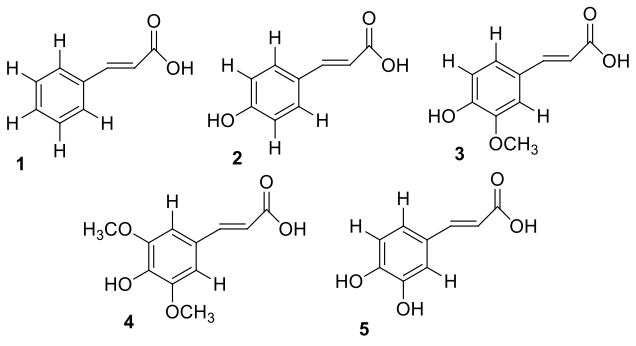


Figure 3. Hydroxycinnamic acid: Cinnamic acid (1), p-coumaric acid (2), Ferulic acid (3), Sinapic acid (4), Caffeic acid (5)

2.2.3 Coumarins

Coumarins comprise a very large class of compounds found throughout the plant kingdom.[109,110,111] They are found at high levels in some essential oils, but they are also found in fruits, green tea and other foods such as chicory.[112] Although distributed throughout all parts of the plant, the coumarins occur at the highest levels in the fruits, followed by the roots, stems and leaves. Environmental conditions and seasonal changes can influence the occurrence in diverse parts of the plant.[113]

Coumarin is classified as a member of the benzopyrone family, which consist of a benzene ring joined to a pyrone ring.[114] The benzopyrones can be subdivided into the benzo-α-pyrones to which the coumarins belong and the benzo-γ-pyrones, of which the flavonoids are the principal members. There are four main coumarin sub-types: the simple coumarins, furanocoumarins, pyranocoumarins and the pyrone-substituted coumarins (**Figure 4**). The simple coumarins (e.g. coumarin, 7-hydroxycoumarin and 6,7-dihydroxycoumarin), are the hydroxylated, alkoxylated and alkylated derivatives of the parent compound, coumarin, along with their glycosides. Furanocoumarins consist of a five-membered furan ring attached to the coumarin nucleus, divided into linear or angular types with substituents at one or both of the remaining benzoid positions. Pyranocoumarin members are analogous to the furanocoumarins, but contain a six-membered ring. Coumarins substituted in the pyrone ring include 4-hydroxycoumarin.[113]

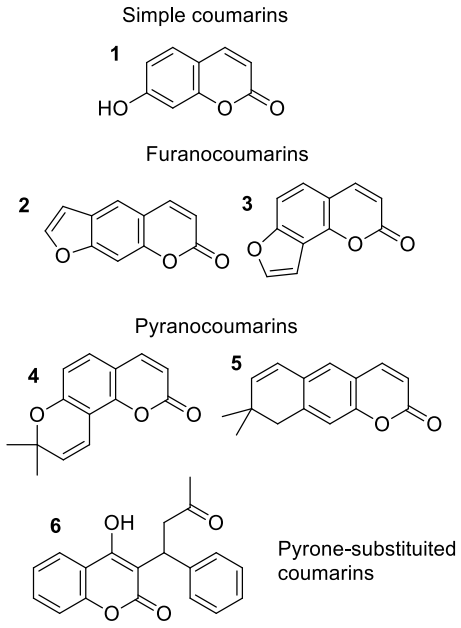


Figure 4. 7-Hydroxycoumarin (1), Psoralen (2), Angelicin (3), Seselin (4), Xanthyletin (5), Warfarin (6)

2.2.4 Stilbenes

Stilbenes are a small group of phenylpropanoids characterized by a 1,2-diphenylethylene backbone. There are two isomeric forms of 1,2-diphenylethylene: (E)-stilbene (trans-stilbene), which is not sterically hindered, and (Z)-stilbene (cis-stilbene), which is sterically hindered and therefore less stable (**Figure 5)**.[115]

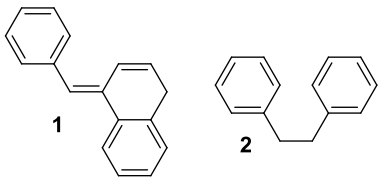


Figure 5. Isomeric form of stilbene: *trans*-stilbene (**1**), *cis*-stilbene (**2**).

Most plant stilbenes are derivatives of the basic unit trans-resveratrol (**Figure 6**), although other structures are found in particular plant families. Hydroxylated derivatives of stilbene (stilbenoids) are secondary products of heartwood formation in trees that can act as phytoalexins (antibiotics produced by plants).[116]

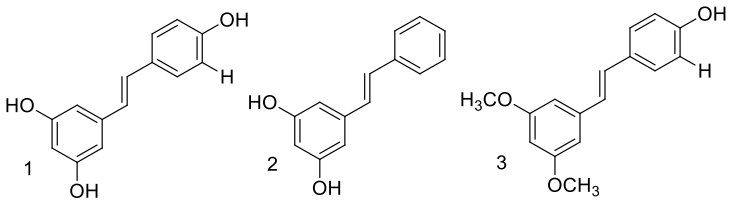


Figure 6. *trans*-Resveratrol (**1**), Pinosylvin (**2**), *trans*-Pterostilbene (**3**)

2.2.5 Flavonoids

Flavonoids are polyphenolic compounds comprising fifteen carbons, with two aromatic rings connected by a three-carbon bridge. (**Figure 7**) They are the most numerous of the phenolics and are found throughout the plant kingdom.[117] They are present in high concentrations in the epidermis of leaves and the skin of fruits and have important and varied roles as secondary metabolites. In plants, flavonoids are involved in such diverse processes as UV protection, pigmentation, stimulation of nitrogen-fixing nodules and disease resistance.[118,119]

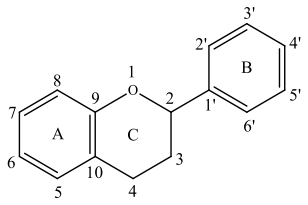


Figure 7. Flavonoid skeleton

Typical flavonoids, have a six-member heterocycle. Flavonoids have an A-, B-, and C-ring, and are typically depicted with the A-ring on the left-hand side. The A-ring originates from the condensation of three malonyl-CoA molecules, and the B-ring originates from p-coumaroyl-CoA. These origins explain why the A-ring of most flavonoids is either meta-dihydroxylated or meta-trihydroxylated. In typical flavonoids one of the meta-hydroxyl groups of the A-ring contributes the oxygen to the six atom-heterocycle. The six member oxygen heterocycle of typical flavonoids may be a pyran, pyrylium, or pyrone ring. The B-ring is typically mono-hydroxylated, orthodihydroxylated, or vic-trihydroxylated. The B-ring may also have methylethers as substituents.

The main subclasses of flavonoids are the flavones, flavonols, flavan-3-ols, isoflavones, flavanones and anthocyanidins. Other flavonoid groups, which quantitatively are in comparison minor components of the diet, are dihydroflavonols, flavan-3,4-diols, chalcones, dihydrochalcones and aurones (**Figure 8**). The basic flavonoid skeleton can have numerous substituents. Hydroxyl groups are usually present at the 4', 5 and 7 positions. Sugars are very common with the majority of flavonoids existing naturally as glycosides. Whereas both sugars and hydroxyl groups increase the water solubility of flavonoids, other substituents, such as methyl groups and isopentyl units, make flavonoids lipophilic.

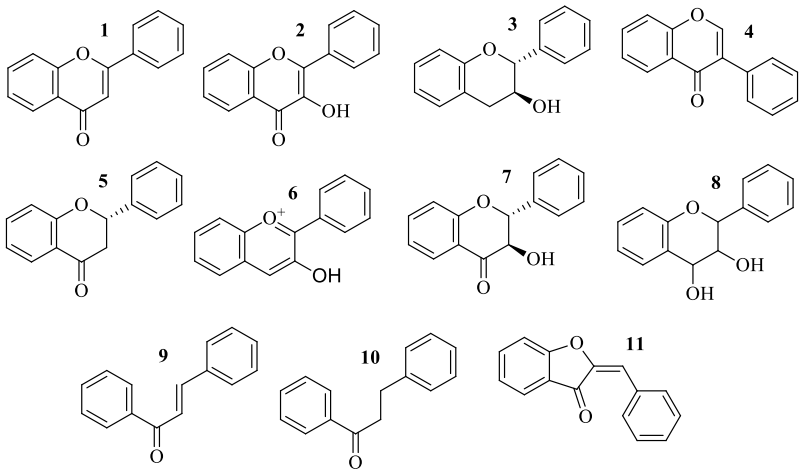


Figure 8. Structures of classes of flavonoids: Flavone (**1**), Flavonol (**2**), Flavan-3-ol (**3**), Isoflavone (**4**), Flavanone (**5**), Anthocyanidin (**6**), Dihydroflavonol (**7**), Flavan-3,4-diol (**8**), Chalcone (**9**), Dihydrochalcone (**10**), Aurone (**11**).

The structures of flavonoids differ greatly within the major classifications and substitutions include glycosylation, hydrogenation, hydroxylation, malonylation, methylation, and sulfation.[120] The pattern of conjugation, glycosylation, or methylation can be very complex, can modify the hydrophilicity of the molecule and its biological properties, and markedly increase the molecular weight of the flavonoid. Flavonoid molecules which are not bonded to sugar moieties are referred as the aglycone.[120] Except for catechins, flavonoids occur in plants most frequently as glycoside derivatives.[120,121,122,123] Flavonols and flavones occur in food usually as *O*-β-glycosides.[124] Among the major flavonoid classes, the flavonols glycosides predominate in fruits, whereas quercetin glycosides predominate in vegetables.[125] When glycosides are formed, the preferred glycosylation site on the flavonol molecule is the C-3

position and, less frequently, the C-7 position.[126] D-glucose is the most usual sugar residue, but other carbohydrate substitutions include arabinose, galactose, glucorhamnose, lignin, L-rhamnose, and xylose.[124] For instance, quercetin can be linked to the 3-*O*-glycoside rhamnose to yield quercitrin, or glucorhamnose to yield rutin.

2.2.5.1 Flavones

Flavone is a class of flavonoids based on the backbone of 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one) scaffold. The molecular formula of flavone molecule is $C_{15}H_{10}O_2$, it has a three-ring skeletons, C6-C3-C6. Flavones have three functional groups, including hydroxy, carbonyl, and conjugated double bond; consequently they give typical reactions of all three functional groups. The heterocycle of flavones contains a ketone group, and has an unsaturated carbon-carbon bond. Although flavones, such as apigenin and luteolin (**Figure 9, compounds 1 and 2**), have A- and C-ring substitutions, they lack oxygenation at C3. A wide range of substitutions is also possible with flavones, including hydroxylation, methylation, O- and C-alkylation, and glycosylation. Most flavones occur as 7-O-glycosides. Flavones are not distributed widely with significant occurrences being reported in only celery, parsley and some herbs. In addition, polymethoxylated flavones, such as tangeretin and nobiletin (**Figure 9, compounds 3 and 4**), have been found in citrus species.[127] Flavones are colorless-to-yellow crystalline substances, soluble in water and ethanol. They give yellow color solution when dissolve in alkali.[128]

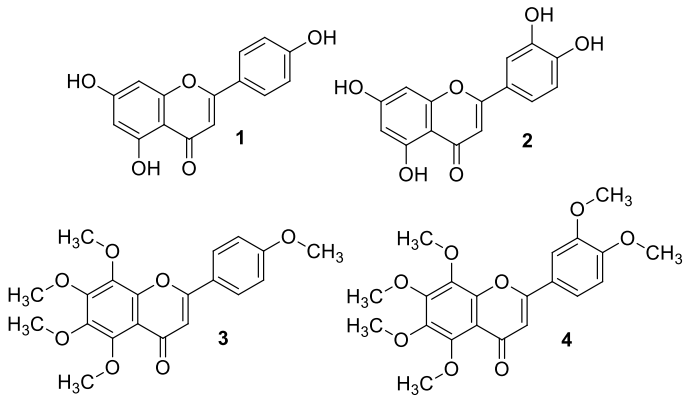


Figure 9. Apigenin (**1**), Luteolin (**2**), Targeretin (**3**), Nobiletin (**4**)

2.2.5.2 Flavonols and Dihydroflavonols

Flavonols are the most widespread of the flavonoids, being dispersed throughout the plant kingdom with the exception of fungi and algae. Many flavonols, such as kaempferol, quercetin, myricetin and isorhamnetin (**Figure 10, comounds 1,2,3,4**), are most commonly found as O-glycosides. Conjugation occurs most frequently at the 3 position of the C-ring but substitutions can also occur at the 5, 7, 4', 3' and 5' positions of the carbon ring. Although the number of aglycones is limited, there are numerous flavonol conjugates with more than 200 different sugar conjugates of kaempferol alone.[129]

Dihydroflavonols are also known as flavanonols and often occur in association with tannins in heartwood. An example is taxifolin, also known as dihydroquercitin (**Figure 10, compound 5**).[130]

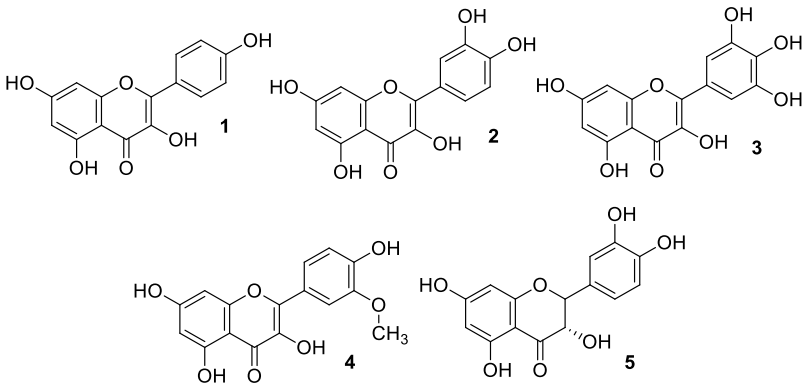


Figure 10. Kaempferol (**1**), Quercetin (**2**), Myricetin (**3**), Isorhamnetin (**4**), Taxifolin (**5**)

2.2.5.3 Flavan-3-ols

Flavan-3-ols are derivatives of flavans and these compounds are the most complex subclass of flavonoids ranging from the simple monomers (+)-catechin and its isomer (−)-epicatechin, to the oligomeric and polymeric proanthocyanidins, which are also known as condensed tannins. Unlike flavones, flavonols, isoflavones and anthocyanidins, which are planar molecules, flavan-3-ols, proanthocyanidins and flavanones have a saturated C3 element in the heterocyclic C-ring, and are thus non-planar. The two chiral centres at C2 and C3 of the flavan-3-ols produce four isomers for each level of B-ring hydroxylation, two of which, (+)-catechin and (−)-epicatechin (**Figure 11, compounds 1 and 2**), are widespread in nature whereas (−)-catechin and (+)-epicatechin (**Figure 11, compounds 3 and 4**) are comparatively

rare. The oligomeric and polymeric proanthocyanidins have an additional chiral centre at C4.[131] Type-B proanthocyanidins are formed from (+)-catechin and (−)-epicatechin with oxidative coupling occurring between the C-4 of the heterocycle and the C-6 or C-8 positions of the adjacent unit to create oligomers or polymers. Type A proanthocyanidins have an additional ether bond between C-2 and C-7. Proanthocyanidins can occur as polymers of up to 50 units. In addition to forming such large and complex structures, flavan-3-ols are hydroxylated to form gallocatechins and also undergo esterification with gallic acid. Proanthocyanidins that consist exclusively of (epi)catechin units are called procyanidins, and these are the most abundant type of proanthocyanidins in plants. The less common proanthocyanidins containing (epi)afzelechin and (epi)gallocatechin subunits are called propelargonidins and prodelphinidins, respectively.[132]

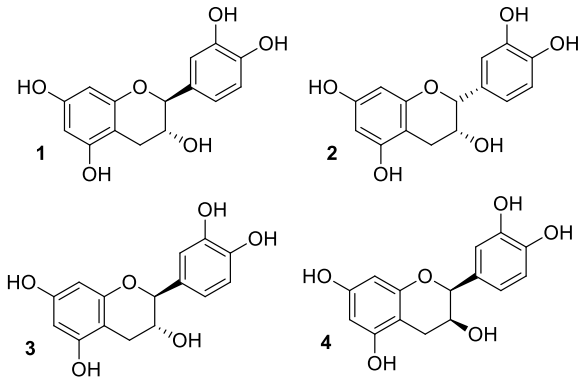


Figure 11. (+)-Catechin (**1**), (-)-Epichatechin (**2**), (-)-Catechin (**3**), (+)-Epicatechin (**4**)

2.2.5.4 Isoflavones

Isoflavones are naturally-occurring plant compounds. More than 300 kinds of plants, in particular roots and seeds, include isoflavones.[133] The sources are the Fabaceae/Leguminosae family. Isoflavones are the secondary metabolite formed by symbiotic relationship with the rhizobial bacteria and the defense responses of leguminous plant.[134] Isoflavones are polyphenolic compounds which exist in twelve differents chemical forms.[135] Daidzein, glycitein and genistein are the aglycone forms of isoflavones (**Figure 12, compounds 1, 2 and 3**). In conjuction with sugars, they build the β-glucosides (daidzin, glycitin and genistein), the 6‘-O-malonyl glucosides (malonyl daidzin, malonyl glycitin, and malonyl genistin) and the 6‘-O-acetyl glucosides (acetyl daidzin, acetyl glycitin, and acetyl genistin). The aglycone structures can be found in very small amounts in soybean, while the glycoside

forms are dominant. However, isoflavones in glycoside forms are inactive, because hydrolysis and the release of the aglycone component are essential for the absorption of isoflavones in the digestive tract.[136] For this reason aglycones are considered to be biologically active forms of isoflavones. The isoflavones, genistein and daidzein, have sufficient oestrogenic activity to seriously affect the reproduction of grazing animals such as cows and sheep and are termed phyto-oestrogens. The structure of these isoflavonoids is such that they appear to mimic the steroidal hormone oestradiol which blocks ovulation.

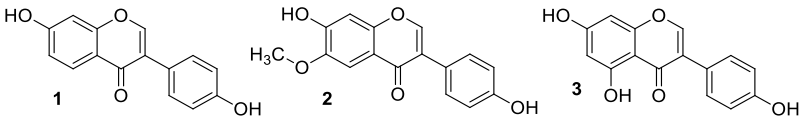


Figure 12. Daidzein (**1**), Glycitein (**2**), Genistein (**3**)

2.2.5.5 Flavanones

Flavanones are characterized by the absence of a $\Delta^{2,3}$ double bond and the presence of a chiral centre at C2. In the majority of naturally occurring flavanones, the C-ring is attached to the B-ring at C2 in the α-configuration. The flavanone structure is highly reactive and have been reported to undergo hydroxylation, glycosylation and O-methylation reactions. Flavanones are dietary components that are present in especially high concentrations in citrus fruits.[137] Examples of flavanones are: naringenin, pinocembrin, eriodictyol and sakuranetin (**Figure 13**).

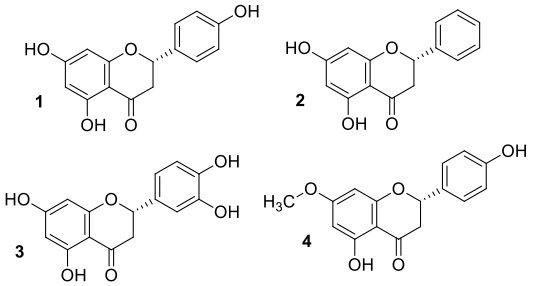


Figure 13. Naringenin (**1**), Pinocembrin (**2**), Eriodictyol (**3**), Sakuranetin (**4**)

2.2.5.6 Anthocyanidins

Anthocyanidins, principally as their conjugated derivatives, anthocyanins, are widely dispersed throughout the plant kingdom, being particularly concentrated in fruit and flower tissue, where they are responsible for red, blue and purple colours. In addition they are also found in leaves, stems, seeds and root tissue. They are involved in the protection of plants against excessive light by shading leaf mesophyll cells and also have an important role to play in attracting pollinating insects. Anthocyanidins are typically not found as free aglycones, with the exception of the following widely distributed, colored compounds: pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (**Figure 14**). In plant tissues these compounds are invariably found as sugar conjugates that are known as anthocyanins. The anthocyanins also form conjugates with hydroxycinnamates and organic acids such as malic and acetic acids. Although conjugation can take place on carbons 3, 5, 7, 3' and 5', it occurs most often on C3.[137]

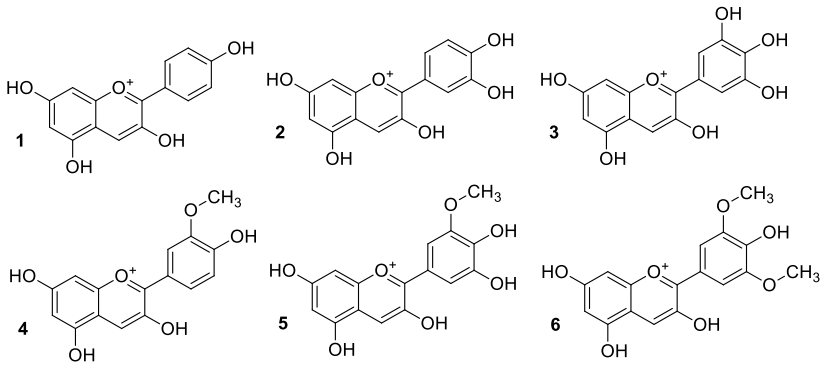


Figure 14. Pelargonidin (**1**), Cyanidin (**2**), Delphinidin (**3**), Peonidin (**4**), Petunidin (**5**), Malvidin (**6**)

2.2.5.7 Chalcones and Dihydrochalcones

Chalcones are natural products belong to flavonoid, are considered as intermediate in the flavonoids biosynthesis, and are widespread in plants. Chalcones (trans-1,3-diaryl-2-propen-1-ones), a biosynthetic product of the shikimate pathway, belonging to flavanoid family are precursors of open chain flavonoids and isoflavonoids, which are abundant in edible plants. Chalcones are also key precursors in the synthesis of many biologically important heterocycles such as benzothiazepine, pyrazolines, 1,4-diketones, and flavones. Chalcones and dihydrochalocones have a linear C3-chain connecting the two rings. The C3-chain of chalcones contains a double bond, whereas the C3-chain of dihydrochalcones is saturated.[138]

2.2.6 Lignins

Lignin is a phenolic polymer. It is the second most abundant bio-polymer on Earth, after cellulose, and plays an important role in providing structural support to plants. Its hydrophobicity also facilitates water transport through the vascular tissue. Finally, the chemical complexity and apparent lack of regularity in its structure make lignin extremely suitable as a physical barrier against insects and fungi.[139,140] Lignin is synthesized primarily from three monolignol precursors: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (**Figure 15**). Additional compounds are incorporated into the lignin, but typically in small quantities. Some of these compounds include: coniferaldehyde,[141,142,143] sinapaldehyde,[141] dihydroconiferyl alcohol,[144] 5-hydroxyconiferyl alcohol,[145,143,146] tyramine ferulate,[147] *p*-hydroxy-3-methoxybenzaldehyde,[148] *p*-hydroxybenzoate,[149] *p*-coumarate[150] and acetate.[151] After polymerization, the different lignin subunits are referred to as *p*-hyrdoxyphenyl, guaiacyl, and syringyl residues, depending on whether they originated from *p*-coumaryl alcohol, coniferyl alcohol, or sinapyl alcohol, respectively. On the basis of the relative content of three monomeric precursors, it is possible to classify lignin in three different types: guaiacyl lignins (or G-lignins), guaiacylsyringyl lignins (or GS-lignins) and guaiacyl-syringyl-p-hydroxyphenyl lignins (or GSHlignins).[152] G-lignins are predominantly composed of coniferyl alcohol (guaiacyl alcohol) and are distinctive of gymnosperms (softwood); GS-lignins are characterized by a large amount of coniferyl and synapyl alcohol are typical of hardwood; GSH-lignins represent the most heterogeneous lignins, containing the same amount of the three monomers, and are distinctive of grass.

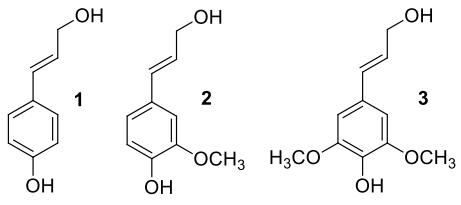


Figure 15. Monomeric precursors: *p*-coumaryl alcohol (**1**), coniferyl alcohol (**2**), sinapyl alcohol (**3**)

2.2.7 Melanins

Melanins are irregular tri-dimensional macromolecules composed of phenolic or indolic monomers that combine randomly to form a polymer.[153,154,155] The monomeric units possibly are linked by several types of non-hydrolyzable bonds.[156,157] Melanins are heterogeneous and even when the starting substrate could be the same, the products may be different because polymerization patterns vary depending on biological or chemical conditions.[155] All have the basic structure of an aromatic ring, usually mixtures of quinone, hydroquinone and semiquinone moieties, complexed with proteins, carbohydrates and lipids.[158,159] The protein component might be essential for its biological activity.[160,161,155] Plant metabolism is characterized by the principle of nitrogen economy, as this element is an important limiting factor for plant growing. In general, the melanin is a polymer devoid of nitrogen and is generically named allomelanin. In plants, the most common precursor is just catechol; so the melanin formed is also called catechol-melanin[162,163] and the enzymatic system involved in the synthesis is named catechol oxidase[164]. The configuration of quinone or quinonimine residues in catechol melanins are para- (polymers of γ-glutaminyl-4-hydroxybenzene) or meta- (polymers of DHN).[165] The color of allomelanin is always from dark brown to totally black, and its structure depends on the nature of the main unit oxidized. Some vegetables use just normal catechol, but others use different catecholic acids (such as caffeic, chlorogenic, protocatechuic, or gallic acids).[166]

2.2.8 Tannins

Tannins are water-soluble polyphenolic compounds with molecular weights ranging from 500 to 20000 Da. The presence of a large number of phenolic hydroxyl groups enables them to form large complexes, mainly with proteins, alkaloids, and polysaccharides.[167,168] Tannins are widespread in the plant kingdom (pteridophytes, gymnosperms, and angiosperms) and are found in leaves, fruits, bark, and wood.[169] Tannins are stored in leaf, bud, seed, root, or stem tissues and are physically located in the vacuoles or surface wax.[170] They are responsible for the typical taste of astringency of fruits and leaves, making them less appetizing for herbivores. These compounds have a range of biological effects on various organisms, ranging from growth inhibition to toxicity.[171] Tannins inhibit the growth of a number of microorganisms, resist microbial attack, and are recalcitrant to biodegradation.[172] Condensed tannins are more resistant to microbial attack than hydrolyzable tannins and are toxic to a

variety of microorganisms. Tannins do not constitute a unified chemical group, but display a variety of molecular structures. On the basis of their structures and properties, they are distributed into two major groups: hydrolysable and condensed tannins. Hydrolyzable tannins are gallic acid derivatives, which are classified as gallo and ellagitannins.[173,174] Gallotannins are gallic acid esters of a core polyol, usually glucose. The simplest hydrolyzable prototannin is pentagalloyl glucose (β-1,2,3,4,6-pentagalloyl-O-D-glucopyranose) (PGG) (**Figure 16**). PGG occurs in different isomers in which one aliphatic hydroxyl group is free and the other is present as a digalloyl ester. Polygalloyl ester chains are formed by a meta- or para-depside bond involving a phenolic hydroxyl rather than an aliphatic hydroxyl group.

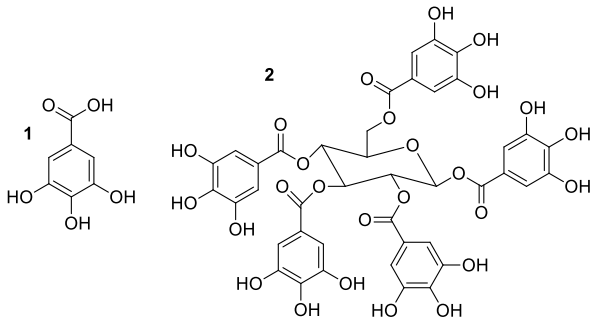


Figure 16. Gallotannins: Gallic acid (**1**), PGG (**2**)

Ellagitannins are esters of hexahydrodiphenic acid (HHDP) that in turn is generated by oxidative cross-linking of two galloyl groups. Upon hydrolysis the ellagitannins release HHDP, which in turn lactonizes to ellagic acid. Coupling occurs preferentially between C-4/C-6 (eugeniin) and C-2/C-3 (casuarictin) (**Figure 17**).

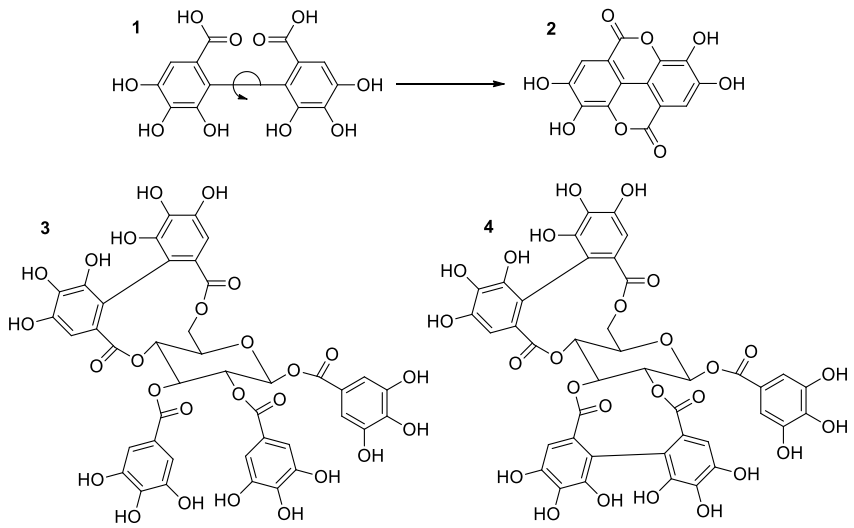


Figure 17. Ellagitannins: Hexahydroxydiphenic acid (**1**), Ellagic acid (**2**), Eugeniin (**3**), Casuarictin (**4**)

Condensed tannins, better known as proanthocyanidins, are polymeric flavonoids. They are usually more abundant in tree barks and woods than their hydrolyzable counterparts. The most abundant and well-studied flavonoids are based on the flavan-3-ol structure of (−)-epicatechin and (+)-catechin. Addition of a third phenolic group on the B ring yields epigallocatechin and gallocatechin. Flavan-3-ols with a single phenolic group on the B ring are less common. **Figure 18** shows the structures of most common procyanidins.[175]

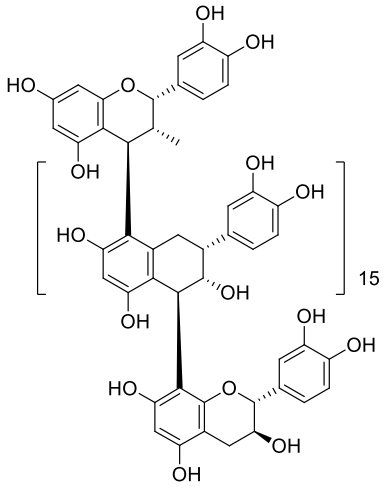


Figure 18. Procyanidins: epicatechin-[(4β->8)-epicatechin]$_{15}$-(4β->8)-catechin

2.3 Extraction, characterization and quantification

Extraction of phenolic compounds in plant materials is influenced by their chemical nature, the extraction method employed, sample particle size, storage time and conditions, as well as presence of interfering substances. The chemical nature of plant phenolics vary from simple to highly polymerized substances that include varying proportions of phenolic acids, phenylpropanoids, anthocyanins and tannins, among others. They may also exist as complexes with carbohydrates, proteins and other plant components; some high-molecular weight phenolics and their complexes may be quite insoluble. Therefore, phenolic extracts of plant materials are always a mixture of different classes of phenolics that are soluble in the solvent system used.[176] Methanol, ethanol, acetone, water, ethyl acetate and, to a lesser extent, propanol, dimethylformamide, and their combinations are frequently used for the extraction of phenolics.[177] The extracts of phenolics, first concentrated under vacuum, may be extracted with petroleum ether, ethyl acetate or diethyl ether in order to remove lipids and unwanted polyphenols.[178,179] A number of spectrophotometric methods for quantification of phenolic compounds in plant materials has been developed. These assays are based on different principles and are used to determine various structural groups present in phenolic compounds. Gas chromatographic (GC) and high performance liquid chromatographic techniques are used widely for both separation and quantitation of phenolic compounds. Structure elucidation is often achieved using combination of GC and HPLC with mass spectrometric analysis, as well as other relevant techniques.[180]

2.3.1 Analisys of tannins

Conversation apart to be done for the tannins, their study has been difficult because of the structural complexity of these compounds. Condensed and hydrolysable tannins are analyzed with different procedures, each of which has advantages but also disadvantages that should be evaluated based on the type of analysis that needs to be done.[181,182]

2.3.1.1 Hydrolyzable tannins

Several solvents have been used to extracts hydrolizable tannins. Hexane or dichloromethane can be used initially to remove lipids and chlorophyll.[183] Hydrolyzable tannins have been extracted with water (RT or 90°C), 50% methanol (RT or boiling) and 50-70% acqueous

acetone (4°C or RT).[184,185] Methanol tends to be the better solvent for tannins of low molecular weight. Acetone is often the preferred solvent as it is less liable to react with hydrolysable tannins than water or methanol. The solubility of hydrolysable tannins is surprisingly variable; for example, castalagin and vescalagin are high water-soluble, whereas pentagalloylglucose is only sparingly water-soluble but highly soluble in ethyl acetate.[186] On a cautionary note: ethyl acetate is often used to remove non-tannin components from crude tannin extracts and may thus cause significant losses of some hydrolysable tannins.

Hydrolyzable tannins are more likely to react with the extracting solvent than condensed tannins. For example, methanol cleaves the bonds in gallotannins at neutral pH and RT,[187,188] but acidified methanol (pH<3) will not cleave these bonds[189]. Large and complex tannins are easily degraded into smaller tannins by water or diluite acids especially at elevated temperatures in just 30 min.[190,191,183] Water at 60°C is likely to liberate gallic acid from the anomeric C1 position of glucose.[187] Water at 100°C may also release ellagic acid from ellagiotannins[192] and cleave the ether bond in valoneoyl group[183]. Although some of the reactions happen under fairly mild conditions, not all bonds in hydrolyzable tannins are readily degraded.[188,193] Following extraction, tannins are frequently subjected tofurther purification by Sephadex LH-20, Toyopearl HW-40 or Diaion HP-20[191]. However, some labile oligomeric hydrolyzable tannins can be degraded during purification on Sephadex LH-20.[194,195,183] It is well known, yet rarely mentioned, that Sephadex LH-20 tends to absorb large molecular weight condensed tannins, and possibly some large hydrolyzable tannins.[191]

2.3.1.1.1 Colorimetric assays

KIO_3-reagent for gallo- and ellagiotannins

Some authors described a procedure for measuring gallo- and ellagiotannins in *Acer* species, which yield a pink reaction product with the KIO_3-reagent.[196] Other authors noted, however, that this assay was not suitable for complex mixtures of tannins, since it yields brown rather than pink products.[197]

Rhodanine reagent for gallotannins

Gallotannins can be detected quite specifically by the rhodanine test.[198] The success of this assay depends on the absence of oxygen and is performed either under nitrogen or in a vacuum inside a sealed test tube at RT. Rhodanine reacts only with gallic acid and not with galloyl esters, ellagic acid, ellagiotannins or other phenolics.[188] Gallotannins are measured by determining the quantity of gallic acid before and after hydrolysis of the tannins. It is important to recognise tha this assay cannot provide an absolute quantification of gallotannins as the number of gallic acid units differs between the various gallotannin molecules that occur in nature.[198] A further complication arise from the fact that many ellagitannins also contain gallic acid.

$NaNO_2$-reagent for ellagitannins

Some authors reported an improved $NaNO_2$ assay for measuring ellagitannins.[193] this assay is selective for ellagic acid only; gallic acid, gallotannins, ellagitannins, condensed tannins, flavonoids do not interfere. Unfortunately, this assay is also sensitive to oxygen and therefore needs to be carried out under nitrogen. Furthermore, the authors recommend that new glass tube be used as residues from glass washing can inhibit the reaction. Clearly, this test would benefit from further development. None of the phenolic constituents of hydrolyzable tannins having molecular weights larger than ellagic acid will react with the above reagents. This means that not all hydrolyzable tannins can be detected by these colorimetric assay.

2.3.1.2 Condensed tannins

Commonly used methods include oxidative depolymerization of condensed tannins, reactions of the A ring with an aromatic aldehyde, and oxidation-reduction reactions.[185] Other methods involve acid cleavage reactions, precipitation reactions, enzyme and microbial inhibition and gravimetric procedures. The initial harvesting, drying and extraction methods have significant effects on condensed tannin analyses.[185,199] Most condensed tannin assay have been done on the soluble material extracted with aqueous acetone or methanol. One may also wish to evaluate tannins insoluble in these solvents. This fraction may include higher molecular weight tannins and tannins bound to fiber. Methods for the analysis of insoluble tannins

include the use of ^{13}C-NMR[200] and application of the butanol-HCl reaction to insoluble plant materials[201,202].

2.3.1.2.1 Colorimetric assays

Acid-butanol assay

This colorimetric reaction is based on a acid-catalyzed oxidative depolymerization of condensed tannins to yield red anthocyanidins. It is diagnostic for the polyflavan structure.[203] Users of this procedure should be aware of the following limitations:

- The amount of water in the reaction medium is reported to be critical in color formation and therefore in the quantitative determination of condensed tannins.[185,204,205] Increasing the water content from 2 to 6% (v/v) in a methanol-based reaction medium suppressed theanthocyanidin yield of condensed tannins. The amount of water present may lead to variable color development when different plant species are used.[205]
- The ease with which the interflavan bond is cleaved by acid varies widely; 4→6 bonds are more resistant to this cleavage than are the more usual 4→8 bonds. The nature of the A ring substituent also affects acid lability. For example, in quebracho tannin, which is frequently used as a standard in this condensed tannin assay, R1 is H rather than OH. This change increase the acid stability of the interflavan bond and reduces the color yield from this tannin.[202]
- The number of phenolic groups in ring A e B affects the wavelength of the absorbance maximum and the extinction coefficients of the anthocyanidin products. For example, cyaniding and delphinidin (2 vs 3 phenolic OH in B) have λ_{max} at 545 and 557 nm, respectively.[206]
- Color yield is not always linear with the amount of tannin input. Larger amounts may give a lower unit colour yield.[185]
- The presence of transition metal ions in the assay medium is an important factor in color development.[205,207,208] Some authors found the use of Fe^{3+} at concentration greater than 15 µg/ml decreased color development from *Leucaena* spp. Condensed tannins. They speculated that excess Fe^{3+} either inhibited tannin depolymerisation or reduced the absorbance of the anthocyanidin products.[205] Inconsistent colour

development has led some investigators to discontinue the use of metal ion catalysts.[209]

- Another important factor is the ratio of acid-butanol to sample medium in the reaction mixture.[185] When the ration of reagent to sample was increased from 4:1 to 6:1, the color yield decreased.[205] Other important considerations in the acid-butanol assay are strict regulation of the temperature and length of the reaction time.[208] The presence of ascorbic acid, an anti.oxidant, in the reaction medium increased color development.[205]
- The choice of standards remains an unresolved issue due to the heterogeneity of condensed tannins and the lack of appropriate standards for their quantification. Cyanidin, delphinidin and quebracho tannin have been used as standards.[210,208,202] While cyaniding would be an appropriate standard for cyaniding-yielding condensed tannin, it is less suitable for prodelphinidins.[202,208] Condensed tannins may be underestimated if cyaniding is used as a standard because the yield of cyaniding from some tannins may not be quantitative.[208]
- The acid-butanol assay is commonly used to quantify soluble condensed tannins. However, it is now well recognized that some condensed tannins are insoluble in common solvents and, therefore, condensed tannins in plant materials may be underestimated. After condensed tannin extraction with 70% acetone, the insoluble fraction was reported to contain between 6 and 20% of the total condensed tannins.[211] Some of this insoluble condensed tannins fraction is fiber-bound and is associated with reduced nitrogen digestibility and increased fecal excretion of nitrogen, fat and water in rats.[212,213] The aid-butanol assay has been proposed as a method for estimating the amount of fiber-bound condensed tannins.[213,214] However, not all bound condensed tannins react quantitatively in the acid-butanol assay, and may thus be underestimated.[200]

The butano-HCl reaction should be used with caution as a quantitative assay. The assay's greatest strength lies in its confirmation of the presence of a polymeric interflavan structure. Hydrolyzable tannins do not react in this assay.

Vanillin assay

The vanillin assay method depends on the reaction of vanillin with condensed tannins and the formation of colored complexes. Critical to successful use of this assay are the type of solvent

used, the nature and concentration of the acid, the reaction time, temperature, vanillin concentration and the standards used.[208,205,215,216,217] In the vanillin assay, sulphuric acid is a better catalyst than hydrochloric acid at the same normality.[208,217] At higher water contents, there is a rapid decrease in A_{500}.[218,216,217] The use of HCl as a catalyst is also an important factor that must be controlled in the vanillin assay because an increase of 1.4°C caused an 11% increase in absorbance.[185] Although the most commonly used standard in the vanillin-acid assay is catechin, under normal reaction conditions the catechin monomers has a different reaction rate than related procyanidin polymers.[216,185] The major problem with the vanillin assay seems to derive from the variable reactivity of the subunits of the tannin polymer. In glacial acetic acid, only terminal units react.[219] Unfortunately, glacial acetic acid is not a good solvent for polymeric condensed tannins. When hydrochloric and sulphuric acids were used as solvents, the color yield per mole from catechin was somewhat greater than from polymeric condensed tannin.[217] This means that only some of the internal flavanol units in the polymer react with vanillin.

2.3.1.3 Gravimetric methods

A primary advantage of gravimetric methods is that they do not require standards. However, they are less sensitive than the common colorimetric methods.[215] Between the gravimetric methods proposed, two approaches described are ytterbium precipitation assay[220] and methods based of tannin binding to insoluble polyvinylpyrrolidone[215]. The ytterbium precipitation assay precipitates condensed tannins. The amount of polyphenols is determined by deducing the amount of ash (ytterbium oxide) after ashing the dry precipitated complex. To validate the ytterbium method, the tannins are isolated by the Sephadex LH-20.[216] The ytterbium extraction method requires less time and is less expensive than the Sephadex procedure.[202] The utility of the ytterbium method was questioned because some model phenolics like rutin were not precipitated by ytterbium.[221] However, most model phenolics are precipitated if the molar ratio of the phenolics to the ytterbium is controlled.[211]

2.3.1.4 Protein precipitation assays

Tannins are, by definition, protein-binding and precipitating agents and many tannins assay are based on the protein precipitation. The most convenient form of this assay used BSA, dyed with Remazol Brilliant Blue.[222] Tannins and proteins are mixed under defined

conditions (pH and ionic strength) and allowed to precipitate. After centrifugation, the pellet is redissolved in an alkaline SDS buffer for measurement of the dyed protein content. Protein precipitation can also be followed in agarose plates.[223] Another precipitation assay is the PEG binding assay. In this method the determination of tannins can be made in native plant samples without extraction, by using ^{14}C-labeled PEG.[224] Polyethylene glycol 4000 (PEG), which binds to a wide range of hydrolysable and condensed tannins, forms a stable PEG-tannin complex over a pH range from 2 to 8.5.[225] The tannin-PEG complex is insoluble in boiling water, neutral and acid detergents and many organic solvents[225,226]. The method is useful for plant materials containing strong tannin-protein complex in which extraction would give low tannin yield. The PEG can disrupt these complexes because its affinity for tannin exceeds that of protein. The primary disadvantages of this methods are safety issue and costs due to radioactive materials.

2.3.1.5 High performance liquid chromatography (HPLC)

Mixture of tannins can be separated on two type of HPLC columns. In normal phase HPLC, the stainless steel columns are tightly packed with polar particles (<5µm). In reverse phase HPLC, the silica particles are coated with non-polar chains, for example C_8 or C_{18} chains. Several workers found that normal phase HPLC was a useful technique for separating hydrolyzable tannins by their molecular weights.[190,227,191,228] Usually, the purification of hydrolizable tannins in normal phase is more efficient than procedures based on reverse phase columns.[227,191]

Both normal-phase and reversed-phase columns have been applied for condensed tannins.[229,230,231] Reversed-phase HPLC has been used for separation of lower molecular weight condensed tannins but the order of eluition is not related to the degree of polymerization.[229] Separation of larger polymers (≥tetramers) with this method is not possible. The presence of many isomers with similar polarity, results in overlapping retention times.[230,231] Normal-phase HPLC was used to separate condensed tannin oligomers and polymers from various food plant products.[230] The order of elution increased by increasing the degree of polymerization.

Various detection methods have been applied in conjunction with HPLC for condensed tannin determination. Ultraviolet (UV) detection is the most commonly used.[185] However, this method is not specific for condensed tannin in presence of other polyphenols.[230] Alternative

methods include electrochemical detection and fluorescence detection.[185,230] Structural information for identification of condensed tannins can be obtained using mass spectrometry (MS), nuclear magnetic resonance (NMR) and chemical hydrolysis.[230]

CHAPTER 3

PHENOLS, POLYPHENOLS AND HEALTH

3.1 Bioavailability, absorption, metabolism and elimination

Most common polyphenols in human diet are not necessarily the most active within the body, either because they have a lower intrinsic activity or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. In addition, metabolites that result from digestive or hepatic activity may differ from the native substances in terms of biological activity.[232]

Absorption and metabolism properties of phenols are primarily determined by the chemical structure, including the parent scaffold (gallic acid, ellagic acid, flavan-3-ol), degree of chemical modification, the presence or absence of glycosylation on hydroxyl groups, the position of glycosylation, the quality of sugar moiety, plant/food matrix, and interactions with proteins, micelles, and emulsifiers, molecular size, degree of polymerization and solubility.[233,234,235,236,237] Molecular weight is another important factor that greatly affects the bioavailability of certain flavonoids, as in the case of polymeric proanthocyanidins for which absorption would be practically impossible.[236,238,239] Relatively small molecular weight phenolic acids, such as gallic acid and isoflavones, are easily absorbed through the gut followed by catechins, flavanones, and quercetin glucosides. Larger polyphenols such as proanthocyanidins are very poorly absorbed as well as galloylated tea catechins and the anthocyanins. Absorption of flavonoids can vary drastically among different classes as well as among different conjugates of the same compound in a particular class. Absorption of some flavonoid glycosides can be very rapid, thus affecting the bioavailability. The associated sugar moiety had a great influence on absorption; quercetin glucosides was absorbed 10 times faster and the plasma concentration peaked 20 times higher than quercetin rutinosides in humans.[240] It was suggested that glucosides were absorbed in the small intestine whereas quercetin rutinosides may be absorbed in the colon after deglycosylation.[241] Most glycosylated flavonoids need to be hydrolyzed to their aglycones for absorption, anthocyanins were absorbed and detected in the circulation without sugars or with their sugars intact.[242]

Metabolism of polyphenols occurs via a common pathway.[243] The aglycones can be absorbed from the small intestine. However, most polyphenols are present in food in the form of esters, glycosides, or polymers that cannot be absorbed in their native form. These substances must be hydrolyzed by intestinal enzymes or by the colonic microflora before they can be absorbed. When the flora is involved, the efficiency of absorption is often reduced because the flora also degrades the aglycones that it released, and produces various simple aromatic acids in the process. During the course of absorption, polyphenols are conjugated in the small intestine and later in the liver. This process mainly includes methylation, sulfation, and glucuronidation. This is a metabolic detoxication process common to many xenobiotics that restricts their potential toxic effects and facilitates their biliary and urinary elimination by increasing their hydrophilicity. The conjugation mechanisms are highly efficient, and aglycones are generally either absent in blood or present in low concentrations after consumption of nutritional doses. Polyphenols are able to penetrate tissues, but their ability to accumulate within specific target tissues needs to be further investigated. Polyphenols and their derivatives are eliminated chiefly in urine and bile. Polyphenols are secreted via the biliary route into the duodenum, where they are subjected to the action of bacterial enzymes, especially β-glucuronidase, in the distal segments of the intestine, after which they may be reabsorbed. This enterohepatic recycling may lead to a longer presence of polyphenols within the body.[232]

3.1.1 Oral cavity

The oral cavity is the first section of the alimentary tract, where the digestion of polysaccharides (by α-amylase) and dietary lipids (by lingual lipase) starts. The food is masticated and mixed with saliva to facilitate swallowing.[244,245] Proline-rich proteins in saliva have strong affinity for polyphenols (e.g. catechins), resulting in their complexation and development of astringent response in the palate.[246] The hydrolysis of flavonoid glycosides could be catalyzed by human enzymes in the mouth or by those of microbial origin.[247]

3.1.2 Stomach

The ingested material is transferred from the mouth to the stomach through the pharynx and esophagus. In the stomach, the masticated food is mixed with secreted enzymes to form a

chyme.[245] The non-enzymatic deglycosylation of flavonoids, such as gastric acid hydrolysis was not found.[248]

Most polyphenols are probably too hydrophilic to penetrate the gut wall by passive diffusion, but the membrane carriers that could be involved in polyphenol absorption have not been identified. To date, the unique active transport mechanism that has been described is a Na^+-dependent saturable transport mechanism involved in cinnamic and ferulic acid absorption in the rat jejunum.[249] Experiments using surgically treated rats in which absorption was restricted to the stomach showed that absorption at the gastric level is possible for some flavonoids, such as quercetin and daidzein, but not for their glycosides.[250,251] Most of the glycosides probably resist to acid hydrolysis in the stomach and thus arrive intact in the duodenum.[252]

3.1.3 Small intestine

The stomach content is emptied into the small intestine, divided into three segments: duodenum, jejunum, and ileum. The intestinal lumen can be considered as the continuation of the external environment, therefore its epithelium functions as a crucial barrier; the substances absorbed from the lumen must first cross the epithelium to reach systemic circulation. Apart from the regulation of absorptive and secretory processes, the epithelium can also modify the substances traversing it.[244] An experiment showing that the intestinal barrier was able to detoxify phenol and that phenol entered the portal blood in conjugated form.[253] Deglycosylation has been shown as the first step of metabolism of some flavonoid glucosides occurring in the small intestinal lumen.[254,255] The epithelial cells of the gastrointestinal tract are the only cells of the body in contact with flavonoid glucosides; the other cells are reached only by flavonoid metabolites and degradation products.[256] Flavonoids passing into the small intestine as glycosides can undergo either luminal deglycosylation catalyzed by membrane bound enzymes[254,256] or enter the enterocytes in the form of glycosides requiring active transport[257,258] followed by intracellular hydrolysis by e.g. broad-specificity cytosolic β-glucosidase[259]. Flavonoids liberated in the lumen can pass into enterocytes via passive diffusion where they are subject to Phase II metabolism. The transport across the intestinal enterocytes depends on the quality of the flavonoid aglycone moiety and the nature and position of the attached sugar. After passage into enterocytes, flavonoid glucosides are susceptible to hydrolysis by intracellular β-glucosidases, e.g. broad-specificity cytosolic β-

glucosidase.[260] After absorption, polyphenols are conjugated to glucuronide, sulphate, and/or methyl groups in the intestinal mucosa and inner tissues.[261] Xenobiotics are detoxified mainly in the liver but apart from it, Phase II reactions—conjugation reactions such as glucuronidation after deglycosylation, and methylation can occur in the jejunal and the ileal part of the small intestine.[262,263,264,265]

3.1.4 Colon

Continuing absorption of water, electrolytes, and nutrients occurs in this part of the gastrointestinal tract. The colon is heavily colonized by microorganisms (~1012/mL) with a strong catalytic and hydrolytic potential[266] against compounds of exogenous (dietary) and endogenous origin. Microbiota also function as a conservator of nitrogen that would otherwise be excreted as urea. In exchange, the flora competes directly with the host tissues for nutrients ingested in the diet.[267,268] Flavonoids neither absorbed in the stomach nor in the small intestine are propelled to the colon. Reaching the colon, they are subject to deglycosylation[269] and deconjugation by colonic bacteria, and are cleaved giving rise to ring fission products. The microflora hydrolyzes glycosides into aglycones and extensively metabolizes the aglycones into various aromatic acids.[270,271] Aglycones are split by the opening of the heterocycle at different points depending on their chemical. Flavonols are degraded to phenylacetic acids and phenylpropionic acids. Flavones and flavanones are cleaved to phenylpropionic acids. Ring fission of catechins gives rise to valerolactones (a benzene ring with a side chain of 5 C-atoms), and phenylpropionic acids which are finally oxidized (beta-oxidation) to benzoic acids.[272,273] These low molecular microbial metabolites of flavonoids exhibit several important biological activities. The arising cleavage products are absorbed from the colon or further metabolized. 3,4-Dihydroxyphenylpropionic acid is degraded in the colon to phenylpropionic, 3-hydroxypropionic, and 4-hydroxypropionic acid, which are further metabolized by the liver giving rise to hippuric, 3-hydroxyhippuric, and 4-hydroxyhippuric acids.[274]

3.1.5 Liver

Compounds absorbed from intestines enter the liver via the portal vein. Once in the liver, the absorbed substances are removed from the blood by the liver parenchymal cells and

biotransformed.[245] The metabolism of xenobiotics is a process in which the compound first undergoes a reaction of oxidation, reduction, or hydrolysis (Phase I), which introduces or discloses within its structure a functional group (examples –OH, –NH2) suitable for linkage with glucuronic or sulfuric acid in the second step called conjugation (Phase II). Compounds with a suitable group can undergo conjugation directly. Xenobiotics can covalently bind to biological molecules as blood or cellular (glyco)proteins, forming xenobiotic-macromolecule adducts with immunological properties.[275] Phase I reactions take place in the smooth endoplasmatic reticulum of hepatocytes. Many of the oxidative reactions are catalyzed by cytochrome P450 systems. The Phase I biotransformation reactions of flavonoids introduce or expose polar groups. Hydroxylation of flavonols and flavones occurs, unless there are two or more hydroxyl groups on the B-ring. Exposure of hydroxyl groups can occur when P450 enzymes demethylate the methyl group in the 4 position.[276] Conjugation of the polar hydroxyl groups with glucuronic acid, sulphuric acid, glycine,[272] or possibly glutathione[277] are Phase II reactions. The arising water soluble conjugates can be excreted into the urine. Moreover, their molecular weight increases, which promotes secretion into the bile. Finally, O-methylation plays an important role in the inactivation of B-ring catechol moiety, the two *ortho*-hydroxyl groups in some flavonoids (i.e. quercetin, catechin).[278] Enzymes of the Phase II reactions are transferases. Glucuronidation is catalyzed by glucuronyltransferases, yielding O- and N-glucuronides. The coenzyme involved is the "active glucuronate", the uridine diphosphate derivative of glucuronic acid (UDP-GlcUA). Similarly, sulfate esters are synthesized with the help of the "active sulfate", phosphoadenosinephosphosulfate (PAPS), catalyzed by phenolsulfotransferase (PST). The relative importance of the 3 types of conjugation (methylation, sulfation, and glucuronidation) appears to vary according to the nature of the substrate and the dose ingested. Sulfation is generally a higher-affinity, lower-capacity pathway than is glucuronidation, so that when the ingested dose increases, a shift from sulfation toward glucuronidation occurs.[279] The balance between sulfation and glucuronidation of polyphenols also seems to be affected by species, sex, and food deprivation.[280] Conjugates are eliminated either from the liver with bile— the gallbladder squirts the bile into duodenum, or renally, i.e., via the urine. One of the factors determining whether a compound/ conjugate will undergo billiary excretion is minimum molecular weight. The molecular weight threshold, depending on particular species, in humans was reported to be around 500–600 Da.[281]

3.1.6 Plasma transport

Polyphenol metabolites are not free in the blood. In vitro incubation of quercetin in normal human plasma showed that quercetin is extensively bound to plasma proteins (99% for concentrations up to 15 µmol/L), whereas binding to VLDL is not significant (<0.5%).[282] Metabolites of quercetin are also extensively bound to plasma proteins in the plasma of rats fed a quercetin-enriched diet.[283] Albumin is the primary protein responsible for the binding. The affinity of polyphenols for albumin varies according to their chemical structure. Kaempferol and isorhamnetin, which differ from quercetin in the nature of the B-ring substitution, have an affinity for human serum albumin that is similar to that of quercetin.[284] In contrast, substitution of 3-OH markedly weakens binding to albumin, as shown for rutin and isoquercitrin, the 3-O-glycosides of quercetin.[285] The effect of sulfation and glucuronidation is unknown, but it probably depends highly on the position of substitution. Hydroxycinnamic acids, especially ferulic and coumaric acids, have a low affinity for bovine serum albumin but may have a different affinity for albumin of human origin.[286] The partitioning of polyphenols and their metabolites between aqueous and lipid phases is largely in favor of the aqueous phase because of their hydrophilicity and binding to albumin. However, in some lipophilic membrane models, some polyphenols penetrate the membrane to various extents.[287,288,289,290,291] Quercetin showed the deepest interaction, probably because of its ability to assume a planar conformation.[292] At physiologic pH, most polyphenols interact with the polar head groups of phospholipids at the membrane surface via the formation of hydrogen bonds that involve the hydroxyl groups of the polyphenols.[293]A high number of hydroxyl groups on the polyphenol structure and an increase in pH that leads to deprotonation of the hydroxyl groups would thus enhance interactions between the polyphenols and the membrane surface. This adsorption of polyphenols probably limits the access of aqueous oxidants to the membrane surface and their initial attack on that surface. LDL is made up of lipophilic structures that, once oxidized, participate in the etiology of atherosclerosis. Many studies have shown that various polyphenols have the ability to protect LDL from oxidation. However, a very small proportion of plasma polyphenols are in fact associated with the LDL fraction after consumption of nutritional doses of these compounds.[294,295] They are associated with lipoproteins only by ionic interactions with charged residues on the surface of the particles. The low integration of polyphenols into LDL has been confirmed by in vitro incubation experiments.[296,297] Protection probably occurs at the interface between lipophilic and hydrophilic phases. However, a recent study in which [^{3}H]genistein was incubated in

human plasma showed that genistein and its lipophilic derivatives were incorporated into HDL and, to a lesser extent, into LDL.[298]

3.1.7 Tissue uptake

When single doses of radiolabeled polyphenols (quercetin, epigallocatechin gallate, quercetin 4'-glucoside, resveratrol) are given to rats or mice killed 1–6 h later, radioactivity is mainly recovered in blood and in tissues of the digestive system, such as the stomach, intestine, and liver.[299,300,301,302] However, polyphenols have been detected by HPLC analysis in a wide range of tissues in mice and rats, including brain,[303,304] endothelial cells,[305] heart, kidney, spleen, pancreas, prostate, uterus, ovary, mammary gland, testes, bladder, bone, and skin.[300,306,307,308] The concentrations obtained in these tissues ranged from 30 to 3000 ng aglycone equivalents/g tissue depending on the dose administered and the tissue considered. The time of tissue sampling may be of great importance because we have no idea of the kinetics of penetration and elimination of polyphenols in the tissues. A few studies seem to indicate that some cells may readily incorporate polyphenols by specific mechanisms. The endothelium is likely to be one of the primary sites of flavonoid action. One research showed that a rapid, energy-dependent transport system is active in aortic endothelial cells for the uptake of morin.[309] This system may also transport other hydroxylated phenolic compounds. The nature of the tissular metabolites may be different from that of blood metabolites because of the specific uptake or elimination of some of the tissular metabolites or because of intracellular metabolism. An experiment showed that the uptake of flavanone glucuronides by rat and mouse brain endothelial cultured cells is much lower than that of their corresponding aglycones.[310] Only 2 studies reported data on polyphenol concentrations in human tissues. The first study measured phytoestrogens in human prostate tissue. Surprisingly, the study showed significantly lower prostatic concentrations of genistein in men with benign prostatic hyperplasia than in those with a normal prostate, whereas plasma genistein concentrations were higher in men with benign prostatic hyperplasia.[311] In addition, concentrations of enterodiol and enterolactone were higher in prostatic tissue than in plasma, whereas the opposite was true for daidzein, genistein, and equol. In the other study, equol concentrations in women who ingested isoflavones were found to be higher in breast tissue than in serum, whereas genistein and daidzein were more concentrated in serum than in breast tissue.

3.1.8 Elimination

Water and water-soluble substances like salts, metabolic wastes, and foreign substances that have to be excreted from the body are released into the urine in the kidneys. Metabolites of polyphenols may follow 2 pathways of excretion, via the biliary or the urinary route. Large, extensively conjugated metabolites are more likely to be eliminated in the bile, whereas small conjugates such as monosulfates are preferentially excreted in urine. In laboratory animals, the relative magnitude of urinary and biliary excretion varies from one polyphenol to another.[312] Several papers report elimination of flavonoid metabolites by the urinary pathway.[313,314,315,316,317] Polyphenols in plasma are in the conjugated form; reactions leading to conjugate formation facilitate the excretion of polyphenols and decrease their potential toxicity.[261] Biliary excretion of polyphenols in humans may differ greatly from that in rats because of the existence of the gall bladder in humans; however, this has never been examined. Intestinal bacteria possess β-glucuronidases that are able to release free aglycones from conjugated metabolites secreted in bile. Aglycones can be reabsorbed, which results in enterohepatic cycling.[308] After consumption of flavonoids only a very small fraction of the dose is typically recovered in urine as forms containing the intact flavonoid ring, and the total amount of metabolites excreted in urine is roughly correlated with maximum plasma concentrations[232]. Indirect evidence of elimination by bile in humans, along with animal models, supports the theory that elimination in bile is quantitatively the most important route of elimination for some or most flavonoids.[318]

3.2 Effects on health

3.2.1 Antioxidant effect

Degenerative diseases, such as cancers, cardiovascular diseases, osteoporosis and cancer, are associated with aging. Oxidative damage of DNA, proteins, and lipids accumulates with age and contributes to degeneration of somatic cells and to pathogenesis of several diseases. Antioxidants present in food can limit this damage by acting directly on reactive oxygen species or by stimulating endogenous defence systems. The phenolic groups in polyphenols can accept an electron to form relatively stable phenoxyl radicals, thereby disrupting chain oxidation reactions in cellular components.[319] The antioxidant potency of polyphenols has

been evaluated in vitro by measuring their ability to trap free radicals and reduce other chemicals. Their potency is compared to that of a reference substance, usually Trolox (that is a water-soluble derivative of vitamin E), gallic acid, or catechin. Polyphenols with catechol groups (aromatic rings with two hydroxyl groups in the *ortho* position) have greater antioxidant potency than those with simple phenol groups (aromatic rings with a single hydroxyl group). However, the value of such measurements is limited for a number of reasons:

- quite different results are observed when the antioxidant properties of different polyphenols are compared using different assays;[320]
- polyphenols are extensively metabolized in the body, and the majority of catechol nuclei are methylated, dehydroxylated, or conjugated by O-glucuronidation and formation of sulphate esters.[243,232,321]
- Antioxidant properties of polyphenols largely depend on their chemical and physico-chemical environment, which varies according to tissues and physiological conditions and that cannot be reproduced in vitro antioxidant assays;
- The impact of polyphenols on antioxidant protection of tissues depends by their bioavailability, which differs considerably from one polyphenol to the other.[232,243]

Health benefits of polyphenols cannot be merely reduced to their antioxidant potency as measured in vitro. However, this does not exclude that the redox properties of polyphenols might be key factors that may trigger various cell responses at the origin of their biological effects.[322]

When ingested, polyphenol-rich foods and beverages increase the antioxidative capacity of plasma. Increased antioxidative capacity is systematically observed over the hours following the intake of polyphenol-rich beverages, such as tea,[323,324,325,326] wine,[326,327,328,329,330,331] and beer,[332] as well as fruit and vegetables rich in polyphenols, such as strawberries and spinach[327]. If it is well established that polyphenol ingestion results in an increase of the plasma antioxidant capacity, there is still some uncertainties about its efficiency to enhance the protection of cellular components, such as lipids or DNA, against oxidative stress in humans. In some studies, polyphenol intake reduced the plasma concentration of lipid oxidation products. The effects of polyphenols on the stability of deoxyribonucleic acids (DNA) have also been examined. In vitro, polyphenols can have either harmful or protective

effects. In the presence of transition metals, such as Cu(II) and Fe(III), phenolic compounds induce the breakage of DNA.[333],[334] Such effects are caused by a reduction of these transition metals that, once reduced, catalyse the formation of hydroxyl radical (OH◦) (Fenton reaction). These reactions have also been noted in cultured cells.[335] Such breakage of DNA has been considered both beneficial (cytotoxic and apoptotic effects on tumor cells) and toxic (mutagenic effects on normal cells). Conversely, polyphenols may also protect DNA against degradation induced by cytotoxic agents. In vitro, they can inhibit the formation of adducts between activated polycyclic hydrocarbons and DNA.[336]

3.2.2 Cardiovascular disease

There is evidence from epidemiological studies that consumption of dietary flavonoids such as quercetin, kaempferol, myricetin, apigenin, and luteolin found in tea, apples, onions and red wine (usually as the glycoside derivatives) may help to protect against cardiovascular disease(CVD). The influence of flavonoid consumption on cardiovascular risk factors including flow-mediated dilatation (FMD; a measure of endothelial function), blood pressure (BP), and total serum cholesterol and lipoproteins has been investigated in a considerable number of dietary studies.[337]

A number of animal studies have demonstrated that the consumption of polyphenols limits the development of atheromatous lesions. These effects are associated with reduced low density lipoprotein (LDL) uptake by macrophages, lower oxidation of isolated LDL (TBARS method), and decreased susceptibility of LDL to aggregation.[338,339,340,341]

Polyphenols can inhibit oxidation of LDL in vitro; this type of oxidation is considered to be a key mechanism in atherosclerosis. These antioxidant effects result in the decreased oxidation of LDL lipids and of α-tocopherol.[342] However, evidence in humans is contradictory. Certain studies have shown that the consumption of beverages and foods rich in polyphenols (red wine, cocoa, tea, or pomegranate juice) resulted in reduced susceptibility of LDL to oxidation induced ex vivo by Cu(II).[329,343,344,345,346,347] The lower levels of oxidation products of phosphatidylcholine (the main lipid found in LDL) observed after consumption of green tea catechins in man suggest that polyphenols effectively protect LDL against oxidation.[348] However, several other studies have shown no effects of polyphenols consumption on the ex vivo oxidation of LDL.[331,349,350,351,352,353,354] In the plasma, polyphenols are largely conjugated with glucuronide and sulfate groups.[232] They are, therefore, polar and, most probably, largely

eliminated during the isolation of LDL preceding the ex-vivo oxidation test. This could explain the lack of protection observed in these last studies. This does not exclude the association of some polyphenol aglycones or of some esters with fatty acids to LDL, as has been suggested in an experiment where [^{3}H]-genistein was added to human plasma.[355]

Polyphenols may exert antithrombotic effects. They inhibit platelet aggregation in vitro.[356,357] They were also shown to inhibit platelet aggregation in several animal models: the consumption of red wine (rich in polyphenols), rather than white wine or alcohol, in the rat prevented the platelet rebound effect (measured by ex-vivo thrombin-induced platelet aggregation), otherwise observed in the hours following alcohol withdrawal.[358]

Polyphenols can improve endothelial dysfunction, an early event in atherogenesis. Endothelial dysfunction is associated with different risk factors for atherosclerosis before the plaque is formed; its use as a prognostic tool for coronary heart disease has been proposed.[359,360] The endothelial-dependent vasorelaxing activity of isolated polyphenols, such as wine anthocyanins,[361] soy isoflavones,[362] resveratrol, quercetin,[363] and cocoa proanthocyanidins,[364] has been observed on isolated rat or rabbit aorta and in female macaques. These effects could be mediated by the protection of the vasorelaxant factor nitric oxide against oxidation. In human subjects, endothelial dysfunction can be assessed by measuring the brachial artery flow-mediated dilation. The consumption of black tea (450 mL) increased artery dilation 2 h after intake by coronary patients.[365]

3.2.3 Cancer

Anticarcinogenic effects of polyphenols are well documented in animals. Polyphenols, when given to rats or mice before and/or after the administration of a carcinogenic agent or the implantation of a human cancer cell line, are most often protective and induce a reduction of the number of tumors or of their growth.[366] These effects have been observed at various sites, including mouth, stomach, duodenum, colon, liver, lung, mammary, or skin. Many polyphenols, such as quercetin, catechins, isoflavones, lignans, flavanones, ellagic acid, red wine polyphenols, resveratrol, or curcumin, showed protective effects.

Different mechanisms have been suggested to explain their anticarcinogenic effects.[367] First, polyphenols may act as blocking agents at the initiation stage. They influence the metabolism of procarcinogens by modulating the expression of cytochrome P450 enzymes involved in

their activation to carcinogens. They may also facilitate the excretion by increasing the expression of phase II conjugating enzymes.[368] This induction of phase II enzymes may have its origin in the toxicity of polyphenols. Polyphenols can form potentially toxic quinones in the body that are, themselves, substrates of these enzymes.[369] The intake of polyphenols could then activate these enzymes for their own detoxication and, thus, induce a general boosting of our defenses against toxic xenobiotics.[370] Polyphenols may also limit the formation of initiated cells by stimulating DNA repair.[371,372] Secondly, polyphenols can act as suppressing agents, and inhibit the formation and growth of tumors from initiated cells; they inhibit cell proliferation in vitro.[373,374] It was also shown that some polyphenols can affect growth-related signal transduction pathways through inhibition of protein kinase C and AP-1-dependent transcriptional activity.[375,376] They inhibit oncogene expression[377] and the activity of ornithine decarboxylase, a key enzyme in the synthesis of polyamines associated to cell proliferation.[378,379] They may also inhibit cell proliferation through their effect on the metabolism of arachidonic acid. Phenolic phytoestrogens could influence the growth of hormone responsive tumors through their estrogenic properties or their capacity to affect the response to endogenous estrogens.[380,381] This may explain the protective effects of isoflavones against mammary and prostate cancers observed in different animal models.[382]

Polyphenols can induce apoptosis of tumor cells and, therefore, reduce the growth of tumors. Evidence has been given both in vitro[374] and in vivo.[383,384] However, the significance of this mechanism in cancer prevention is not clear, as polyphenols may also have opposite effects. They were shown to inhibit apoptosis of some non-tumorigenic cells when induced by hydrogen peroxide.[385,386] Lastly, polyphenols, such as those of green tea, can also inhibit angiogenesis and, therefore, limit the growth of the tumors,[387,388] or prevent tumor invasion through inhibition of the matrix metalloproteinases.[389,390]

The anticarcinogenic properties of polyphenols could, thus, be explained by many different mechanisms. To explain their protective effect by their antioxidant properties and inhibition of DNA oxidative damage is certainly an oversimplification. However, various antioxidants, including polyphenols, inhibit NF-kB activation, probably through triggering a redox-sensitive signal in the cells.[391,392] The inhibition of such transcription factors by polyphenols may play an essential role in the prevention of cancers.[393,394]

The question of doses is essential, as opposite effects have been observed at different exposure levels. Caffeic acid induces hyperplasia and tumors in the forestomach and kidney when administered in the diet of rats or mice at a dose of 0.5–2% of the diet, whereas it shows

anticarcinogenic effects at doses of 0.05– 0.15 %.[395] On the contrary, genistein at high doses (50–100 μM) inhibits the growth of human breast cancer cells in vitro, whereas it induces proliferation at lower doses (0.01–10μM), effects that were explained by their estrogenic properties at low doses and cytotoxicity at higher doses.[396] A similar influence of the dose of genistein was observed on the expression of prostate-specific antigen by prostate cancer cells.[397]

The final evidence on the prevention of cancers by polyphenols will come from clinical and epidemiological studies. Tumor biomarkers are useful tools for prognosis, for the monitoring of therapy in various cancers, and for the evaluation of the influence of diet on the disease.[398] Some polyphenols have been shown to reduce the levels of tumor biomarkers in different cancer cell lines. Genistein decreased the expression of protein-specific antigen (PSA) in prostate cancer cells[397] and EGCG, epicatechin gallate, or genistein significantly reduced in a human lung cancer cell line, the levels of heterogeneous nuclear ribonucleoprotein B1, a new biomarker for early clinical diagnosis of lung cancer.[399]

Polyphenol supplements might be useful as adjuvants in chemotherapy or radiotherapy treatments. Some polyphenols were shown to reinforce the antiproliferative activities of anticancer drugs. EGCG showed synergistic effects with sulindac or tamoxifen on apoptosis of the lung cancer cell line PC-9,[400] and quercetin potentiated the growth inhibition of ovarian cancer cells and leukemia cells by cisplatin.[401,402] However, such adjuvant effects vary widely between polyphenols. Galangin, when tested on leukemia cells, showed opposite effects to quercetin and inhibited the anti-apoptotic effects of cisplatin.[401] Tangeretin, a citrus polymethoxylated flavonoid, when added to the diet of nude mice, inhibited the cytotoxic effects of tamoxifen on MCF-7 breast cancer cell inoculated subcutaneously.[403]

The associations between the consumption of coffee, tea, and wine and the risk of cancer have been studied in different epidemiological studies. The consumption of coffee has been associated to a reduced risk of colorectal cancer but not with cancers at other sites.[404] Experimental evidence on tea strongly suggests a protective role of tea consumption against cancers, but the epidemiological evidence is inconclusive.

A prospective study has suggested that wine polyphenols may protect against the deleterious effects of alcohol on cancers of the upper digestive tract. The consumption of alcoholic beverages, such as beer or spirits, increased the risk of upper digestive tract cancer in a Danish cohort, whereas a moderate consumption of wine did not increase this risk.[405] Another

study on the same cohort also suggested the protective effects of wine consumption against lung cancer.[406]

The consumption of dietary sources of phytoestrogens has been repeatedly associated to a lower cancer risk. Epidemiological studies have suggested a protective role of the consumption of soy products, rich in isoflavones, against various cancers and, more particularly, hormone-related cancers.[407] The consumption of whole-grain cereals, a major source of lignans, has also been associated to a reduced risk of various cancers.[408]

3.2.4 Neurodegenerative disease

Neurodegenerative diseases represent an increasing burden to our aging societies. About 15% of the population over 65 are afflicted by Alzheimer's disease and 1% by Parkinson's disease, not including other type of dementia resulting from ischemic injury.[409] Such diseases are dependent of oxidative stress, which particularly affects brain tissues,[410] and antioxidants may, therefore, contribute to their prevention.[409] Feeding aging rats a diet supplemented with aqueous extracts of spinach, strawberry, or blueberry rich in polyphenols improved their cognitive functions and neuronal signal transduction.[411,412] Blueberries rich in anthocyanins were particularly effective.

Intravenous injection of epicatechin or catechin to mice improved the memory impairment induced by cerebral ischemia.[413] Polyphenols also protect experimental animals against some neurotoxic drugs whose toxicity is linked to a stimulation of oxidative stress. Dietary supplementation with grape polyphenols reduced the neurodegenerative changes induced by chronic ethanol consumption, and improved the synaptic function measured on isolated synaptosomes.[414] The oral administration of EGCG restored the dopaminergic neurotransmission in rats injected with N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a drug used to reproduce a parkinsonian syndrome, and prevented the increase of SOD and catalase induced by this drug.[415] The chronic consumption of ferulic acid with the drinking water protected mice from the deleterious effects of an intracerebral injection of β-amyloid peptide, a component of senile plaques postulated to be involved in the pathogenesis of Alzheimer's disease.[416] It prevented the drop of learning and memory performance and the increase in the level of inflammation markers in the brain induced by the β-amyloid peptide, possibly via a transitory activation of hippocampal astrocytes. Similar protective effects were observed with curcumin in an Alzheimer transgenic mouse model.[417]

At low doses (0.1–10μM), epigallocatechin gallate protects neuronal cells against oxidative damage and improves cell survival, whereas at higher doses (50 μM), it appears pro-oxidant and toxic.[418] Therefore, low polyphenol concentrations would be more effective to prevent neurodegenerative diseases. Little is known about the polyphenol concentrations in the brain. The concentrations reached in the brain after feeding rats with genistein are much lower (0.04 nmol/g tissue) than those reached in the plasma (2 μmol/L) and other tissues.[419] The poor permeability of the blood-brain barrier to polyphenols was confirmed in other studies with naringin or quercetin.[420,421] The glucuronide conjugate of epicatechin was unable to protect cortical neurons against oxidative stress induced by H_2O_2.[422] However, the low amounts of polyphenols present in the brain may be only aglycone, as has been shown for genistein, and due to the poor permeability of the blood-brain barrier, to anionic conjugates.[419,423]

3.2.5 Diabetes

Many plants have been traditionally used in the treatment of diabetes. Polyphenols contained in these plants may explain some of their therapeutic activity.[424,425] The acute or chronic administration of polyphenols to experimental animals influences glycemia. Caffeic acid and isoferulic acid, when administered intravenously to rats, reduce the fasting glycemia and attenuate the increase of plasma glucose in an intravenous glucose tolerance test.[426,427,428]

More interestingly, some hypoglycemic effects were also observed with polyphenols administered orally, shortly before consumption of the glucose source. A diacylated anthocyanin reduced the peak of glycemia induced by maltose consumption in normal rats.[429] An ill-defined leucodelphinidin (probably a mixture of prodelphinidins) reduced fasting glycemia in rats and lowered the plasma glucose peak in a glucose tolerance test.[430] Similar effects were observed with 4-hydroxybenzoic acid.[431] Catechin improved the tolerance to glucose induced by starch or sucrose ingestion in rats.[432] A fermented tea extract showed hypoglycemic effects in mice.[433]

Polyphenols may affect glycemia through different mechanisms, including the inhibition of glucose absorption in the gut or of its uptake by peripheral tissues. Polyphenols could not only inhibit the glucose absorption in the small intestine, but they could also limit their reabsorption in the kidney, as has been shown for phlorizin.[434] Polyphenols may exert different actions on peripheral tissues that would diminish glycemia. They include the

inhibition of gluconeogenesis,[427,435,436] adrenergic stimulation of glucose uptake,[437] or the stimulation of insulin release by pancreatic β-cells.[438]

Polyphenols could, thus, limit the risk of diabetic complications, as advanced glycation end (AGE) products are known to generate oxidative stress. This effect on AGE products could explain the reduction of renal damage by curcumin observed in streptozotocin-treated rats.[439]

3.2.6 Osteoporosis

Osteoporosis is a degenerative bone disease, characterized by low bone mass and structural deterioration of bone tissue, leading to an increased risk of fracture. Bone is a dynamic organ that is constantly renewed through a process of remodelling and modelling involving bone resorption by osteoclasts and bone formation by osteoblasts.[440]

Estrogen deficiency in postmenopausal women is an important cause of osteoporosis, and hormone replacement therapy is often recommended to prevent bone loss. Isoflavones with weak estrogen-like activity have attracted much attention as a possible alternative treatment to prevent osteoporosis.[441] Their osteoprotective effects have been evaluated in mice or rats in which an estrogen deficiency has been induced by ovariectomy. The supplementation of the diet with genistein, daidzein, or their glycosides during several weeks prevents the loss of bone mineral density and trabecular volume caused by the ovariectomy.[442,443,444,445] These effects were observed at daily doses of 10–50 mg/kg body weight. The highest doses also induced uterine hypertrophy, but the lowest protective doses did not affect the uterine weight.[443,445] Feeding soy proteins with either normal or reduced isoflavone content to ovariectomized rats also suggested that the osteoprotective effects of soy proteins were due to their isoflavones.[446] The restoration of the bone mineral density was not observed when the isoflavones were administered 80 d after the ovariectomy of the rats, suggesting that they may prevent bone loss, but not reverse an established osteopenia.[447] In vitro, daidzein was shown to inhibit the differentiation of osteoclasts developping on dentine slices and to diminish the dentine resorption.[448] On the other hand, the daily subcutanous injection of genistein to ovariectomized rats increased the number of osteoblasts associated to bone formation, but had no effects on bone resorption.[449] Structurally related isoflavones may protect bones through different mechanisms of action,[444] and with different magnitude.[445]

The consumption of soybeans, a major source of calcium in Japan, was associated to a higher bone mineral density in Japanese women.[450] A soy-rich diet followed by postmenopausal women stimulated their bone osteoblastic activity.[451,452] Such effects of soy food consumption could also be explained by the intake of isoflavones, as soy foods are their main dietary sources.

Much less is known on the possible impact of other polyphenols on osteoporosis. Rutin, a glycoside of quercetin, added to the diet of ovariectomized rats restaured the loss of bone mineral density induced by the ovariectomy and was even more efficient than isoflavones.[453,454] It also reduced the urinary excretion of deoxypyridinoline, a marker of bone resorption, and increased osteocalcinemy, a marker of osteoblastic activity. Such an effect of rutin likely explains the inhibition of bone resorption observed in rats fed a diet rich in onions (the main source of quercetin in Western diets), whereas several other vegetables were without effects.[455]

The consumption of tea has been associated with a higher bone mineral density in a cohort of English older women.[456] Catechins abundant in tea could possibly counteract the effects of tea caffeine, knownfor its adverse effects on bone metabolism. No effect of the consumption of coffee was observed in the same study. A rat experiment also showed no effect of coffee on bone metabolism. Feeding rats during 140 d with a diet supplemented with instant coffee containing both chlorogenic acid and caffeine had no influence on bone histomorphometry, deoxypyridinoline urinary excretion, and osteocalcinemy.[457]

3.2.7 Antiviral effects

Flavonoids shown antiviral activity, some of the virus reported to be affected by flavonoids are herpes simplex virus, respiratory syncytial virus, parainfluenza virus and adenovirus. For example, quercetin was reported to exhibit both antiinfective and antireplicative abilities. Some flavonoids work on the intracellular replication of viruses, whereas others inhibit the infectious properties of the viruses. Morever, there is some evidence that flavonoids in their glycone form seem to be more inhibitory on rotavirus infectivity than are flavonoids in their aglycone form.[458]

3.3 Pro-oxidant effects of high-dose

Commonly used dietary compounds can exert deleterious effects at pharmacological concentrations.[459] Dietary polyphenols are purportedly potent antioxidants as demonstrated, in large part, through in vitro studies. Demonstration in vivo has been problematic although an increasing body of evidence suggests detrimental pro-oxidant effects. Under certain conditions (high concentrations of phenolic antioxidants, high pH, presence of iron) phenolic antioxidants can initiate an auto-oxidation process and behave like pro-oxidants.[460] Hot beverages contain high H_2O_2 concentrations because polyphenols oxidize rapidly at high temperatures. Probably the most compelling supportive evidence involves studies of EGCG commonly found in tea. H_2O_2 has been measured up to 10 μmol/L in saliva of those chewing green tea leaves.[461] EGCG undergoes, in vitro, oxidative polymerization and generation of toxic H_2O_2 at concentrations up to 334 μmol/L when adding 1 mmol/L EGCG to culture medium.[460,462,463] In hepatocytes given high-dose EGCG (200 μmol/L), cell viability was significantly reduced and associated with increased ROS production and antioxidant, viz, glutathione, depletion.[464] In human lung and esophageal cancer cells, EGCG (20 μmol/L for 24 hours), presumably via ROS production, interrupted phosphorylation of epidermal growth factor receptor and the second messenger signaling cascade.

Interruption of cell signaling cascades can be detrimental to cells and contribute to chronic disease. Epidemiological data support an inverse relationship between increased isoflavone consumption and a decreased risk of breast cancer. However, data also suggest that dietary isoflavones can promote breast cancer.[465] For example, resveratrol, a polyphenol stilbene in red wine and available as a dietary supplement, can bind the estrogen receptor and foster breast cancer cell growth at concentrations of 0.1–1000 μmol/L.[466] However, this effect is biphasic depending on the estrogen concentrations. Resveratrol is protective in microenvironments with high hormone (estrogens and androgens) concentrations, but stimulatory in low hormone environments. The isoflavone genistein also inhibits the growth of most cancer cells but in estrogen-responsive (ER+) cells the response is biphasic where genistein inhibits human breast cancer cell growth at higher concentrations yet stimulates proliferation of ER+ breast cancer cells at lower concentrations consistent with physiologically relevant levels in humans.[467,468]

Phenolic acids comprise ~30% of dietary polyphenols. High-dose phenolic acids promote carcinogenicity in some studies through H_2O_2-induced DNA damage and resultant genotoxicity.[469] Moreover, studies show administration of high doses (up to 50 μmol/L) of

purified flavonoids for six hours could cause chromosome translocation and clastogenicity in human cell line studies.[470,471]

In many studies, the effect of polyphenol dose leading to toxicity becomes apparent. Ferulic acid, a phenolic acid, is an antioxidant which neutralizes free radicals (superoxide, nitric oxide and hydroxyl radical) and is found in the leaves and seeds of many plants and cereals such as brown rice, whole wheat and oats, coffee, apple, artichoke, peanut, orange and pineapple.[472] Research results have indicated that ferulic acid when fed to rats at 500 mg/kg diet is carcinogenic to liver but anticarcinogenic to tongue, skin and colon.[473] Dietary caffeic acid, a precursor of ferulic acid, has been shown to be carcinogenic at 2% (20 g/kg diet), a tumor promoter at 0.5%–1% (5–10 g/kg diet), and anticarcinogenic at 0.05%–0.5% (0.5–5 g/kg diet).[473,474,475]

CHAPTER 4

BIOCATALYSIS AND LACCASE

4.1 Biocatalysis

Biocatalysis may be broadly defined as the use of enzymes or whole cells as biocatalysts for industrial synthetic chemistry. Similar to other catalysts, biocatalysts increase the speed in which a reaction takes place but do not affect the thermodynamics of the reaction. However, they offer some unique characteristics over conventional catalysts. The most important advantage of a biocatalyst is its high selectivity. This selectivity is often chiral (i.e., stereo-selectivity), positional (i.e., regio-selectivity), and functional group specific (i.e., chemo-selectivity). Such high selectivity is very desirable in chemical synthesis as it may offer several benefits such as reduced or no use of protecting groups, minimized side reactions, easier separation, and fewer environmental problems. Other advantages, like high catalytic efficiency and mild operational conditions, are also very attractive in commercial applications. Among the disadvantages: many enzymes can accept non-natural substrates and convert them into desired products, the solvent usually used are water and they have limiting operating region as enzymes typically denatured at high temperature and pH.[476]

4.1.1 Biocatalyst immobilization

Immobilization is the process of adhering biocatalysts to a solid support. The solid support can be an organic or inorganic material, such as derivatized cellulose or glass, ceramics, metallic oxides, and a membrane. Immobilized biocatalysts offer several potential advantages over soluble biocatalysts, such as easier separation of the biocatalysts from the products, higher stability of the biocatalyst, and more flexible reactor configurations. In addition, there is no need for continuous replacement of the biocatalysts. As a result, immobilized biocatalysts are now employed in many biocatalytic processes. More than one hundred techniques for immobilizing enzymes have been developed, based on the interactive force linking an enzyme to its solid support, enzyme immobilization can be performed physically

(via adsorption and entrapment) or chemically (via cross-linking and carrier binding). (**Figure 19.**)

Enzyme adsorption involves the adhesion of an enzyme to the active surface of an adsorbent, such as carbon, ionic-exchange resins, celluloses, and clays. While the immobilization reaction is simple and unlikely to affect enzyme activity, enzyme leakage is a common occurrence due to weak bonding. Immobilization yield can also be quite low since the enzyme is fully exposed and can be sensitive to the reaction environment. Enzyme entrapment is an alternative physical immobilization method typically achieved through gel entrapment or microencapsulation of the biocatalyst. Entrapped enzymes offer physical protection against the reaction environment, but still suffer from occasional enzyme leakage. Specifically, gel entrapment is achieved by the formation of a polymer network in the presence of an enzyme solution. Polymeric materials, such as polyacrylamide, gelatin, and alginate are commonly employed but can potentially affect the physical, chemical, and kinetic properties of the immobilized biocatalyst. Hence, the entrapment matrix should be carefully selected to optimize the immobilization and reaction efficiency while minimizing negative effects on the enzyme. Yet another method involves confining an enzyme within a semipermeable microcapsule. Liquid microencapsulation entails an organic polymer solution mixing with an aqueous enzyme solution in the presence of a surfactant. A polymer membrane is formed at the liquid–liquid interface and the aqueous phase containing the enzyme is entrapped within the membrane. Accordingly, solid microencapsulation involves the entrapment of an enzyme within a solid membrane, hollow fiber, or nanostructure such as silica sol–gel.

Chemical immobilization occurs through the formation of covalent bonds between certain enzyme amino acid residues and the matrix support. While covalent bond formation can prevent enzyme leakage from a biocatalystmatrix, enzyme activity can be affected as the immobilization reaction sometimes results in significant conformational changes in the enzyme structure. A common method for enzyme immobilization involves chemical attachment of enzyme molecules to a water-insoluble support. Carriers can be made from inorganic materials (e.i., silica gel and alumina), natural organic materials (e.i., proteins (albumin and collagen) and carbohydrates (alginate, chitosan, and cellulose), and synthetic organic materials (e.i., polystyrene, polyacrylamide, and polypropylene). Smart polymers (e.i., poly-N-isopropylacrylamide) are novel carriers of particular importance because a dramatic conformational change can be induced by a minor perturbation in environmental conditions, such as pH, temperature, or ionic strength.[477] The carrier properties, primarily

determined by the characteristics of the functional groups, can affect the efficiency of immobilization, the yield of the biocatalytic reaction, and the stability of the biocatalyst. Other properties, such as size, permeability, surface area, mechanical stability, affinity to proteins, toxicity, and biocompatibility, are also important.

With the presence of functional groups on both enzyme and carrier, chemical bonds between enzyme molecules (carrier-free cross-linking methods) or between enzyme and carrier molecules (carrier cross-linking methods) can be generated. Among various multifunctional cross-linking agents, glutaraldehyde is the most commonly used due to its low cost, high efficiency, and superior stability. The carbonyl group of glutaraldehyde is the functional group involved in the immobilization reaction, which occurs rapidly even at room temperature. Because the linkages are irreversible and relatively strong, the cross-linking system is rather stable against environmental challenges. However, the activity of a chemically immobilized enzyme is generally lower due to the occurrence of side reactions during cross-linking and/or by diffusion limitation within the immobilized system. In addition, the carrier must be selected carefully as it can drastically affect the kinetic behavior of the immobilized system. Immobilization efficiency can be affected by several factors, including the nature and concentration of the enzyme, pH, ionic strength, temperature, and the cross-linking agent utilized. For carrier-free cross-linking methods, the immobilized system can be established by direct cross-linking of different enzyme forms. Soluble enzymes are used to form cross-linked enzymes (CLEs), which can suffer from dramatic loss of enzyme activity and poor mechanical stability. Crystalline enzymes are used to form cross-linked crystalline enzymes (CLCEs), which are capable of preserving a higher level of enzyme activity through increased stability against environmental challenges. Insoluble enzyme aggregates, prepared by mixing suitable aggregating agents with enzyme solutions, have also been explored for cross-linking to generate cross-linked enzyme aggregates (CLEAs), which exhibit similar activity and stability to CLECs. Finally, spray-dried enzymes can be cross-linked to form cross-linked spray-dried enzymes (CLSDEs). However, this method is less common since the enzyme is liable to significant deactivation during the spray-drying process.[478]

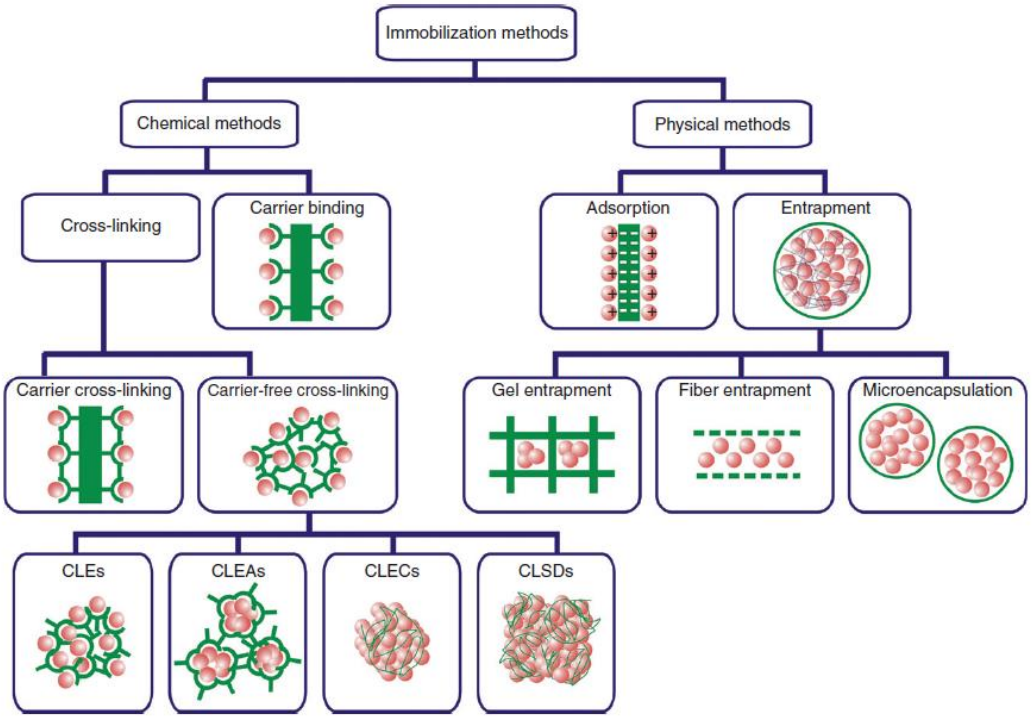


Figure 19. Methods used for enzyme immobilization.[479]

Immobilization may have a profound impact on essential properties of the enzyme like activity, stability, substrate specificity, and selectivity.

Immobilization may produce structural changes in the enzyme molecule affecting its activity. This usually reduces it, but the opposite may occur as is the case of lipases when immobilized onto hydrophobic matrices where hyperactivation is observed as the consequence of a configuration more prone to catalysis; also, a favorable microenvironment may keep deleterious substances apart from the active site so enhancing activity and immobilization may prevent enzyme aggregation and autolysis. Enzyme activity losses and incomplete protein capture during immobilization should also be considered and may vary considerably from one procedure to another.

Stability (preservation of enzyme activity through time) may be considerably affected by immobilization. Even though far from a general rule, immobilization usually improves enzymestability bymaking the enzymemoleculemore robust and sometimes protected from an aggressive environment. Again, this effect may vary considerably according to the immobilization method used.

Enzymes are increasingly being used as catalysts for organic synthesis where substrate specificity and reaction selectivity are major issues. Enzymes are highly chemo-, regio-, and enantioselective, being attractive catalysts for the production of pharmaceuticals and fine chemicals. Chiral drugs are produced as racemic mixtures when chemically synthesized, being this a problem in terms of process efficiency and safety because only one of the isomers (eutomer) is effective whereas the other (distomer) is inert or, even worse, harmful.[480]

4.1.2 Biocatalysis based on enzyme classes

According to the International Union of Biochemistry and Molecular Biology (IUBMB) nomenclature system, all enzymes are classified into six classes on the basis of the general type of reactions that they catalyse (**Table 2.**). Within each class are subclasses and the enzymes themselves. The result is an ordered system of enzymes and the reaction(s) that each catalyzes. It is important to note that, in biological processes, every class of enzyme is utilized in the cell to a large extent. However, this is not the same in industrial processes, where certain classes of enzymes are used more often than others. Most of the enzymes that have been used as biocatalysts in industry are hydrolases (65%), even though oxidoreductases are typically much more useful than hydrolases as catalysts. (**Figure 20.**) The utility of an enzyme class depends on the relative commercial importance of the products that each enzyme produces, the accessibility of the enzymes, and the specific characteristics of the enzymes (e.g., stability, activity, and selectivity).[481]

Enzymes	Type of reactions	Representative subclasses
Oxidoreductases	Catalyze the transfer of hydrogen or oxygen atoms or electrons from one substrate to another	Oxidases, oxygenases, peroxidase, dehydrogenases
Transferases	Catalyze the group transfer reactions	Glycosyltransferases, transketolases, methyltransferases, transaldolases, acyltransferases, transaminases
Hydrolases	Catalyze hydrolytic reactions	Esterases, lipases, proteases, glycosidases, phosphatases

Lyases	Catalyze the nonhydrolytic removal of groups	Decarboxylases, aldolases, ketolases, hydratases, dehydratases
Isomerases	Catalyze isomerization reactions	Racemases, epimerases, isomerases
Ligases	Catalyze the synthesis of various types of bonds with the aid of energy-containing molecules	Synthetases, carboxylases

Table 2. Classification of enzymes[481]

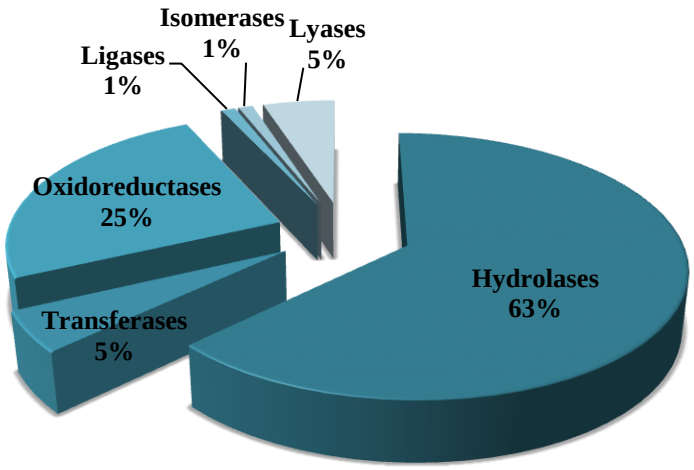


Figure 20. The relative use of enzyme classes in industry.[482]

Oxidoreductases

Oxidoreductases catalyze oxidation and reduction reactions that occur within the cell. They are very appealing for industrial uses because of the reactions that they are able to catalyze. However, they often need expensive cofactors such as nicotinamide adenine dinucleotides (example, NAD^+=NADH) and flavines (example, FAD=$FADH_2$) in the reactions. In fact, nicotinamide adenine dinucleotides are required by about 80% of oxidoreductases. Fortunately, several NAD(H) regeneration systems have been developed, the most widely used being the formate=formate dehydrogenase (FDH) system.[483]

An example of a pharmaceutical synthesis reaction involving an oxidoreductase is the synthesis of 3,4-dihydroxylphenyl alanine (DOPA).[482] 3,4-Dihydroxylphenyl alanine is a chemical used in the treatment of Parkinson's disease. The industrial process that synthesizes DOPA utilizes the oxidoreductase polyphenol oxidase. The monohydroxy compound is oxidized by the regio-specific addition of a hydroxyl group. It is worth mentioning that epinephrine (adrenaline) can also be synthesized by a similar reaction path using the same enzyme.[482]

Transferases

Transferases catalyze the transfer of functional groups such as methyl, hydroxymethyl, formal, glycosyl, acyl, alkyl, phosphate, and sulfate groups by means of a nucleophilic substitution reaction. They are not widely used in industrial processes; however, there are a few examples of industrial processes that utilize transferases. A classical example of industrial application of transferases is the use of various glycosyltransferases for the synthesis of oligosaccharides. Oligosaccharides and polysaccharides are important classes of naturally occurring compounds, which play vital roles in cellular recognition and communication processes.[484] Because of the required use of many protection and deprotection groups, chemical synthesis of complex oligosaccharides represents a daunting challenge in synthetic organic chemistry. By contrast, enzymatic synthesis of oligosaccharides by glycosyltransferases requires very few protection and deprotection steps because of the high regio- and stereoselectivity of glycosyltransferases, thus offering an attractive alternative.[482]

Hydrolases

Hydrolases catalyze the addition of water to a substrate by means of a nucleophilic substitution reaction. Hydrolases (hydrolytic enzymes) are the biocatalysts most commonly used in organic synthesis. They have been used to produce intermediates for pharmaceuticals and pesticides, and chiral synthons for asymmetric synthesis. Of particular interest among hydrolases are amidases, proteases, esterases, and lipases. These enzymes catalyze the hydrolysis and formation of ester and amide bonds. Several industrial processes using lyases as catalysts, the most prominent lyasecatalyzed process is the production of acrylamide from acrylnitrile.[485] Another example is the use of a fumarase for the production of (*S*)-malic acid

from fumaric acid. A water molecule is added to the double bond in fumarate by means of an addition reaction. The result is a cleavage of the carbon–carbon double bond, and a formation of a new carbon–oxygen bond. A third example is biocatalytic production of a cyanohydrin from a ketone. This reaction is catalyzed by a lyase called oxynitrilase. It consists of the cleavage of one carbon–oxygen bond, and the addition of a HCN molecule. The chirality of the product is based on the form of the enzyme used (*R*-oxynitrilase or *S*-oxynitrilase).[482]

Isomerases

Isomerases catalyze isomerization reactions such as racemization and epimerization. They have not been used in many industrial applications. However, one of the most successful enzyme based biocatalytic processes involves an isomerase: the use of glucose isomerase for the production of high-fructose corn syrup (HFCS). High-fructose corn syrup is used as an alternative sweetener to sucrose in the food and beverage industry. The isomerization of glucose to HFCS on an industrial scale is carried out in continuous fixed-bed reactors using immobilized glucose isomerases. The total amount of HFCS produced by glucose isomerase exceeds a million tons per year.[486]

Ligases

Ligases catalyze reactions that involve the creation of chemical bonds with nucleotide triphosphates. They are important in certain cellular processes, such as connecting nucleotides in DNA replication. However, similar to isomerases, ligases have very few industrial applications.[482] It is important to note that DNA ligases are essential tools in recombinant DNA technology and are used almost in every biology-related laboratory.

4.1.3 Industrial biotechnology

Enzymes are the most proficient catalysts, offering much more competitive processes compared to chemical catalysts. A number of enzyme-based processes have been commercialized for producing several valuable products since the biocatalysis was first introduced almost century.[487,488,489,490] Despite great potential of enzymes, however, their industrial applications have been hampered mainly owing to undesirable property in terms of

stability, catalytic efficiency, and specificity. To overcome such shortcomings, a variety of approaches have been attempted, including screening of enzymes from natural sources, random mutations, immobilization.[491,492] During 1980s and 1990s, engineering of enzymes based on structural information allowed extension of their substrate ranges, enabling the synthesis of unusual intermediates.

Fine and bulk chemical industries

Applications of enzymes and whole cell biocatalysis for producing diverse types of chemical and biological substances have become a proven technology in chemical and pharmaceutical industries because enzyme-based processes usually lead to a reduction in the process time, number of reaction steps, and amount of waste.[493] In particular, enzymes provide a more powerful way of producing enantiomerical pure compoundsmainly through high chemoselectivity, regioselectivity, and streoselectivity.[494]

Acrylamide is an important commodity chemical for synthesizing polyacrylamide used for petroleum recovery, wastewater treatment, papermaking, pesticide formulation, soil erosion prevention, and gel electrophoresis.[495,496] Traditionally, acrylamide can be produced chemically by oxidizing acrylonitrile using copper and sulfuric acid as a catalyst at high temperature.[497] However, both methods are known to cause several types of environmental pollution. The discovery of nitrile hydratase (EC 4.2.1.84) and its application in nitrile hydration has offered a novel process for the production of acrylamide.[498,499] *Rhodococcus rhodochrous* J1 overexpressing nitrile hydratase efficiently converts acrylonitrile into acrylamide at up to 45% (W/W) undermild conditions.[500,501,502]

Glycolic acid is a C2 chemical building block that has found a wide range of applications in cosmetics, food industry and as a precursor for biopolymers.[503,504] Glycolic acid can be polymerized into polyglycolic acid (PGA), which has high strength and thermo-tolerance as well as low gas permeability suitable as an ideal packaging material for food and other goods.[503] The conventional method of glycolic acid production relied on the reaction of formaldehyde and carbon monoxide through an acid catalysis at high pressure and temperature.[504] An alternative method is the use of heterologous host expressing nitrilase (EC 3.5.5.1), lactoaldehyde reductase (EC 1.1.1.77), and lactoaldehyde dehydrogenase (EC 1.2.1.22) for the hydrolysis of glycolonitrile and the oxidation of ethylene glycol, followed by the conversion of glycolic acid.[505] However, conventional chemical and biotransformation

methods for glycolic acid have certain drawbacks such as high impurity. For the production of high-purity glycolic acid, some researchers attempted a chemo-enzymatic process using *E. coli* cells overexpressing nitrilase from *Acidovorax facilis* 72 W.[504] This chemo-enzymatic process comprises the synthesis of glycolonitrile from formaldehyde and hydrogen cyanide using NaOH, followed by the conversion of glycolonitrile into ammonium glycolate by nitrilase at room temperature, which is further converted to glycolic acid by ion exchange chromatography (IEC). This process enables the productivity of more than 1 kg of glycolic acid/g dry cell weight. In addition, to further increase the catalytic activity of nitrilase, a directed evolution was attempted, resulting in a 125-fold increase.[506]

1,3-Propanediol is a valuable chemical as a C3 chemical building block, and is polymerized with terephthalates for the synthesis of polymethylene terephthalates used in the manufacturing of textile fiber, film, and plastic.[507] The market for 1,3-propanedol is over 100 million tons/year, and is growing rapidly.[508] Currently, the production of 1,3-propanediol is produced from glycerol by metabolically engineered *Saccharomyces cerevisiae* and *Klebsiella pneumonia*.[509,510] This bioconversion process was shown to result in a maximum yield of about 50–60% (mol/mol), but requiring a supply of coenzyme B_{12} as a cofactor for enzymes involved in 1,3-propanediol biosynthesis in the microbial fermentation process. For the economic and eco-friend production of 1,3-propanediol, Rieckenberg et al. developed a novel biosynthetic process using glycerol dehydratase (EC4.2.1.30) and oxidoreductase-isoenzyme (EC 1.1.1.202).[511] In this process, glycerol dehydratase converts glycerol into 3-hydroxypropionaldehyde (3-HPA), which is further transformed into 1,3-propanediol by NADPH-dependent propanediol oxidoreductase-isoenzyme from *E. coli*. Interestingly, the conversion yield of glycerol into the target product, 1,3-propanediol, reached almost 100%.

5-hydroxymethylfurfural (HMF) is considered a promising building block because it allows diverse synthetic processes leading to various chemical compounds such as dimethylfuran (biofuel), 2,5-diformylfuran and 2,5-furandicarboxyllic acid (polymer monomers), levulinic acid, adipic acid, caprolactam, and caprolactone, above and beyond many other molecules, including pharmaceutical ingredients.[512] Traditionally, HMF is produced through the acid-catalyzed dehydration of monosaccharides such as fructose or glucose.[513] It was reported that the production yield of HMF from fructose at higher than 70–100% (w/w) can be achieved using hydrochloride and Amberlst-15.[514,515] However, fructose is less stable than glucose. For amore economical production of HMF, attempts to directly use glucose or glucose-based carbohydrates have been made, leading to the development of a process for the production of

HMF through a combination of glucose–fructose isomerization using glucose isomerase (EC 5.3.1.5) followed by fructose dehydration into HMF by acid. This process results in a production yield of about 63–87% (w/w).[513]

(R),(S)-Epichlorohydrin is a chiral building block for synthesizing pharmaceuticals and agrochemicals.[516] Epichlorohydrin is usually produced from allyl chloride through a two-step process, starting with addition of hypo-chlorous, which produces 1,3- and 2,3-dichlorohydrin. In the second step, this mixture is reacted with a base to generate epoxide.[517] One alternative method for producing (*R*),(*S*)-epichlorohydrin is to biotransform the starting substrate, 1,3-dichloro-2-propanol, using halohydrin dehalogenase (EC 4.5.1) and epoxide hydrolases (EC 3.3.2.3). Although this process was shown to result in efficient production of enantio-pure epichlorohydrin, it gave rise to severe problems. The water-insoluble epichlorohydrin caused an inhomogeneous reaction mixture, and epichlorohydrin spontaneously hydrolyzes in aqueous media. To overcome these shortcomings, some researchers. used organic solvents as reaction medium for the production of (*R*)-epichlorohydrin with an *ee* of 99% and a yield of 28.5%, from 20 mM of a racemic substrate using recombinant epoxide hydrolases.[518] Similarly, Jin et al. employed epoxide hydrolases from *Aspergillus niger* to hydrolyze racemic epichlorohydrin at the substrate concentration of up to 153.6 mM, and produced (*S*)-epichlorohydrin at the yield of 18.5% with *ee* of 98% in organic solvents.[519] Based on the results, the use of organic solvents appeared to solve the problem of instability and low solubility of epichlorohydrin. However, these processes are not practically feasible at the industry scale because of a low yield and ee value owing to the substrate and product inhibition. In an effort to tackle this problem, Jin et al. attempted an intermittent feeding of the substrate in a two-phase system, achieving a 42.7% yield of (*R*)-epichlorohydrin and an ee value of above 99%.[520] Furthermore, a great deal of effort has been made to increase the productivity, stability and enantioselectivity through the engineering and discovery of epoxide hydrolase.[521,522,523,524]

Pharmaceutical industry

Over the decades, the pharmaceutical substances have become increasingly complex, and public and environmental quests for green technologies have increased. Therefore, the industry is seeking low-cost, safer, and greener biocatalytic processes as alternatives to traditional chemical catalysis.[525,526] Specific reactions that can be replaced with biocalaysis

have been identified in the synthesis of pharmaceuticals, including chiral amine synthesis, stereo and regio-specific hydroxylation of complex molecules, and other redox reactions.[527,528]

One of the most successful examples in the practical application of enzymes in the pharmaceutical industry is the anti-diabetic compound, sitagliptin.[529,530] Sitagliptin is a drug for type II diabetes that has been marketed under the trade name Januvia by Merck.[529] Researchers had engineered *R*-selective transaminase (*R*-ATA, ATA-117) from *Arthrobacter* sp. for the asymmetric amination of prositagliptin ketone. By applying a substrate walking, modeling, and mutation approach, they were able to overcome the limitation of the substrate's size for the enzyme. A combination of the further directed enzyme evolution and process engineering yielded a variant that converts 200 g/L of prositagliptin ketone into sitagliptin at an enantio-purity of greater than 99.95% even in the presence of 1 M i-PrNH2, with 50% DMSO at a higher temperature than 40 °C.[530] Comparedwith the rhodium (Rh)-catalyzed process, the biocatalytic process not only reduces the total waste and eliminates the requirement of a rare heavy metal (Rh), it also increases the overall yield by 10% and the productivity (kg/L per day) by 53%.[529,531] Immobilization of engineered (R) selective-ATA enables the maintenance of the enzyme activity and stability in an organic solvent, simplifying the workup and allowing a repetitive use of the enzyme.[532] The use of several R- or S-selective-ATAs have been reported in a large-scale synthesis of potential drugs such as niraparib,[533] an orexin receptor antagonist,[534] and Janus kinase 2 (JAK2) inhibitor.[535,536]

Another example of a chiral amine synthesis is boceprevir,[537] which is a clinically used drug for chronic hepatitis C infections under the trade name Victrelis by Merck. In the synthesis of boceprevir,an efficient and enantio-pure desymmetrisation of a bicyclic proline intermediate is highly required. Was employed monoamine oxidase (MAO) from *Aspergillus niger* for the asymmetric amine oxidation of the intermediate. Although the activity, solubility, and thermal stability of the enzyme were sufficiently improved to sustain the manufacturing process through protein engineering, an irreversible product inhibition remained a challenge. However, this problem was successfully solved through the trapping of an imine product by the addition of bisulfite,which demonstrates the importance of process engineering in the industrial application of a biocatalysis. Compared with the resolution method, the biocatalytic process not only increases the product yield by 150%, but also reduces the use of raw materials by 59.8%, the consumption of water by 60.7%, and the overall process waste by 63.1%.[537] Although the process has yet to be scaled up to the industrial scale, the same

asymmetric amine oxidation by MAO is currently used in the synthesis of another drug for hepatitis C infection, i.e., telaprevir.[538] Recently, the substrate spectrum for MAO has expanded to accommodate amine substrates with bulky aryl substituents through a rational structure-guided design and high-throughput screening approaches. Engineered MAO was applied in the synthesis of the drugs solifenacin and levocetirizine, as well as alkaloid natural products including coniine, eleagnine, leptaflorine, and harmicine.[531]

Another example involving KRED is the synthesis of hydroxynitrile, which is a key intermediate for atorvastatin, using amulti-enzyme process. Atorvastatin is a member of the statin family that lowers cholesterol by blocking the cholesterol synthesis in the liver, and is currently marketed by Pfizer under the trade name Lipitor.[539] Using pre-evolved enzymes, Codexis developed a two-step process composed of three enzyme steps including halohydrin dehalogenase (HHDH), glucose dehydrogenase (GDH), and KRED. In this process, KRED is involved in the first step of ethyl-4-chloroacetoacetate reduction coupled with GDH for cofactor regeneration. Advances in protein engineering technology have enabled a large-scale synthesis of hydroxynitrile intermediates. This multienzyme process has been proven to be not only environmentally attractive, but also economically viable compared to a traditional chemical process.

With a growing number of enzymes available for the synthesis of pharmaceutical compounds, attempts at developing a "one-pot" processes based on multi-enzyme cascade reactions are also increasing. Compared to a traditional chemical process and a single-enzyme process, a one-pot process is highly enantioselective and efficient by circumventing the need for multiple steps.[540,541,542,543]

Food industry

In the food industry, biocatalysis has been used to produce raw materials and final products for a long time.[544] However, most uses of biocatalysis have focused on hydrolytic reactions for debranching, improving the solubility, and clarification.With the increasing request for nutritional aspects, a significant amount of attention has been paid to the functionality of foods beyond the primary function of nutrient supply. A recent trend in the food industry is to develop functional foods such as prebiotics, low-calorie sweeteners, and rare sugars.[545] Prebiotics are a dietary substance composed of non-starch polysaccharides and oligosaccharides, including inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides

(GOS), lactulose, and breast milk oligosaccharides. However, most of them are not digested well by human enzymes,[546] and ingredients that selectively promote the growth of intestinal microorganisms have yet to be elucidated. With the increasing demands for prebiotics, the food industry has become interested in the use of enzymes for high-production yields at low cost and using simple processes.

Difructose anhydride (DFA) III is a non-cariogenic sweetener and non-digestible disaccharide that promotes the absorption of calcium, magnesium, and other minerals in the intestine.[547] DFA III is produced from inulin by the exo-acting inulin fructotrnasferase (EC 4.2.2.18) from *Arthrobacter ureafaciencs*. Since then, inulin fructotrnasferase from *Arthrobacter* sp. and other bacteria has been identified.[548,549] However, the industrial use of DFA III was limited by a low thermal stability and expensive inulin.[550] A great deal of effort has been made to isolate heat-stable inulin fructotrnasferase from various microorganisms and to develop a novel process using a cheap substrate. Recent reports have shown a novel inulin fructotrnasferase *Arthrobacter pascens* T13-2, *Arthrobacter* sp. L68-1, and other *Nonomuraea* species, stable up to 70–80 °C after 1 h of heat treatment.[547,551] Fructooligosaccharides (FOS) as a prebiotic can be synthesized fromsucrose using fructosyltransferase (EC 2.4.1.19). Inulin has a similar fructofuranosidic linkage to FOS, which is the smallest substrate for inulin fructotransferase. The utilization of sucrose as a substrate to produce DFA III, resulting in about a 10% (w/w) yield, was attempted through a coupled enzyme reaction as a novel approach.[550]

Galacto-oligosaccharides are health promoting ingredients that show prebiotic properties, but are poorly digestible sugars.[552] Additionally,many other health benefits have been reported, including an improvement in defecation, the stimulation of mineral absorption, colon cancer prevention, and protection against certain pathogenic bacterial infections.[553] The production of galacto-oligosaccharides was achieved through an enzymatic reaction of lactose with β-galactosidases from various microbial, yeast, and fungal sources, leading to the structural diversity of galactooligosaccharides.[554,555] The basic structure of galacto-oligosaccharides contains a lactose (galatose–glucose) backbone at the reducing end, which is expanded up to six galactose residues. In general, production yield of galacto-oligosaccharides using β-galactosidase reached around 30–35% (w/w).[556] To increase the production yield, a great deal of effort has focused on the isolation and application of thermostable β-galactosidase from thermophilic microorganisms such as *Geobacillus stearothermophilus*, *Pyrococcus furiosus*, *S. solfataricus*, *T. maritima*, and *Thermus* sp. because thermostable enzymes lead to a high reaction velocity, lower contamination, and high lactose solubility at high temperature.[557,558]

Like GOS, fructo-oligosaccharides (FOS) are used as an artificial sweetener and dietary fiber with low caloric levels, promoting the growth of *Bifidobacterium* in the human colon. In addition, it has an important role in the stimulation of calcium and magnesium absorption, and a lowering of the cholesterol, phospholipid, and triglyceride levels in human serum.[559,560] FOS is produced from sucrose by enzymes showing transfructosylation activity. Such enzymes are β-fructofuranosidase (EC 3.2.1.26) and fructosyltransferase (EC 2.4.1.9), which originate from fungi and bacteria.[561,562] The reaction of FOS by enzymes is one D-glucose unit (G) and one fructose units (F) in each sucrose (GF) bound together by β (2 → 1) glycosidic linkages ($GF + GF \rightarrow GF_{n-1} + GF_{n+1}$). The production yield of FOS at an industrial scale was reported to reach 55–60% (w/w) based on the initial sucrose concentration.[563] A further increase in yield was shown to be difficult because a high level of glucose was also produced during the reaction, inhibiting the transfructosylation activity. To solve this problem, additional glucose oxidasewas added to the reaction mixture in an attempt to convert glucose into gluconic acid, which resulted in a production yield of up to 90–98% (w/w).[564] As an approach to remove glucose in a reaction mixture, glucose dehydrogenase and calcium carbonate were simultaneously used to precipitate the gluconic acid.[565]

Cosmetic industry

A variety of the ingredients used in the cosmetic industry are produced from petrochemical-based raw materials.[566] Recently, however, the cosmetic industry has faced a challenge because of increasing consumer demands for natural and eco-friendly cosmetics.[567] Accordingly, the cosmetic industry promotes basic research and eco-friendly processes using enzymes for developing more effective cosmetic products.

Arbutin is the most common skin-lightener, and is known to inhibit melanogenesis without causingmelano-cytotoxicity.[568] As an enzymatic approach to producing arbutin, various enzymes have been used, including α-amylase, α-glucosidase, transglucosidase, sucrose phosphorylase, and dextransucrase.[569] Most enzymatic processes, however, have certain drawbacks such as a high substrate cost and low conversion yield.[570] Recently, a high production yield was achieved using amylosucrase (EC 2.4.1.4), which belongs to glycoside hydrolase family that catalyzes the synthesis of amylose-like glucans from sucrose.[571] Amylosucrase from *Deinococcus geothermalis* was shown to catalyze a glycosyltransferase reaction using sucrose and hydroquinone as a donor and an acceptor, respectively. The

maximum conversion yield of α-arbutin was higher than 90% in the presence of 0.2 mM ascorbic acid.

Emollient esters are multi-functional oleochemicals that are widely used in cosmetic products owing to their moisturizing property. Emollient esters such as myristyl myristate were conventionally produced using tin oxalate as catalyst at a high temperature through trans-esterification of vegetable oils and alcohols.[572,573] An esterification reaction was carried out without a solvent in the presence of equal amounts of reactants at 75 °C using Novozym 435 lipase, and a space time yield of 6,731 g d^{-1} L^{-1} was achieved for myristyl myristate.[574]

Textile industry

In the textile industry, prior to conversion into fabric and yarn, cotton under goes various processes including refining, bleaching, dyeing, and polishing.[575] These processes consume large amounts of energy, water, and resources, discharging huge amounts of waste. For the development of cleaner processes, the use of enzymes is rapidly growing. Typical examples include the staining of jeans using cellulase from *Trichoderma viride*, and a bio-carbonization process in the case of wool.[576] Cellulase and protease are used in the polishing step for clear dyeing, the improvement of color and surface vividness, and resistance to wrinkles.[491,577]

Pulp and paper industry

In the pulp and paper industries, xylanase and ligninase are used to enhance the quality of the pulp by removing lignine and hemicelluloses, which are typical impurities.[578] In pulp production, lipase is also employed for degrading the pitch in wood, the presence of which causes a serious problem in the manufacturing process. The recycling of printed papers such as newspaper using cellulase was also developed.[579] In the paper making process, lignin causes a dark color, and the removal of lignin is required for making bright paper. The chemical pulping process requires the addition of a large amount of alkali chemicals and chlorine.[580] The use of laccase was shown to avoid elemental chlorine, and significantly reduces the amount of waste that causes ozone depletion and acidification, as well as high energy consumption.

4.2 Carbon nanotubes

Nanomaterials can serve as excellent supporting materials for enzyme immobilization, because they offer the ideal characteristics for balancing the key factors that determine the efficiency of biocatalysts, including surface area, mass transfer resistance, and effective enzyme loading.[581,582,583,584] Carbon nanotubes (CNTs) are produced by various methods, such as arc discharge,[585,586,587] laser ablation,[588] and chemical vapour deposition[589,590]. CNTs consist of graphitic sheets that have been rolled up into a cylindrical shape with lengths in the micrometers, and diameters up to 100 nm.[591] Enzyme immobilization is a promising biotechnological application of CNTs.[592,593,594,595] Two main types of CNTs, single-walled carbon nanotubes (SWNTs) and multiwalled carbon nanotubes (MWNTs), have been used to immobilize enzymes.A MWNT is comprised of several layers of graphite surrounding a central tubule, whereas a SWNT only has the central tubulewithout the graphitic layer. SWNTs are attractive for their higher surface area for enzyme interaction, but MWNTs are desirable for their easier dispersibility and lower cost.[596] Noncovalent attachment preserves the unique properties of both enzymes and CNTs, but the immobilized protein can be gradually lost during the use of the CNT-enzyme complex.[593] Covalent conjugation provides durable attachment, but the enzyme structure may be more disrupted. Functionalization of CNTs with organic, polymeric, and biologicalmolecules can provide biocompatible nanotube composites with specific groups on their surface. CNT composites can provide a basis for specific immobilization of an enzyme.

Non-covalent enzyme immobilization

For the immobilization of enzymes on CNTs, compared to covalent methods, the noncovalent approach is considered to be a more promising technique, because it preserves the conformational structure of the immobilized enzymes.[597,598] Enzymes can be adsorbed on a range of surfaces of CNTs; high surface loadings of enzymes or the crowding of the enzyme on the surface may help to prevent inactivation due to surface spreading.[599] Higher activity was reported by enzymes physically adsorbed onto CNTs.[600] Adsorption is a commonly used noncovalent approach, which involves the enzyme being physically adsorbed onto the carbon nanotubes. The adsorption typically involves bathing the CNTs in a solution of the enzyme and shaking the sample to allow time for the physical adsorption onto the surface to occur and then rinsing away enzyme that is not adsorbed.

With the direct physical adsorption method, the interacting force between the enzyme and CNT is predominantly a hydrophobic interaction.[593] An enzyme including hydrophobic regions on its exterior can interact with the wall of a CNTthrough hydrophobic interactions.

Polymers and biomolecules have been used to functionalize CNTs. The functionalized CNTs (f-CNTs) have good aqueous dispersibility, and the formation of the enzyme–CNT complex is facilitated. f-CNTs can have molecular recognition and binding specificity for enzymes,[592,601] due to the combination of many molecular properties such as hydrophobicity, electrostatic interactions, hydrogen bonding, and steric properties. Polymers coated on CNTs can provide negatively and positively charged functional groups on the surface of CNT–polymer complexes.

A layer-by-layer (LBL) approach has been adopted for immobilization of enzymes. It permits the coating of various enzymes, producing multilayer enzyme films on CNTs. The biocatalytic activity can be increased by increasing the number of enzyme layers assembled on CNTs.[602,583,603] The electrostatic LBL adsorption based on the alternating assembling of oppositely charged layers has been demonstrated to be an effective method for the coating of enzyme on CNTs by the formation of ordered and stable multilayer films.[602]

Covalent linking

Covalent immobilization of enzymes on CNTs has been demonstrated by inducing the reaction of the free amine groups on the surface of a protein with carboxylic acid groups that are generated by sidewall oxidation of CNTs and subsequent activation using carbodiimide.[604,596,605]

Linking molecules are frequently used for covalent immobilization of enzymes onto CNTs. They bind to CNTs through hydrophobic and π–π interactions,[606,607] and also covalently bind the enzyme through, for example, an amide bond.[608,609] Linking molecules present advantages in the immobilization of enzymes.

4.3 Laccase

Laccases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) belong to the superfamily of multicopper oxidases.[610] Laccases are widely distributed in many eukaryotes e.g. fungi, plants[611] as well as in prokaryotes e.g. bacteria[612] and exhibit various functions, depending on their source organism, physiological and pathological conditions. Range of functions exhibited by laccases is broadly divided into three categories: cross-linking of monomers, degradation of polymers, and ring cleavage of aromatic compounds.[613] Thus, the various functions carried out by laccases include lignification, wound healing and iron oxidation (in plants), delignification, pigmentation, fruiting body formation as well as pathogenesis (in fungi) and melanin formation, endospore coat protein synthesis (in bacteria).[614,615,616,617,618,619] The localization of plant and fungal laccase is extracellular, while in bacteria most of the laccases are intracellularly localized.[620] Laccases are dimeric or tetrameric glycoproteins. To perform their catalytic function, laccases depend on Cu atoms that are distributed at the three different copper centers Type-1 or blue copper center, Type-2 or normal copper and Type-3 or coupled binuclear copper centers, differing in their characteristic electronic paramagnetic resonance (EPR) signals.[621,622] At three dimensional structure level, laccases (bacterial, fungal and plant) have been suggested to have three sequentially arranged cupredoxin-like domains. From wet lab analysis as well as from in silico studies, it is evident that despite their wide taxonomic distribution and substrate diversity, molecular architecture of catalytic site of laccases exhibits commonality with that of multicopper oxidases. The ability of laccases to catalyze the oxidation of various phenolic as well as non-phenolic compounds, coupled to the reduction of molecular oxygen to water, makes them valuable from the standpoint of their commercial application.[623]

4.3.1 Properties of laccases

Laccase is a glycosylated monomer or homodimer protein generally having fewer saccharide compounds (10-25%) in fungi and bacteria than in the plant enzymes. The carbohydrate compound contains monosaccharides such as hexoamines, glucose, mannose, galactose, fucose and arabinose. On SDS-PAGE, most laccases show mobilities corresponding to molecular weight of 60-100 kDa, of which 10-50% may be attributed to glycosylation. Mannose is one of the major components of the carbohydrate attached to laccase.

Glycosylation in laccase is responsible for secretion, proteolytic susceptibility, activity, copper retention and thermal stability.[672]

Most laccases studied are extracellular proteins, although intracellular laccases have been detected in several fungi and insects. Fungal laccases have isoelectric points 8 pI) ranging from 3 to 7, whereas plant laccase pI value range to 9. The main difference between the two enzymes is that fungal enzymes have their pH optima between pH 3.6 and 5.2, while laccase from *Rhus vernicifera* have pH optima between *6*.8 and 7.4. The low pH optima of the fungal enzyme may be because they are well adpted to frow under acidic conditions, while the plant laccase being intracellular have their pH optima nearer to the physiological range. Thus the differences in pH optima may be because of the dissimilarity in physiological functions. In addition to their variation in pH, these enzymes also differ in their functions. Fungal enzyme is responsible in mechanism for removing toxic phenols from the medium in which these fungi grow under natural conditions, while the plant enzymes are involved in synthetic process such as lignin formation.[624]

Purified laccases exhibit a characteristic blue appearance from their electronic absorption around 600 nm. Typical UV-Visible spectra of laccase (at resting state) show two maxima around 280 and 600 nm and one shoulder near 330 nm. The ratios of the absorbance at 280 nm to that at 600 nm are generally 14 to 30, and the ratio of the absorbance at 330 nm to that at 600 nm is 0.5 to 2.[671] In the holoenzyme form, most laccases have four copper atoms per monomer,[625] although the laccase from *Phlebia* was reported to have two copper and one pyrroloquinoline quinone as the prosthetic group. These copper atoms are classied in three groups using UV/visible and electron paramagnetic resonance (EPR) spectroscopy). The type I copper (T) is responsible for the intense blue colour of the enzymes and has a strong electronic absorption around 600 nm and is EPR detectable. The type II copper (T2) is colourless, but EPR detectable, and the type 3 copper (T3) consists of a pair of copper atoms that give a weak absorbance near the UV spectrum but no EPR signal. The T2 and T3 copper sites are close together and form a trinuclear centre in which binding dioxygen and four electron reduction to water occur.[626]

Copper of the first type can be acted upon by solvents including water, and removed from the enzyme molecule by various complexes. Copper of the second type is easy to eliminate, which can frequently be done during purification procedures. On the other hand, type 2 copper can be reconstructed both in aerobic and anaerobic conditions. The study of laccase derivatives showed that type 2 copper is bound to three nitrogen atoms. A water molecule is

the fourth copper ligand. Type 2 copper has proven to play an important role in structural nonspecific stabilization of anionic bonding in the copper 3 active site. It has also been found that a type I copper center, which lacks a liganding methionine, is relatively unstable. This is true for all fungal laccaes.[627]

Although most laccases adhere to these descriptions, there are certain highly purified laccases that do not show these typical characteristics. It was demonstrated that the laccases isolated from solid state fungal cultures were yellow-brown and did not have typical blue oxidase spectra, showing simultaneously atypical EPR spectra. The comparison of N- terminal amino sequences of the *Phlebia radiate*, *Panus tigrinus*, *Coriolus versicolor* and *Phlebia tremellosus* laccases showed high homology between blue and yellow-brown laccase forms. The yellow enzyme seems to have an altered oxidation state of copper in the active center, which is probably caused by the integration of aromatic lignin-degradation products. Interestingly, evidence has been provided that yellow laccases are capable of oxidizing nonphenolic lignin models and veratryl alcohol directly in presence of O_2 but in the absence of any diffusible mediator.[627] Not all laccases are reported to possess four copper atoms per monomeric molecule.[628] One of the laccases from *Pleurotus ostreatus* is said to confer no blue colour and was described by the author to be a white laccase.[664] It was determined by atomic absorption that the laccase consisted of 1 copper atom, 1 zinc atom and 2 iron atoms instead of the typical four coppers.

4.3.2 Classification of laccases

Laccase (EC 1.10.3.2) is a lue copper protein, but it also falls within the broader description of polyphenol oxidases. Polyphenol oxidases are copper proteins with the common feature that they are able to oxidize aromatic compounds with molecular oxygen as the terminal electron acceptor.[629]

Polyphenol oxidases are associated with three types of activities:

- Catechol oxidase or *o*-diphenol: oxygen oxidoreductase,
- Laccase or *p*-diphenol: oxygen oxidoreductase,
- Cresolase or monophenol monooxygenase.

These different enzyme can therefore be differentiated on the basis of substrate specificity.[630] There is, however, difficulty in defining laccase according to its substrate specificity, because laccase has an overlapping range of substrates with tyrosinase, Catechol oxidases or tyrosinase have *o*-diphenol as well as cresolase activity (oxidation of L-tyrosine). Laccases have ortho and paradiphenol activity, usually with more affinity towards the second group. Only tyrosinases possess cresolase activity and only laccases have the ability to oxidize syringaldazine.[628,631] There has been only one report of an anzyme exhibiting both tyrosinase and laccase activity.[632] Secondly, laccases are remarkably nonspecific as to the inducing substrate, and the range of substrate oxidized varies from one laccase to another.[633]

4.3.3 Fungal laccases

In fungi, laccases are widely distributed in ascomycetes, duteromycetes, and basidiomycetes. These laccase producing fungi (called as wood-degrading fungi) include *Trametes (Coriolus) versicolor*, *Trametes hirsute*, *Trametes ochracea*, *Trametes villosa*, *Trametes gallica*, *Cerrena maxima*, *Phlebia radiata*, *Coriolopsis polyzona*, *Lentinus tigrinus*, *Plreurotus eryngii*, etc. Laccases are also reported in saprophytic ascomycetes of composts (*Myceliophthora thermophila, Aspergillus, Curvularia*, *Penicillium and Chaetomium thermophile*) and in the soil hyphomycete *Mycelia sterlia* INBI 2-26.[634,635,636] Laccases have also been purified and characterized from a few fungi-forming ectomycorrhiza e.g. *Cantharellus cibarius*,[637] *Lactarius piperatus*,[638] *Russula delica*[639] and *Thelephora terestris*[640] or orchideoid mycorrhiza such as *Armillaria mellea* [641,642] as well as from the species of genera that contain both saprotrophic and mycorrhizal fungi e.g. *Agaricus, Marasmius, Tricholoma and Volvariella*. Better penetrative ability, due to extensive hyphal organization, has been suggested to be the reason for efficient wood degradation by fungi in nature.[643] Furthermore, the high activity of laccases in wood-rotting basidiomycetes fungi suggests that the main role of fungal laccases is to depolymerize the complex cell-wall constituents such as lignin. This degradation process also involves the synergistic effects of some other enzymes and non-enzymatic components that help to establish equilibrium between enzymatic polymerization and depolymerization.[644,645,646,647] In addition to laccases, the other enzymes implicated in lignin degradation are: lignin peroxidase, which catalyzes the oxidation of both phenolic and non-phenolic units, manganese-dependent peroxidase, glucose oxidase and glyoxal oxidase for H_2O_2 production and cellobiose-quinone oxidoreductase for quinone reduction.[648]

White-rot fungi, most efficient lignin degraders, are characterized by high laccase activity. It has been postulated that almost all white-rot fungi produce laccase[611,623] except for *Phanerochaete chrysosporium*, which is reported to produce a range of isoenzymes of lignin peroxidase and manganese peroxidase[649]. In white-rot fungi, manganese peroxidase in combination with either laccase or lignin peroxidase may be the minimum necessary enzymatic component for the lignin biodegradation.[650] Based on the enzyme production patterns followed, three categories of fungi may be postulated: lignin–manganese peroxidase group, manganese peroxidase–laccase group and lignin peroxidase–laccase group.

Besides degradation of biopolymers, fungal laccases are also reported to perform several other functions, such as development associated pigmentation (dihydroxynaphthalene melanins, that are produced against environmental stress), fruiting body formation, fungal morphogenesis, detoxification, sporulation, and pathogenesis.[651] Plant-pathogenic fungi are reported to produce laccases that are proposed to detoxify the toxic components generated by the plant defence systems.[652] Botytis cinerea that causes soft rot infections in many crop plants such as carrot, cucumbers as well as the noble rot and grey rot in grapes produces extracellular laccases, which are involved in the pathogenesis.[653,654,655] In *Cryptococcus neoformans*, laccases are expressed as virulence factors, thus they are considered as amajor fungal pathogen in immuno-compromised individuals such as AIDS patients, organ transplant recipients and high doses corticosteroid treated patients.[656] This fungal laccase is thought to convert host catecholamines into melanin, which protects *C. neoformans*, allowing it to cause more damage to the host.[657] Laccase production in fungi is sensitive to the nitrogen concentration. Usually high nitrogen concentration is required to obtain greater amounts of laccases. For example, when *Lentinula edodes*,[658] and *Rigdoporus lignonus* [659] were grown in a high nitrogen (24–26mM) containing medium, laccase production becomes highest. In contrary to this, enhanced production of the laccase in nitrogen-limited media is also reported in *Pycnoporus cinnabarinus*[660] and *Phlebia radiate*[661].

4.3.4 Structure of fungal laccases

Fungal laccases often occur as isoenzymes that oligomerize to form multimeric complexes. The molecular mass of the monomer ranges from about 50 to 100 kDa. An important feature is a covalently linked carbohydrate moiety (10–45%), which may contribute to the high stability of the enzymes.

For the catalytic activity a minimum of four copper atoms per active protein unit is needed:

- Type 1: paramagnetic ‘blue’ copper, absorbance at 610 nm (ox.).
- Type 2: paramagnetic ‘non-blue’ copper.
- Type 3: diamagnetic spin-coupled copper-copper pair, absorbance at 330 nm (ox.).

Type 1 copper has a trigonal coordination, with two histines and a cysteine as conserved equatorial ligands and one position usually variable. This axial ligand is leucine or phenylalanine in fungal laccases. It has been widely argued that this axial position ligand strongly influences the oxidation potential of the enzyme, possibly providing the mechanism for regulating its activity. A mutation from phenylalanine to methionine significantly lowered the oxidation potential of a fungal laccase from *Trametes villosa*.[662] Type 2 and type 3 copper form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place. Type 2 copper is coordinated by two and type 3 copper atoms by six histidines. The strong anti-ferromagnetical coupling between the two type 3 copper atoms, is maintained by a hydroxyl bridge. (**Figure 21.**)

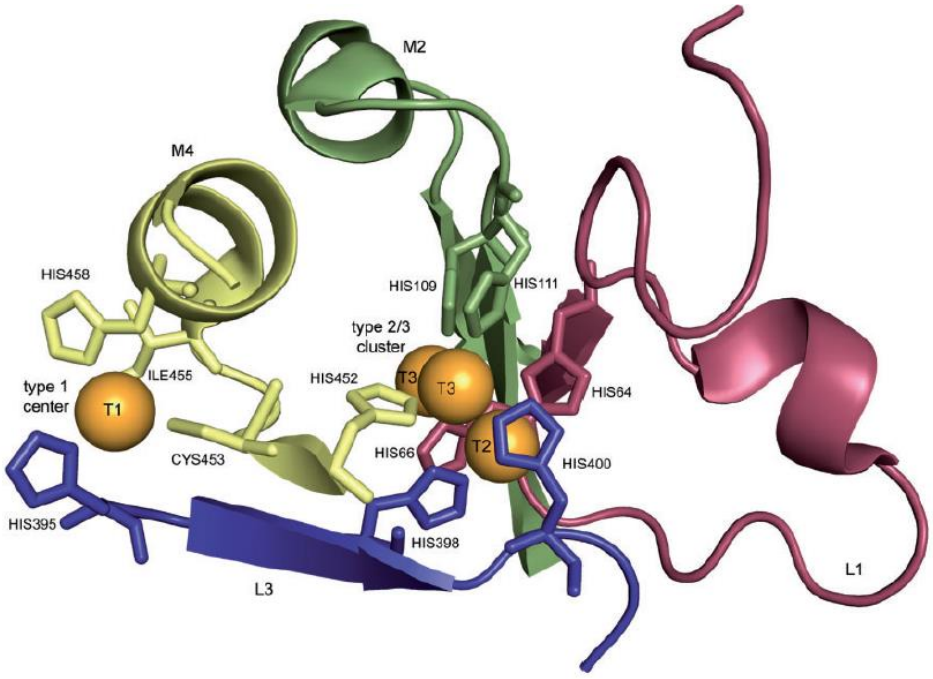


Figure 21. Cooper binding residues of laccase from *Trametes versicolor*. The copper centers are shown in orange.[663]

Multiple sequence alignments of more than 100 laccases resulted in identification of four ungapped sequence regions, L1–L4, as the overall signature of laccases, distinguishing them within the broader class of multicopper oxidases. The 12 amino acid residues in the enzymes serving as the copper ligands are housed within these conserved regions. The amino acid ligands of the trinuclear cluster are the eight histines, which occur in a highly conserved pattern of four HXH motifs. In one of this motifs, X is the cysteine bound to the T1 copper while each of the histines is bound to one of the two type 3 coppers. Intraprotein homologies between signatures L1 and L3 and between L2 and L4 suggest the occurrence of duplication events.[662]

4.3.5 Mechanism of action of laccases

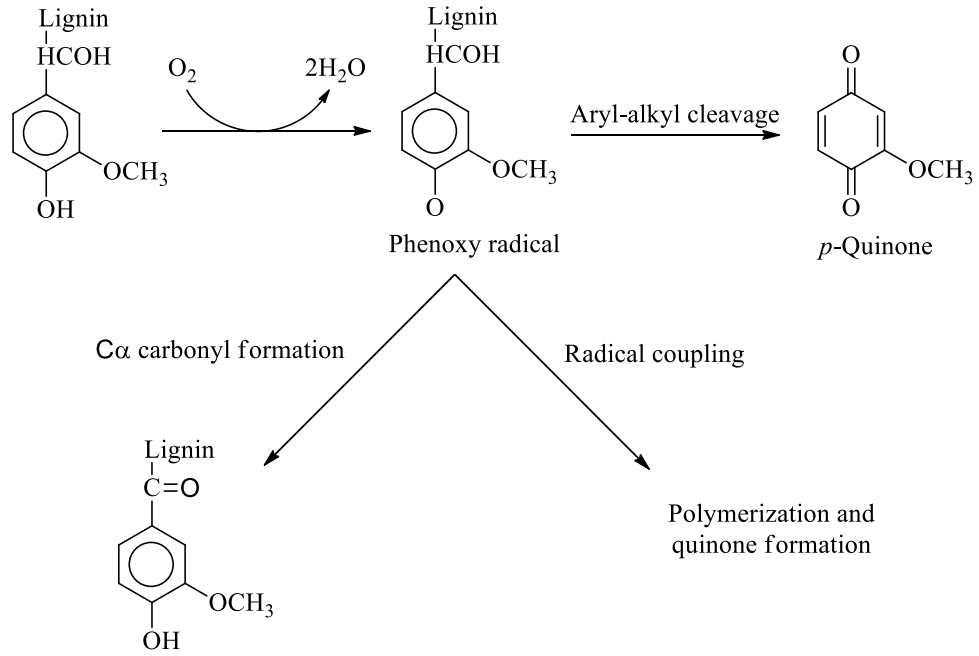
Laccases consume O_2 instead of H_2O_2 to oxidize the monolignols.[664,665] To perform catalytic function, laccase depends on Cu atoms that are distributed at the three different copper centers. The laccase enzyme withdraws the electron from the substrates and converts them in free radicals, which can be polymerized. After receiving four electrons, the enzyme donates them to molecular oxygen to form water molecule.[666] Overall, there are three major steps in laccase catalysis:

1. Type-1 Cu reduction by reducing substrate,
2. Internal electron transfer from Type-1 Cu to Type-2 and Type-3 Cu trinuclear cluster,
3. Reduction of oxygen (to water) at Type-2 and Type-3 Cu.

The overall reaction is as follows:

$4RH + O_2 \rightarrow 4R\bullet + 2H_2O$[651]

Laccase only attacks the phenolic subunits of lignin, leading to Cα oxidation, Cα-Cβ cleavage, and aryl-alkyl cleavage. (**Scheme.**)



Scheme. Oxidation of phenolic subunits of ligni by laccase.[669]

The oxidation of a reducing substrate by laccase typically involves the loss of a single electron and the formation of a free (cation) radical. The radical is in general unstable and may undergo further laccase-catalyzed oxidation (e.g. to form quinone from phenol) or nonenzymatic reactions (e.g. hydration, disproportion or polymerization).[672] The electron transfer from substrate to type 1 copper is probably controlled by redox potential difference. A lower oxidation potential of substrate or a higher redox potential of laccase (type 1 site) often results in a higher rate for substrate oxidation.

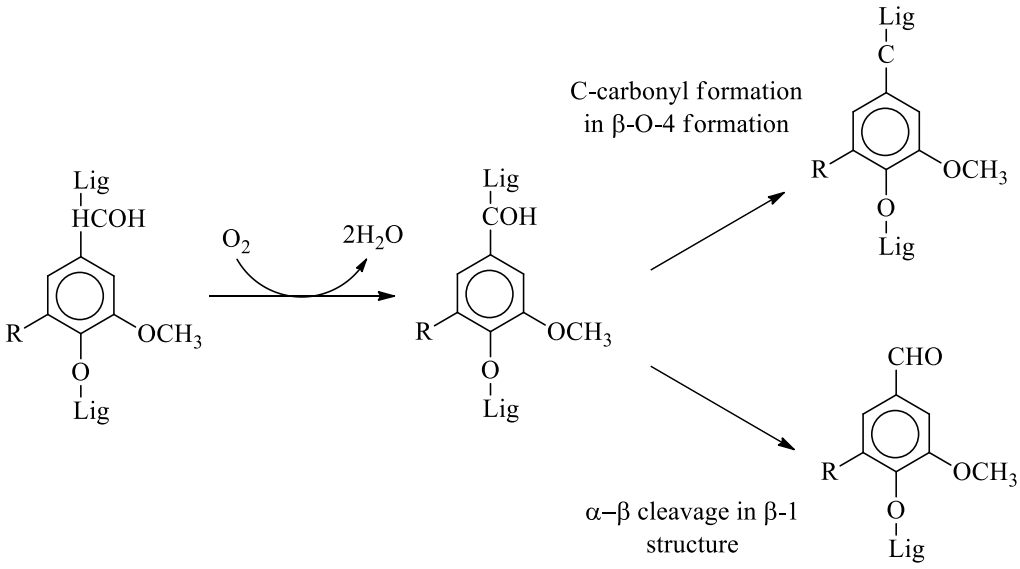
It seem that the binding pocket of reducing substrate (or type 1 copper site) is quite shallow and has limited stearic affect on simple phenol substrate. In contrast, in the O_2 binding pocket (or the type 2 and type 3 copper sites) appears to restrict the access of oxidizing agents other than O_2. Activation of O_2 likely involves chemical bond formation on the trinuclear copper cluster. Some authors proposed that under turnover conditions, the electron transfer from the substrate to the type 1 site (the initial electron acceptor from substrate) is the rate-determining step.

Laccase are similar to other phenol-oxidizing enzymes, which preferably polymerize lignin by coupling of the phenoxy radicals produced from oxidation of lignin phenolic groups. However, the substrate range of laccase can be extended to non.phenolic subunits of lignin by

the inclusion of a mediator such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS).[667]

4.3.6 Laccase Mediator System

Laccases were thought to play a role in the biodegradation of lignin, but it was restricted to phenolic compounds because of the low oxidation potentials of these enzymes.[668] Application of these enzymes in the presence of mediator compounds results in a high oxidation capability, leading to the oxidation of non-phenolic lignin model compounds. (**Scheme.**)



Scheme. Oxidation of nonphenolic lignin model compounds by Laccase Mediator System.[669]

The laccase mediator system (LMS) was originally developed to solve problems in bio-bleaching of wood pulps with the use of ABTS s the first mediator.[667] The first attempt at using laccase mediator couples for delignification in the pulp industry was the development of Lignozym process, the delignification of kraft pulp by laccase can be supported by a number of external synthetic, low molecular mass dyes or other aromatic hydrogen donors. ABTS was the firt mediator shown to be effective in the delignification of kraft pulp and lignin transformation by laccase.

The reaction mechanism mediated by ABTS appears to proceed as follows: Oxigen activates laccase, and the mediator is oxidized by the enzyme. The oxidized mediator diffuses into pulp and oxidized lignin, disrupting it into smaller fragments, which are easily removed from the pulp by means of alkaline extraction. The application of the laccase mediator system on hardwood kraft pulp resulted in a reduction of kappa number, demethylation and depolymerisation of kraft lignin.[669]

The LMS was successfully applied to the oxidation of aromatic methyl groups, benzyl alcohols, polycyclic aromatic hydrocarbons and bleaching of texile dyes (Rodriguez 2004a).[670,677]

4.3.7 Laccase characterization

Influence of pH on laccase activity

The pH optima of laccases are highly dependent on the substrate. For phenols, the optimal pH can range from 3 to 7 for fungal laccases and up to 9 for plant laccases. When using ABTS as substrate, the pH optima are more acidic and are found in the range between pH 3 and pH 5.[671] In general, laccase activity has a bell-shaped profile with an optimal pH that varies considerably. This variation may be due to changes to the reaction caused by the substrate, oxygen, or the enzyme itself.[672] The difference in redox of the substrate at high pH value, but the hydroxide anion (OH^-) binding to the T2/T3 coppers results in an inhibition of the laccase activity due to the disruption of the internal electron transfer between the T1 and T2/T3 centres. These two opposing effects can play an important role in determining the optimal pH of the biphasic laccase enzymes.[672] The role of the T1 copper in the pH optima of the anzyme was confirmed by some authors,[673] who found that the T1 copper was absent in laccase enzymes exhibiting more neutral pH optima.

Influence of temperature on laccase activity

The optimal temperature of laccase can differ greatly from one strain to another. Laccase isolated from *Ganoderma lucidum* showed optimum temperature 20-25°C and was found to be stable between 10-50°C for 4 hours.[674] Laccases isolated from *Marasmius quercophilus*,

were found to be stable for 1 h at 60°C. Some researchs further found that pre-incubation of enzymes at 40°C and 50°C greatly increased laccase activity.[675]

Influence of inhibitors on laccase activity

In general, laccaes respond similarly to several inhibitors of enzyme activity.[676] In their study, it was found that azide, thioglycolic acid, and diethyldithiocarbamic acid all inhibited laccase activity, whereas EDTA affected laccase activity to a lesser extent. Small anions such as halides (excluding iodide), azide, cyanide, and hydroxide bind to the type 2 and type 3 copper, rsulting in an interruption of the internal electron transfer and activity inhibition. Other inhibitors include metal ions (e.g. Hg^{+2}), fatty acids, sulfhydryl reagents, hydroxyglycine, kojic acid, and cationic quaternary ammonium detergents.[677]

4.3.8 Laccase immobilization

Laccases are excellent biocatalysts for biotechnological and environmental applications because of their high activity, selectivity and specificity, which permit them to perform complex chemical processes under experimental and natural conditions (Mateo et al., 2007). However, the use of these enzymes for practical applications is still limited due to their low stability and high production costs.[678,679] The immobilization of laccases can overcome some of the aforementioned limitations by improving some of the properties of the enzyme .[680] The major advantages of laccase immobilization are the increase in the thermostability of the enzyme and its resistance to extreme conditions and chemical reagents. In addition, immobilized laccases may be easily separated from the reaction products, allowing the enzymes to be employed in continuous bioreactor operations.[681,682,683] However, the immobilization processes could result in conformational alterations of the enzyme, the heterogeneity of the enzyme on the support and a slight loss of activity.[682]

Entrapment

Several examples of laccases immobilized by this method are presented in the literature, mainly for dye decolorization applications. For instance, *Cerrena unicolor* laccase was immobilized in a hydrogel matrix of poly(*N*-isopropylacrylamide) and attached to an indium

tin oxide (ITO) film electrode. The laccase entrapped in this matrix allowed for the control of the catalytic efficiency of the film by changing the temperature.[684] (**Figure 22)**

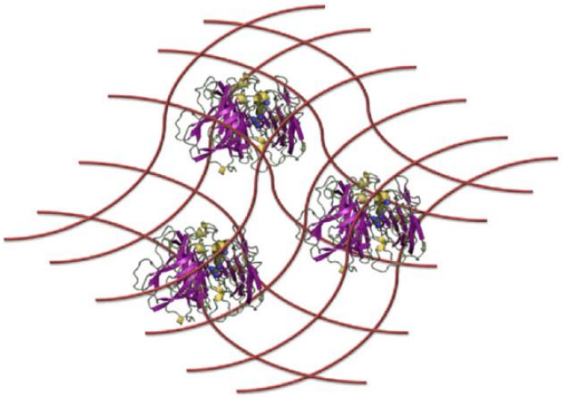


Figure 22. Immobilization of enzymes by physical interactions: entrapment.[685]

Encapsulation

Microencapsulated laccases are surrounded by semipermeable membranes, such as polymers (e.g., polyethyleneimine) or inorganic materials (e.g., SiO_2) (**Figure 23**).[686] Since 2002, a few studies have been performed to investigate the different methods and applications of laccase encapsulation. Two groups have recently used a sol–gel silica matrix for laccase encapsulation.[687,688] Some authors demonstrated the change of the optimum pH (4–5) of laccase from *Trametes* sp., and the authors also showed that the quantity of the immobilized laccase influenced the activity.[687] The layer-by-layer (LbL) technique is another microencapsulation method that has been employed during recent years.[689,690] For example, some authors microencapsulated *Trametes trogii* laccase by LbL in gold electrodes that had been previously functionalized with dithiobis-N-succinimidyl propionate.[690]

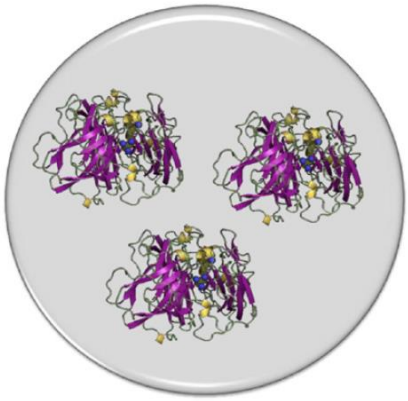


Figure 23. Immobilization of enzymes by physical interactions: encapsulation.[685]

Absorption

The adsorption of laccase onto a support is based on ionic and/or other weak forces of attraction (**Figure 24**). Adsorption is a relatively simple and inexpensive method for laccase immobilization and may therefore have a higher commercial potential than other methodologies.[691,692] Several adsorption studies have focused on the immobilization of *T. versicolor* laccase. Silicate-based supports have been the main carriers for laccase adsorption. Porous supports such as the mesoporous molecular sieve MCM-41 have been used to adsorb laccase, improving its thermal, pH and operational stability (40% of residual activity after 10 cycles).[693] Mesoporous silicate particles, such as Mobil composition of matter (MCM), cyano-modified silica (CNS) and Santa Barbara amorphous (SBA-15), have also been used to immobilize *T. hirsute* and *M. thermophila* laccases. The surfaces used for adsorption in these studies were previously modified by bifunctional (glutaraldehyde (GLU) and ethyleneglycol-N-hydroxysuccinimide (NHS)) and monofunctional (citraconic anhydride) methods. The best results were achieved by the adsorption of the *M. thermophila* laccase with GLU onto SBA-15 silica.[694]

Some studies have shown that adsorption is preferable to other techniques for the immobilization of some particular laccases. Some authors immobilized the laccase from *T. versicolor* by electrostatic adsorption and covalent binding.[695] The former was conducted by treating nanoporous gold (NPG) with lipic acid and methylene blue, and the latter was accomplished by treating NPG with lipic acid and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). Adsorption was demonstrated to be the best and easiest method of those tested. NPG has several advantages for laccase

immobilization and its subsequent application as a support because it offers a wide range of porous diameter and may be completely cleaned with a nitric acid treatment, rendering it reusable. Other metal-based supports, such as magnetic Cu2+ nanoparticles, have also been used as a support for metal-chelated adsorption. The laccase from *Pycnoporus sanguineus* has been shown to recover 65% of its activity following immobilization on this new carrier and to maintain 87% of the laccase activity after ten operation cycles. In addition, the immobilization of laccase in Cu^{2+} nanoparticles improved the catalytic capacity and stability of the enzyme toward various parameters such as pH, temperature, reuse and storage time.[696] Magnetic chitosan microspheres have also been employed as a support for the laccase from *P. sanguineus* by using GLU as a cross-linker. The optimal conditions for the immobilized enzyme were shown to be different from those of the free enzyme. Additionally, the thermal, operational and storage stabilities were greatly improved.[697,698]

An important aspect that should be noted is the general improvement in laccase activity and stability observed with all supports. However, there are some particular supports that cannot produce this effect, although they present advantages for other specific situations. For example, aluminum hydroxide has been tested as a support for *T. villosa* laccase but did not provide promising results, as the adsorbed laccase had similar activity to the free enzyme but lower resistance to thermal and proteolytic degradation. However, the immobilized laccase proved to be less sensitive to inhibition by humic acids, and no significant changes were detected in its secondary structure due to its adsorption to aluminum hydroxide. These results suggest that laccases in soil may be predominantly available in complex species with aluminum hydroxide. This study suggests that laccase could be a practical tool for soil remediation.[699] Another adsorption technique proposed for laccase immobilization is based on the use of ion exchange resins with several functional groups and mobile ions, such as dextran, agarose and chitosan.[682,691,700,701,702,703,704] For instance, T. versicolor laccase has been immobilized on poly(hydroxyethylmethacrylate-n-methacryloly-(I)-histidinemethylester) (PHEMAH) nanospheres by Cu-chelation. These histidine-containing nanospheres exhibited favorable adsorption properties, and the immobilized laccase had higher thermostability and activity than the free enzyme over a wide range of pH and temperature. Metal-based supports, such as ITO films, polycrystalline gold, glass covered by gold and silver and gold electrodes, have also been tested as ion exchange supports for laccase adsorption after treatment with $ZrOCl_2$, in which Zr^{4+} is coordinated with the carboxyl termini of proteins (via zirconium phosphonate/carboxylate). High Km values were observed with this support, indicating conformational changes of the enzyme.[704] An ion exchange method has also been employed

with a layered double hydroxide (LDH) produced by the co-precipitation of crystals of Al and Mg. The commercial laccase from Denilite II Base was adsorbed in this synthetic clay with positive electrostatic charges, resulting in the recovery of 92% of the activity.[700]

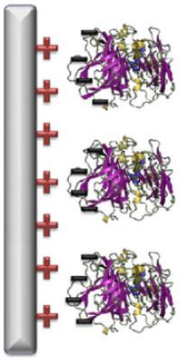


Figure 24. Chemical interactions for enzyme immobilization: absorption of enzymes onto a support by ionic forces.[685]

Covalent binding

The most interesting method of laccase immobilization for industrial applications is covalent binding. In this technique, chemical groups on the support surface are activated and react with nucleophilic groups on the protein (**Figure 25**).[682] Most enzymes are covalently attached using their lysine amino groups because of their frequent presence on the protein surface and high reactivity.[692] Covalent immobilization methods are based either on inert or on commercially available active carriers.[705] The optimal support for immobilization should contain short spacer arms and a high density of reactive groups. These characteristics are required for the multipoint attachment of the laccase, providing its rigidity. Many different supports have been used for covalent laccase immobilization, including silica-based supports such as kaolinite or mesoporous silica nanoparticles.[706],[707],[708],[709] Some authors used silanized and GLU-activated silica nanoparticles as a support.[708] The thermal and operational stabilities of laccase were improved by the retention of 61% of the residual activity after 4 h at 60 °C and the retention of 55% of the activity after 10 cycles of operation. Epoxy-activated resins such as Eupergit and Sepabeads have been frequently employed.[710,711] For instance, some authors used this type of support for the comparison of the covalent and entrapment methods of immobilization.[711] In this study, better results were achieved by immobilizing *T. villosa*

laccase with Eupergit C than with activated carbon and entrapment on a Cu–alginate matrix. Many different types of electrodes based on carbon, glass, gold, silver or graphite have been designed to act as suitable supports for laccase immobilization.[685] Silver and gold surfaces have been employed as supports after the modification of the surface by thiol monolayers with carboxylic and amino groups (SAMs). Immobilization has also been studied using resonance Raman (RR) and surface-enhanced Raman scattering spectroscopy (SERS), both of which are surfacesensitive techniques that can detect single molecules adsorbed or covalently bonded onto rough metal surfaces.[712] A gold electrode with organothiol monolayers has also been used for the immobilization of *C. unicolor* laccase. Covalent bonds were formed between the amino groups of the enzyme and the carboxylic groups of mercaptoundecanoic or mercaptopropionic acids, which were activated by immersing the electrode in EDC and NHS. The catalytic site of the immobilized protein was studied using SERS, along with the electrical connectivity of the enzyme with the electrode in the presence of mediators. The authors concluded that the use of smaller acids allowed for the transfer of a higher proportion of the catalytic activity to the electrode.[713]

The use of magnetic supports, such as nanoparticles or beads, for covalent immobilization may offer the advantage of quick separation in a magnetic field.[685] The most interesting examples were reported by one researchers group, that prepared a magnetic nanoparticle composite of copper tetraaminophthalocyanine-Fe_3O_4 that was used with the laccase from *P. sanguineus* with an immobilization yield of 20%. After 1 month of storage, the immobilized laccase was found to retain 85% of the residual activity, in contrast with the free laccase, which retained only 30% of its residual activity. The operational stability was also improved, as 80% of the initial activity was retained after 5 cycles. The same authors later designed a similar composite of zinc tetraaminophthalocyanine-Fe_3O_4. The immobilization yield obtainedwith this new designwas only 25%, but good levels of thermal, storage and operational stability were achieved.[714] The magnetic support properties have been combined with polymeric particles to improve the support characteristics. In particular, polystyrene particles (PS) with reactive β-diketone groups (AAEMs), which represent the binding site for the enzyme, have been combined with maghemite nanoparticles on the surface or in the core. Better immobilization performance was obtained when the polymeric particles had the maghemite nanoparticles on the surface,most likely due to the additional chemisorption of the enzyme as a consequence of the electrostatic interactions between the iron oxide nanoparticles and the protein. However, this chemisorption effect resulted in a partial loss of the recovered

activity, which was improved when the maghemite was present in the core of the PS particles.[715]

Several types of fibers and polymers have also been employed for covalent immobilization by means of a previous linkage between the enzyme and the support. In addition, the support may be cross-linked.[685] With this approach, some authors used the copolymer of butyl acrylate and ethylene glycol dimethacrylate to immobilize *C. unicolor* laccase.[716] Three bifunctional agents were employed for carrier activation: GLU, divinyl sulfone and carbodiimide. GLU proved the most effective because an enhancement in operational stability was obtained using a packed bed reactor at 30 °C. Nylon was used for the immobilization of *R. vernicifera* laccase after grafting with glycidyl methacrylate (nylon-poly (GMA)-HMDA-GLU). Non-isothermal assays were conducted to demonstrate an increase in the catalytic activity under a temperature gradient.[717] Other authors proposed that woven nylon offers several advantages over nylon membranes, as the former is inexpensive, chemically inert, non-toxic,mechanically stable, insoluble in water, readily available and can be obtained in several forms.[718] Woven nylonwas pretreatedwith protease for amine activation, and a spacer (1,6-hexanediamine) was included to increase the flexibility of the immobilized enzyme. Laccase has also been immobilized using chitosan. One research group determined the optimal conditions for this process: 5% GLU for 8 h and 20mg of laccase per gram of support with a 6 h reaction time.[719] The stability and reusability of the laccase were considerably improved through this immobilization process, and 52.2% of the original activity was recovered. *A. bisporus* laccase was immobilized on a ceramic-chitosan support using GLU, resulting in a 51% immobilization yield and improvements in the thermal, operational and storage stabilities.[720,721] Recently, another polymer, poly(vinyl alcohol) cryogel particles (PVA), was used for the covalent immobilization of the commercial laccase Roglyr Lite 1540. GLU was employed as the crosslinking agent and β-alanine as a spacer. The immobilized laccase exhibited a lower specific activity than the free enzyme, but enhancements in the stability and operational pH range could be observed.[722] Alumina [685] and Granocel have been commonly used as covalent supports. The latter was selected for the study of *C. unicolor* laccase immobilization after the modification of several support characteristics, including the functionalization, surface density and pore size. GLU and divinyl sulfone were employed as bifunctional agents. The least favorable results were obtained with carriers containing –OH or –OH and –COOH groups.[723]

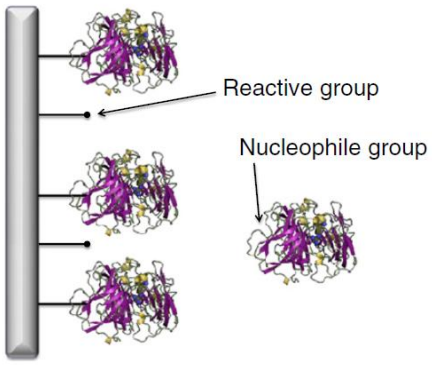


Figure 25. Chemical interactions for enzyme immobilization: Covalent binding between the nucleophilic groups of the enzyme and the support.[685]

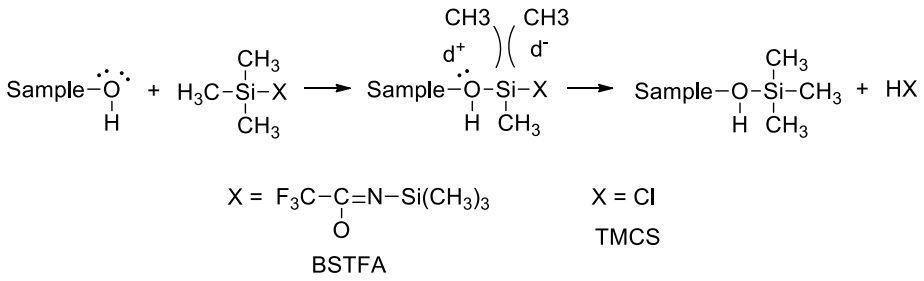
RESULTS AND DISCUSSIONS

CHAPTER 5

CHARACTERIZATION OF PHENOL LOW-MEDIUM MOLECULAR WEIGHT

The extracts were characterized by GC-MS analysis. The samples, before of analysis, were silylated with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1% Trimethylchlorosilano (TMCS), this reaction leads to the formation of trimethyl silyl ether on the hydroxyl functions (active hydrogen) present on the molecule. (**Scheme 5.1**) These products silyl derivatives are more volatile, less polar and more thermally stable.

BSTFA is very versatile, reacting with a range of polar organic compound and replacing active hydrogens with a $–Si(CH_3)_3$ (trimethylsilyl) group. BSTFA and its by-products (trimethylsilyltrifluoroacetamide and trifluoroacetamide) are more volatile than many other silylating reagents, causing less chromatographic interference, and the hydrogen fluoride, a by-product of silylation reduces detector (FID) fouling. TMCS increases the reactivity of BSTFA, and amides, many secondary amines and hindered hydroxyls, incompletely derivatized by BSTFA alone, can be derivatized by adding TMCS.



Scheme 5.1. Mechanism of silylation reaction.

The silylation products were analysed by GC-MS analysis and the chromatograms were processed by MS Data Review program. Each peak with related fragmentation spectrum was compared with fragmentation spectrums present in a library specially built and a commercial libraries.

The quantification was made through the internal standard method. Each molecule, used for library construction, was silylated in the same conditions of the samples and a rate known internal standard (oleic acid) is added to the silylation reaction. From the areas and mmol of molecules and the internal standard was calculated the conversion factor according to the relation

$$f = \frac{\text{Area molecule}}{\text{Area Standard}} \times \frac{\text{mmol standard}}{\text{mmol molecule}}$$

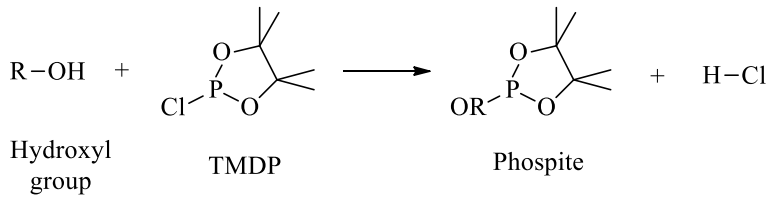
The conversion factors were used for the quantification of molecules on the each chromatograms.

CHAPTER 6

CHARACTERIZATION OF OLIGOPHENOLS AND POLYPHENOLS HIGH MOLECULAR WEIGHT

6.1 Lignins

The extracts were characterized by ^{31}P-NMR analysis. In particular, the different hydroxyl moieties in isolated lignins have been identified by a ^{31}P–NMR technique that involves derivatization with the phosphorylating agent 2–chloro–4,4,5,5–tetramethyl–1,3,2–dioxaphospholane (TMDP). (**Schema 6.1**). TMDP reacts with hydroxyl moieties to give phosphite products which are resolvable by ^{31}P–NMR into separate regions arising from aliphatic hydroxyl, phenolic, and carboxylic acids groups. (**Figure 6.1)**



Schema 6.1. Phosphorylation reaction

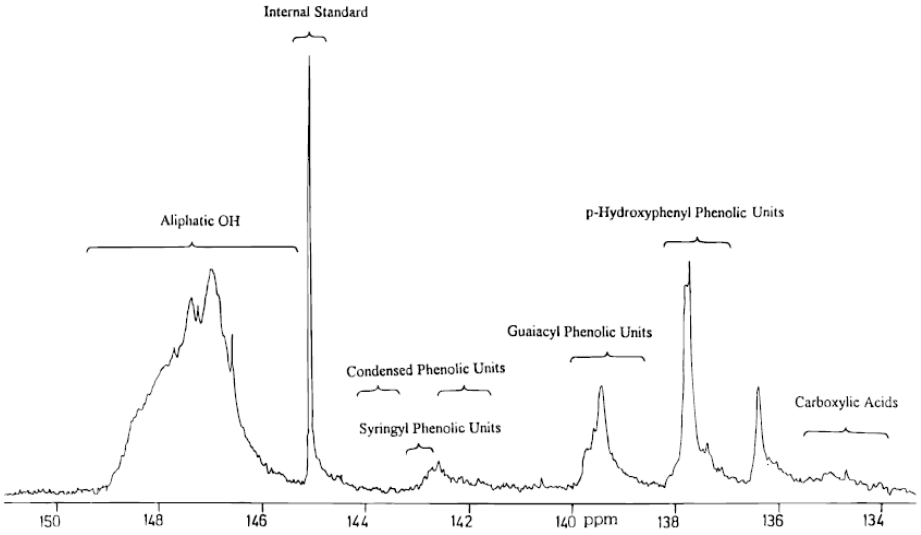


Figure 6.1. 31P-NMR of a tipically lignin phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. (C.Crestini, D.S. Argyropoulos, *J. Agric. Chem.*, **1997**, 45, 1212-1219)

6.2 Tannins

The extracts were characterized by ^{31}P-NMR analysis after derivatization with the phosphorylating agent 2–chloro–4,4,5,5–tetramethyl–1,3,2–dioxaphospholane (TMDP). (**Schema 6.1**)

Assignment of signals was accomplished by comparison between the spectrum obtained with reference spectra known in the literature.[724,725] The classification of tannins into hydrolyzable (gallo- and ellagic-tannins) and condensed tannins was performed by comparison with authentic samples.

In the first analysis, we compared the spectra of some basic components (**Figures 6.2, 6.3** and **6.4**) of tannins, and subsequently those of mixtures of tannins(**Figures 6.4** and **6.5., 6.6** and **6.7**).

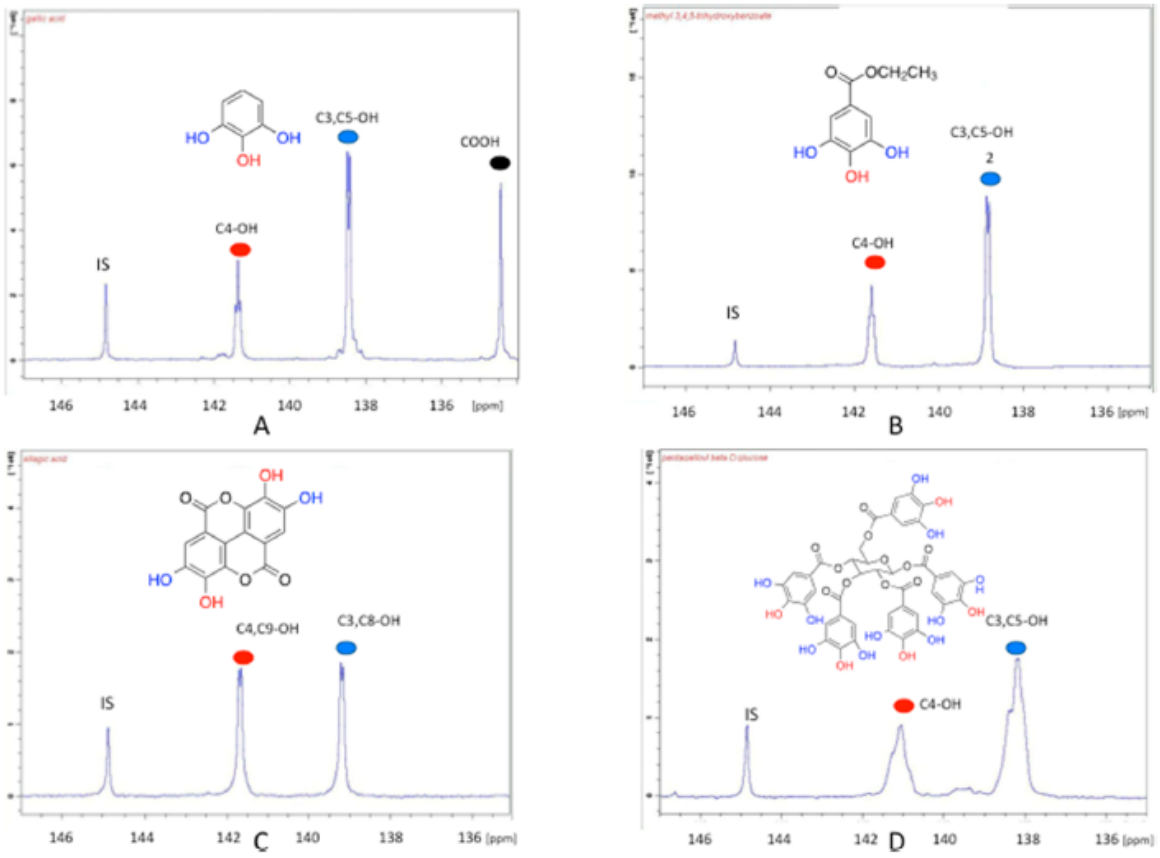


Figure 6.2. ^{31}P-NMR spectra of hydrolyzable tannin model compounds phosphitylated (**A**) gallic acid, (**B**) methyl gallate, (**C**) ellagic acid, (**D**) pentagalloyl D-glucose.

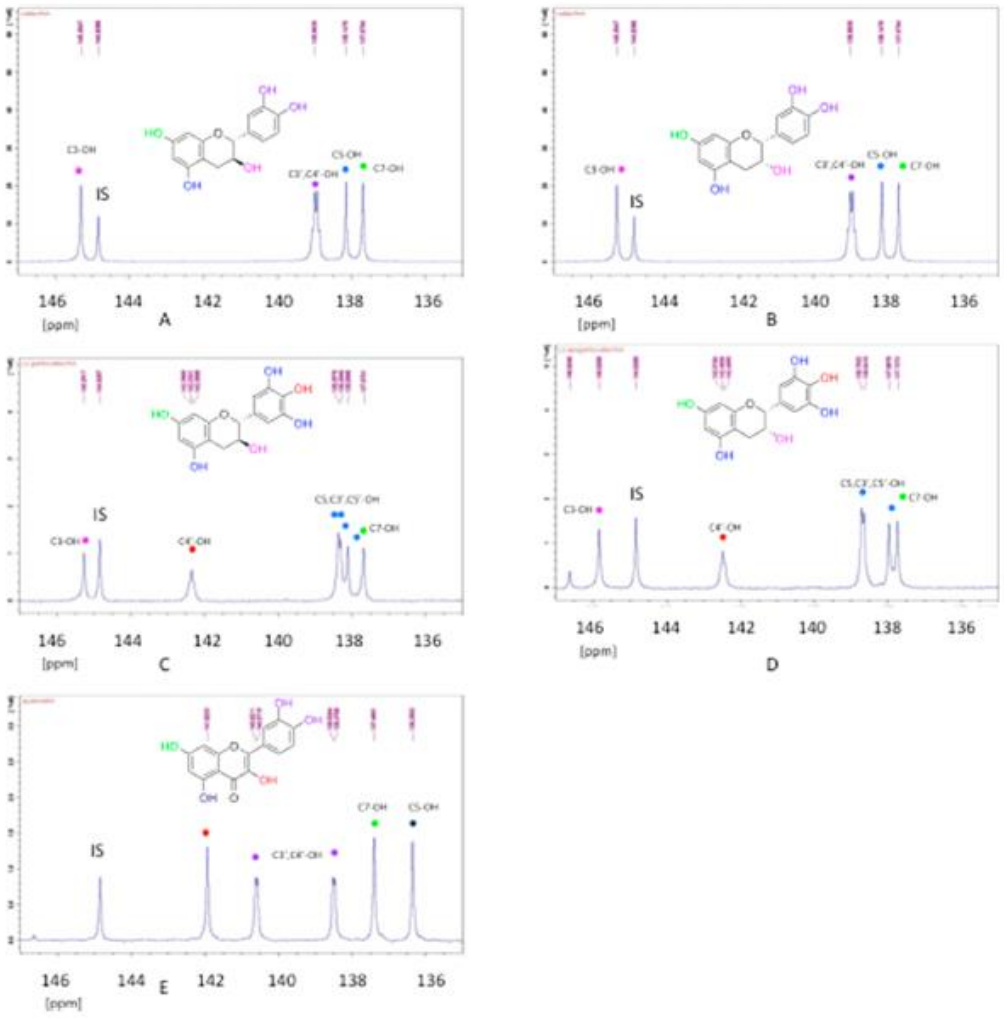


Figure 6.3. ^{31}P-NMR spectra of condensed tannin model compounds phosphitylated (**A**) catechin, (**B**) epicatechin, (**C**) gallocatechin, (**D**) epigallocatechin, (**E**) quercetin.

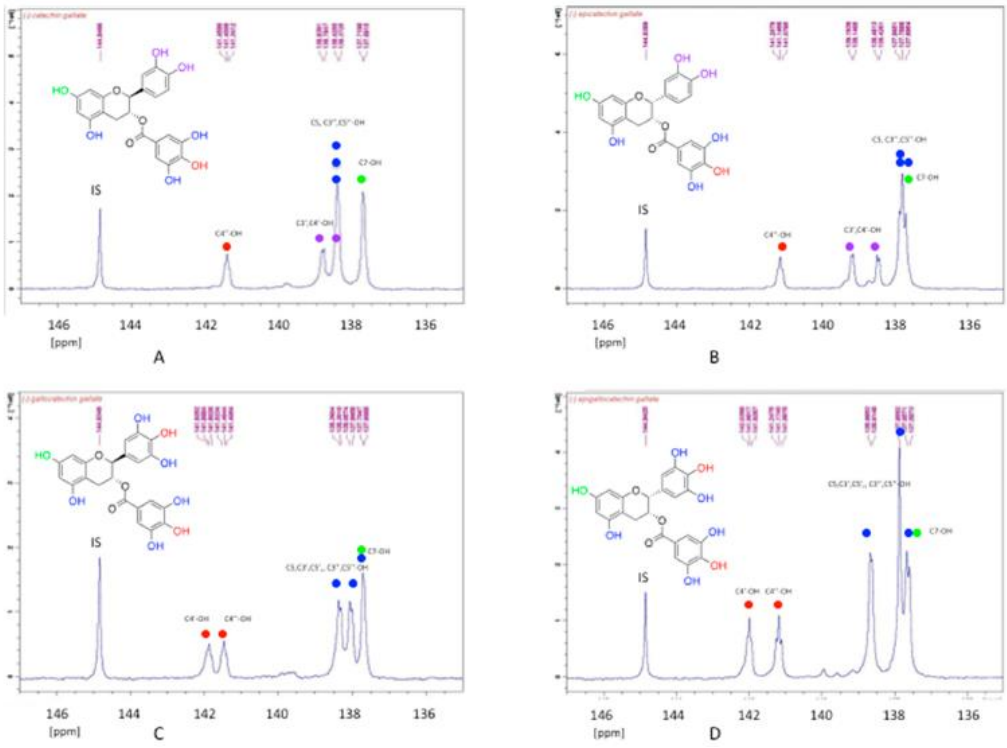


Figure 6.4. ^{31}P-NMR spectra of tannin model compounds phosphitylated (**A**) catechin gallate, (**B**) epicatechin gallate, (**C**) gallocatechin gallate, (**D**) epigallocatechin gallate.

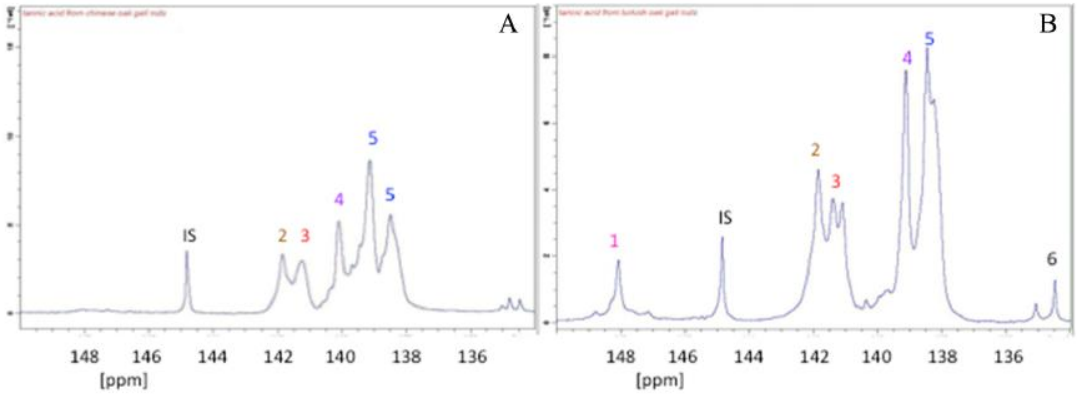


Figure 6.5. ^{31}P-NMR spectra of tannic acid extracted from (**A**) natural Chines oak gall and (**B**) natural Turkish oak gall after phosphitylation. Signal assignment: **1** aliphatic OH; **2** ortho-disubstituted phenol, internal gallate; **3** ortho-disubstituted phenol, terminl gallate; **4** ortho-substituted phenol; **6** COOH; **IS** internal standard.

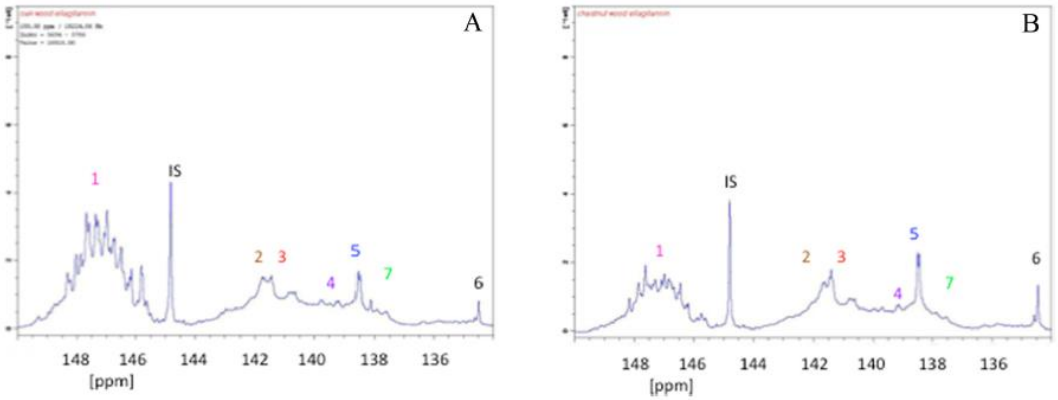


Figure 6.6. ^{31}P-NMR of ellegitannin extracted from (**A**) chestnut and (**B**) oak wood after phosphitylation. Signal assignment: **1** aliphatic OH; **2,3** ortho-disubstituted phenol; **4,5** ortho-substituted phenol; **6** COOH; **7** ortho-unsubstituted phenol; **IS** internal standard.

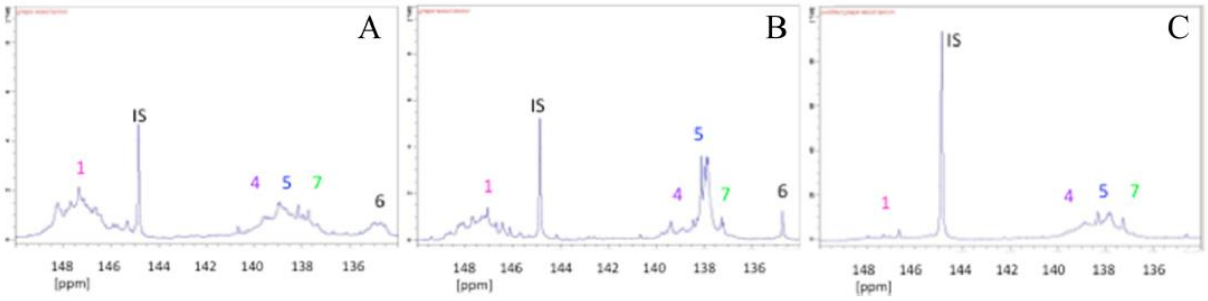


Figure 6.7. ^{31}P-NMR of tannin extracted from (**A**) grape seed, (**B**) grape wood and (**C**) purified grape wood tannin after phosphitylation. Signal assignment: **1** aliphatic OH; **4,5** ortho-substituted phenol; **6** COOH; **7** ortho-unsubstituted phenol; **IS** internal standard.

CHAPTER 7

CHARACTERIZATION OF SAMPLES

7.1 Buds of poplar

The genus poplar (*Populus* L.) belongs to the Salicaceae family; this plant is of interest because of its diversity, great resources, and wide distribution. The poplar possesses unique biological features (such as high rate of growth of aboveground organs, vigorous development of the root system, transpiration, and the photo synthesizing surface of leaves), indicating a high stability, high productivity, and exceptional competitiveness to slowly growing plants.[726] Several species of *Populus* have been used traditionally in medicine,[727,728] especially for their anti-inflammatory properties.[729,730] Poplar buds are coated with a viscous substance, an exudate, which was reported to contain different varieties of phenolic compounds, depending on the species studied: terpenoids, flavonoid aglycons and their chalcones and phenolic acids and their esters.[731,732,733] Among the *Populus* species, the black poplar (Populus nigra) is widely distributed in Europe. The chemical characterization of its bud exudate has allowed the identification among the flavonoid aglycons of some flavanones such as pinocembrin and pinostrobin, some flavonols such as galangin, quercetin and kaempferol, some flavones such as chrysin and apigenin,[734,735] and some esters of phenolic acids.[736] These compounds have also been reported in propolis,[737] the product collected by honeybees from tree buds, especially poplar buds.[738] Propolis has long been used in popular medicine to cure many diseases,[739] owing to its antimicrobial, anti-inflammatory and antioxidant properties.[740]

7.1.1 Sample characterization: Buds of poplar 146 (ABO-AR-2013-146)

Phenols medium-low molecular weight

1 gr of lyophilized samples was extracted in soxhlet for 6 h with petroleum ether, dichloromethane and methanol (80% water solution). The total amount for 1 gr of sample of three fractions was found to be: petroleum ether 160 mg, dichlorometane 536.5 mg and methanol 80% 46 mg. The fractions (dichloromethane and methanol 80%) were analysed

through GC-MS analysis after derivatization with silylating reagents, the chromatograms of each fraction are shown in **Figures 7.1.1 and 7.1.2**.

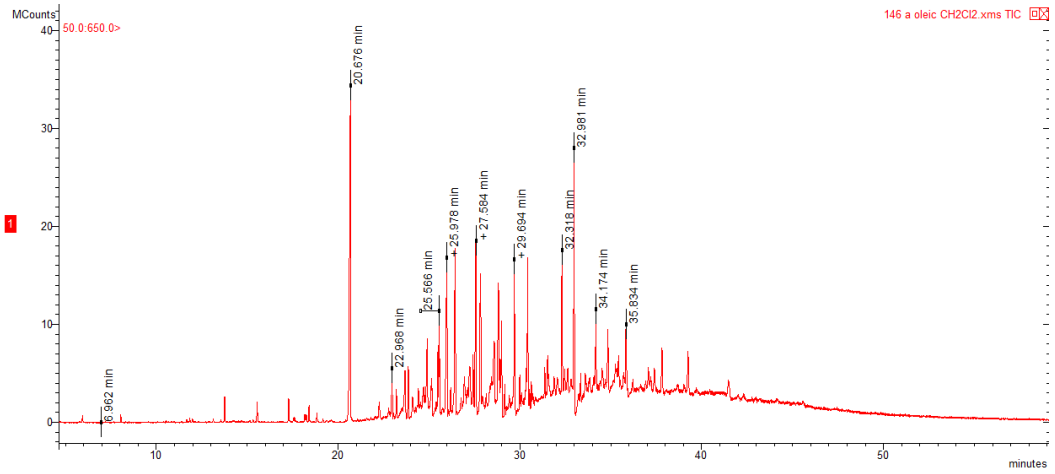


Figure 7.1.1. Chromatogram dichloromethane fraction.

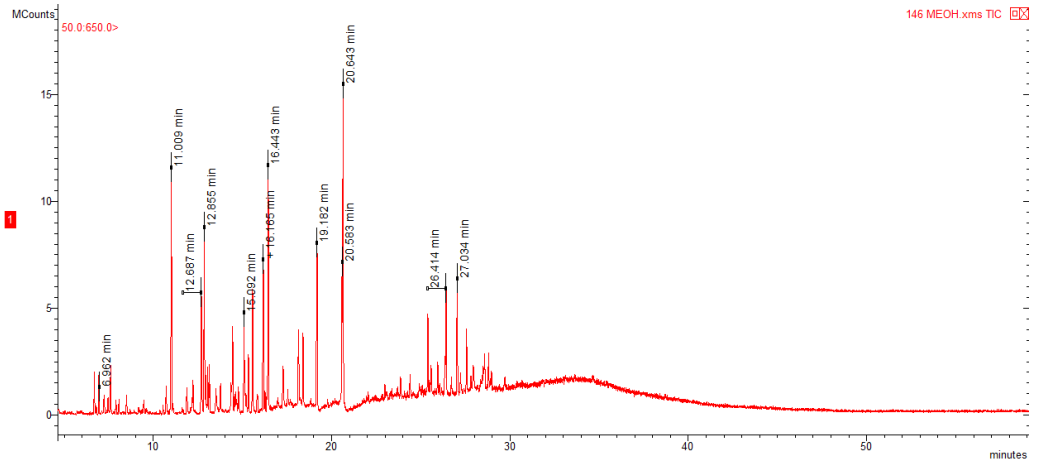


Figure 7.1.2. Chromatogram methanol (80% water solution) fraction.

The results of GC-MS analysis, grouped by family, are reported in **Table 7.1.1.** The quantities and the percentages referred to the total amount of phenols quantified for each family of compounds

Buds of poplar 146 Compound classes	Amount (mg/gr)	%
Aromatic acids and derivatives	2,053	0.94
Flavonols and Dihydroflavonols	70,363	32.11
Phenols and catechols	0,518	0.24
Flavones	25,263	11.53
Flavanones	15,821	7.22
Hydroxycinnamic acid and derivatives	57,026	26.02
Terpenophenols and terpenes	11,93	5.44
Catechins	0,488	0.22
Stilbenes	0,272	0.12
Hydroxychalcones	25,487	11.63
Isoflavones	0,061	0.03
Lignans	6,486	2.96
Glycosides	3,356	1.53

Table 7.1.1. Quantities and percentages for family of compounds.

Oligophenols and high molecular weight polyphenols

The sample extracted according to general procedure reported in previously paragraph was analysed through ^{31}P-NMR, after phosphitilation, for the detection of lignins and tannins (**Figure 7.1.3 and 7.1.4**). Note that melanins were not present in the sample. Starting from 1 gr of lyophilized sample we obtained: lignins 10.26 mg, tannins 31.5 mg and melanins 0 mg.

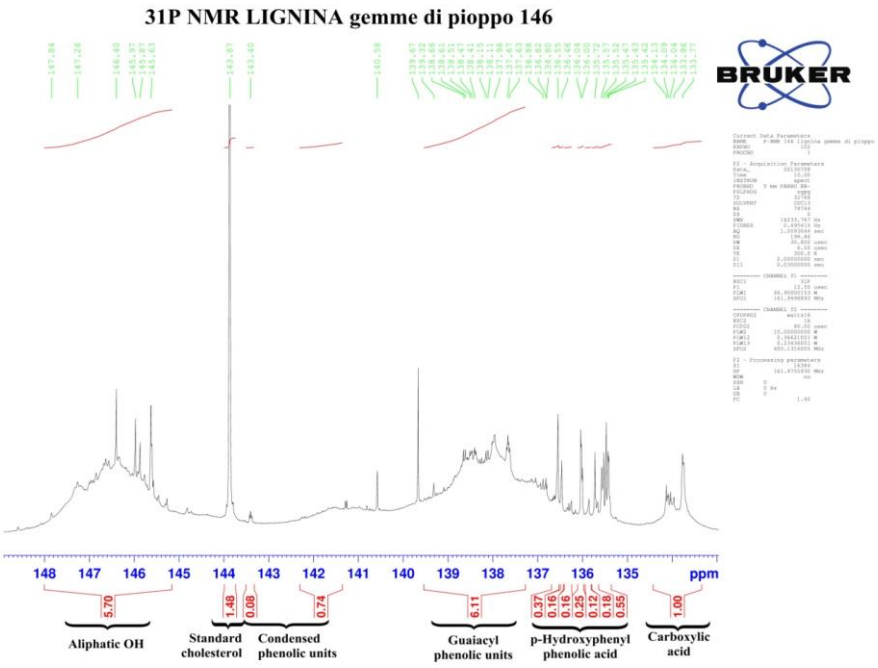


Figure 7.1.3. ^{31}P-NMR spectra of lignins

Table 7.1.2 shows the data for the assignment of the main families of phenolic and non-phenolic hydroxyl moietis in lignin, on the basis the comparison with what reported in the literature. The family of phenolic hydroxyl groups were found to be prevalent in quantity as phenolic units of guaiacyl type, followed by hydroxyl of aliphatic type, characteristic of the side and terminal chain of the polymer and the *p*-hydroxyphenyl units. The condensed phenolic units, characteristics of the degree of polymerization of the molecule, and the carboxylic acid units were present in similar amounts.

OH Groups	Chemical shift	mmol OH/gr of lignins
Aliphatic	147.84-145.63	1.27
Internal Standard (cholesterol)	143.87	
Condensed phenolic units	143.40-144.35	0.19
Syringyl phenolic units	-	-
Guaiacyl phenolic units	139.67-137.3	1.36
p-Hydroxyphenyl phenolic	136.55-135.52	0.39

units		
Carboxylic acids	134.19-133.91	0.19

Table 7.1.2. Assegment of ^{31}P-NMR spectra of lignins and OH mmol of groups.

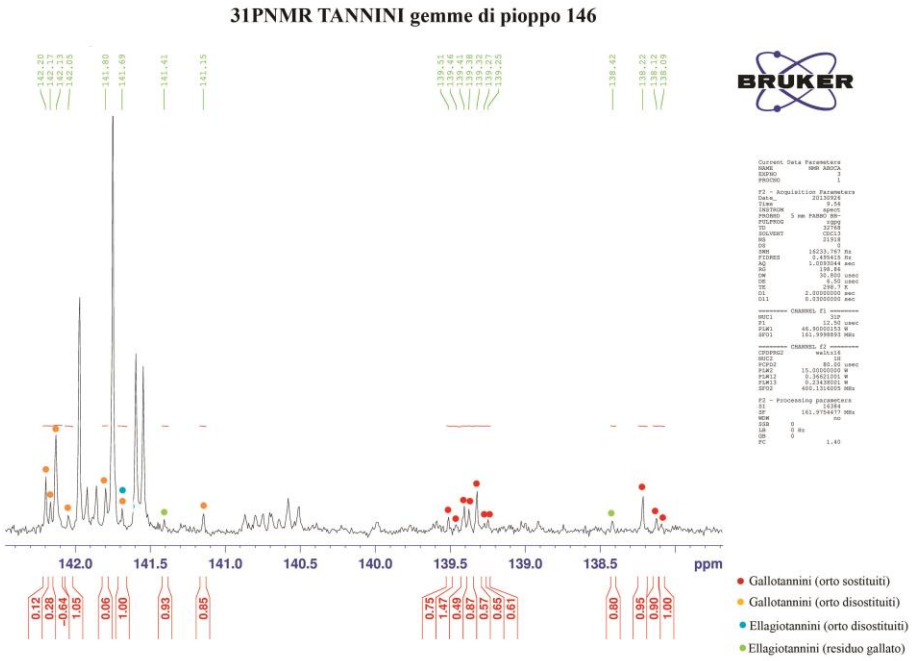


Figure 7.1.4. ^{31}P-NMR spectra of tannins.

Table 7.1.3 shows the qualitative and quantitative data of tannins. The sample is exclusively characterized by tannins of hydrolizable type, while there are not nuclear magnetic resonance signals attributable to condensed tannins. As it regards, the subclasses of hydrolizable tannins are predominating, the gallotannins (identified with a percentage of 81,75%) beging in higher amount than ellegiotannins (18,25%, on the total of hydrolizable tannins).

Tannins		**mg/g of tannins**	**% tannins classes**
Hydrolizable	Gallotannins	25.75	81.75%
	Ellagiotannins	5.75	18.25%
Condensed		0	0%

Table 7.1.3. Classification of tannins classes.

7.1.2 Cosideration data: Buds of poplar 146

On the basis of data previously reported it is possible to make some general considerations. Firstly, the total amount of phenols (including low and high molecular weigth derivatives) correspond to about 27% in weight of the sample (**Figure 7.1.5**). The remaining 73% is attributable to other families of natural substances whose presence is known in the exudates of poplar, including aminoacids, proteins, carbohydrates, terpenoids, oligomineral elements and others.

Analyzing in detail the total phenolic and polyphenolic fractions, it is observed that the phenolic compounds with low/medium molecular weight predominate on polyphenols. (**Figure 7.1.6**) In particular, the most abundant phenolic families (**Figure 7.1.7**), were: flavonols and dihydroflavonols, followed by hydroxycinnamic acids and their derivatives, and then flavones and hydroxychalcones. Other families of natural phenols have been identified in smaller percentages.

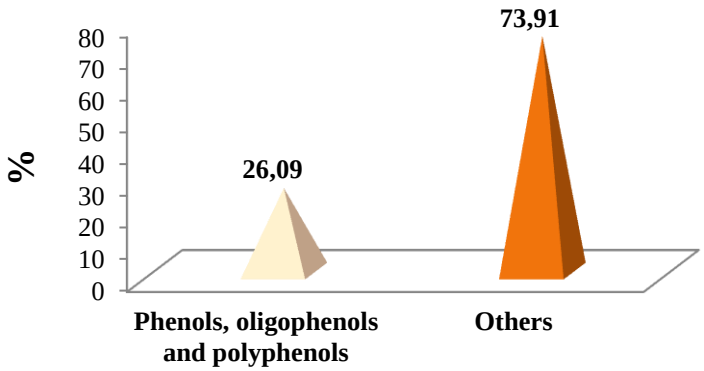


Figure 7.1.5. Percentage of phenols, oligophenols and polyphenols identified compared to the total sample.

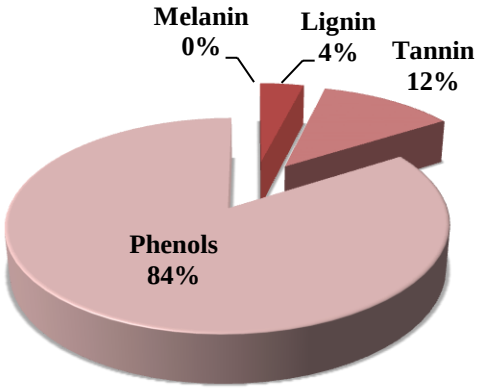


Figure 7.1.6. Percentage of phenols, tannins, lignins and melanins compared to the total amount identified.

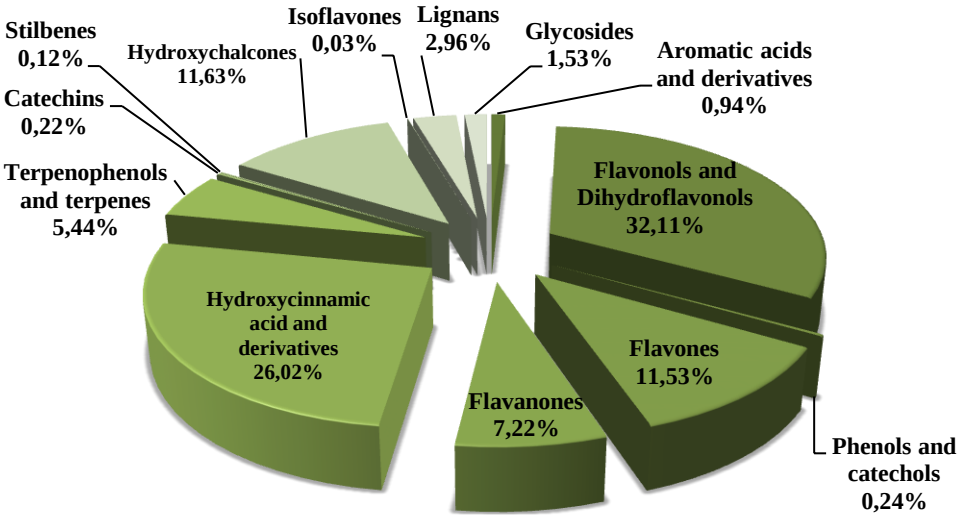


Figure 7.1.7 Percentage of families of phenols compared to the total amount phenols identified.

7.1.3 Sample characterization: Buds of poplar 147 (ABO-AR-2013-147)

Phenols medium-low molecular weight

1 gr of lyophilized samples was extracted in soxhlet for 6 h with petroleum ether, dichloromethane and methanol (80% water solution). The total amount for 1 gr of sample of three fractions was found to be: petroleum ether 198 mg, dichlorometane 520 mg and methanol 80% 47 mg. The fractions (dichloromethane and methanol 80%) were analysed

through GC-MS analysis after derivatization with silylating reagents, the chromatograms of each fraction are shown in **Figure 7.1.8 and 7.1.9**.

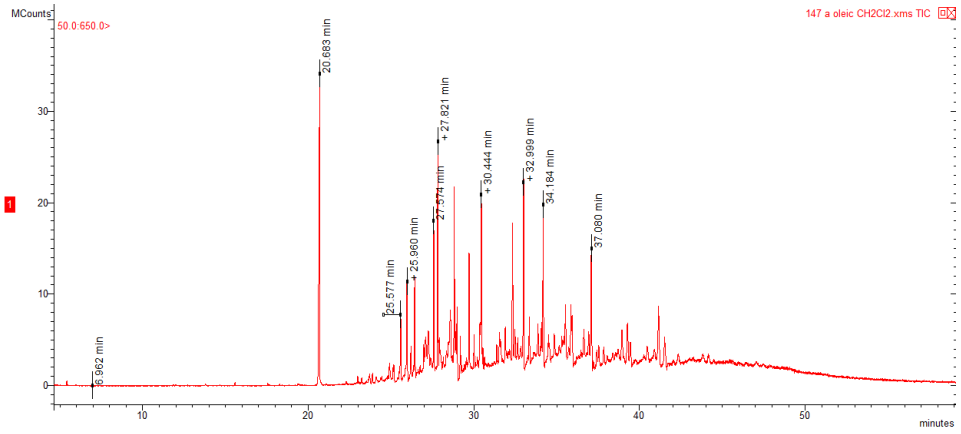


Figure 7.1.8. Chromatogram dichloromethane fraction.

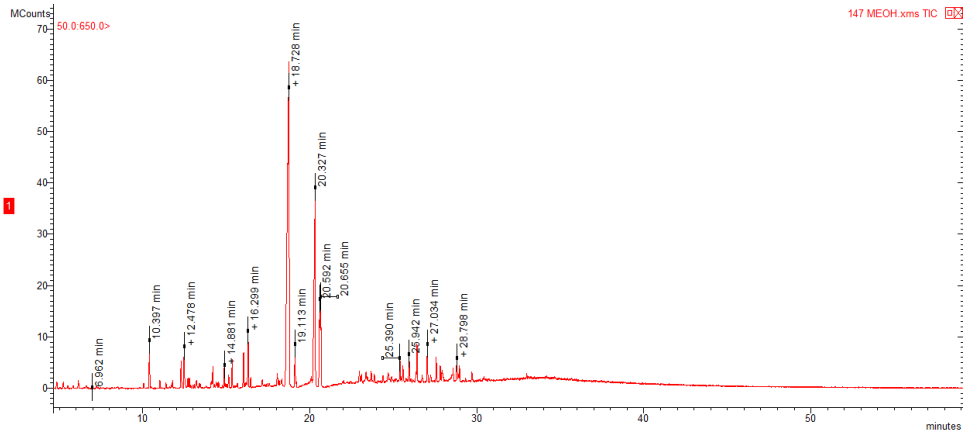


Figure 7.1.9. Chromatogram methanol (80% water solution) fraction.

The results of GC-MS analysis, grouped by family are reported in **Table 7.1.4**. The quantities and the percentages referred to the total amount of phenols quantified for each family of compounds.

Buds of poplar 147 Compound classes	Amount (mg/gr)	%
Aromatic acids and derivatives	0,214	0.07
Hydroxycinnamic acid and derivatives	9,376	3.00
Flavones	21,635	6.93
Stilbenes	25,071	8.03
Flavonols and Dihydroflavonols	192,771	61.76
Catechins	0,034	0.01
Terpenophenols and terpenes	3,84	1.23
Hydroxychalcones	3,271	1.05
Phenols and catechols	0,26	0.08
Isoflavones	2,208	0.71
Lignans	0,284	0.09
Flavanones	52,546	16.84
Glycosides	0,613	0.20

Table 7.1.4. Quantities and percentages for family of compounds.

Oligophenols and high molecular weight polyphenols

The sample treated according to general procedure reported in the previously paragraph was analysed through: ^{31}P-NMR, after phosphitilation, for the detection of lignins and tannins (**Figures 7.1.10 and 7.1.11**), and solubility assay and UV/Visible spectrophotometric analysis for the component of melanins (**Figures 7.1.12 and 7.1.13**). For 1 gr of lyophilized sample we obtained: lignins 8.26 mg , tannins 0.0052 mg and melanins 0.4 mg.

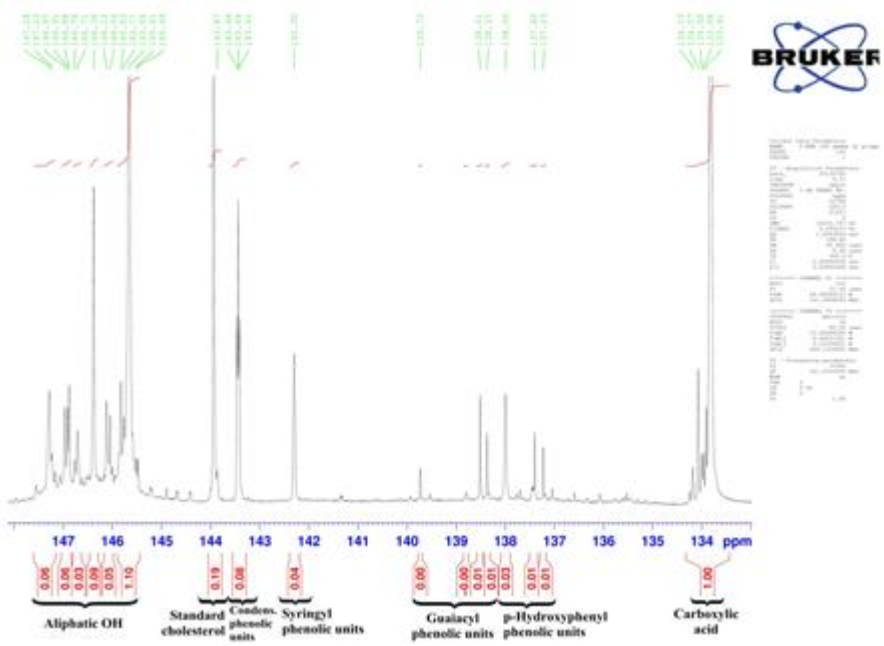


Figure 7.1.10. ^{31}P-NMR spectra of lignins

Table 7.1.5 shows the data for the assignment of the main families of phenolic and non-phenolic hydroxyl moietis in lignin,on the basis the comparison with what reported in literature. The family of phenolic hydroxyl groups were found to be in quantity as hydroxyl groups of aliphatic type. The condensed phenolic units, characteristics of the degree of polymerization of the molecule, and the carboxylic acid units are present in similar amounts. We found very low amounts of phenolic units of syringyl and *p*-hydroxyphenyl type, in the same amount, followed by phenolic units of guaiacyl type

OH Groups	Chemical shift	mmol OH/gr of lignins
Aliphatic	147.28-145.48	0.25
Internal Standard (cholesterol)	143.87	
Condensed phenolic units	143.46-143.41	0.15
Syringyl phenolic units	142.30	0.01
Guaiacyl phenolic units	139.73, 138.8, 138.51, 138.37	0.003
p-Hydroxyphenyl phenolic	138.00, 137.40, 137.23	0.01

units		
Carboxylic acids	134.19-133.91	0.18

Table 7.1.5. Assegment of ^{31}P-NMR spectra of lignins and OH mmol of groups.

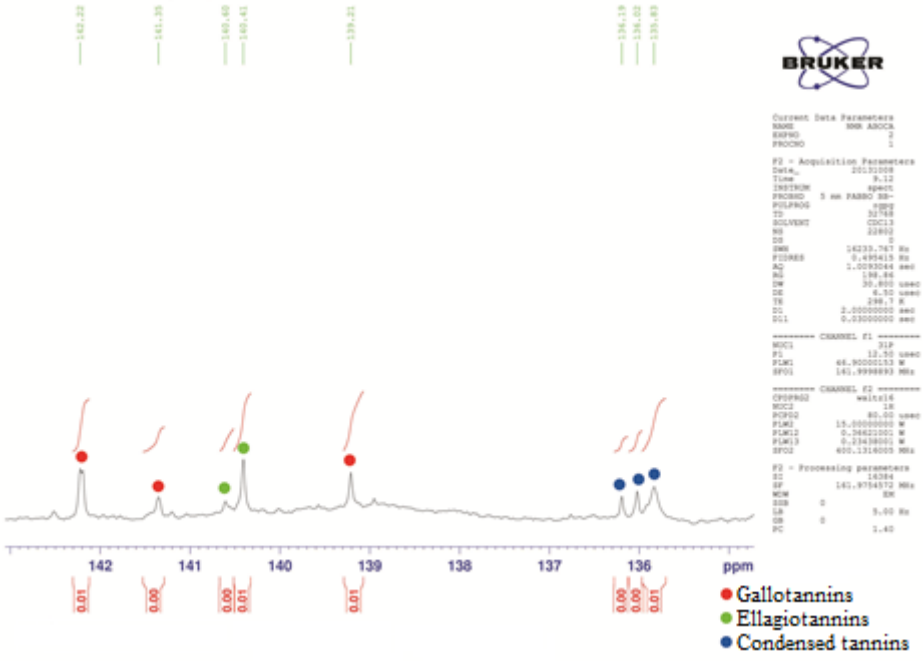


Figure 7.1.11. ^{31}P-NMR spectra of tannins.

Table 7.1.6 shows the qualitative and quantitative data of tannins. The sample present a predominant percentage of tannins of hydrolizable type (71%), compared to the condensed tannins (29%). As it regards, the subclasses of hydrolizable tannins are predominating, the gallotannins (identified with a percentage of 44.75%), being in higher amount than ellagiotannins (26.25%, on the total of hydrolizable tannins).

Tannins		**mg/g of tannins**	**% tannins classes**
Hydrolizable	Gallotannins	0.023	44.23%
	Ellagiotannins	0.014	26.92%
Condensed		0.015	28,85%

Table 7.1.6. Classification of tannins classes.

In the case of the sample of exudate poplar 147 (unlike the sample 146) it was possible to identify a significant amount of melanins equal to 0.4 mg/gr of analysed sample. As you can see in **Figure 7.1.12** the sample of melanin gave a positive response at all quanlitative standard tests. In particular, it was insoluble in H_2O and in acid solution (HCl 7 M), while an appreciable solubility was observed in basic conditions (KOH). Confirmation of melaninic structure was obtained through spectrophometric assay, by applying the conditions indicated previously, on which case the peak of maximum absorption at 220 nm was clearly evident (**Figure 7.1.13**).

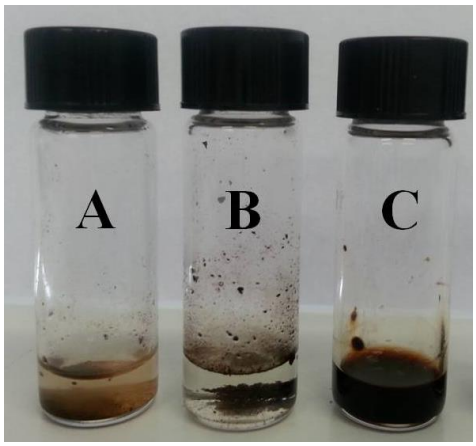


Figure 7.1.12. Solubility assay in: **A** H_2O, **B** HCl 7M, **C** KOH 1M

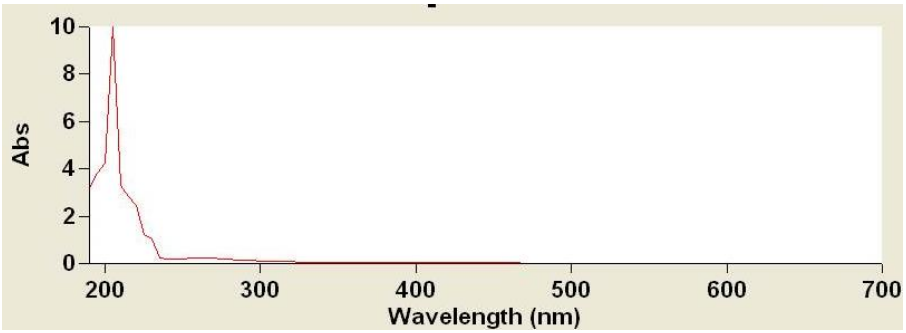


Figure 7.1.13. UV/Visible spectra of melanins

7.1.4 Consideration data: Buds of poplar 147

On the basis of data previously reported it is possible to make some general considerations. Firstly, the total amount of phenols (including low and high molecular weigth derivatives)

correspond to about 32% in weight of the sample (**Figure 7.1.14**). The remaining 68% is attributable to other families of natural substances whose presence is known in the exudates of poplar, including aminoacids, proteins, carbohydrates, terpenoids, oligomineral elements and others.

Analyzing in detail the total phenolic and polyphenolic fractions, it is observed that the phenolic compounds at low/medium molecular weight predominate on polyphenols. (**Figure 7.1.15**) In particular, in the most abundant phenol families (**Figure 7.1.16**), it was possible to identify: flavonols and dihydroflavonols followed from flavanones and then from stilbenes and flavones. Other families of natural substance have been identified in smaller percentages.

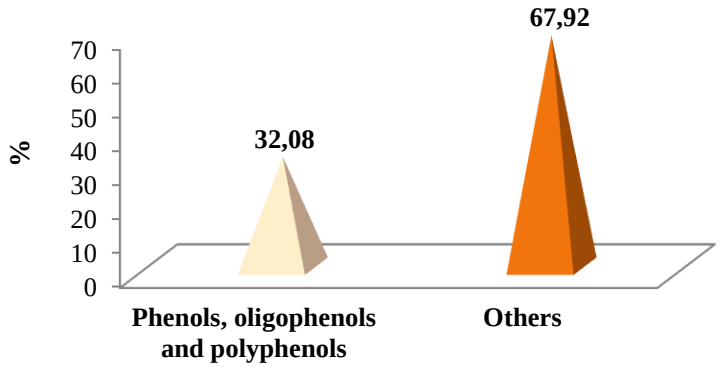


Figure 7.1.14 Percentage of phenols, oligophenols and polyphenols identified compared to the total sample.

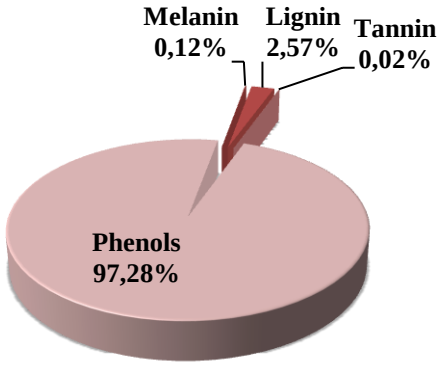


Figure 7.1.15. Percentage of phenols, tannins, lignins and melanins compared to the total amount identified.

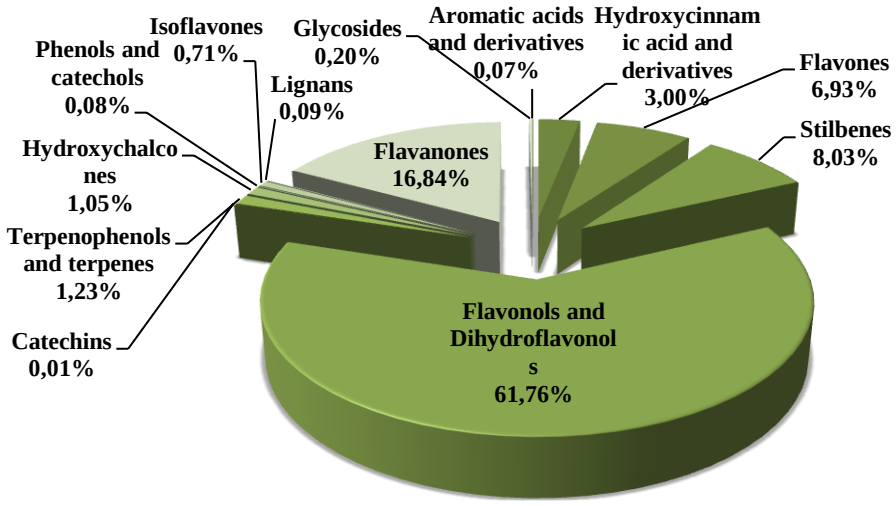


Figure 7.1.16. Percentage of families of phenols compared to the total amount phenols identified.

7.2 Propolis (XXPRODL0)

Propolis is a natural resinous substance collected by bees from parts of plants, buds and exudates.[741] Bees use it as a sealer for their hives[742] and, more importantly, to prevent the decomposition of creatures which have been killed by bees after an invasion of the hive[743]. Characteristically, it is a lipophilic material, hard and brittle when cold but soft, pliable, and very sticky when warm, hence the name beeglue.[744] It possesses a pleasant aromatic smell, and varys in color, depending on its source and age.[745] The main types of chemical substances found in propolis are waxes, resins, balsams, aromatic and ethereal oils, pollen and other organic matter.[746] The proportion of these types of substances varies and depends on the place and time of collection.[746,747] In general, propolis is composed of 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances, including organic debris.[748] The compounds identified in propolis resin originate from 3 sources: plant exudate collected by bees; secreted substances from bee metabolism; and materials which are introduced during propolis elaboration.[741,749]

Propolis shows antibacterial, antiviral, antifungal, antioxidant, antiinflammatory, immunostimulating and cytostatic properties.[750,751,752] The antimicrobial property of propolis has been widely investigated, confirming its antibacterial, antiviral, antifungal and antiprotozoan activities.[753,754] Some authors reported that propolis is active against Gram-positive bacteria, showing only limited activity against Gram-negative bacteria.[755] Propolis

extracts have been reported to potentiate some antibiotic effects attributing the antibacterial propolis activity mainly to flavonoids or to a synergism between some phenolic components.[756]

Many other biological and pharmacological properties of propolis have been described by various authors, including regeneration of cartilaginous tissue, bone tissue and dental pulp, anaesthetic activity, hepatoprotective activity, increasing the number of plaque-forming cells in the spleen of populations of immunized males, immunomodulatory action, immunogenic properties, liver detoxifying action, choleretic and antiulcer action in vitro, antioxidant activity, anticaries in rats, protection agent against gamma irradiation in mice, antileishmaniosis in hamster, antitrypanosomal agent and inhibition of dihydrofolate reductase activity.[757]

Besides pharmacological activity, an important point refers to its botanical origin and the consequent variation in chemical composition in samples when samples from different locations, and even from the same locality, are compared.[758] Despite the chemical differences, it is well known that samples of different geographical origin and chemical composition usually demonstrate similar biological activity.[762,759]

More than 300 compounds, among which polyphenols, terpenoids, steroids, sugars and amino acids have been detected in raw propolis. Their abundance is influenced by botanical and geographical factors, as well as by the collection season.[760,761,762] Literature survey revealed that flavonoids, aromatic acids, diterpenic acids and phenolic compounds appear to be the principal components responsible for the biological activities of propolis samples.[763]

7.2.1 Sample characterization

Phenols medium-low molecular weight

1 gr of lyophilized samples was extracted in soxhlet for 6 h with petroleum ether, dichloromethane and methanol (80% water solution). The total amount for 1 gr of sample of three fractions was found to be: petroleum ether 136.75 mg, dichlorometane 717.25 mg and methanol 80% 102.5 mg. The fractions were analysed through GC-MS analysis after

derivatization with silylating reagents, the chromatograms of each fraction are shown in **Figures 7.2.1 and 7.2.2**.

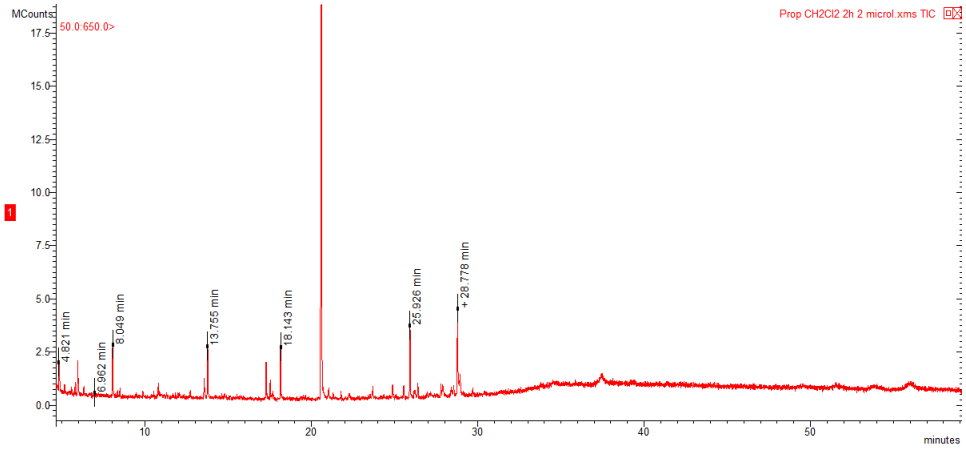


Figure 7.2.1. Chromatogram dichloromethane fraction.

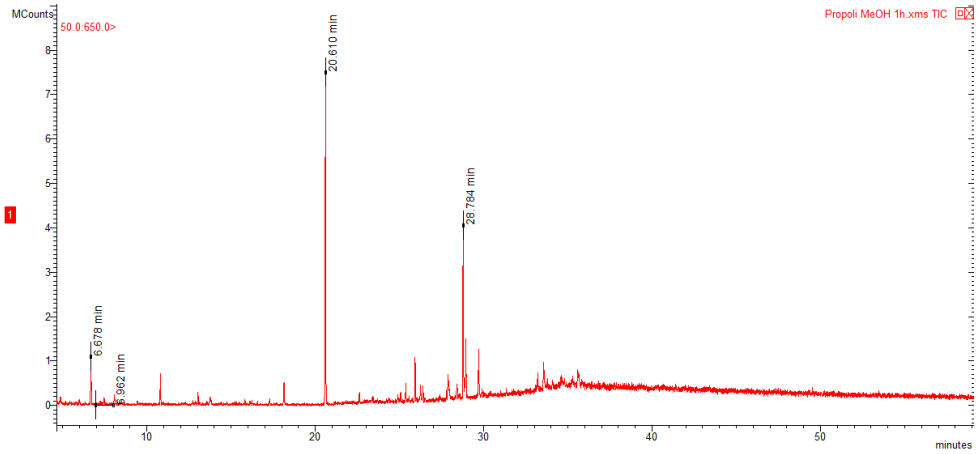


Figure 7.2.2. Chromatogram methanol (80% water solution) fraction.

The results of GC-MS analysis, grouped by family, are reported in **Table 7.2.1**. The quantities and the percentages referred to the total amount of phenols quantified for each family of compounds.

Propolis Compound classes	Amount (mg/gr)	%
Aromatic acids and derivatives	0,139	0.06
Flavonols and Dihydroflavonols	62,841	25.24
Flavones	45,07	18.10
Flavanones	46,54	18.69
Hydroxycinnamic acid and derivatives	37,984	9.83
Terpenophenols and terpenes	15,015	6.03
Isoflavones	16,08	6.46
Hydrochalcones	24,471	9.83
Glycosides	0,83	0.33

Table 7.2.1. Quantities and percentages for family of compounds.

Oligophenols and high molecular weight polyphenols

Starting from 1 gr of lyophilized sample we obtained: tannins 244.58 mg , lignins 0 mg and melanins 0 mg.

On the basis of data previously reported it is possible to make some general considerations. Firstly, the total amount of phenols (including low and high molecular weigth derivatives) correspond to about 27% in weight of the sample (**Figure 7.2.3**). The remaining 50,65% is attributable to other families of natural substances.

Analyzing in detail the total phenolic and polyphenolic fractions, it is observed that the phenolic compounds with low/medium molecular weight and tannins, belonging at polyphenols class are present in similar amounts. (**Figure 7.2.4**). In particular, among the most abundant (**Figure 7.2.5**) phenol families, it was possible to identify: flavonols and dihydroflavonols, followed from flavanones, flavones and then from hydroxychalcones, isoflavones and terpenophenols and terpenes. Other families of natural substance have been identified in smaller percentages.

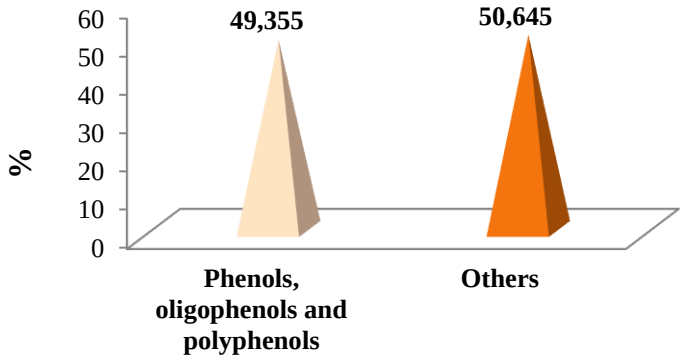


Figure 7.2.3. Percentage of phenols, oligophenols and polyphenols identified compared to the total sample.

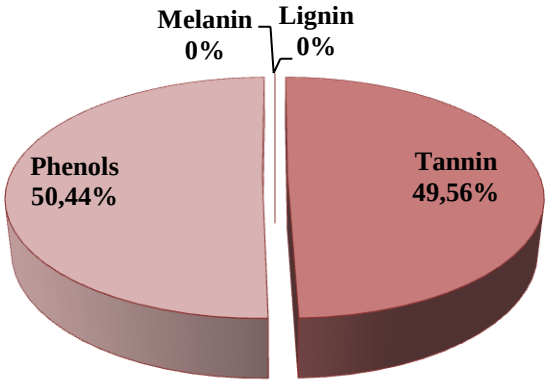


Figure 7.2.4. Percentage of phenols, tannins, lignins and melanins compared to the total amount identified.

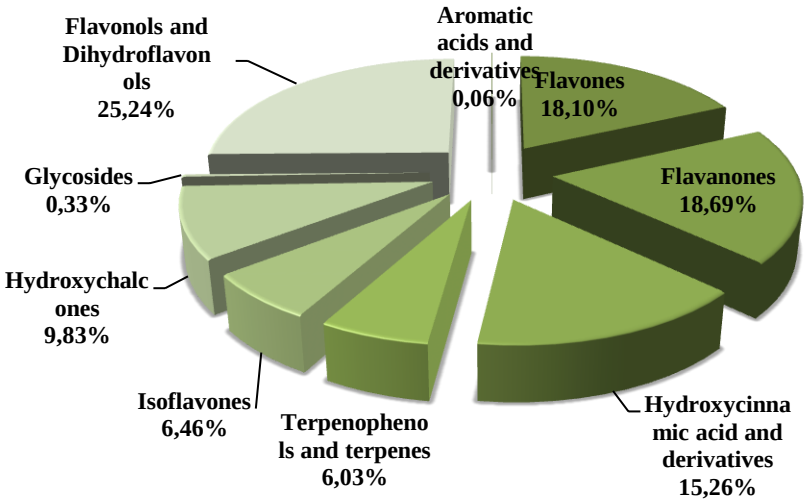


Figure 7.2.5. Percentage of families of phenols compared to the total amount phenols identified.

7.3 Comparison between buds of poplar and propolis

Regarding total quantity of phenols present in the samples, propolis is the sample more representative, it contains a quantity of 49,36% of these substances, following of two exudates of poplar, that present comparable quantities, slightly higher in the case of 147 sample. (**Figure 7.3.1**)

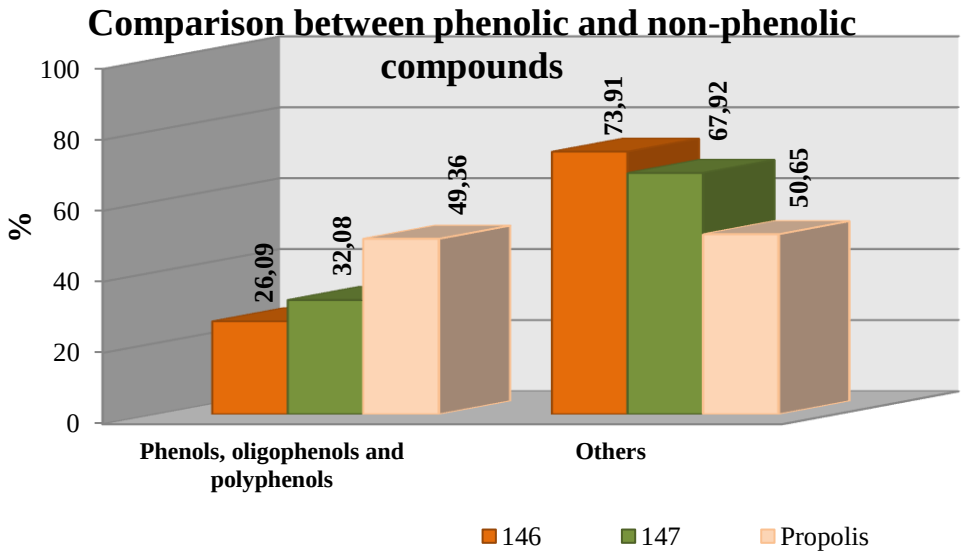


Figure 7.3.1. Percentage of phenols, oligophenols and polyphenols identified compared to the total sample identified in samples of buds of poplar 146 and 147, and propolis.

Other informations can be obtained by comparing the different polyphenols families and low/medium molecular weight phenols. From this comparison, is evident that propolis contains greater amount of polyphenols, compared to exudates of poplar, and significantly lower quantity of phenols at low/medium molecular weight. In particular, tannins were more abundant in propolis compared to exudates of poplar. (**Figure 7.3.2**)

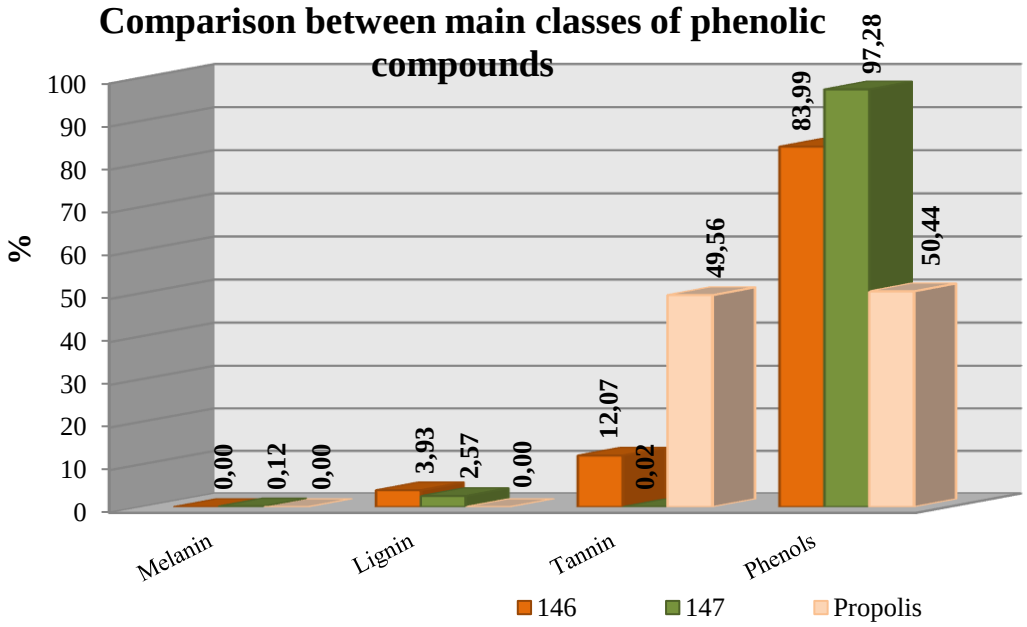


Figure 7.3.2. Percentage of phenols, tannins, lignins and melanins compared to the total amount identified in samples of buds of poplar 146 and 147, and propolis.

Histograms (**Figures 7.3.3 and 7.3.4**) reported the results of comparison for the single subclasses of phenols at low-medium molecular weight. The general trend of phenols in propolis and in exudates of poplar is similar, confirming the relation between such exudates and the origin of propolis. This relationship is highlighted in the literature, as regards to propolis product by bees in the temperate zone of Mediterranean, including falls also our country. In detail, we can observe that the families more abundant in propolis and in the exudates of poplar are flavonols and dihydroflavonols, hydroxycinnamic acids and derivatives and flavanones with flavones. Propolis shoes the highest amount of isoflavones, while the lignans are characteristic of two exudates of poplar, begin not present in the sample of propolis. Also glycosides have a significant presence, although of the same order of greatness of other subclasses, as catechins, aromatic acids and hydroxychalcones.

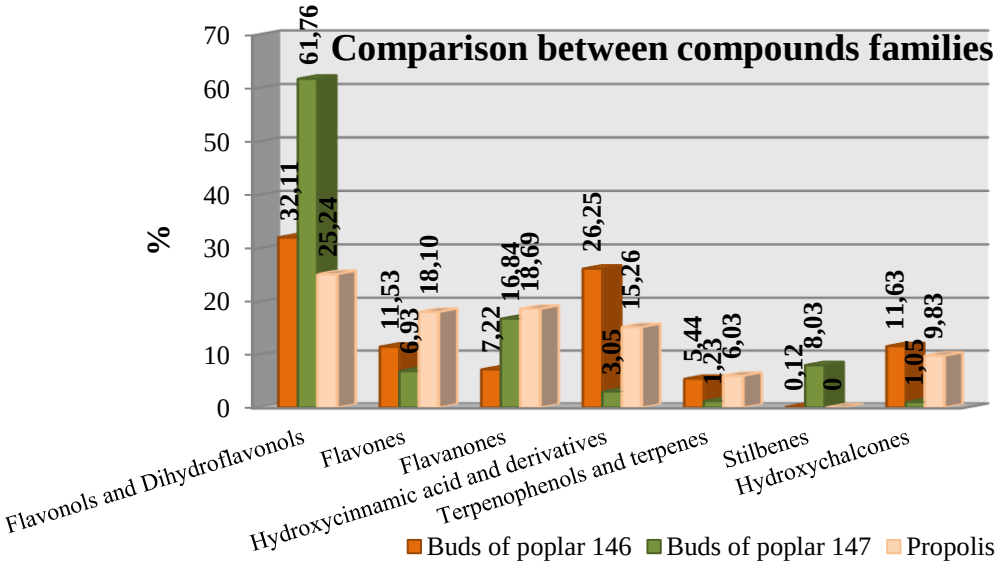


Figure 7.3.3 Results of comparison for the single subclasses of phenols at low-medium molecular weight.

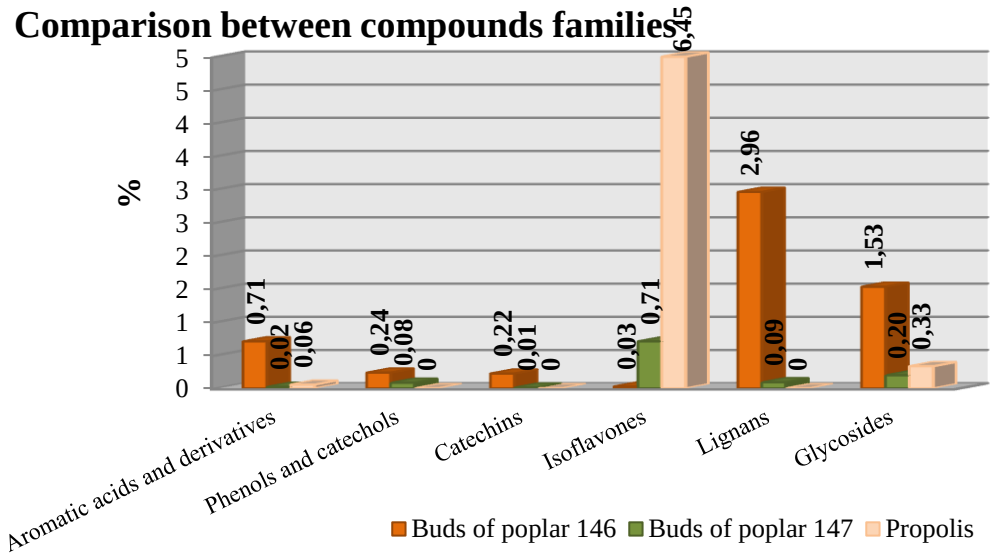


Figure 7.3.4 Results of comparison for the single subclasses of phenols at low-medium molecular weight.

7.4 *Filipendula* (ABO-AR-2013-511)

Genus *Filipendula* Mill. Includes 10 species which occur in temperate and subartic regions of the Northern hemisphere. Some of them are used in phytotherapy, others are grown as decorative or even fodder plants.[764] It is a medicinal plant that has been used in folk medicine for centuries in Europe and Asia. Flowers, herb and underground organs (rhizomes and tubers forming on the roots) are used as medicinal raw materials since they are rich in tannins and

polyphenolic acids. Flowers also contain flavonoids and essential oil.[765,766] The raw materials reveal antibacterial, antiinflammatory and antipyretic activity.[765,767] A heparin-like complex found in Filipendulae ulmariae flos had anticoagulant and fibrinolytic effects in several in vivo experiments.[768,769] Various extracts of meadowsweet flowers showed strong immunomodulatory activity towards the classical pathway of complement activation.[770] The flavonoid complex is considered to be responsible for the antiulcerogenic activity of the drug.[771,772] Furthermore, results of experiments showed that extracts of this plant decrease the permeability of capillaries and produce pronounced anticoagulant, antiulcerous, antidiabetic, and antitumor effects.[773]

7.4.1 Sample characterization

Phenols medium-low molecular weight

1 gr of lyophilized samples was extracted in soxhlet for 6 h with petroleum ether, dichloromethane and methanol (80% water solution). The fraction of methanol was resuspended in ethyl acetate and it was washed with water. The total amount for 1 gr of sample was found to be: petroleum ether 4.2 mg, dichlorometane 3.45 mg, methanol 80% 842.9 mg, and only 241,07 mg after treatment with ethyl acetate.

The fractions were analysed through GC-MS analysis after derivatization with silylating reagents, the chromatograms of each fraction are shown in **Figures 7.4.1, 7.4.2, 7.4.3** and **7.4.4**.

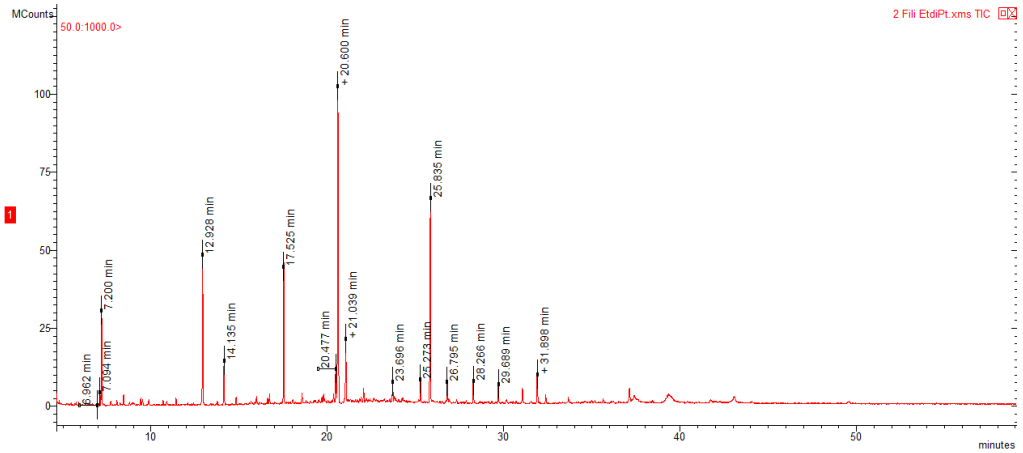


Figure 7.4.1. Chromatogram of petroleum ether fraction.

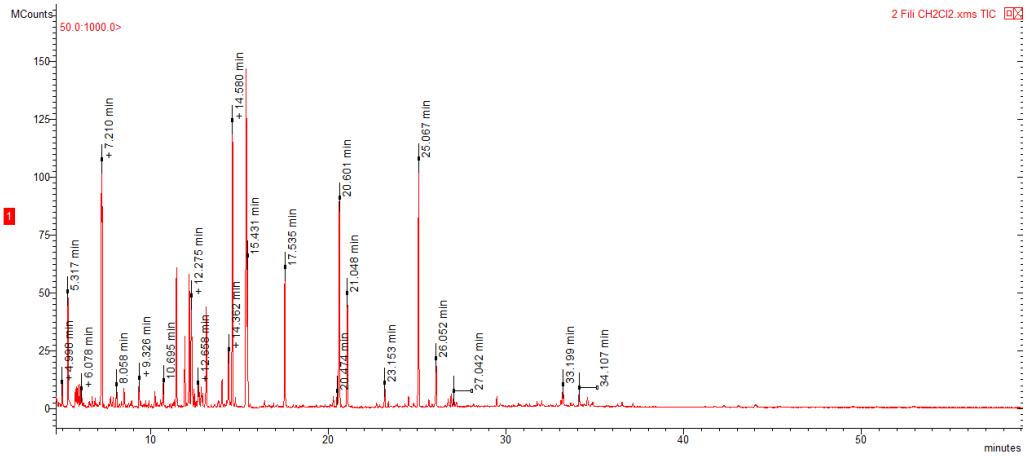


Figure 7.4.2. Chromatogram dichloromethane fraction.

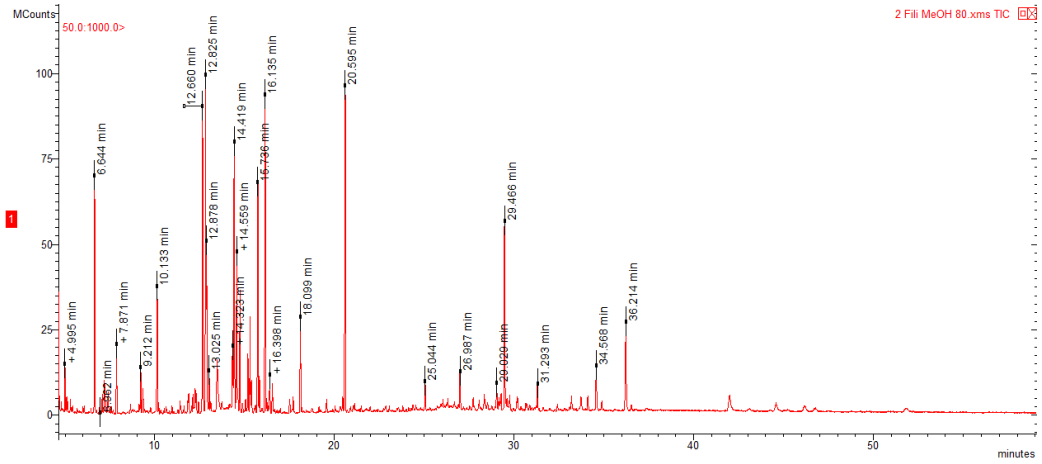


Figure 7.4.3. Chromatogram methanol (80% water solution) fraction.

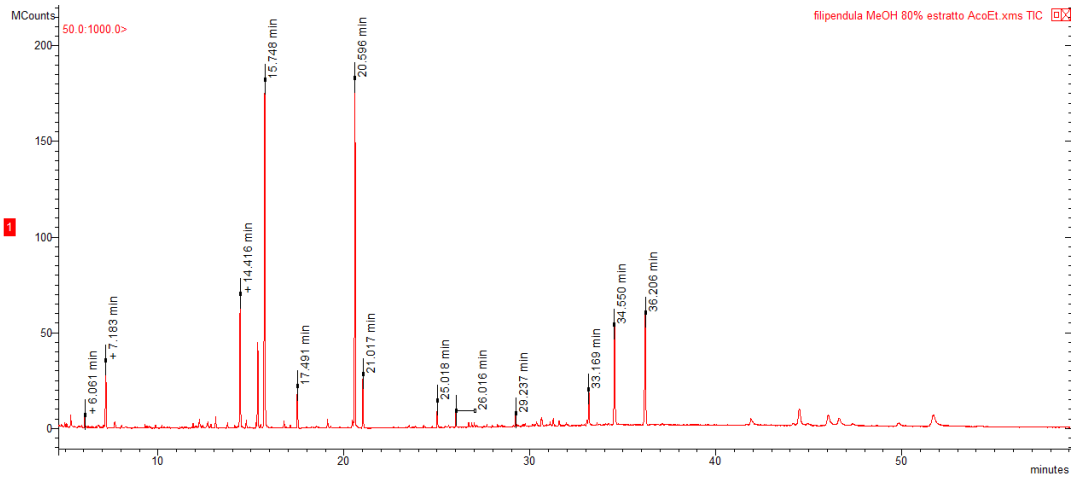


Figure7.4.4. Chromatogram of ethyl acetate fraction.

The results of GC-MS analysis, grouped by family, are reported in **Table 7.4.1**. The quantities and the percentages referred to the total amount of phenols quantified for each family of compounds.

Filipendula Compound classes	**Amount (mg/gr)**	**%**
Aromatic acids and derivatives	205,842	46,92
Hydroxycinnamic acid and derivatives	2,305	0,53
Flavonols and Dyhydroflavonols	149,636	34,11
Phenols and catechols	0,0004	0,0001
Flavanones	0,007	0,002
Isoflavones	0,001	0,0002
Catechins	5,771	1,32
Flavanonols	0,041	0,01
Stilbenes	0,004	0,001
Glycosides	75,089	17,12

Table 7.4.1. Quantities and percentages for family of compounds.

Oligophenols and high molecular weight polyphenols

The sample treated according to general procedure reported in previously paragraph was analysed through ^{31}P-NMR, after phosphitilation, for the detection of tannins (**Figures 7.4.5**)

Note that melanins were not present in the sample. Starting from 1 gr of lyophilized sample were obtained: lignins 4.66 mg, tannins 104.41 mg and melanins 0 mg.

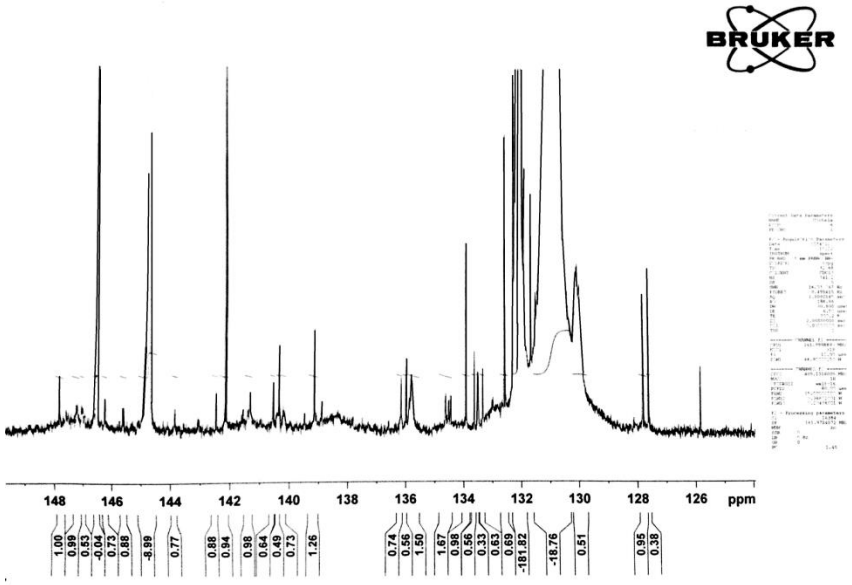


Figure 7.4.5. ^{31}P-NMR spectra of tannins.

Table 7.4.2 shows the qualitative and quantitative data for the structural characterization of tannins. The sample present predominant amount of tannins of hydrolizable type (63,95%), compared to that of condensed tannins (36,05%). As it regards, the subclasses of hydrolizable tannins are predominating, the gallotannins (identified with a percentage of 61,82%) being in higher amount than ellagiotannins (38,18% on the total of hydrolizable tannins).

Tannins		**mg/g of tannins**	**% tannins classes**
Hydrolizable	Gallotannins	41,27	39,53%
	Ellagiotannins	25,49	24,41%
Condensed		37,64	36,05%

Table 7.4.2. Classification of tannins classes.

Glycosides

The qualitative and quantitative analysis of glycosides was performed by high performance liquid chromatograpy –mass spectrometry (LC-MS) due to their structural complexity and high polarity, which is a limiting factor in gas chromatography - mass spectrometry (GC-MS) also after applying the silylation procedure. The sample analyzed was that relative to the fraction methanol (80% water solution), that is the sample characterized by the highest concentration of glycosides. Analyses were conducted using the conditions reported in the **Table 7.4.3**, operating in gradient with acetonitrile and water acidified for the presence of formic acid.

Time	**Acetonitrile acid (C_2H_3N + CH_2O_2 0.5%)**	**Water acid (H_2O + CH_2O_2 0.5%)**	**Flow**	**Column**	**UV**	**MS**
0	5	95		Phenomenex		
20	25	75		(kinetex)		100-800
44	90	10	0.4 ml/min	100x4.6	280 nm	*m/z*
46	99	1		2.6 µm particle size		neg.
48	5	95		25° C		

Table. 7.4.3. Conditions of analysis LC-MS.

Figures 7.4.6 and 7.4.7 show the chromatogram in the optimal conditions and the ion current spectra at two fragmentation energies, 94 V and 150 V.

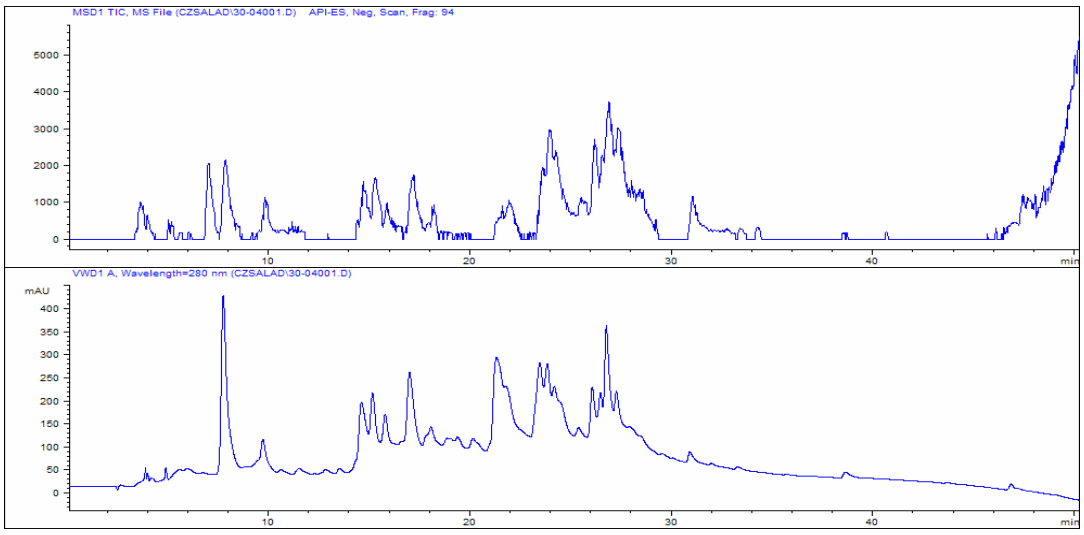


Figure 7.4.6. MS-chromatographic and UV profiles of sample at 94 V fragmentation energy.

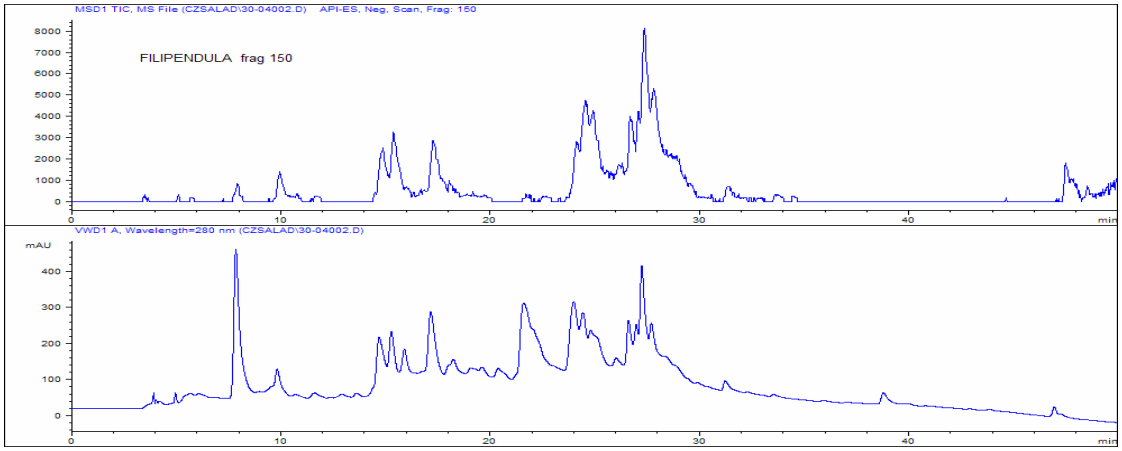


Figure 7.4.7. MS-chromatographic and UV profiles of sample at 150 V fragmentation energy.

The assignment of the glycosides was performed both by the addition method using appropriate standards, and by the method of the "search by ion" that allows to identify all those peaks in the chromatogram which corresponds to a mass fragmentation ion with the mass/charge ratio corresponding to substances to be detected.

Then we proceeded to final assignment of quantitative analysis of individual components applying the procedure of the "calibration line" and the "internal standard method". Below are two examples of assignment of chlorogenic acid and Quercetin-3-β-D glucoside, in which is shown the chromatographic profile analysis of sample (**Figures 7.4.8** and **7.4.10**) and the quantification of any molecule (**Figures 7.4.9** and **7.4.11, Tables 7.4.4** and **7.4.5**).

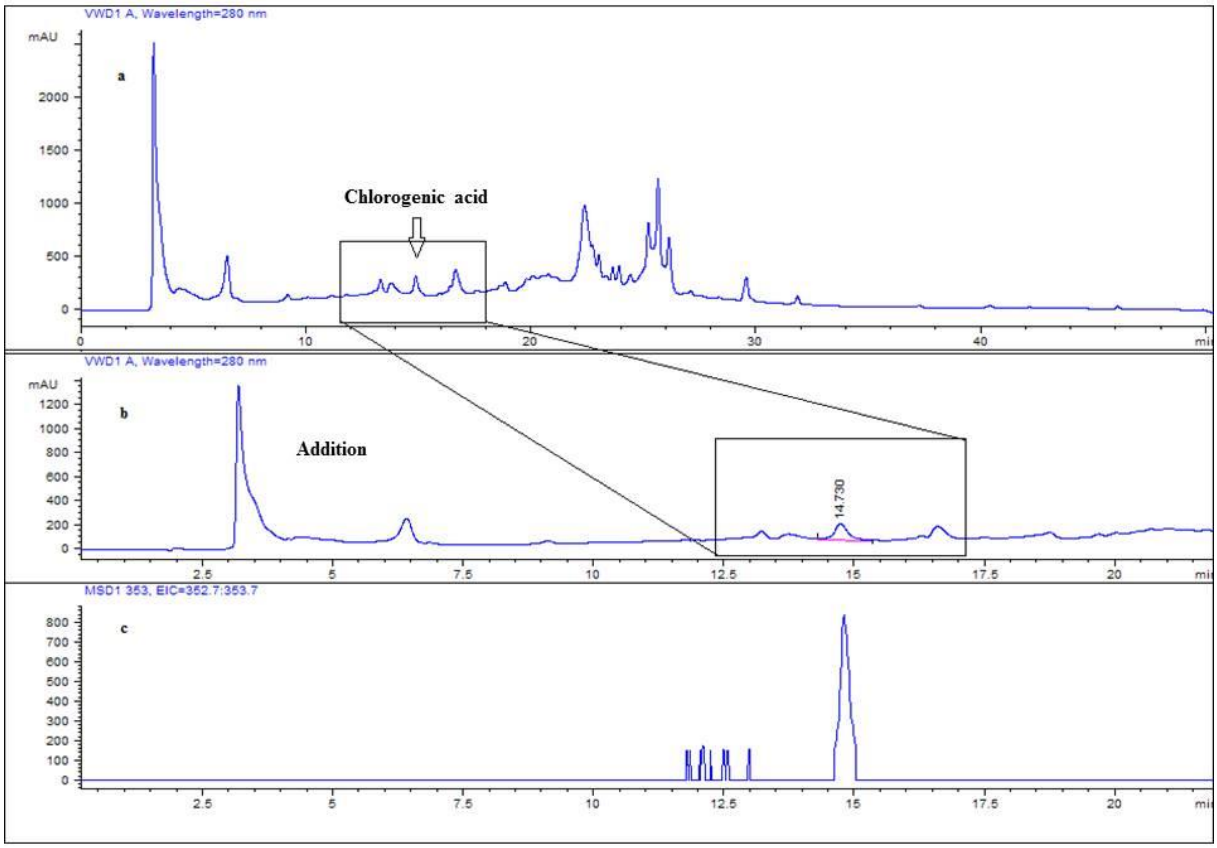
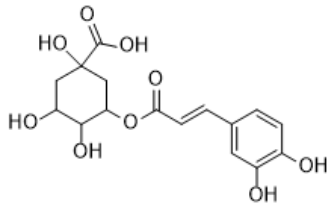


Figure 7.4.8. Chromatographic profile analysis of chlorogenic acid: **a** sample of filipendula 10 mg/ml; **b** sample addition; **c** ion extraction ion 353 m/z.



µg/ml	Average area
1	28,175
5	119,3
10	230,8
50	1170,1
100	2516,325

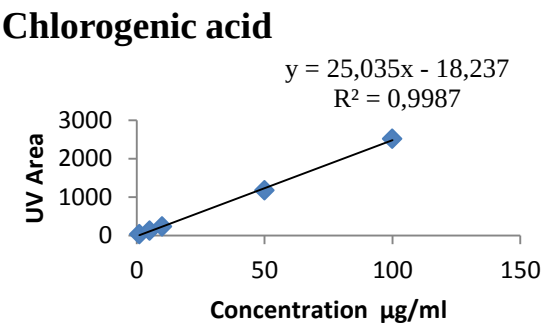


Figure 7.4.9. The average of the areas and the calibration curve.

Sample 1	2507.13	Addition 1	2238.00	Concentration from	100.60

				calibration line	
Sample 2	2493.56	Addition 2	2329.88	Concentration from addition	141.57
Average	2500.34	Media	2283.94	Average µg/mL	121.09±28.97

Table 7.4.4. Concentration calculation of chlorogenic acid.

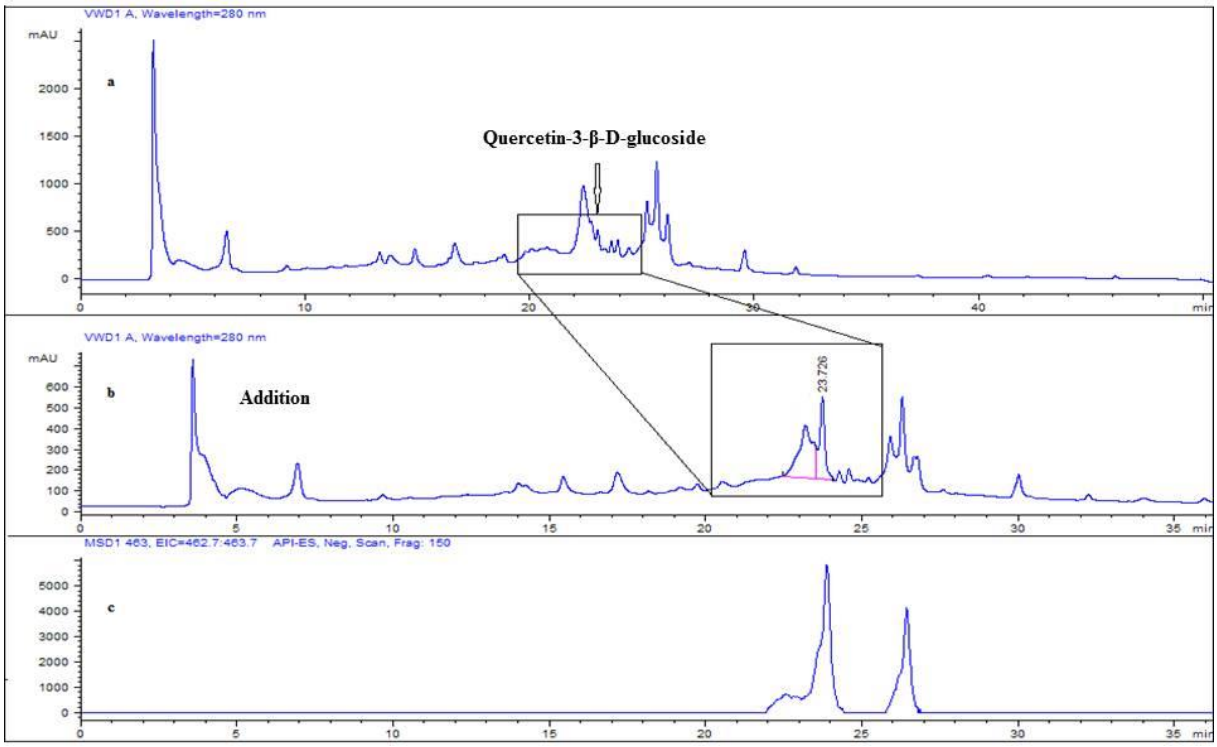
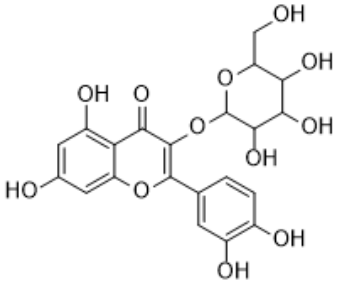


Figure 7.4.10. Chromatographic profile analysis of Quercetin-3-β-D glucoside: **a** sample of filipendula 10mg/mL; **b** sample addition; **c** ion extraction ion 463 m/z.



µg/ml	Average area
1	17,55
10	206,3
100	2154,87
500	11921,2

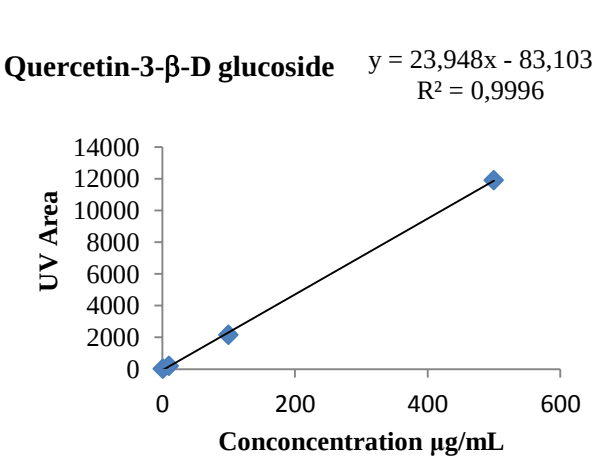


Figure 7.4.11. The average of the areas and the calibration curve.

Sample 1	1788.01	Addition 1	4133.59	Concentration from calibration line	75.75
Sample 2	1674.01	Addition 2	3988.33	Concentration from addition	82.79
Average	1731.01	Media	4060.96	Average µg/mL	79.27±4.98

Table 7.4.5. Concentration calculation of Quercetin-3-β-D glucoside.

The results of LC-MS analysis identified the presence of: Chlorogenic acid, Quercetin-3-D-Galactoside, Quercitrin, Kaempferol-3-O-rutinoside and Quercetin-3-β-D-glucoside. The quantities and the percentages refer to the total amount of glycosides quantified shows in **Table 7.4.1**.

7.4.2 Consideration data

On the basis of data previously reported it is possible to make some general consideration. Firstly, the total amount of phenols (including low and high molecular weigth derivatives) correspond to about 54,78% in weight of the sample (**Figure 7.4.12**). The remaining 45,22% is attributable to others families of natural compounds.

Analyzing in detail the total phenolic and polyphenolic fractions, it is observed that the phenolic compounds at low/medium molecular weight (80%) predominate on polyphenols. In the polyphenol class, the tannins represent 19% of total sample. (**Figure 7.4.13**) In particular, in the most abundant (**Figure 7.4.14**) phenol families, it was possible to identify: aromatic acids and their derivatives, followed by flavonols and dihydroflavonols, and then glycosides. Other families of natural substance have been identified in smaller percentages.

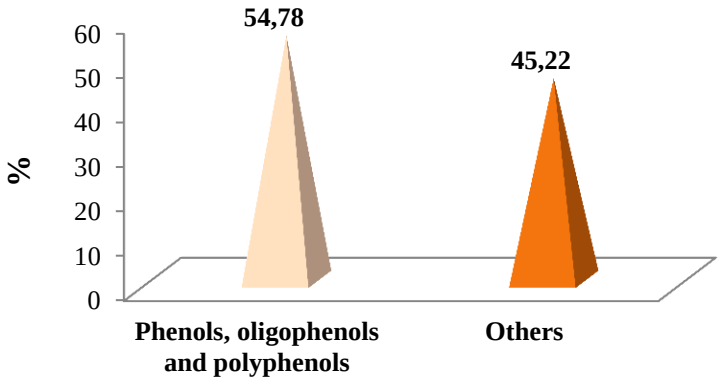


Figure 7.4.12. Percentage of phenols, oligophenols and polyphenols identified compared to the total sample.

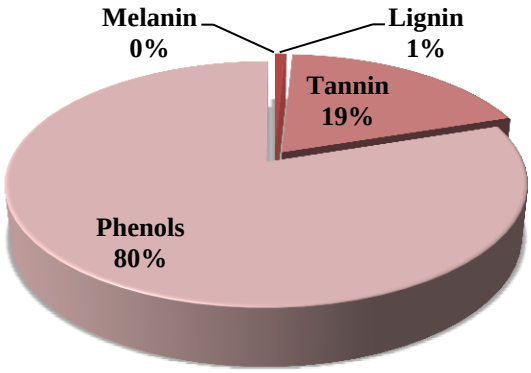


Figure 7.4.13. Percentage of phenols, tannins, lignins and melanins compared to the total amount identified.

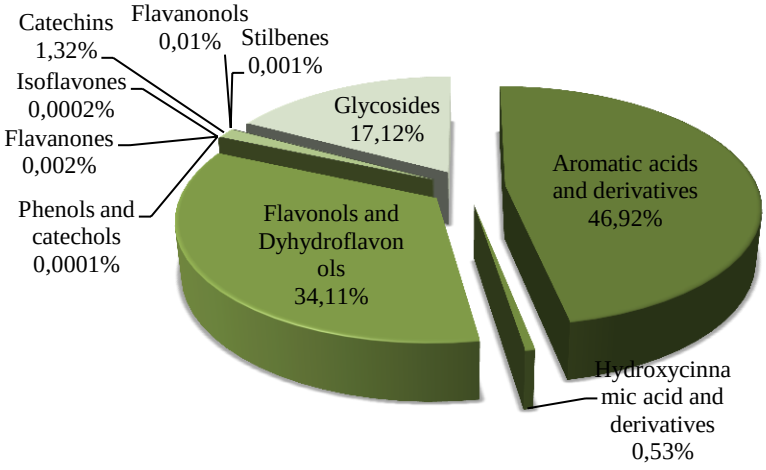


Figure 7.4.14. Percentage of families of phenols compared to the total amount phenols identified.

7.4.3 Biological and pharmatological activities of main phenolic compounds

For some of the most abundant phenolic compounds (gallic acid, methyl gallate, ellagic acid, quercetin, quercitrin and quercetin-3-D-galactoside) we report in the follow the main biological and pharmacological activities.

Gallic acid is a well known antioxidant compound with benign effects against various degenerative diseases, such as cardiovascular diseases, inflammation and cancer.[774] Gallic acid is described as an excellent free radical scavenger and as an inducer of differentiation and programmed cell death in a number of tumor cell lines. Recently, gallic acid from rose flowers exhibits antioxidant effects in senescence accelerated mice and can inhibit the activities of catalase and glutathione peroxidase.[775]

Methyl gallate is gallotannins found in a number of medical herbal and food plants. It has been reported to have various in vitro pharmacological activities, including anti-oxidant, anti-infiammation, anti-bacterial, anti-human immunodeficiency virus (HIV-1) and anti-cancer activities.[776] Furthermore, it was shown to be an anti-herpetic agent (Herpes simplex type 2).[777]

Ellagic acid is a naturally occurring polyphenolic compound which is found in many fruits, nut galls and plant extracts such as: strawberries, grapes, pomegranate, black currants,camu-camu, mango, guava, walnuts, almonds, longan seeds and green tea. Ellagic acid has been reported to possesses antimutagenic,antigenotoxic, anti-apoptotic, anticarcinogenic, antibacterial, antiviral, antimalarial, antiallergic, anti-inflammatory, antiatherogenic; antidiabetic, antiepileptic, antidepressant, antianxiety ,neuroprotective, pneumoprotective, nephroprotective, cardioprotective and hepatoprotective activities. Recently, it has been shown that urolithins are not only potent antioxidants, but that also have anti-inflammatory, anticarcinogenic, antimalarial, antidiabetic andantiaromatase properties. It exerts its beneficial effects by regulating multiple pathwaysincluding: activation of the antioxidant response through thenuclear erythroid 2-related factor 2 (Nrf2); inhibitionof proinflammatory agents, such as cyclooxygenase (COX-2) andcytokines by nuclear factor-kappa B (NF-κB); alteration of several growth factors expression, as the platelet-derived growthfactor (PDGF), transforming growth factor beta (TGF-β), hepaticgrowth factor (HGF); depletion of adhesion molecules,like vascular cell adhesion molecule-1 (VCAM-1) and intercellularadhesion molecule-1 (ICAM-1) among others; modulation of several cell survival/cell-cycle genes such

as cyclin D1 and E, p21, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), tumor suppressors (p53, DUSP6, Fos), oncogenes (K-Ras,c-Myc); regulation of kinases, like mitogen-activated pro-tein kinase (MAPK), phosphoinositide 3-kinase (PI3-K), glycogensynthase kinase 3 beta (GSK-3β).[778]

Regarding the quercetin, this compound shows to be an excellent in vitro antioxidant. Within the flavonoid family, quercetin is the most potent scavenger of ROS, including $O_2^{-\bullet}$, and RNS like NO and $ONOO^-$. These antioxidative capacities of quercetin are attributed to the presence of two antioxidant pharmacophores within the molecule that have the optimal configuration for free radical scavenging, i.e. the catechol group in the B ring and the OH group at position 3 of the AC ring. Moreover, it is known to possess strong anti-inflammatory capacities. Several in vitro studies using different cellines have shown that quercetin inhibits LPSinduced TNFα production in macrophages and LPS-induced IL8 production in lung cells (A549). Moreover, in glial cells it was even shown that quercetin can inhibit LPS-induced mRNA levels of two cytokines, i.e. TNFα and IL-1α. Furthermore, it has been shown in vitro that quercetin also possesses anti-fibrotic, anti-coagulative, anti-bacterial, anti-atherogenic, anti-hypertensive and anti-proliferative properties. Furthermore, quercetin is reported to directly modulate the gene expression of enzymes involved in biotransformation and to inhibit cell proliferation by interacting with estrogen binding sites.[779]

Quercitrin is a bioflavonoid with antioxidant properties and is better absorbed than other forms of quercetin. For example, studies have demonstrated that the absorption of quercetin glycosides contained in onions was higher (52%) than that of quercetin aglycones (24%).[780] In addition to its chemopreventive activity against a variety of tumors, quercitrin also functions as an agent against bacterial infection, allergic reaction, and H_2O_2 and UV-induced cell death and apoptosis. Moreover, some studies have proven the effectiveness of quercitrin in reducing ROS generation involved in the skin aging process and UVB-irritated inflammation,[781] and has been found to inhibit oxidation of low-density lipoproteins.[782]

Hyperoside (quercetin-3-D-galactoside) is a flavonoid compound, that has been shown to possess various biological functions against ROS induced damage, such as the antidepressant effect by inhibiting nitric oxide synthase in rat blood and cerebral homogenate, the inhibitory effect of linoleic acid peroxidation or deoxyribose degradation induced by ROS, the partial uncoupling effect of oxidative phosphorylation in cardiac mitochondria, and the protection of PC 12 rat pheochromocytoma against cytotoxicity induced by hydrogen peroxide and tert-butyl hydroperoxide.[783] Moreover, it preventing the free radicalinduced oxidation of vitamin

E in human low density lipoprotein, decreasing the total cholesterol, increasing superoxide dismutase activity and high density lipoprotein, protecting the apoptosis in rat cardiomyocyte induced by ischemia and reperfusion injury, protecting gastric mucosal injury in mice induced by ethanol, inhibiting Ca^{2+} influx induced by activation of G protein-coupled receptors, blocking voltage-dependent calcium to attenuate KCl induced increase of [Ca^{2+}], blocking N-methyl-D-aspartate receptor-linked Ca^{2+} channels to decrease [Ca^{2+}] in the neonatal rat brain cells.[784]

7.5 Artichoke (ABO-AR-2013-512)

Artichoke (*Cynara cardunculus* (L.) subsp. *scolymus* Hayek) is an herbaceous perennial plant native of North Africa, Canary isles, and Southern Europe. Artichoke leaves were used in ancient herbal medicine for a variety of diseases: it was widely used in Europe mainly for the treatment of dyspepsia; in fact it possesses a choleretic effect documented both in healthy volunteers and in people suffering from non-specific digestive disorders.[785,786] It is an important component of the Mediterranean diet, a good source of health-promoting compounds, and is recognized as being hepatoprotective, anticarcinogenic, antioxidative, antibacterial, anti-HIV, and bileexpelling, and having diuretic properties as well as the ability to inhibit cholesterol biosynthesis and low density lipoprotein (LDL) oxidation.[787] The bioactive compounds of artichoke are mainly polyphenols, in addition to inulin, fibres and minerals that provide nutritional and pharmaceutical properties.[787] The amounts of single bioactive compounds are extremely variable in relation to the portion of artichoke analysed (head, internal or external bracts, and leaves),[788] to harvest date, to genetic, physiologic and ambient factors, and is also affected by cooking.[789]The potential bioactivity of the artichoke is mainly related to the antioxidant properties of polyphenols.

7.5.1 Sample characterization

Phenols medium-low molecular weight

1 gr of lyophilized samples was extracted in soxhlet for 6 h with petroleum ether, dichloromethane and methanol (80% water solution). The fraction of methanol 80% was

resuspended in ethyl acetate and it was washed with water. The total amount for 1 gr of sample of four fractions was found to be: petroleum ether 5.3 mg, dichlorometane 4.5 mg and methanol 80% 803.65 mg, and only 221,81 mg after treatment with ethyl acetate.

The fractions were analysed through GC-MS analysis after derivatization with silylating reagents, the chromatograms of each fraction are shown in **Figures 7.5.1, 7.5.2, 7.5.3,** and **7.5.4**.

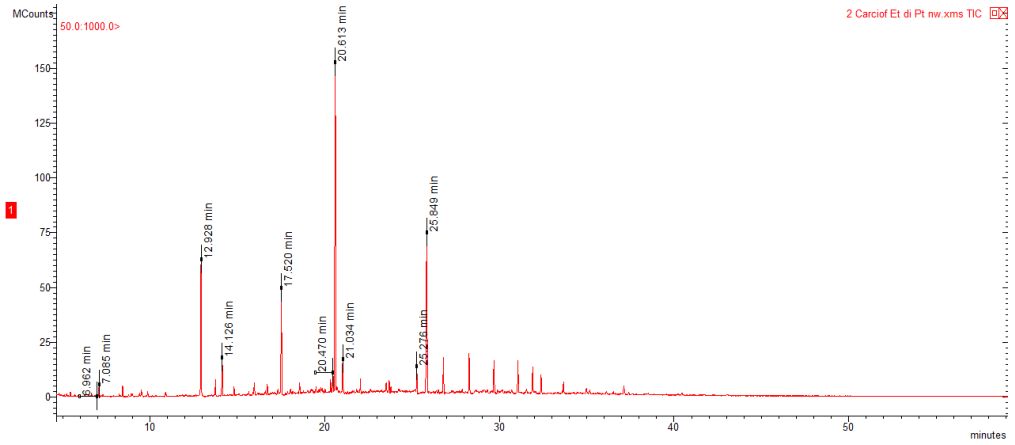


Figure 7.5.1. Chromatogram petroleum ether fraction.

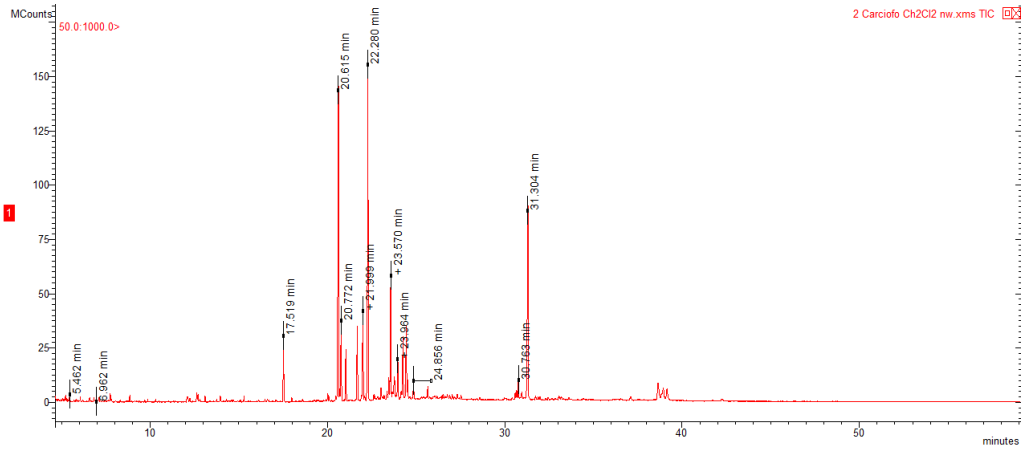


Figure 7.5.2. Chromatogram dichloromethane fraction.

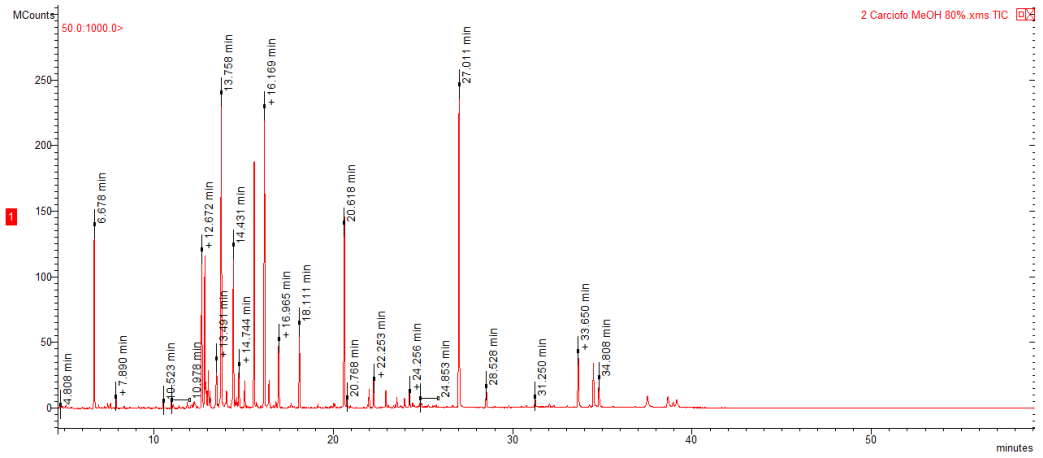


Figure 7.5.3. Chromatogram methanol (80% water solution) fraction.

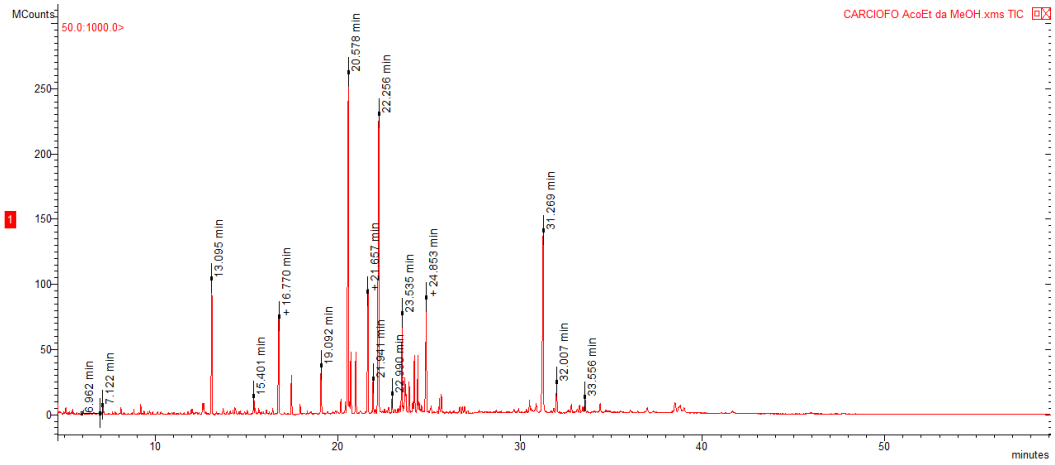


Figure 7.5.4. Chromatogram of ethyl acetate fraction.

The results of GC-MS analysis, grouped by family, are reported in **Table 7.5.1**. The quantities and the percentages referred to the total amount of phenols quantified for each family of compounds.

Artichoke **Compound classes**	**Amount** **(mg/gr)**	**%**
Aldehydes	0,003	0,001
Aromatic acids and derivatives	7,443	3,06
Flavonols and Dihydroflavonols	0,247	0,10
Phenols and catechols	0,011	0,005

Flavones	0,031	0,01
Flavanones	1,364	0,56
Hydroxycinnamic acid and derivatives	46,757	19,25
Glycosides	187,005	77,00

Table 7.5.1 Quantities and percentages for family of compounds.

Oligophenols and high molecular weight polyphenols

The sample treated according to general procedure reported in paragraph was analysed through: ^{31}P-NMR, after phosphitilation, for the component of lignins and tannins (**Figure 7.5.5. and 7.5.6**). Note that melanins were not present in the sample. Starting from 1 gr of lyophilized sample were obtained: lignins 19.8 mg, tannins 59.55 mg and melanins 0 mg.

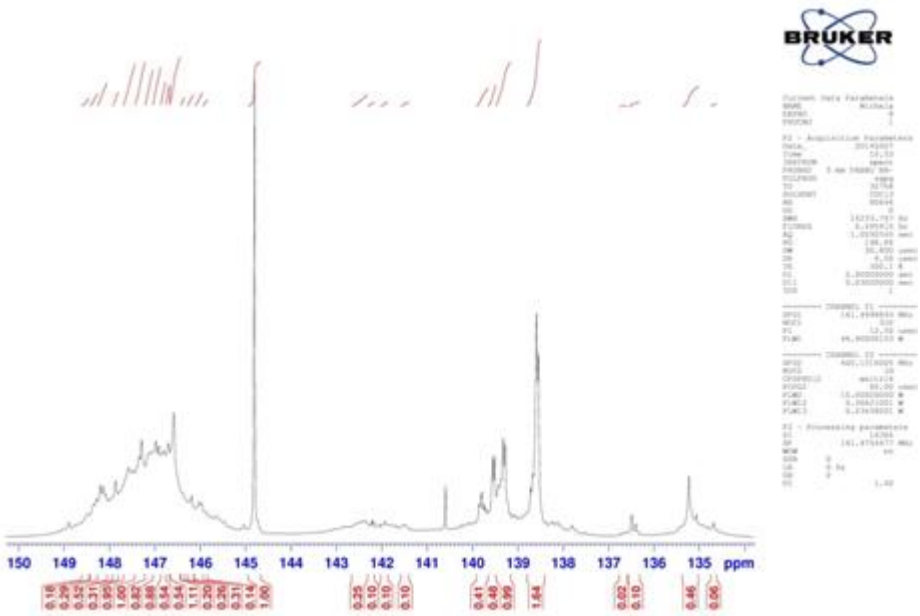


Figure 7.5.5. ^{31}P-NMR spectra of lignins

Table 7.5.2 shows the data for the assignment of the main families of phenolic hydroxyl moietis in lignin, on the basis the comparison with what reported in literature. The family of phenolic hydroxyl groups were found to be prevalent in quantity as hydroxyl of aliphatic type, followed by phenolic units of guaiacyl type. The condensed phenolic units, characteristics of the degree of polymerization of the molecule, and the carboxylic acid units are present in

similar amounts. The *p*-hydroxyphenyl and syringyl phenolic units are present a lessere amount and the quantities are similar.

OH Groups	Chemical shift	mmol OH/gr of lignins
Aliphatic	148.7-146.8	2.69
Internal Standard (cholesterol)	144.88	
Condensed phenolic units	142.6-141.4	0.18
Syringyl phenolic units	143.0-142.6	0.05
Guaiacyl phenolic units	139.9-138.4	1.18
p-Hydroxyphenyl phenolic units	136.8-136.3	0.04
Carboxylic acids	135.5-134.6	0.17

Table 7.5.2. Assegment of ^{31}P-NMR spectra of lignins and OH mmol of groups.

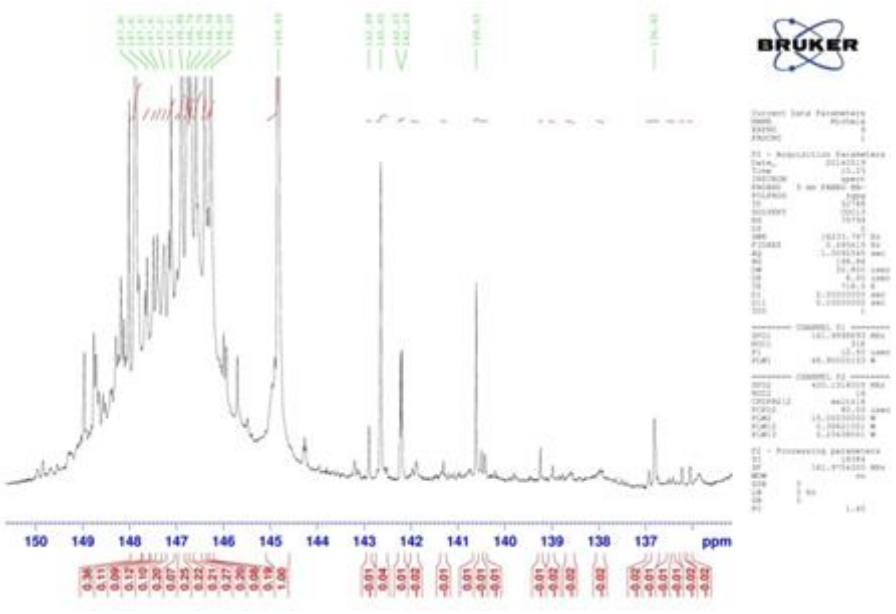


Figure 7.5.6. ^{31}P-NMR spectra of tannins.

Table 7.5.3. shows the qualitative and quantitative data of structural characterization of tannins. The sample presents percentage widely predominant of tannins of hydrolizable type

(96,79%), compared to the condensed tannins (3,21%). As it regards, the subclasses of hydrolizable tannins are predominating the ellagiotannins (identified with a percentage of 96,18%), being in higher amount than gallotannins (3,82%, on the total of hydrolizable tannins).

Tannins		**mg/g of tannins**	**% tannins classes**
Hydrolizable	Gallotannins	2.202	3.7%
	Ellagiotannins	55.434	93.09%
Condensed		1.914	3.21%

Table 7.5.3. Classification of tannins classes.

Glycosides

The qualitative and quantitative analysis of glycosides took a different experimental technique as these substances due to their structural complexity are too polar to be analyzed in gas chromatography-mass spectrometry (GC-MS) also applying a procedure of silylation, for this reason and in agreement with what reported in literature the main glycosides present in the sample of Artichoke were analyzed mediated liquid chromatograpy–mass spectrometry (LC-MS). The sample analyzed was that relative to the fraction methanol (80% water solution) as it is the sample in which it is contained highest concentration of glycosides. Analyzes were conducted using the conditions reported in the **Table 7.4.4**, operating in gradient with acetonitrile and water acids for the presence of formic acid.

Time	**Acetonitrile acid (C_2H_3N + CH_2O_2 0.5%)**	**Water acid (H_2O + CH_2O_2 0.5%)**	**Flow**	**Column**	**UV**	**MS**
0	5	95	0.4 mL/min	Phenomenex (kinetex) 100x4.6 2.6 µm particle size	280 nm	100-800 *m/z* neg.
10	25	75				
15	25	75				
20	50	50				

30	60	40	25° C

Table. 7.5.4. Conditions of analysis LC-MS.

Figure 7.5.7 report the UV and MS spectra of the sample with related extract ions of standards mass.

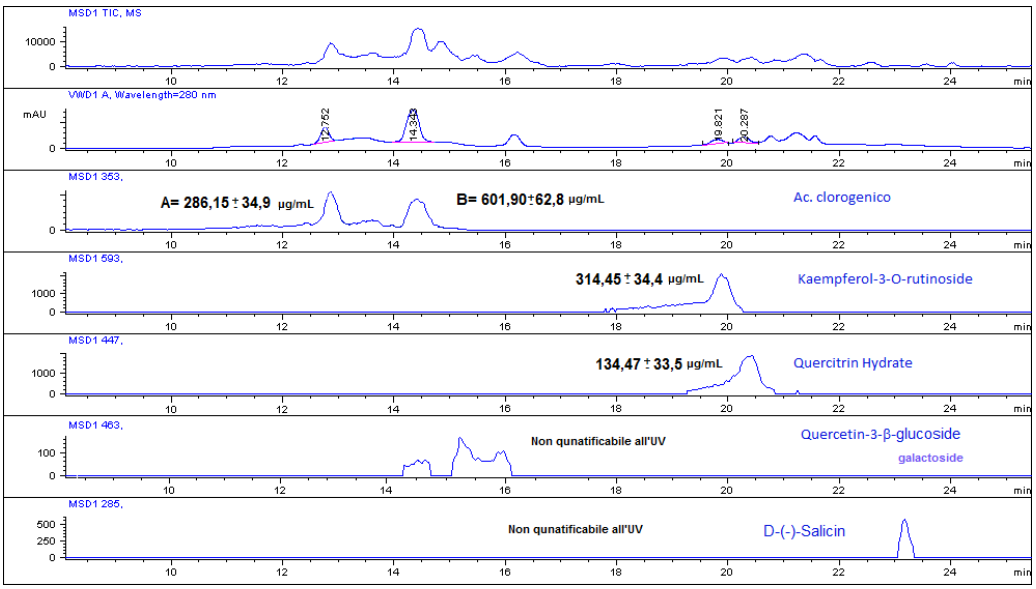


Figure 7.5.7. UV and MS spectra

The assignment of the glycosides was performed by the addition method using appropriate standard, and by the method of the search by ion that allows to identify all those peaks in the chromatogram which corresponds to a mass fragmentation ion with the ratio mass/charge corresponding to the substances which you are checking the presence.

Figure 7.5.8 report some extracts of chromatograms that shown the retention time of analytes and related standards.

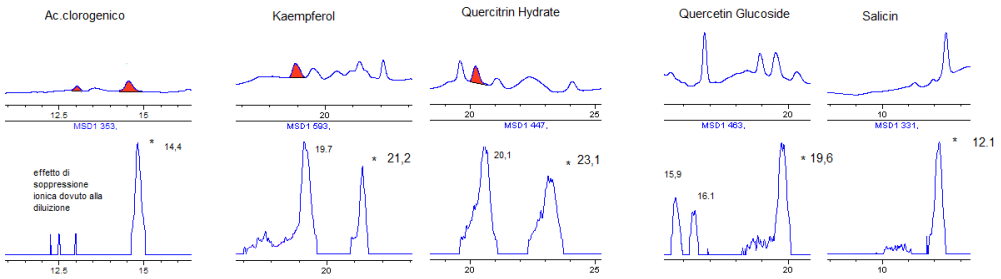


Figure 7.5.8. In the top panel are shown the Uv spectra and in red are highlighted the signals of molecules unknowns, in the bottom panel are shown the extract ions and the mass signals of standard are highlighted.

Figure 7.5.9 focuses attention on the extract ions of compound with pm 515, are noticed at least 4 signals belonging to as many isomers of this family.

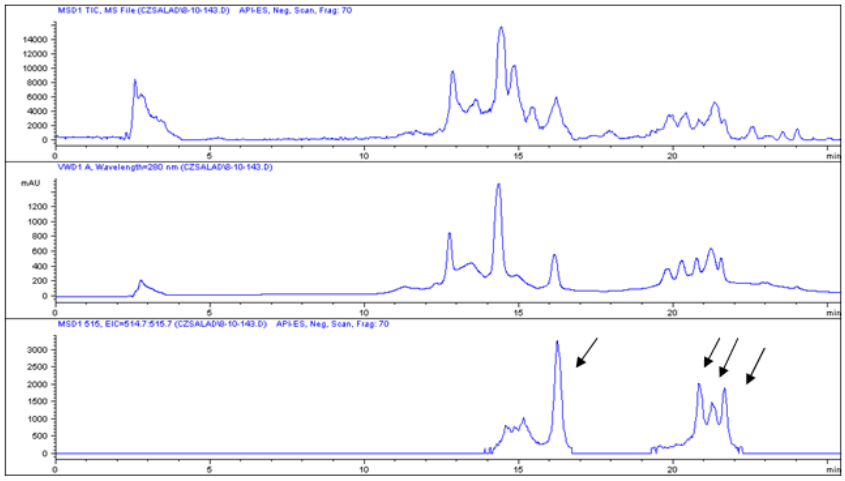


Figure 7.5.9. Analysis of compounds with pm 515.

Then we proceeded to the final assignment qualitative/quantitative analysis of individual components of the mixture, both applying the procedure of the calibration line and the internal standard method.

Below assignment of standard compounds and their quantification. (**Table 7.5.4**)

For the chlorogenic acid we highlight three main peaks (peak1 8.9 min, peak3 10.6 min and peak4 10.8 min) that correspond at MW 353, the peak3 is enriched with the addition of standard and it is the chlorogenic acid. The others peak correspond at its isomers.

For the cynarin we highlight five main peak (peak1 12.4 min, peak2 15.3 min, peak3 15.5 min, peak4 16.0 min and peak5 17.2 min) that correspond at the MW 515, the peak1 is enriched with the addition of standard and it is the cynarin. The others peak correspond at its isomers.

The UV signal at 19.8 min, that correspond at 593 mass, is not enriched with the standard of Kaempferol-3-O-rutinoside, that have a retention time greater (21.3 min), on the basis of literature could be of Luteolin-7-O-neohesperoside.

The UV signal at 13.7 min, that correspond at 593 mass, is not enriched with the standard of Tiliroside, that have a retention time greater (21.5 min), could be of its isomer.

The UV signal at 20.2 min, that correspond at 447 mass, is not enriched with the standard of Quercitrin, that have a retention time greater (23.1 min), on the basis of literature could be of Luteolin-7-O-galactoside or cynaroside.

The UV signal at 14.6 min, that correspond at 447 mass, is not enriched with the standard of Kaempferol 7-O-glucoside, that have a retention time greater (16.7 min), could be of its isomer.

Observed two signals in traces that correspond at 463 mass (Quercetin-3-β-D glucoside, Quercetin-3-β-D galactoside), unquantifiable at UV and in addition the signals of standard Quercetin-3-β-D glucoside and Quercetin-3-β-D galactoside have greatest retention time. On the basis of literature could be of Eriodictyol-glucoronide.

Compound	**MW**	**Peak 1 (mg/gr)**	**Peak 2 (mg/gr)**	**Peak 3 (mg/gr)**	**Peak 4 (mg/gr)**	**Peak 5 (mg/gr)**
Chlorogenic acid	354	25,868 (isomer)	NQ	40,681 (Chlorogenic acid)	31,573 (isomer)	-
Cynarin	516	8,831 (Cynarin)	3,181 (isomer)	1,518 (isomer)	3,885 (isomer)	3,005 (isomer)
Luteolin-7-O-neohesperidoside	594	31,445 (Luteolin-7-O-				

		neohesperidoside)
Tiliroside		10,350 (Isomer)
Luteolin-7-O-galactoside/cynaroside	448	13,447 (Luteolin-7-O-galactoside/cynaroside)
Kaempferol 7-O-glucoside		13,221 (Isomer)
Eriodictyol-glucoronide	464	Traces (Eriodictyol-glucoronide)

Table 7.5.5. Results of glycosides analysis. (NQ: unquantifiable)

The results of LC-MS analysi of glycosides were: Chlorogenic acid, two isomers of Chlorogenic acid, Cynarin, four isomers of Cynarin, one isomer of Kaempferol-7-O-Glucoside, Luteolin-7-O-neohesperidoside, one isomer of Tiliroside, Luteolin-7-O-galactoside/cynaroside, Eriodictyol-glucoronide. The quantities and the percentages refer to the total amount of glycosides quantified shows in **Table 7.5.5**.

7.5.2 Consideration data

On the basis of data previously reported it is possible to make some general considerations. Firstly, the total amount of phenols (including low and high molecular weigth derivatives) correspond to about 32,22 % in weight of the sample (**Figure 7.5.10**). The remaining 67,78% is attributable to other families of natural compounds.

Analyzing in detail the total phenolic and polyphenolic fractions, it is observed that the phenolic compounds at low/medium molecular weight (75%) predominate on polyphenols. In the polyphenols class, the tannins and lignin represent respectively 19% and 6% of total sample. (**Figure 7.5.11**) In particular, in the most abundant (**Figure 7.5.12**) phenol families, it was possible to identify: glycosides, followed by hydroxycinnamic acids and their derivatives and aromatic acids and their derivatives. Other families of natural substance have been identified in smaller percentages.

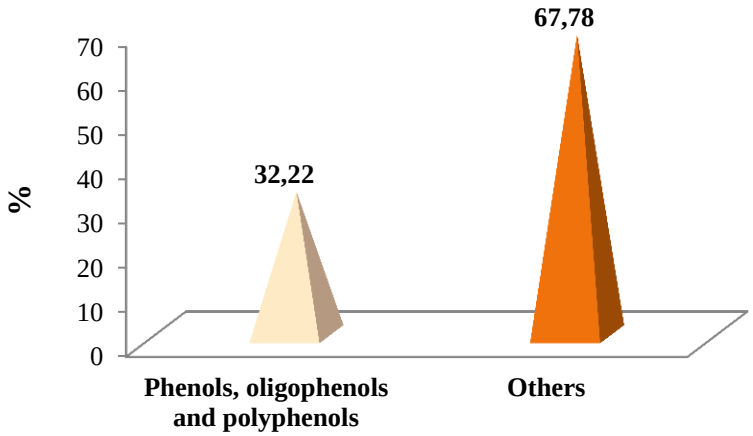


Figure 7.5.10. Percentage of phenols, oligophenols and polyphenols identified compared to the total sample.

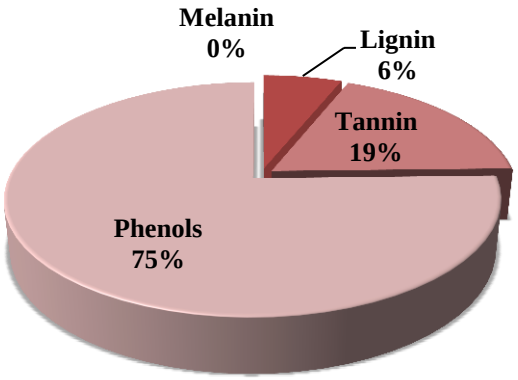


Figure 7.5.11. Percentage of phenols, tannins, lignins and melanins compared to the total amount identified.

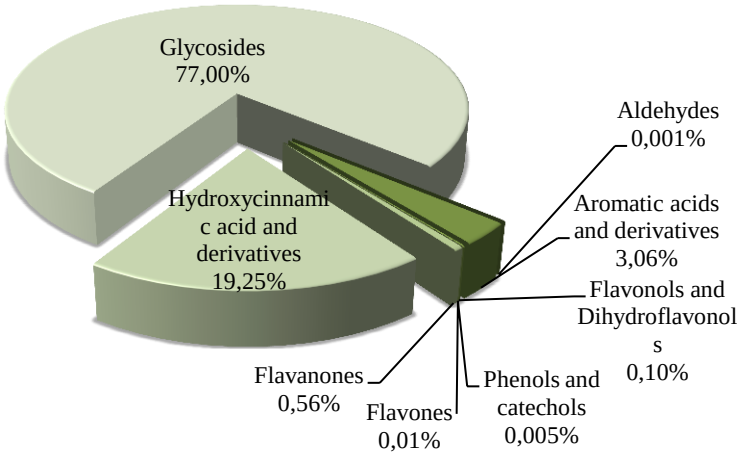


Figure 7.5.12. Percentage of families of phenols compared to the total amount phenols identified.

7.5.3 Biological and pharmacological acitivities of main phenolic compounds

It is of particular interest to highlight the pharmacological activities of chlorogenic acid and cynarin. Chlorogenic acid is a common thermosensitive polyphenol, which is an ester compound produced by caffeic acid and quinic acid with unsaturated double bond. chlorogenic acid is used as antioxidant, antidiabetic, and antihyperglycemic. It is also a promising precursor compound for the development of drugs active againt resistant forms of AIDS virus HIV.[790] Several other pharmacological activities have been reported for this compounds: free radical scavenging, anti-inflammatory, radioprotective, antiulcerogenic, and analgesic properties. Furthermore, the antidiabetic activities of CGA in diabetes have been shown recently. It has been reported to modulate blood glucose levels and exhibit protective effects against tissue changes.[791] Moreover, chlorogenic acid inhibition of the mutagenicity of carcinogenic compounds,[792] reduce the risk of cardiovascular disease and type two diabetes, and there are suggested improvements in cognitive function.[793] Instead, Cynarin is the main derivative of caffeoylquinic acid, found in leaves and heads of artichoke. It may have hepatoprotective, antiatherosclerotic, antioxidative, choleretic, and cholesterol-lowering effects.[794] Moreover cynarin showed cytoprotective action, whose activity is attributable to the caffeoyl moiety, regarding the antioxidant activity, cynarin prevents the CCl_4-induced oxidation of the phospholipids that are constituents of the hepatocyte membranes.[795]

7.6 Green tea and Grape seed extract (ABO-AR-2013-133)

Green tea

Tea, a product made up from leaf and bud of the plant *Camellia sinensis*, is the second most consumed beverage in the world, well ahead of coffee, beer, wine and carbonated soft drinks.[796,797] Originating from China, tea has gained the world's taste in the past 2000 years. Green tea is non-fermentaded tea because it is produced by drying and steaming the fresh leaves to inactive the polyphenol oxidase and thus, non oxidation occur.[798] Green tea has been considered a medicine and a healthful beverage since ancient times. The traditional Chinese medicine has recommended this plant for headaches, body aches and pains, digestion,

depression, detoxification, as an energizer and, in general, to prolong life. Green tea leaves contain three main components which act upon human health: xanthic bases (caffeine and theophylline), essential oils and especially, polyphenolic compounds. Caffeine acts mainly upon the central nervous system, stimulating wakefulness, facilitating ideas association and decreasing the sensation of fatigue.[799] Some of the effects caused by caffeine are influenced by theophylline tea content. Theophylline induces psychoactive activity, it also has a slightly inotrope and vasodilator effect, and a much higher diuretic effect than caffeine. However, its most interesting effects can be seen at the bronchopulmonar and respiratory level. Theophylline causes a non-specific relaxation on the bronchial smooth muscle, and respiratory stimulation is also observed. Among their properties, the one of facilitating digestion must be highlighted.[800,801] Green tea is the type of tea with the higher percentage of essential oils.[799,801] The chemistry of tea is complex: polyphenols, alkaloids, amino acids, glucides, proteins, volatile compounds, minerals and trace elements.[802] Polyphenols are the most biologically active group of tea components which have antioxidative, antimutagenic and anticarcinogenic effects.[803,804] But there are other compounds in green tea with interest for human health like fluoride, caffeine, minerals and trace elements like, chromium and manganese.[805,806,807] However, green tea has received a great deal of attention especially due to its content of polyphenols, which are strong antioxidants and present important biological properties. Numerous studies have also demonstrated that the aqueous extract of green tea polyphenols possesses antimutagenic, antidiabetic, antibacterial, anti-inflammatory, and hypocholesterolemic properties.[808,809,810,811,812,813] Beneficial effects in oral diseases such as protection against dental caries, periodontal disease, and tooth loss (which may significantly affect a person's overall health) have been also described.[800] Among all green tea polyphenols, catechins and gallic acid have been especially considered to be the main players in the beneficial effects on human health.

Grape seed

Grapes have a long and abundant history. During the ancient Greek and Roman civilizations, grapes were revered for their use in winemaking. Nowadays, there are three main species of grapes: European grapes (Vitis vinifera), North American grapes (Vitis labrusca and Vitis rotundifolia) and French hybrids. Grapes are classified as table grapes, wine grapes (used in viniculture), raisin grapes, and so on, with edible seeds or seedless.[814] Grape fruit contains various nutrient elements, such as vitamins, minerals, carbohydrates, edible fibers and

phytochemicals. Polyphenols are the most important phytochemicals in grape because they possess many biological activities and health-promoting benefits.[815,816,817] The phenolic compounds mainly include anthocyanins, flavanols, flavonols, stilbenes (resveratrol) and phenolic acids.[818,819,820] Anthocyanins are pigments, and mainly exist in grape skins. Flavonoids are widely distributed in grapes, especially in seeds and stems, and principally contain (+)-catechins, (−)-epicatechin and procyanidin polymers. Anthocyanins are the main polyphenolics in red grapes, while flavan-3-ols are more abundant in white varieties.[821,822,823] Grape seed polyphenols have been reported to possess a broad spectrum of pharmacologic and medicinal properties, such as mitigating breast cell carcinogenesis,[824] modulating blood pressure in individuals with prehypertension,[825] maintaining glucose homeostasis in diabetic conditions,[826] protecting against ischemia-related injuries,[827] promoting dermal wound healing,[828] and protecting against diabetic nephropathy[829]. Grape seed polyphenols (as well as most other polyphenolic compounds) are potent antioxidants. Indeed, the health benefits of polyphenolic compounds, including grape seed proanthocyanidins, have traditionally been attributed to their antioxidant activities.[830] For example, antioxidant activities might be a major contributory factor to the role of grape seed polyphenols in protection against breast cancer,[824] wound healing,[828] and ischemic injuries[827]. However, grape seed polyphenols may induce additional beneficial disease-modifying mechanisms. For example, grape seed polyphenols might mitigate diabetic nephropathy by mechanisms involving reduced expression of advanced glycation end products and connective tissue growth factor in the kidney.[829] In another example, grape seed polyphenols might promote the maintenance of glucose omeostasis in diabetic conditions, in part, by activating insulin receptor signaling pathways.[831] Recent observations from in vitro experimental studies[832,833] and preclinical studies[834] demonstrated that grape seed polyphenols may interfere with specific neuropathogenic mechanisms underlying Alzheimer's disease and suggest a potential novel role of grape seed polyphenols for treating Alzheimer's disease.

7.6.1 Sample characterization

Phenols medium-low molecular weight

1 gr of lyophilized sample was extracted in soxhlet for 6 h with petroleum ether, dichloromethane and methanol (80% water solution). The fraction of methanol 80% was resuspended in ethyl acetate and it was washed with water. The total amount for 1 gr of sample of four fractions was found to be: petroleum ether 4.75 mg, dichlorometane 11.4 mg, methanol 80% 761.75 mg, and 316,89 mg, after treatment with ethyl acetate.

The fractions were analysed through GC-MS analysis after derivatization with silylating reagents, the chromatograms of each fraction are shown in **Figures 7.6.1, 7.6.2, 7.6.3, 7.6.4**.

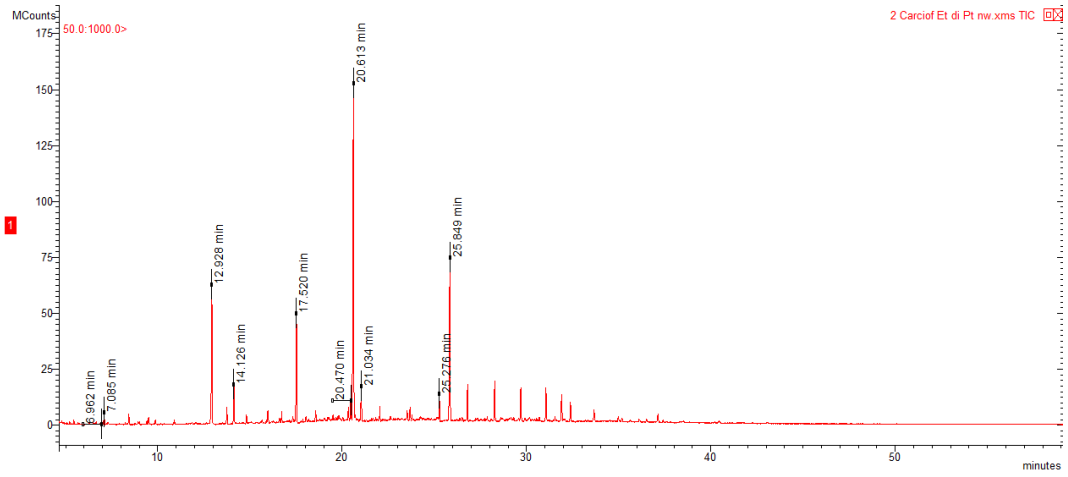


Figure 7.6.1. Chromatogram of petroleum ether fraction.

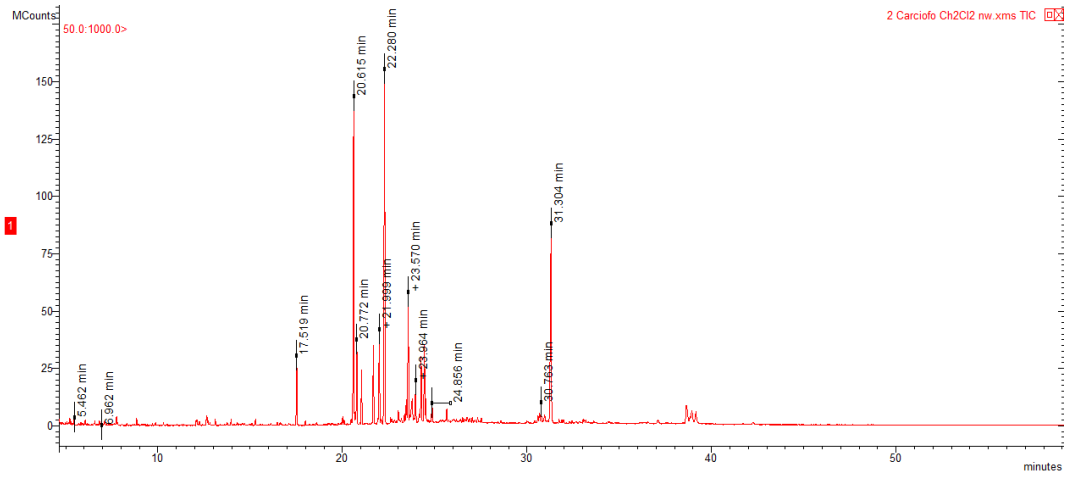


Figure 7.6.2. Chromatogram dichloromethane fraction.

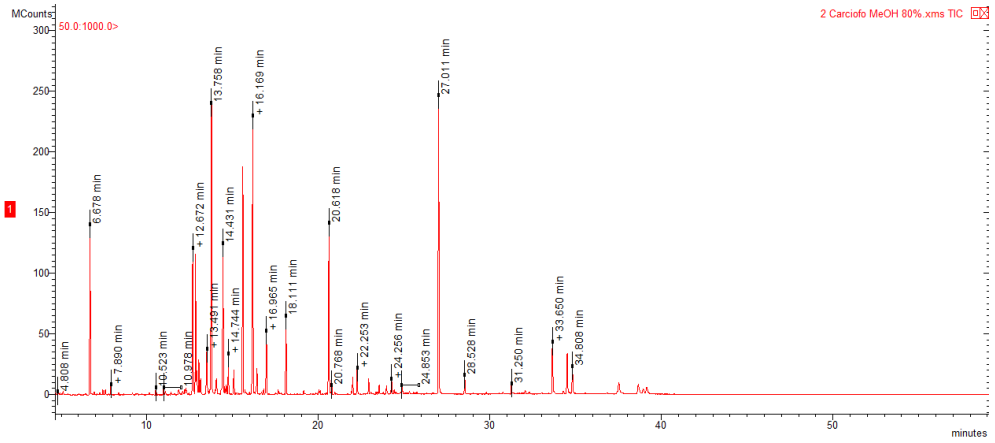


Figure 7.6.3. Chromatogram methanol (80% water solution) fraction.

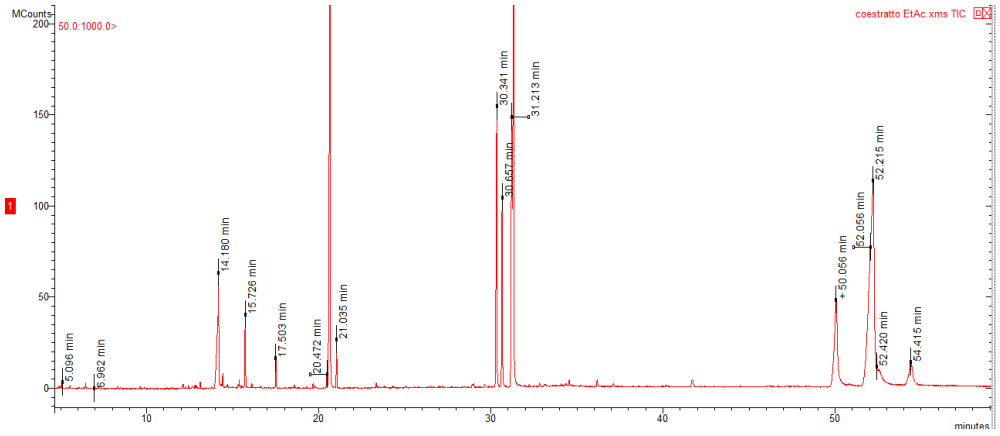


Figure 7.6.4. Chromatogram of ethyl acetate fraction.

The results of GC-MS analysis, grouped by family, are reported in **Table 7.6.1**. The quantities and the percentages referred to the total amount of phenols quantified for each family of compounds.

Green tea and Grape seed Compound classes	**Amount (mg/gr)**	**%**
Aromatic acids and derivatives	15,109	6,63
Flavonols and Dihydroflavonols	16,672	7,32

Phenols and catechols	0,930	0,41
Flavanones	0,015	0,01
Hydroxycinnamic acid and derivatives	1,817	0,80
Catechins	193,155	84,80
Stilbenes	0,071	0,03

Table 7.6.1. Quantities and percentages for family of compounds.

Oligophenols and high molecular weight polyphenols

Starting 1 gr of lyophilized sample were obtained: lignins 57.72 mg, 25.589 mg and melanins 0 mg.

7.6.2 Consideration data

On the basis of data previously reported it is possible to make some general consideration. Firstly, the total amount of phenols (including low and high molecular weigth derivatives) correspond to about 11,11% in weight of the sample (**Figure 7.6.5**). The remaining 68,89% is attributable to other families of natural compounds.

Analyzing in detail the total phenolic and polyphenolic fractions, it is observed that the phenolic compounds at low/medium molecular weight (73%) predominate on polyphenols. In the polyphenols class, the tannins and lignin represent respectively 8% and 19% of total sample. (**Figure 7.6.6**) In particular, the most abundant phenol families (**Figure 7.6.7**) were: catechins, followed by flavonols and dihydroflavonols and aromatic acids and their derivatives. Other families of natural substance have been identified in smaller percentages.

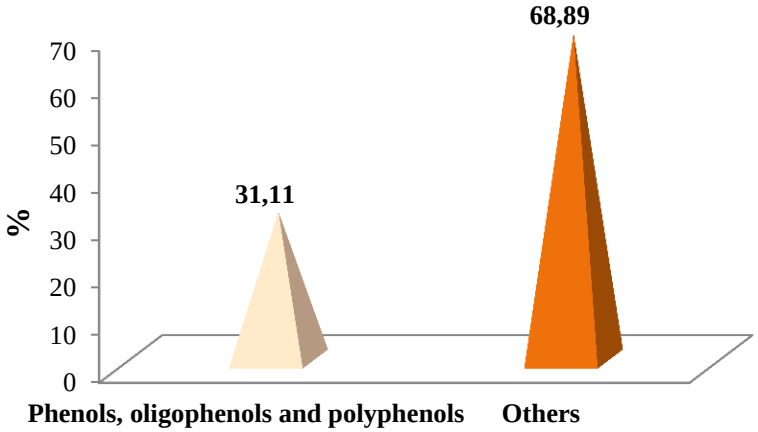


Figure 7.6.5. Percentage of phenols, oligophenols and polyphenols identified compared to the total sample.

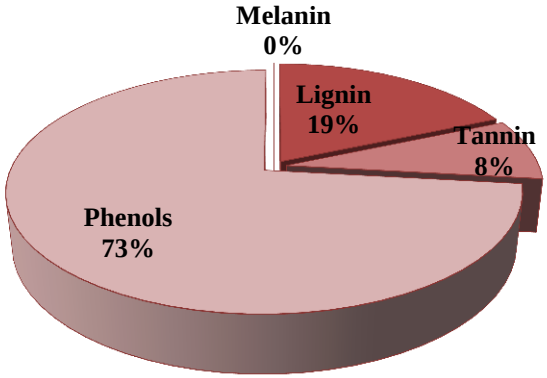


Figure 7.6.6. Percentage of phenols, tannins, lignins and melanins compared to the total amount identified.

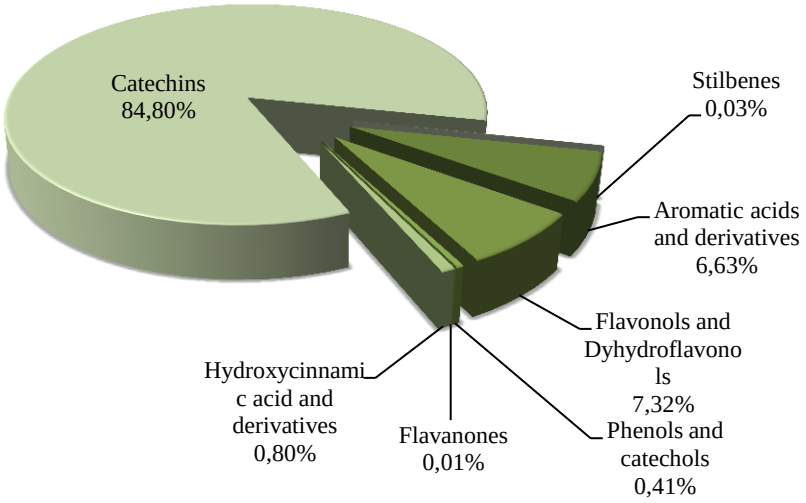


Figure 7.6.7. Percentage of families of phenols compared to the total amount phenols identified.

7.6.2 Biological and pharmacological activities of main phenolic compounds

For some of the most abundant phenolic compounds: catechin, epicatechin and epigallocatechin, belonging to catechins family, caffeine and gallic acid (already reported in **paragraph 7.4)**; we report in the follow the main biological and pharmacological activities.

Catechins (epigallocatechin gallate, epicatechin gallate, epicatechin and epigallocatechin) have beneficial health properties such as the prevention of cardiovascular diseases and cancer as well as their anti-hypertensive, anti-inflammatory, anti-obesity and anti-aging effects.[835] Between them epigallocatechin gallate: growth inhibition of human cancer cell lines in cultures, induction of apoptosis, inhibition of tumor promotion and carcinogenesis in animal experiments, antimutagenic and anti-oxidant activities, inhibition of tumor necrosis factor-α (TNF-α) release from cells induced by a tumor promoter, and modulation of gene expression.[836] Moreover it have anti-endothelial dysfunction effect, epigallocatechin gallate activates endothelial nitric oxide synthase by a phosphatidylinositol-3-OH-kinase-, cAMP-dependent protein kinase-, and Akt-dependent pathway and leads to endothelial-dependent vasorelaxation. In a rat carotid artery balloon injury model, some authors showed that catechins inhibit neointimal hyperplasia and suppress proliferation of vascular smooth muscle cells. These findings have obvious clinical implications, because vascular smooth muscle cell proliferation is one of the major mechanisms of restenosis following percutaneous coronary interventions. Catechins have antithrombogenic effects. In the mode of action it did not change the coagulation parameters such as activated partial thromboplastin time, prothrombin time, and thrombin time using human citrated plasma. Therefore, the modes of antithrombotic action may be due to the antiplatelet activities, rather than to anticoagulant activities. Respect the anti-infiammatory effect, they inhibit NF-κB and AP-1 pathway and this has been reported for the treatment of reperfusion-induced myocardial damage.[837]

Caffeine, an alkaloid, is present at relatively high concentrations in many beverages like coffee, tea, cola and in chocolate-based food products. Caffeine is an effective scavenger of reactive oxygen species, particularly hydroxyl radicals, singlet oxygen and to some extent peroxyl radicals. It was shown that the antioxidant effect of caffeinewas similar to that of glutathione and significantly higher than that of ascorbic acid. In the human body, caffeine is demethylated by the hepatic cytochrome P450 1A2 to the main metabolites 1-methylxanthine

and 1-methyl uric acid which also have been shown to have significant antioxidant activity.[838] The popularity of these beverages stems from the mild stimulatory effects (eg, increased wakefulness, improvedcognition, and decreased fatigue) that caffeine has on the central nervous system when ingested in moderate quantities. Because of its central nervous system stimulant properties, purified caffeine can also be found in various non prescription drug products (eg, NoDozs [caffeine] and Excedrins [acetaminophen, aspirin, and caffeine]). When consumed in moderation, caffeine-containing products have an excellent safety profile. Apart from central nervous system stimulation,moderate caffeine consumption can, in many instances, transiently increase blood pressure and reflexively lower heart rate. Long term ingestion of caffeine, however, can lead to pharmacologic tolerance of some central nervous system effects but not necessarily its cardiovascular effects. Excessive amounts of caffeine can give rise to significant toxic effects, including nausea, vomiting, tachycardia, severe hypertension, arrhythmia, seizures, and even death; however, individuals sensitive to caffeine may exhibit adverse effects at lower doses.[839]

7.7 *Helichrysum*

Helichrysum Miller genus belongs to the family of Asteraceae. This genus includes more than a thousand taxa that have a higher occurrence in the Mediterranean areas of Europe.[840,841,842]The name of the genus is derived from the Greek words "helios" and "chryos", which mean, respectively, "sun" and "gold", that are in direct relationship to the fact that the plant species of this genus typically have inflorescence so far bright yellow color.[841] The plant has a high level of polymorphism, mainly in the habitus, the leaves and the typical capitulum flowers. The most widespread species in Italy is *H. italicum* (Roth) G. Don (syn. *H. angustifolium* DC), which is present all around the country and includes two sub-species, *H. italicum* ssp. *italicum* and *H. italicum* ssp. *microphyllum* (Willd.) Nyman.[843] One of the earlier mentions of the medicinal uses of plants from the *Helichrysum* genus appears in the work of the Greek Theophrastus of Eresos "HistoriaPlantarum" (3rd –2nd century B.C.). There, here ports that "*Heleiochrysos*" may be used in the treatment of burns(mixed with honey) and stings/bites of venomous animals.[844] Another example of an ancient report of *Helichrysum* medicinal properties comes in book four of "De Materia Medica" (1st century A.D.),written by the Greek Pedanius Dioscorides, where the decoction of the filaments of *Helichrysum* flowers macerated in wine is described as possessing diuretic properties and

being useful in the treatment of urinary disorders, snake bites, sciatica and hernias.[845] Concerning the Renaissance period, the first written record of the medicinal uses of *Helichrysum* species in South Africa is attributed to the Dutch botanist Herman Boerhaave, who reported their use in the treatment of nervousness and hysteria in 1727.[846] *Helichrysum* species are traditionally used in the treatment of wounds, infections and respiratory conditions.[847,848,849] Infection often leads to inflammation, and free radicals are released from phagocyte cells during the inflammatory process.[850] Since many respiratory conditions and skin ailments such as atopic dermatitis[851] are associated with inflammation (and hence release of free radicals) as well as infection, the presence of anti-inflammatory, anti-oxidant and anti-microbial agents could explain the effectiveness of plants from this genus in the treatment of these conditions. The extracts as well as the essential oils from *Helichrysum* species have exhibited promising biological activities in many in vitro assays which include anti-oxidant,[852] anti-microbial[853] and anti-inflammatory activity[854]. This genus has furthermore been a source of many fascinating (and bioactive) compounds. *Helichrysum* species were also reported to relief abdominal pain, heart burn, cough, cold and wounds and to treat female sterility and menstrual pain.[855] Chemical studies on *Helichrysum* species have been carried out by many investigators and the presence of flavonoids, phloroglucinols, α-pyrones, coumarins and terpenoid compounds has been reported.[856] However, various biological activities of the members of this genus were reported elsewhere; choleretic,[857] chologogue,[858] antimalarial,[859] antidiabetic,[860] antiproliferative,[861] antiviral and antituberculous[862].

7.7.1 Sample characterization: *Helichrysum* total extract (ABO-AR-2013-157)

Phenols medium-low molecular weight

1 gr of lyophilized sample was extracted in soxhlet for 6 h with petroleum ether, dichloromethane and methanol (80% water solution). The fraction of methanol 80% was resuspended in ethyl acetate and it was washed with water. The total amount for 1 gr of sample of four fractions was found to be: petroleum ether 102.95 mg, dichlorometane 58.8 mg, methanol 80% 733.75 mg, and only 227.46 mg after treatment with ethyl acetate.

The fractions were analysed through GC-MS analysis after derivatization with silylating reagents, the chromatograms of each fraction are shown in **Figures 7.7.1, 7.7.2, 7.7.3** and **7.7.4**.

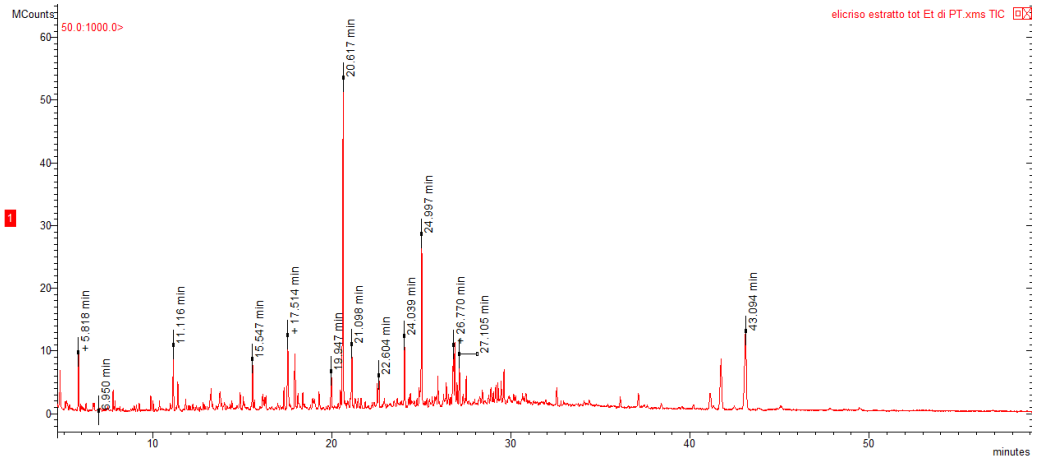


Figure 7.7.1. Chromatogram of petroleum ether fraction.

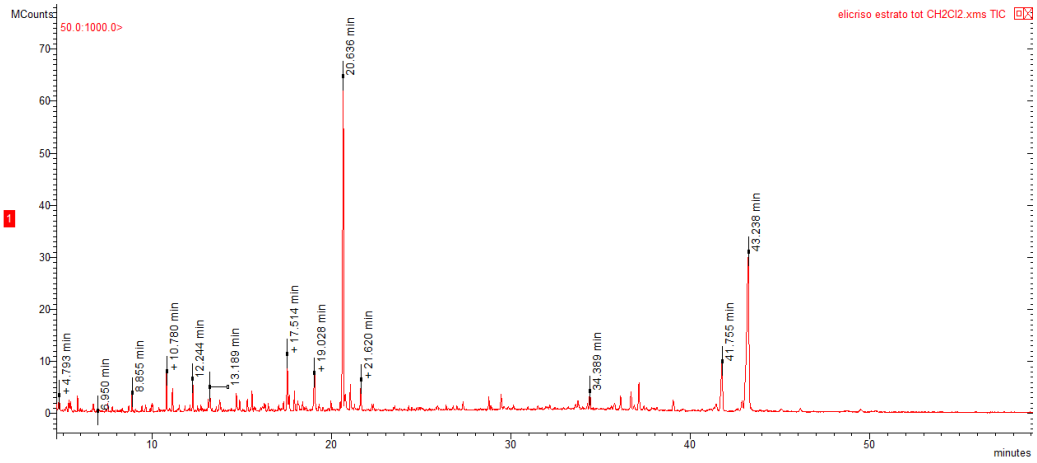


Figure 7.7.2. Chromatogram dichloromethane fraction.

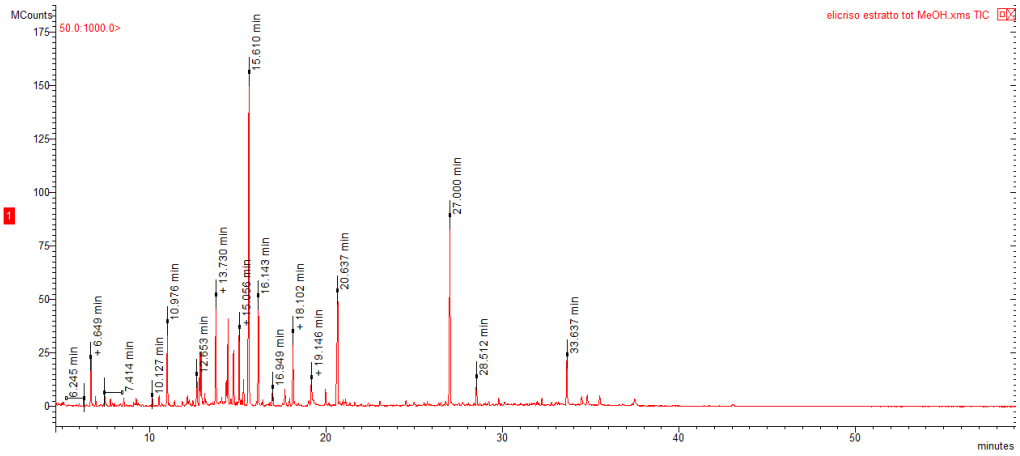


Figure 7.7.3. Chromatogram methanol (80% water solution) fraction.

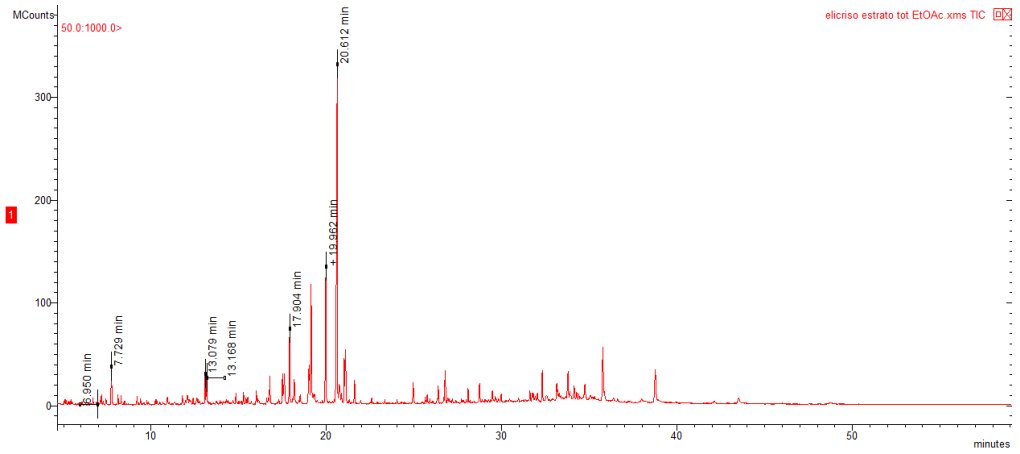


Figure 7.7.4. Chromatogram of ethyl acetate fraction.

The results of GC-MS analysis, grouped by family, are reported in **Table 7.7.1**. The quantities and the percentages referred to the total amount of phenols quantified for each family of compounds.

Helichrysum total extract **Compound classes**	**Amount** **(mg/gr)**	**%**
Aromatic acids and derivatives	5,912	11,09
Flavonols and Dyhydroflavonols	4,844	9,08
Phenols and catechols	0,256	0,48
Flavones	0,447	0,84

Flavanones	2,030	3,81
Hydroxycinnamic acid and derivatives	38,687	72,53
Glycosides	0,426	0,80
Chalcones and Dihydrochalcones	0,074	0,14
Xanthones	0,659	1,24

Table 7.7.1. Quantities and percentages for family of compounds.

Oligophenols and high molecular weight polyphenols

Starting from 1 gr of lyophilized sample were obtained: lignins 158.41 mg, tannins 21.79 mg and melanins 0 mg.

7.7.2 Condieration data: *Helichrysum* total extract

On the basis of data previously reported, it is possible to make some general considerations. Firstly, the total amount of phenols (including low and high molecular weigth derivatives) correspond to about 23,35% in weight of the sample (**Figure 7.7.5**). The remaining 76,65% is attributable to other families of natural compounds.

Analyzing in detail the total phenolic and polyphenolic fractions, it is observed that polyphenols (77%) largely predominate on others phenolic compounds (23%). In the polyphenols family, lignin and tannins represent respectively 68% and 9% of total sample. (**Figure 7.7.6**). The most abundant low/medium molecular weight phenol groups (**Figure 7.7.7**) were: hydroxycinnamic acid and their derivatives, followed by aromatic acids and their derivatives, flavonols and dihydroflavonols and flavanones. Other families of natural substance have been identified in smaller percentages.

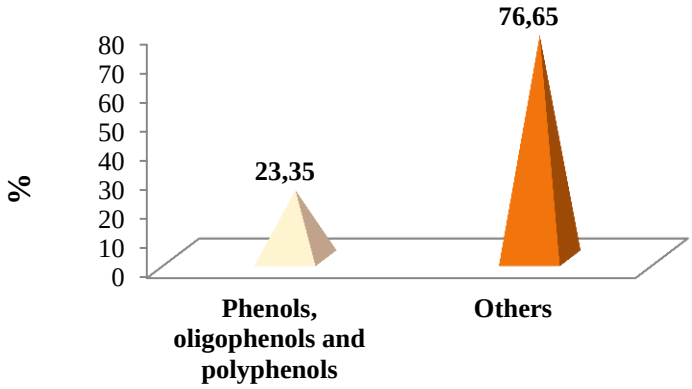


Figure 7.7.5. Percentage of phenols, oligophenols and polyphenols identified compared to the total sample.

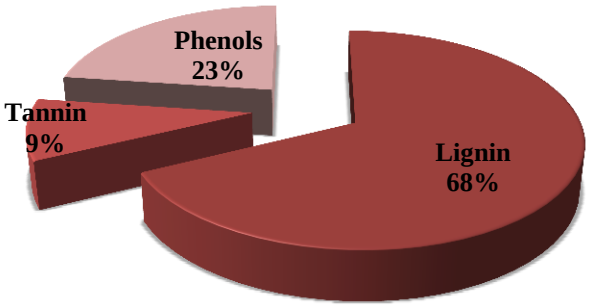


Figure 7.7.6. Percentage of phenols, tannins, lignins and melanins compared to the total amount identified.

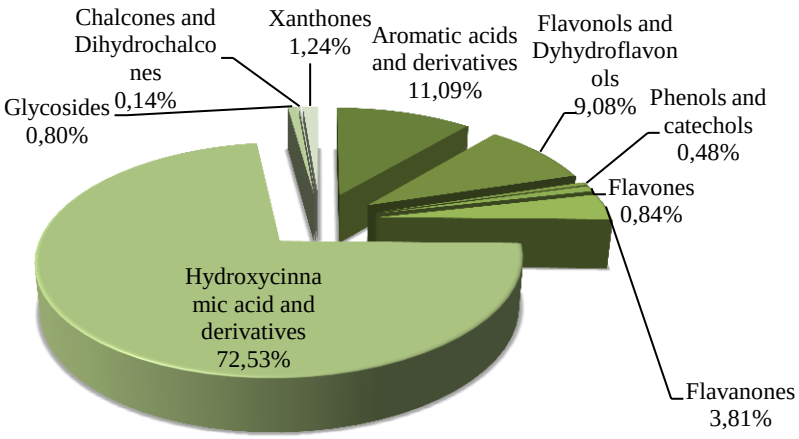


Figure 7.7.7. Percentage of families of phenols compared to the total amount phenols identified.

7.7.3 Sample characterization: *Helichrysum* hydrophilc fraction (ABO-AR-2014-155)

Phenols medium-low molecular weight

1 gr of lyophilized sample was extracted in soxhlet for 6 h with methanol (80% water solution). The fraction of methanol (80% water solution) was suspended in ethyl acetate and washed with water. The total amount of different fractions found for 1 gr of sample was: methanol (80% water solution) 879.3 mg, and only 497,68 mg after treatment with ethyl acetate.

Two fractions were analysed through GC-MS analysis after derivatization with silylating reagents, the chromatograms of fractions are shown in **Figure 7.7.8** and **7.7.9**.

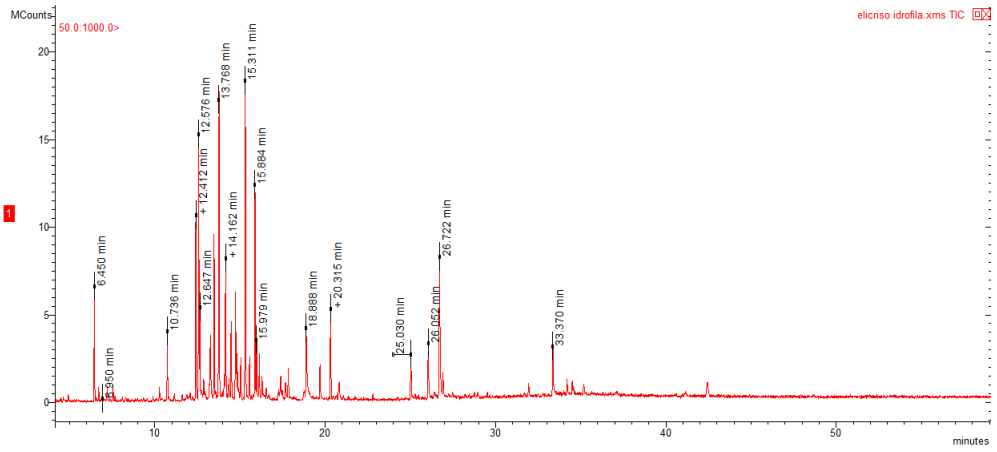


Figure 7.7.8. Chromatogram methanol (80% water solution) fraction.

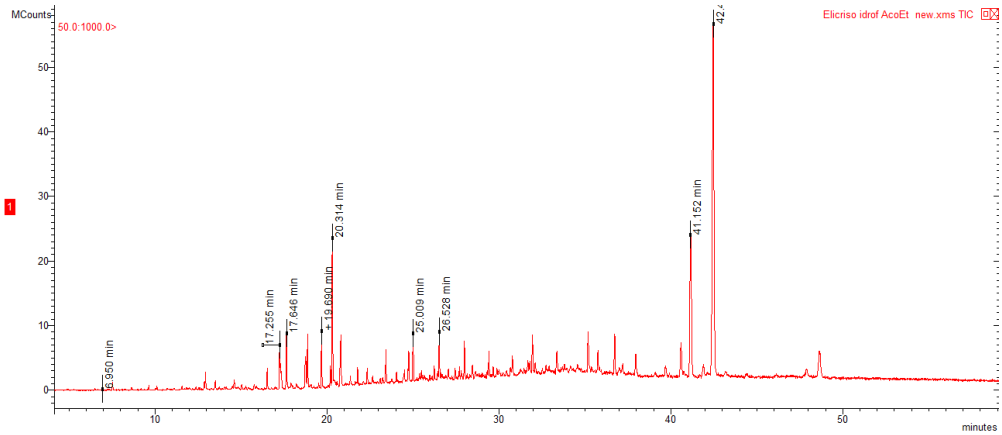


Figure 7.7.9. Chromatogram of ethyl acetate fraction.

The results of GC-MS analysis, grouped by family, are reported in **Table 7.7.2**. The quantities and the percentages referred to the total amount of phenols quantified for each family of compounds.

Helichrysum hydrophilic fraction Compound classes	**Amount (mg/gr)**	**%**
Aromatic acids and derivates	7,243	27,01
Hydroxycinnamic acid and derivatives	13,533	50,47
Flavones	0,948	3,54
Flavonols and Dyhydroflavonols	0,489	1,82
Flavanones	1,783	6,65
Xanthones	1,873	6,98
Glycosides	0,946	3,53

Table 7.7.2. Quantities and percentages for family of compounds.

Oligophenols and polyphenols high molecular weight

Starting from 1 gr of lyophilized sample were obtained: lignins 93.75 mg and tannins 66.22 mg.

7.7.4 Consideration data: *Helichrysum* hydrophilc fraction

On the based of data previously reported it is possible to make some general considerations. Firstly, the total amount of phenols (including low and high molecular weigth derivatives) correspond to about 18,68% in weight of the sample (**Figure 7.7.10**). The remaining 81,32% is attributable to other families of natural compounds.

Analyzing in detail the total phenolic and polyphenolic fractions, it is observed that the polyphenols (86%) predominate on the phenolic compounds at low-medium molecular weight (14%) Within the polyphenols lignin and the tannins represent respectively 50% and 36% of total sample. (**Figure 7.7.11**) In particular, the most abundant phenol families (**Figure 7.7.12**)

were: hydroxycinnamic acids and their derivatives, followed by aromatic acids and their derivatives. Other families of natural substance have been identified in smaller percentages.

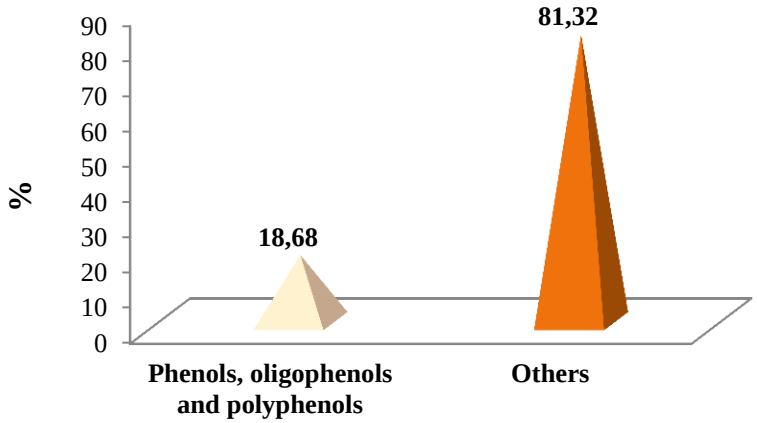


Figure 7.7.10. Percentage of phenols, oligophenols and polyphenols identified compared to the total sample.

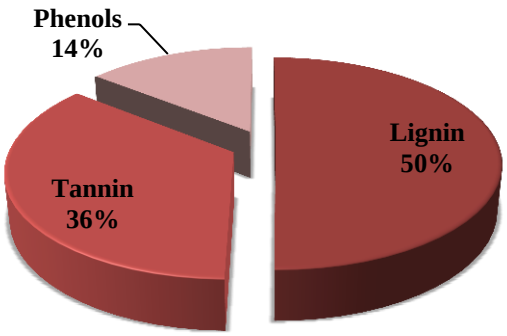


Figure 7.7.11. Percentage of phenols, tannins, lignins and melanins compared to the total amount identified.

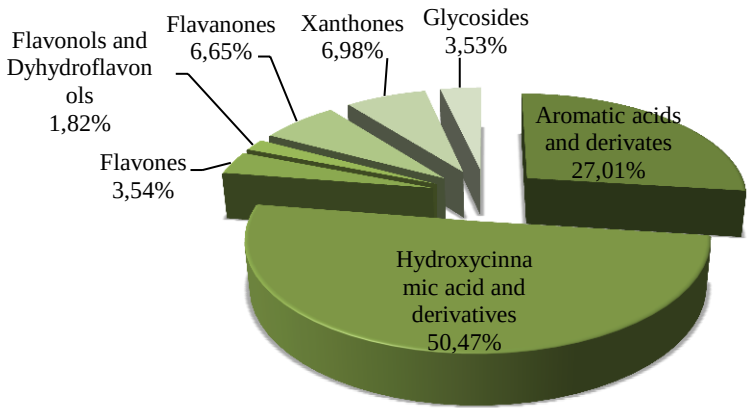


Figure 7.7.12. Percentage of families of phenols compared to the total amount phenols identified.

7.7.5 Sample characterization: *Helichrysum* lipophilic fraction (ABO-AR-2013-156)

Phenols medium-low molecular weight

1 gr of lyophilized sample was extracted in soxhlet for 6 h with dichloromethane. The total quantity for 1 gr of sample of fraction in dichloromethane was 985.1 mg.

The fraction was analysed through GC-MS analysis after derivatization with silylating reagents, the chromatograms of fraction is shown in **Figure 7.7.13**.

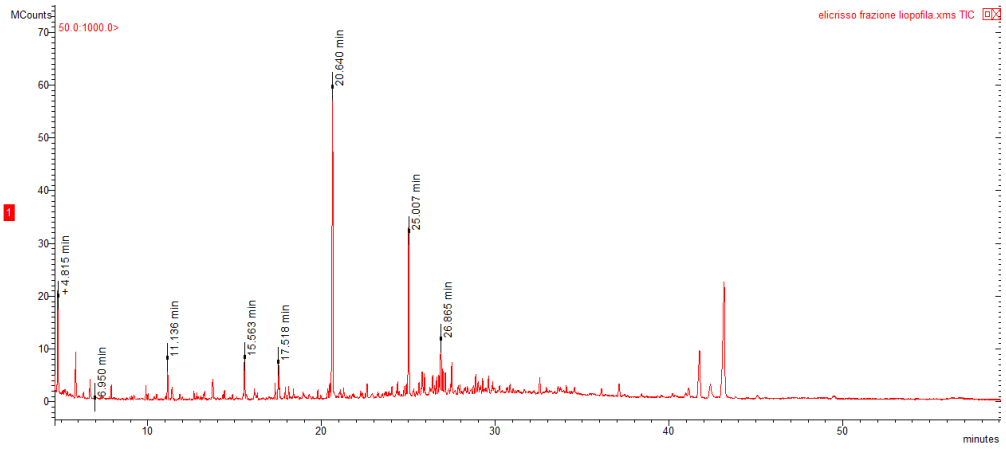


Figure 7.6.13. Chromatogram dichloromethane fraction.

The results of GC-MS analysis, grouped by family, are reported in **Table 7.7.3**. The quantities and the percentages referred to the total amount of phenols quantified for each family of compounds.

***Helichrysum* lipophilic fraction** **Compound classes**	**Amount (mg/gr)**	**%**
Aromatic acids and derivatives	2,310	10,59
Hydroxycinnamic acid and derivatives	0,901	4,13
Flavones	1,227	5,62
Flavonols and Dihydroflavonols	5,800	26,58
Flavanones	6,328	29,00
Xanthones	5,256	24,09

Table 7.7.3. Quantities and percentages for family of compounds.

Oligophenols and high molecular weight polyphenols

Starting from 1 gr of lyophilized sample were obtained: lignins 1.35 mg and tannins 10.18 mg.

7.7.6 Consideration data: *Helichrysum* lipophilic fraction

On the based of data previously reported it is possible to make some general consideration. Firstly, the total amount of phenols (including low and high molecular weigth derivatives) correspond to about 3,34% in weight of the sample (**Figure 7.1.14**). The remain 96,66% is attributable to other families of natural substances.

Analyzing in detail the total of phenolic and polyphenolic fractions, it is observed that the phenolic compounds at low-medium molecular weight (65%) predominate on polyphenols (35%). Within polyphenols tannin and lignin represent respectively 31% and 4% (**Figure 7.7.15**) In particular, the most abundant phenol families (**Figure 7.7.16**) were: flavanones followed by flavonols and dihydroflavonols, xanthones, aromatic acids and their derivatives and then by flavones and hydroxycinnamic acids and their derivatives.

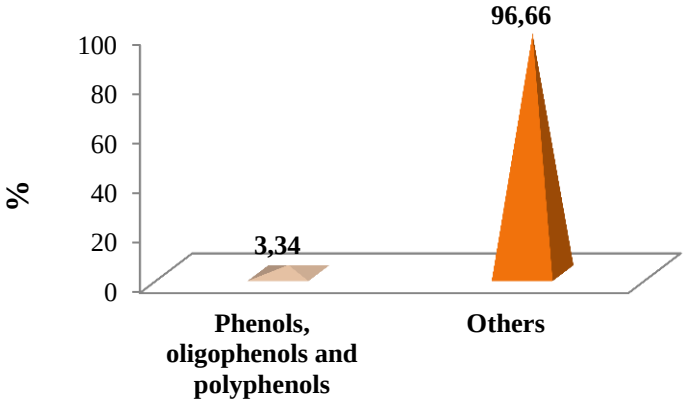


Figure 7.7.14. Percentage of phenols, oligophenols and polyphenols identified compared to the total sample.

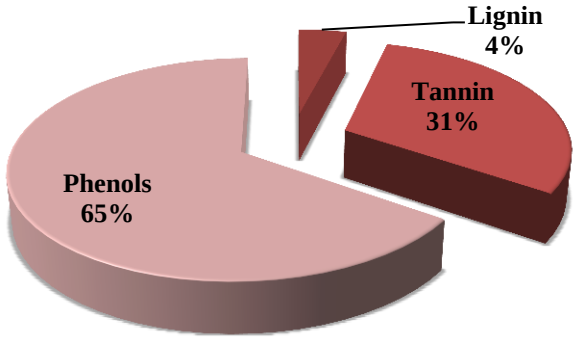


Figure 7.7.15. Percentage of phenols, tannins, lignins and melanins compared to the total amount identified.

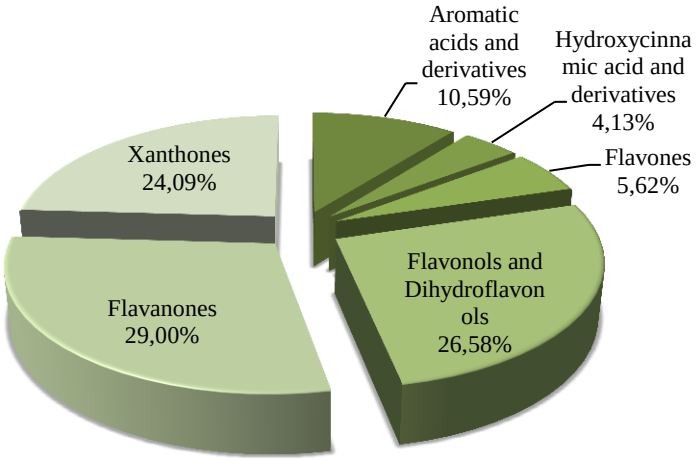


Figure 7.7.16. Percentage of families of phenols compared to the total amount phenols identified.

7.7.7 Comparison between the different *Helichrysum* samples

As reported in Table, phenol compounds are mainly concentrated in the hydrophilic fraction, which amount is similar to that recovered in the total exytract. A low amount of phenols was detected in the lipophilic fraction, mainly due to the low polarity of this medium.

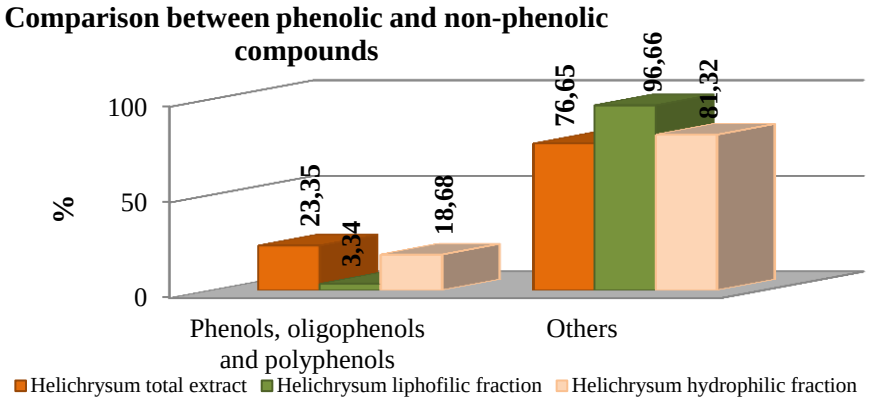


Figure 7.7.17. Percentage of phenols, oligophenols and polyphenols identified compared to the total sample identified in samples of Helichrysumtotal extract, Helichrysum liphofilic extract and Helichrysum hydrophilic extract.

About the specific distribution of polyphenols, lignin and tannins were detected in highest amount in the hydrophilic fraction. Apparently, low molecular weight phenols are prevalent in the lipophilic fraction.

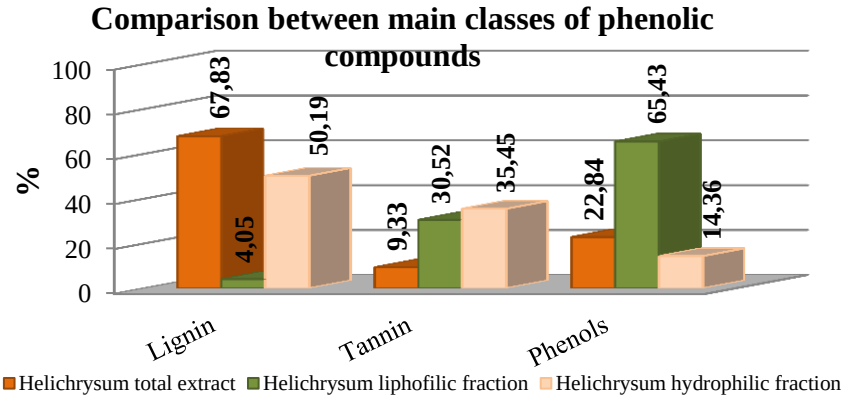


Figure 7.7.18. Percentage of phenols, tannins, lignins and melanins compared to the total amount identified in samples of Helichrysumtotal extract, Helichrysum liphofilic extract and Helichrysum hydrophilic extract.

7.7.8 Biological and pharmacological activities of main phenolic compounds

For some of the most abundant phenolic compounds: caffeic acid, pinocembrin and quercetin (already reported in **paragraph 7.4)**; we report in the follow the main biological and pharmacological activities.

Caffeic acid (3,4-dihydroxycinnamic acid) is a non-flavanoid catecholic compound present in many plants and occurs in the diet as part of fruits, tea and wine. Even though caffeic acid is a phenolic compound especially abundant in coffee, it is chemically unrelated to caffeine. It is known that caffeic acid has a broad spectrumof pharmacological activities including anti-inflammatory, antioxidant, antibacterial, antitumor and immunomodulatory effects. Pharmacology studies have also shown that the caffeic acid exerts a protective effect against hydrogen peroxide-induced oxidative damage in the brain, and cerebral ischemia and prevents brain damage aswell as behavioral and biochemical changes caused by aluminum.[863,864] Moreover, caffeic acid had been found to have antiproliferative and cytotoxic properties in a variety of cancer cell lines without displaying significant toxicity toward healthy cells.[865]

Pinocembrin (5, 7-dihydroxyflavanone) is a flavonoid abundant in propolis and extracted as a pure compound. It has been reported to possess antimicrobial, anti-inflammatory, anti-oxidant, anti-apoptotic and vasorelaxant properties.[866] Pinocembrin has shown to be effective in the protection of brain injury from ischemic and Aβ impairment. It was approved by the State Food and Drug Administration of China for treatment of stroke in 2008. We showed that pinocembrin protected against ischemic injury and reduced the area of cerebral infarction in ischemia models. Moreover, pinocembrin has been investigated for the ability to express neurovascular unit protection by decreasing oxidative damage and inhibiting inflammatory responses. Recently, we found that pinocembrin alleviated cognitive deficits in the vascular dementia model and intracerebroventricular Aβ-injected model. In the $A\beta_{1-42}$-treated receptor for advanced glycation end products overexpressing cell model, pinocembrin inhibited the overexpression of receptor for advanced glycation end products. Moreover, pinocembrin attenuated neuronal apoptosis through downregulating receptor for advanced glycation end products expression and inhibiting receptor for advanced glycation end products downstream pathways, both in SH-SY5Y cells overexpressing the Swedish mutant form of human Aβ-

precursor protein and in the $A\beta_{25-35}$-induced amnesia model. These results suggest that pinocembrin has potential therapeutic effects on Aβ-related cognitive deficits and might have prospects as an Alzheimer's disease therapeutic agent.

7.8 Thyme (ABO-AR-2014-260)

Thymus vulgaris, also known as common thyme, a plant native to the Mediterranean region (Spain, Italy, France, Greece, etc.), has long been used as a source of the essential oil (thyme oil) and other constituents (e.g. thymol, flavanoid, caffeic acid and labiatic acid) derived from the different parts of the plant.[867] The pharmacological properties of the plant and of its different extracts, in particular the essential oils, has been thoroughly studied and afforded the many industrial (mainly as food additive) and medical applications of the plant. In addition to their numerous traditional uses, the plant (herb) and its essential oil have found diverse applications in pharmacy and medicine.[867,868] Thymus has many uses, from phytotherapy to aromatheraphy, homeopathy and folk medicine. The pharmacological effects are: antibacterial, antifungal, antiviral, spasmolytic, antioxidant, antiparasitic and insecticidal. Furthermore, it is use: as a food preservative, in the cosmetics and in the culinary.[869]

7.8.1 Sample characterization

Phenols medium-low molecular weight

1 gr of lyophilized sample was extracted in soxhlet for 6 h with petroleum ether, dichloromethane and methanol (80% water solution). The fraction of methanol 80% was resuspended in ethyl acetate and it was washed with water. The total amount for 1 gr of sample of four fractions was found to be: petroleum ether 60.8 mg, dichlorometane 114.05 mg, methanol 80% 773 mg, and only 209,48 mg, after treatment with ethyl acetate.

The fractions were analysed through GC-MS analysis after derivatization with silylating reagents, the chromatograms of each fraction are shown in **Figures 7.8.1, 7.8.2, 7.8.3** and **7.8.4**.

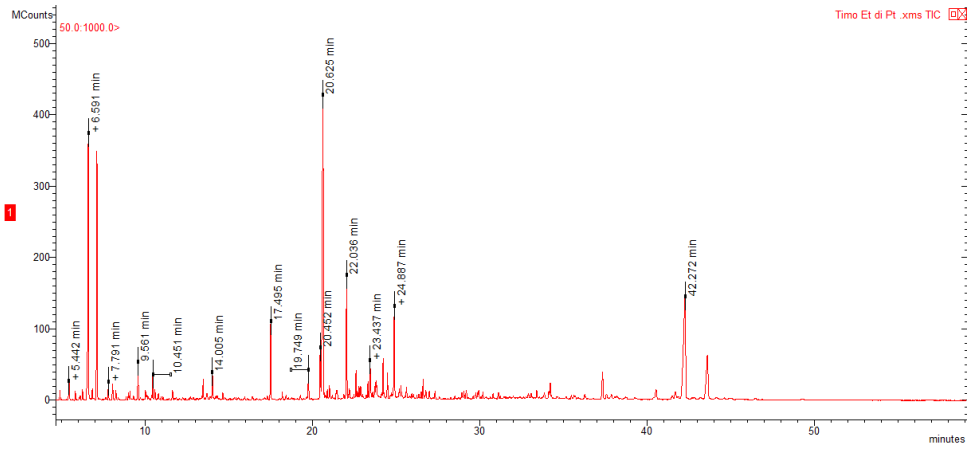


Figure 7.8.1. Chromatogram of petroleum ether fraction.

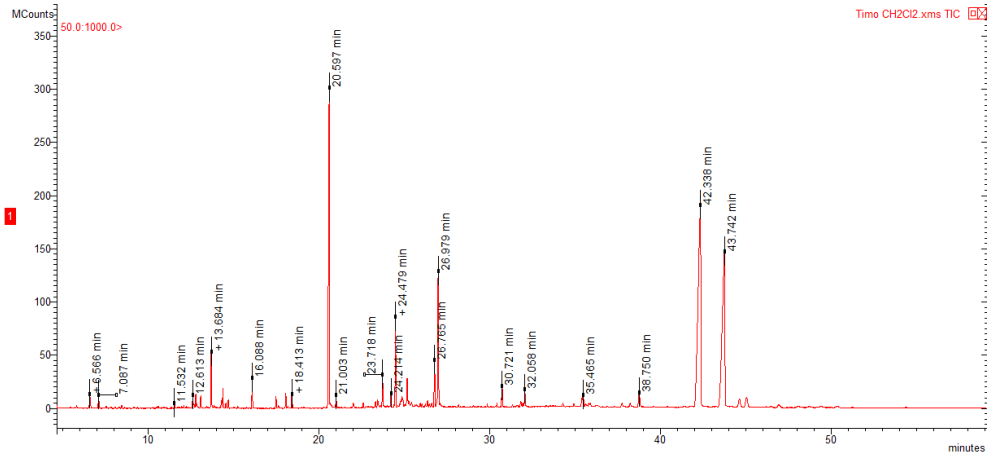


Figure 7.8.2. Chromatogram dichloromethane fraction.

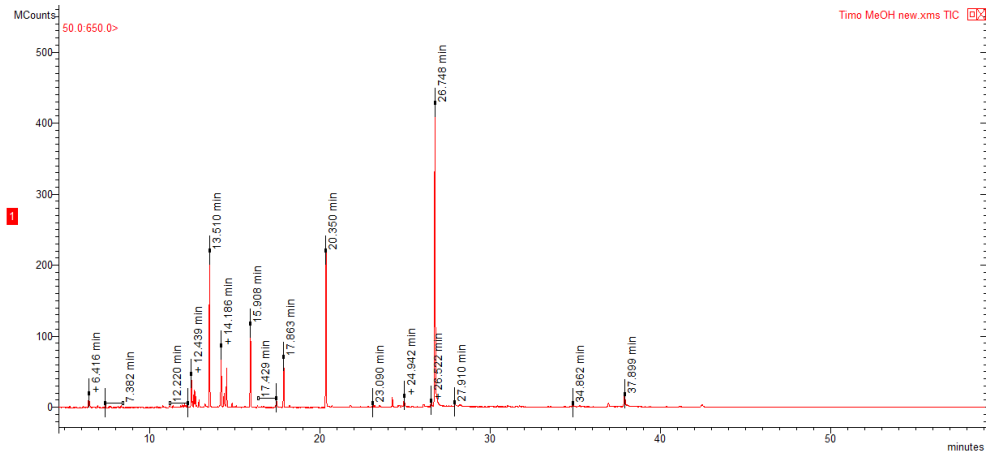


Figure 7.8.3. Chromatogram methanol (80% water solution) fraction.

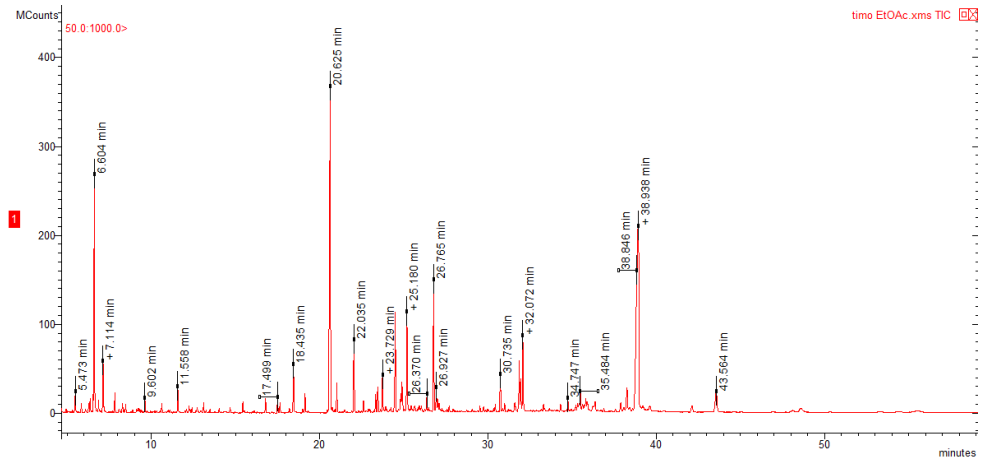


Figure 7.8.4. Chromatogram of ethyl acetate fraction.

The results of GC-MS analysis, grouped by family, are reported in **Table 7.8.1**. The quantities and the percentages referred to the total amount of phenols quantified for each family of compounds.

Thymus **Compound classes**	**Amount** **(mg/gr)**	**%**
Aromatic acids and derivatives	0,974	1,03
Flavonols and Dihydroflavonols	12,333	13,04
Flavones	14,881	15,73
Flavanones	13,500	14,27
Hydroxycinnamic acid and derivatives	41,216	43,56
Glycosides	9,230	9,76
Catechins	0,255	0,27
Flavanonols	2,224	2,35

Table 7.8.1. Quantities and percentages for family of compounds.

Oligophenols and high molecular weight polyphenols

The sample extracted treated according to general procedure reported in paragraph was analysed through: ^{31}P-NMR, after phosphitilation, for the component of tannins (**Figure**

7.8.5). Note that melanins were not present in the sample. Starting from 1 gr of lyophilized sample were obtained: lignins 5.9 mg, tannins 10.24 mg and melanins 0 mg.

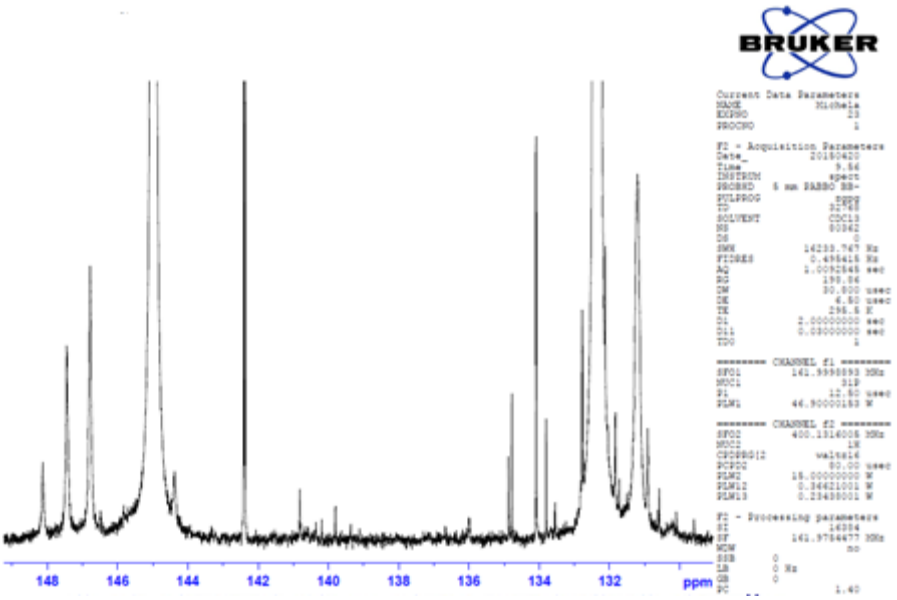


Figure 7.8.5. ^{31}P-NMR spectra of tannins.

Table 7.8.2 shows the qualitative and quantitative data of tannins. The sample present similar percentage of hydrolizable (58,27%), and condensed (41,73%) tannins. As it regards, the subclasses of hydrolizable tannins are predominating, the ellagiotannins (identified with a percentage of 61,83%) beging in higher amount than gallotannins (38,17%, on the total of hydrolizable tannins).

Tannins		**mg/g of tannins**	**% tannins classes**
Hydrolizable	Gallotannins	13,68	38,17
	Ellagiotannins	22,14	61,83
Condensed		25,62	41,73

Table 7.8.2. Classification of tannins classes.

7.8.2 Cosideration data

On the basis of data previously reported it is possible to make some general consideration. Firstly, the total amount of phenols (including low and high molecular weigth derivatives) correspond to about 11,08% in weight of the sample (**Figure 7.8.6**), the remain 88,92% is attributable to other families of natural substances.

Analyzing in detail the total of phenolic and polyphenolic fractions, it is observed that the phenolic compounds at low-medium molecular weight (86%) predominate on polyphenols (14%). Within polyphenols tannin and lignin represent respectively 9% and 5% (**Figure 7.8.7**) In particular, in the most abundant phenol families (**Figure 7.8.8**) were: hydroxycinnamic acids and their derivatives, flavanones and flavones followed by flavanones, flavonols and dihydroflavonols and then by glycosides. Other families of natural substance have been identified in smaller percentages.

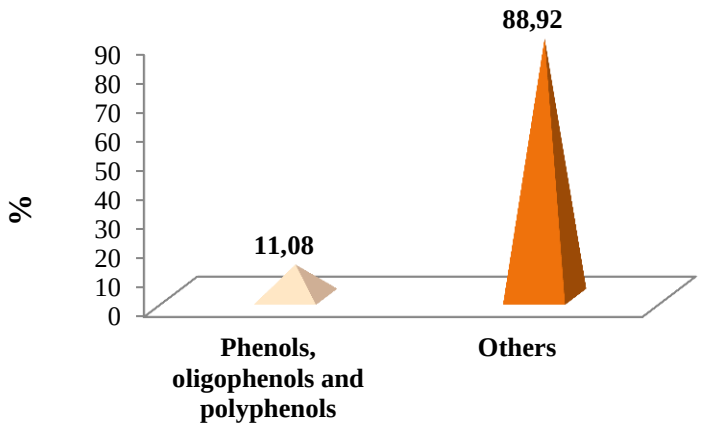


Figure 7.8.6. Percentage of phenols, oligophenols and polyphenols identified compared to the total sample.

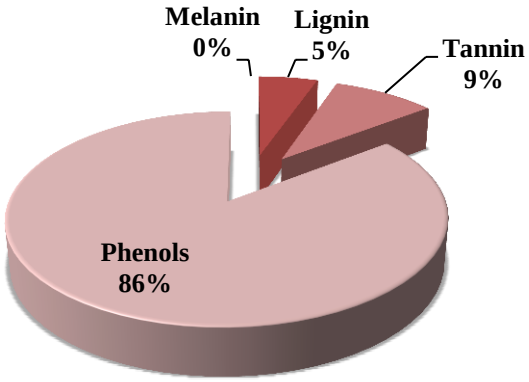


Figure 7.8.7. Percentage of phenols, tannins, lignins and melanins compared to the total amount identified.

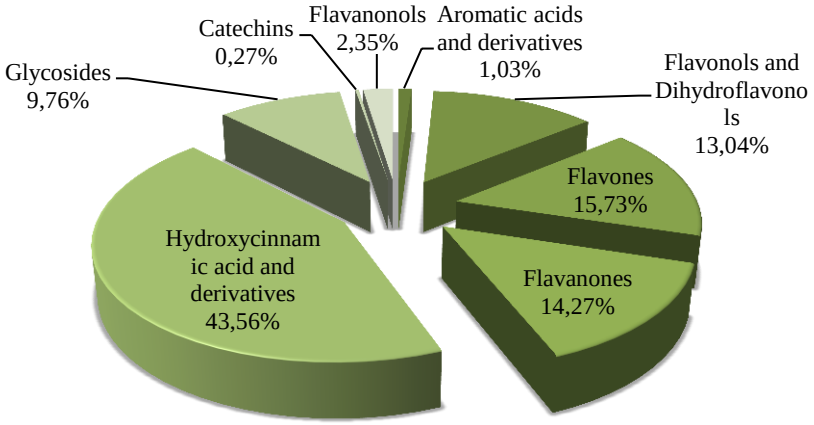


Figure 7.8.8. Percentage of families of phenols compared to the total amount phenols identified.

7.8.3 Bilogical and pharmacological activities of main phenolic compounds

For some of the most abundant phenolic compounds: apigenin, naringenin, salicin and rosmarinic acid (the latter, already reported in **paragraph 7.5)**; we report in the follow the main biological and pharmacological activities.

Apigenin (4',5,7-trihydroxyflavone) belongs to a less toxic and non-mutagenic flavone subclass of flavonoids, and is ubiquitously distributed in common fruits and vegetables. Apigenin exhibits a variety of biological effects, including anticarcinogenic, anti-inflammatory, and free radical-scavenging activities, anti-osteoporotic. It is a potent inhibitor of several protein kinases and has the ability to modulate the expression of PI3K-Akt,

MAPKs, casein kinase-2 and other upstream kinases. It is also noted that apigenin has vaso-relaxing, anti-platelet and anti- oxidant properties, which could actually reduce the risk of coronary heart disease and improve endothelial function. One that to be mentioned is that this compound is well metabolized and can circulate in the body after oral administration and intravenous injection. It has a relative rapid absorption and extensive metabolism, and its concentration appears as a one-compartment model after the single oral dose. Especially, apigenin has a high distribution level in the brain, which provides the solid evidence regarding the neuroprotective action.[870,871]

Naringin has been found to exhibit antioxidant activity, although this activity is not as strong as that of many other polyphenols. Among the studied health benefits of naringenin, some are attributed to its antioxidant, radical scavenging and metal chelation activities but others were suggested to occur through different mechanisms such as enzyme activity regulation, gene expression regulation and phytohormonal activity. The antiatherogenic and fat metabolism effects of naringenin is its most studied properties. Additional health benefits suggested in the literature include: anticancer potential, antimicrobial effects, antiviral effects with a unique inhibitory effect against the Hepatitis C virus, and a neuroprotective and anti-amnestic activity.[872] Moreover, studies proved that naringenin improves obesity-related diseases such as atherosclerosis in disease-model animals.[873]

Salicin is a glycoside with a several pharmacological roles, including analgesic and antipyretic. It si involved in modulating of infiammatory process through inhibition of the activation of NF-κB, and subsequent down regulating COX-2 expression. Moreover, the elucidation of chemical structures of salicin and its metabolite, salicylic acid, leads the discovery of the most common anti-infiammatory drug, acetylsalicyl acid or aspirin.[874,875]

7.9 *Plantago* (ABO-AR-2014-159)

Plantago major L. (*Plantago major* ssp. *major* L.) is a perennial plant that belongs to the Plantaginaceae family. Research on pollen has shown that *P. major* was introduced to Nordic countries parallel to the introduction to the first primitive cultivated fields in the stone age nearly 4000 years ago.[876] P. major was spread by man from Europe throughout the world. The Indians named it ‘White man’s footprint’ because it was found everywhere the Europeans had been. This has been adapted into the genus name *Plantago* that is from Latin *planta*, meaning

sole of the foot. *P. major* is a plant that many people know only as a weed, but *P. major* is also an old medicinal plant that has been known for centuries. In Scandinavia this plant is mostly known for its wound healing properties. The common Norwegian and Swedish name for *P. major* is groblad meaning 'healing leaves'. More recent ethnopharmacological studies show that *P. major* is used in many parts of the world and in the treatment of a number of diseases: skin diseases, infectious diseases, problems concerning the digestive organs, respiratory organs, reproduction, the circulation, against tumours, for pain relief and for reducing fever.[877] *P. major* is used for different purposes in traditional medicine around the world, therefore, researchers have tested it for different types of biological activities, most of the which gave successful: antiulcerogenic,[878] anticancer,[879] immunomodulatory,[880] antibiotic and antifungal, antigiardiasic,[881] antimalarial,[882] anti-inflammatory and analgesic,[883] antioxidant and free radical scavenger,[884] diuretic effect,[885] hypotensive effect,[886] hypoglycaemic[887].

7.9.1 Sample characterization

Phenols medium-low molecular weight

1 gr of lyophilized sample was extracted in soxhlet for 6 h with petroleum ether, dichloromethane and methanol (80% water solution). The fraction of methanol 80% was resuspended in ethyl acetate and it was washed with water. The total amount for 1 gr of sample of four fractions was found to be: petroleum ether 15.5 mg, dichlorometane 36.35 mg, methanol 80% 432.9 mg, and only 53,896 mg, after treatment with ethyl acetate.

The fractions were analysed through GC-MS analysis after derivatization with silylating reagents, the chromatograms of each fraction are shown in **Figures 7.9.1, 7.9.2, 7.9.3** and **7.9.4.**

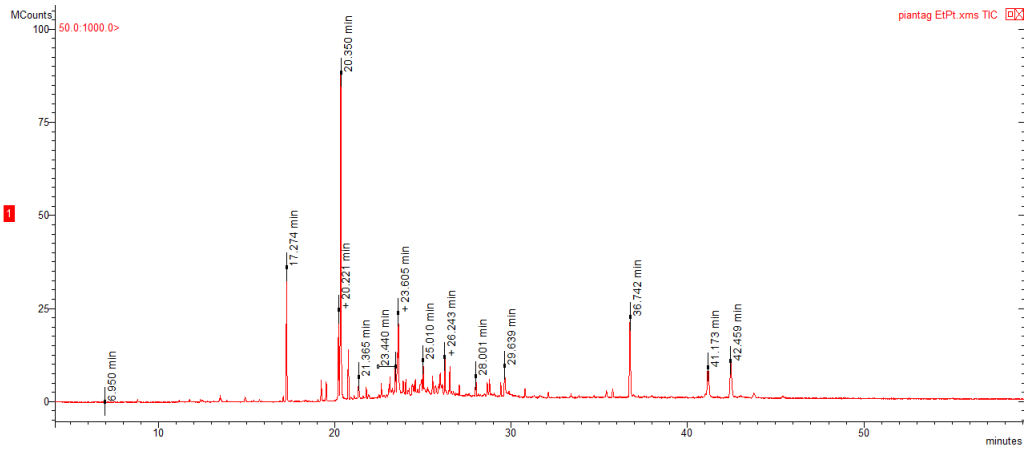


Figure 7.9.1. Chromatogram of petroleum ether fraction.

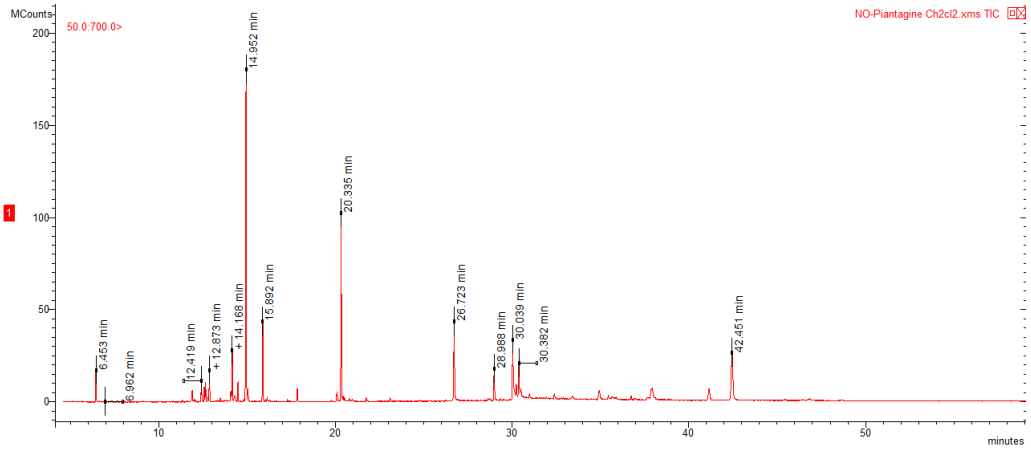


Figure 7.9.2. Chromatogram dichloromethane fraction.

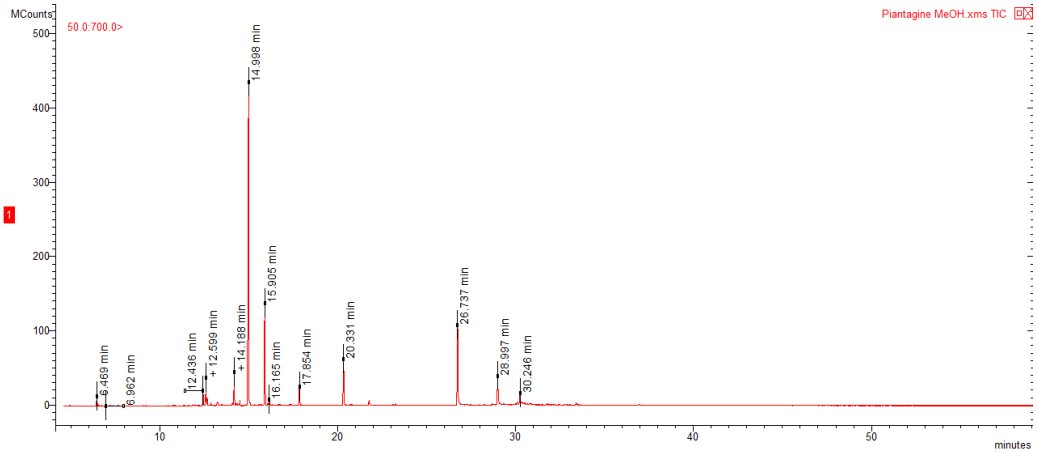


Figure 7.9.3. Chromatogram methanol (80% water solution) fraction.

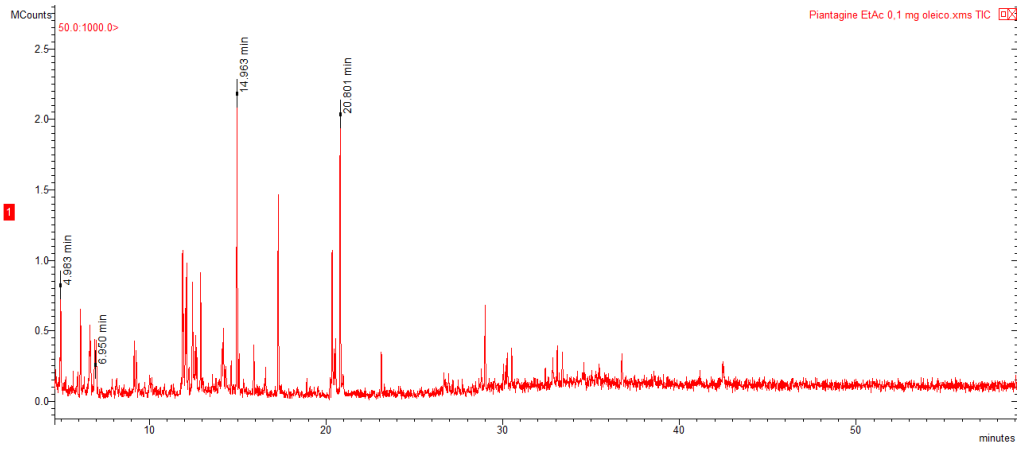


Figure 7.9.4. Chromatogram of ethyl acetate fraction.

The results of GC-MS analysis, grouped by family, are reported in **Table 7.9.1**. The quantities and the percentages refer to the total amount of phenols quantified for each family of compounds.

Plantago **Compound classes**	**Amount** **(mg/gr)**	**%**
Aromatic acids and derivatives	0,152	12,90
Flavonols and Dihydroflavonols	0,096	8,16
Flavones	0,672	56,94
Flavanones	0,024	2,05
Hydroxycinnamic acid and derivatives	0,086	7,31
Catechins	0,127	10,72
Coumarins	0,023	1,91

Table 7.9.1. Quantities and percentages for family of compounds.

Oligophenols and polyphenols high molecular weight

The sample extracted according to general procedure reported in previously paragraph was analysed through: ^{31}P-NMR, after phosphitilation, for the component of tannins (**Figure 7.9.5**). For 1 gr of lyophilized sample were obtained: lignins 25.29 mg, tannins 158,7 mg and melanins 0 mg.

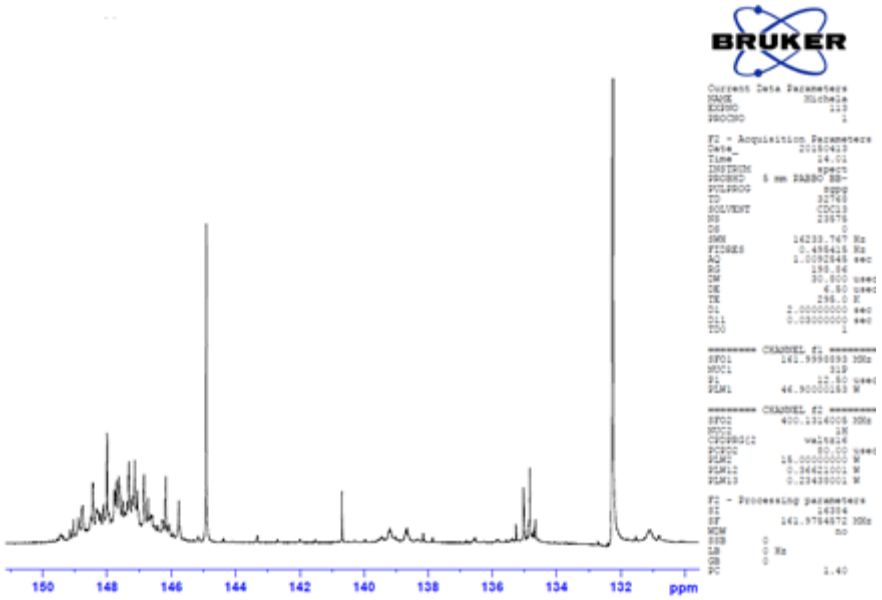


Figure 7.9.5. ^{31}P-NMR spectra of tannins.

Table 7.9.2 shows the qualitative and quantitative data of tannins. The sample present comparable percentage of hydrolizable (43,6%), and condensed (56,4%) tannins. As it regards, the subclasses of hydrolizable tannins, gallotannins are predominant (identified with a percentage of 71,12%) , regard at ellagiotannins (identified with a percentage of 28,88%).

Tannins		**mg/g of tannins**	**% tannins classes**
Hydrolizable	Gallotannins	49,21	30,01
	Ellagiotannins	19,98	12,59
Condensed		89,51	56,4

Table 7.9.2. Classification of tannins classes.

7.9.2 Consideration data

On the basis of data previously reported it is possible to make some general considerations. Firstly, the total amount of phenols (including low and high molecular weigth derivatives) correspond to about 18,55% in weight of the sample (**Figure 7.8.6**). The remaining 81,45% is attributable to other families of natural substances.

Analyzing in detail the total of phenolic and polyphenolic fractions, it is observed that the polyphenols (99,36%) predominate on phenolic compounds at low-medium molecular weight (0,64%). Within polyphenols tannin and lignin represent respectively 85,57% and 13,80% (**Figure 7.9.7**) In particular, in the most abundant phenol families (**Figure 7.9.8**) were: flavones followed by aromatic acids and their derivatives, catechins and then by flavonols and dihydroflavonols and hydroxycinnamic acids and theirderivatives. Other families of natural substance have been identified in smaller percentages.

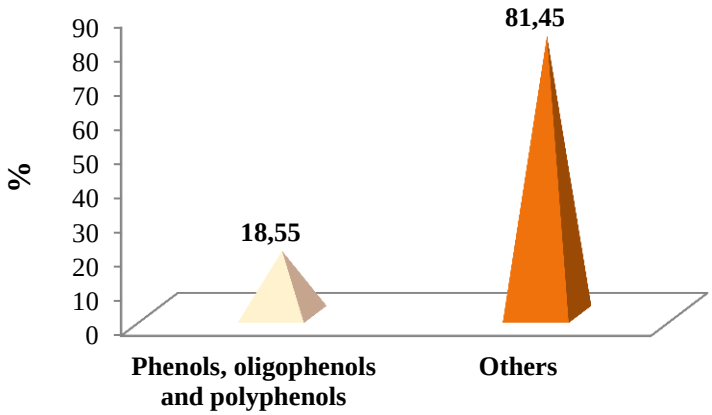


Figure 7.9.6. Percentage of phenols, oligophenols and polyphenols identified compared to the total sample.

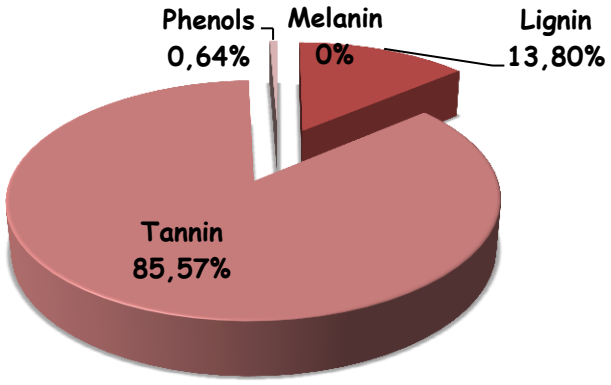


Figure 7.9.7. Percentage of phenols, tannins, lignins and melanins compared to the total amount identified.

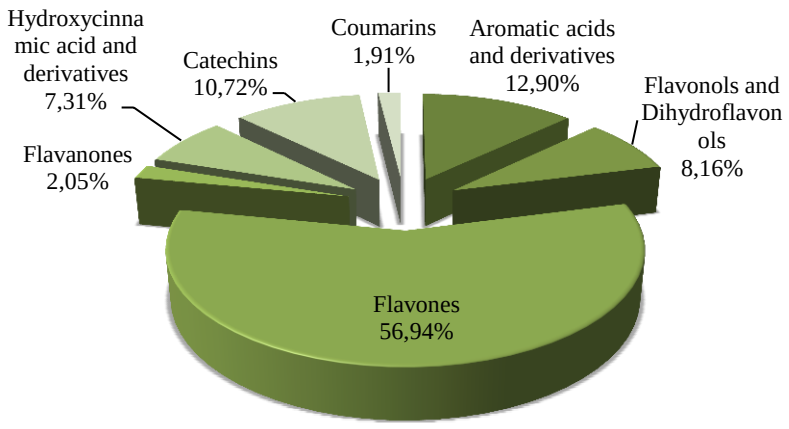


Figure 7.9.8. Percentage of families of phenols compared to the total amount phenols identified.

7.9.3 Biological and pharmacological activities of main phenolic compounds

For some of the most abundant phenolic compounds: luteolin, caffeic acid (the latter, already reported in **paragraph 7.7)** and caffeic acid phenethyl ester, we report in the follow the main biological and pharmacological activities.

Caffeic acid phenethyl ester is the ester of caffeic acid and phenethyl alcohol. Between the pharmacological activities there are: free radical scavenging, anti-oxidant, anti-infiammatory, cytoprotective, immunomodulatory, antiviral and anticancer.More over caffeic acid phenethyl ester had shown to ameliorate blood coagulation abnormalities and disturbed oxidative stress in endotoxic model of acute liver failure, increase cerebral blood flow and improve ischemic stroke in neurovascular disease, protect peripheral blood mononuclear cells against hyperthermal stress, prevent drugs to induce toxic and damage effects on red blood cells, and potently inhibit the synthesis of inflammatory and atherosclerotic leukotrienes in human polymorphonuclear leukocytes and whole blood.[888] Clinically, it has been shown to inhibit the growth and metastasis of different types of tumor cells, to protect tissues from reperfusion injury in various ischemia-reperfusion models, and to suppress inflammation in a variety of tissues. At a molecular level, caffeic acid phenethyl ester modulates the activity of enzymes such as matrix metallopeptidase 9, focal adhesion kinase, inducible nitric oxide synthase, HIVintegrase, lipoxygenase and cyclooxygenase-2 and of transcription factors such as nuclear factor-erythroid 2 p45(NF-E2)-related factor 2(Nrf2) and nuclear factor kappaB, which may be the molecular mechanisms underlying its biological activities.[889]

Luteolin (3,4,5,7-tetrahydroxy-flavone), is a bioflavonoid present in many medicinal plants as well as in some commonly consumed fruits and vegetables including green leafy spices such as parsley, sweet peppers and celery. Previous studies showed that luteolin possess numerous beneficialmedicinal properties including antioxidant, anti-inflammatory and antiallergic activity. Data from in vitro studies also suggest a protective role of luteolin in the vascular systemand the beneficial effect of luteolin on inflammatory process and inflammatory-associated cardiovascular disease. In this context, luteolin at higher doses (≥25 μM) inhibited oxidized low-density lipoprotein and TNF-α-induced VCAM-1 expression. Luteolin at pharmacological concentrations showed lowering plasma lipids, inhibiting cholesterol biosynthesis and increasing eNOS gene expression. Luteolin also protected against Fe(2+)-induced lipid peroxidation and dose dependently showed potent radical scavenging ability and Fe(2+)-chelating ability, but those effects require higher doses that are unachievable by dietary intake of this compound.[890,891]

7.10 *Grindelia* (ABO-AR-2014-158)

Grindelia is a genus of Asteraceae with about 45 species native to North America and Mexico and 26 to Austral South America, more precisely, to Peru, Chile, Bolivia, Brazil, Argentinaand Uruguay.[892] The herbal tea prepared from *Grindelia robusta* Nutt., one of the species growing naturally in northern North America, has antitussive, expectorant, sedative and anti-asthmatic properties.[893] Several classes of compounds have been identified, such as terpenoids (example grindelan-type diterpenes), flavonoids, methylated flavonoids, phenolic acids, acetylenes, essential oil, saponins and tannins.[893,894,895,896,897,898,899,900,901] Concerning its biological properties, extracts rich in saponins and polyphenols[902] and in methylated flavonoids[894] displayed anti-inflammatory activity and the essential oil proved to have antioxidant capacity[893]. Recently a relevant antioxidant activity has been shown for the essential oil.[903]

7.10.1 Sample characterization

Phenols medium-low molecular weight

1 gr of lyophilized sample was extracted in soxhlet for 6 h with petroleum ether, dichloromethane and methanol (80% water solution). The fraction of methanol 80% was resuspended in ethyl acetate and it was washed with water. The total amount for 1 gr of sample of four fractions was found to be: petroleum ether 110.95 mg, dichlorometane 57.30 mg, methanol 80% 682.70 mg, and only 73,732 mg, after treatment with ethyl acetate.

The fractions were analysed through GC-MS analysis after derivatization with silylating reagents, the chromatograms of each fraction are shown in **Figures 7.10.1, 7.10.2, 7.10.3** and **7.10.4.**

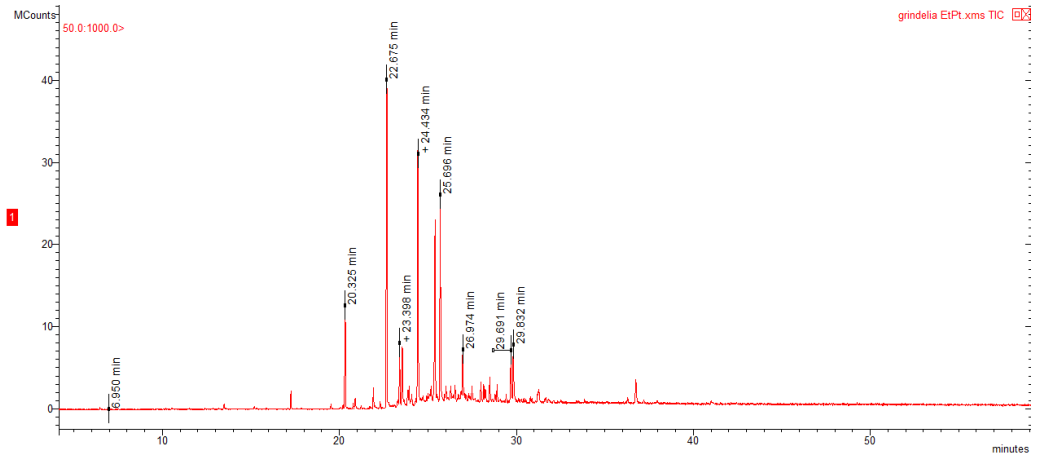


Figure 7.10.1. Chromatogram of petroleum ether fraction.

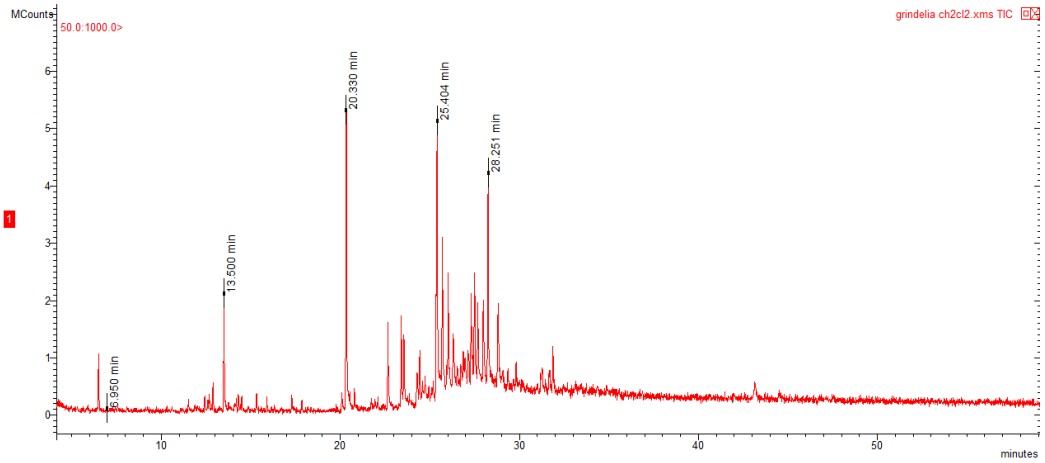


Figure 7.10.2. Chromatogram dichloromethane fraction.

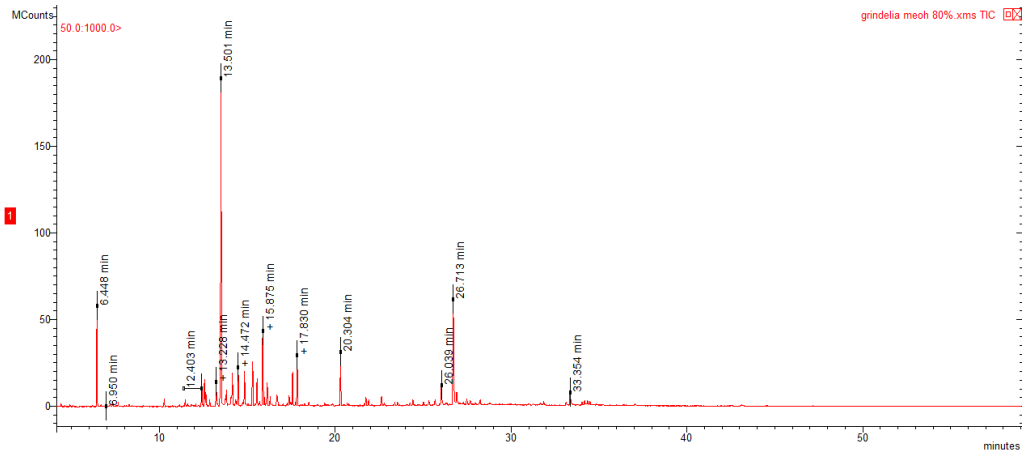


Figure 7.10.3. Chromatogram methanol (80% water solution) fraction.

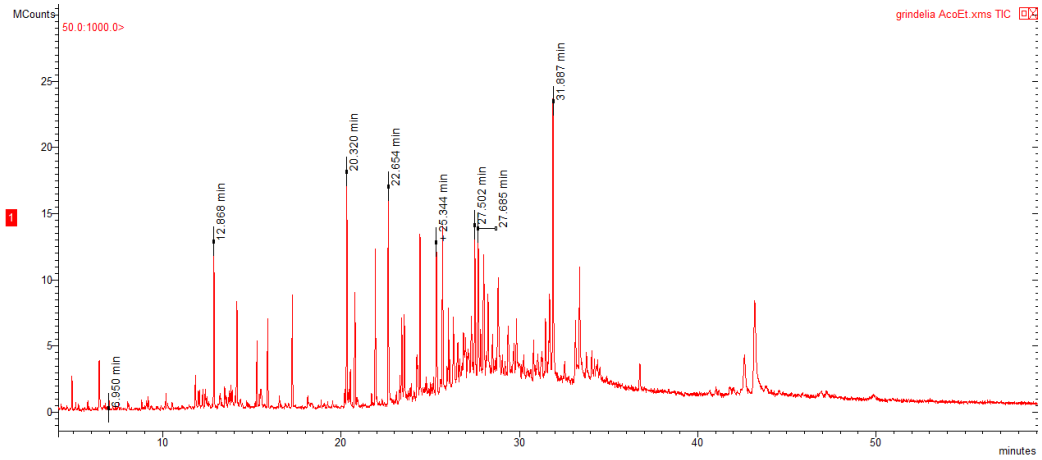


Figure 7.10.4. Chromatogram of ethyl acetate fraction.

The results of GC-MS analysis, grouped by family, are reported in **Table 7.10.1**. The quantities and the percentages refer to the total amount of phenols quantified for each family of compounds.

Grindelia **Compound classes**	**Amount** **(mg/gr)**	**%**
Aromatic acids and derivatives	4,770	20,77
Flavonols and Dyhydroflavonols	0,889	3,87
Flavones	11,487	50,03
Hydroxycinnamic acid and derivatives	5,269	22,95

Terpenophenols and terpenes	0,309	1,34
Catechins	0,238	1,04

Table 7.10.1. Quantities and percentages for family of compounds.

Oligophenols and polyphenols high molecular weight

For 1 gr of lyophilized sample were obtained: lignins 96.1 mg, tannins 14.38 mg and melanins 0 mg.

7.10.2 Cosideration data

On the basis of data previously reported it is possible to make some general considerations. Firstly, the total amount of phenols (including low and high molecular weigth derivatives) correspond to about 19,17% in weight of the sample (**Figure 7.10.5**), the remain 80,83% is attributable to other families of natural substances.

Analyzing in detail the total of phenolic and polyphenolic fractions, it is observed that the polyphenols (88%) predominate on phenolic compounds at low-medium molecular weight (12%). Within polyphenols tannin and lignin represent respectively 38% and 50% (**Figure 7.10.6**) In particular, in the most abundant phenol families (**Figure 7.10.7**) were: flavones followed by hydroxycinnamic acids and their derivatives and aromatic acids and their derivatives. Other families of natural substance have been identified in smaller percentages.

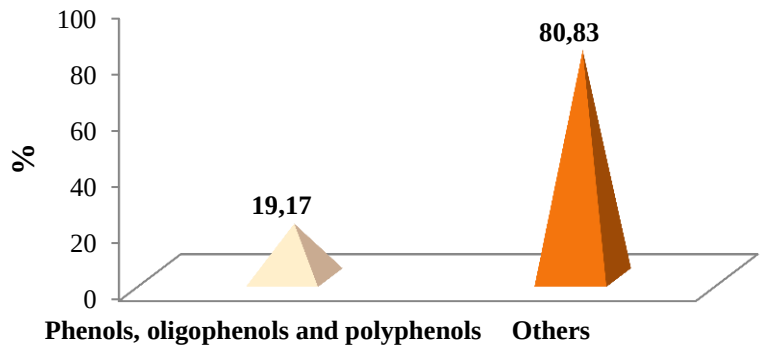


Figure 7.10.5. Percentage of phenols, oligophenols and polyphenols identified compared to the total sample.

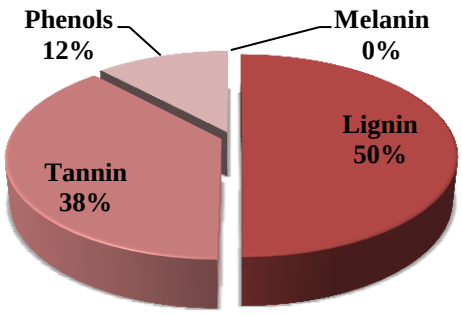


Figure 7.10.6. Percentage of phenols, tannins, lignins and melanins compared to the total amount identified.

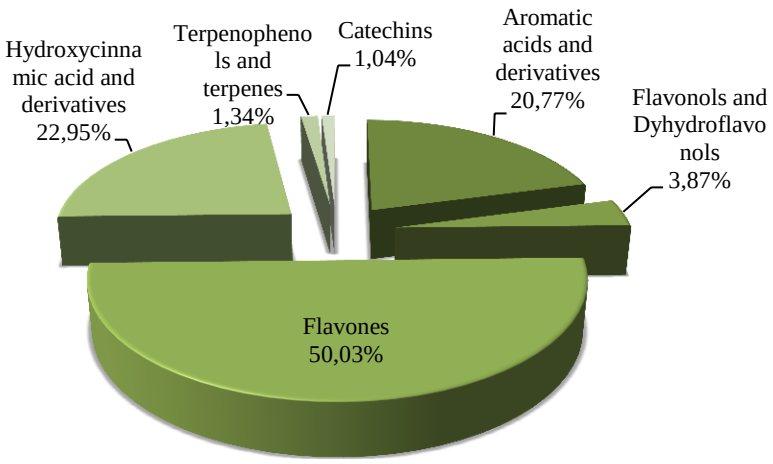


Figure 7.10.7. Percentage of families of phenols compared to the total amount phenols identified.

7.10.2 Biological and pharmacological acitivities of main phenolic compounds

For some of the most abundant phenolic compounds: protocatechuic acid, coumaric acid and gallic acid (the latter, already reported in **paragraph 7.4)**; we report in the follow the main biological and pharmacological activities.

Protocatechuic acid is one of the biologically active substances isolated from a number of popular medicinal plants growing in different parts of the world. Research conducted over the past several years indicates that it may be used in conventional medicine to prevent

cardiovascular diseases and cancer. The mechanism of the preventive action of protocatechuic acid is based on its antioxidant properties, that is, inhibition of the generation of free radicals, and their ability to scavenge and increase the catalytic activity of endogenous enzymes involved in the neutralization of free radicals. It is important that the impact of protocatechuic acid on the activity of enzymes involved in Phase I and II biotransformation of carcinogens and, possibly, direct blocking of specific binding sites of carcinogens with DNA molecule. Other aspects regarding the impact on the activity of cyclooxygenase-2, iNOS, inflammatory cytokines, and the proteins regulating cell cycle process are poorly understood.[904] Some authors have reported that protocatechuic acid could inhibit LPS-stimulated TNF-α and IL-1β secretion in RAW264.7 cells via NF-κB and MAPK pathways activation.[905] Other effects of protocatechuic acid are: antioxidative, antibacterial and it is beneficial in the treatment of breast, lung, liver, cervix and prostate cancers.[906]

p-Coumaric acid (4-hydroxycinnamic acid), a phenolic acid, is a hydroxyl derivative of cinnamic acid. It decreases low density lipoprotein (LDL) peroxidation, shows anti-mutagenesis, anti-genotoxicity, and anti-microbial activities, inhibits cellular melanogenesis, and plays a role in immune regulation in humans. p-Coumaric acid (p-CA) widely used in the chemical, food, health, cosmetic, and pharmaceutical industries. Some authirs reported that antioxidants, such as *p*-Coumaric acid and other hydroxycinnamic acids, function as chemoprotective agents by quenching carcinogenic nitrosating agents in several biological compartments, including salivary and gastric fluids. Moreover, recent research showed that *p*-coumaric acid killed pathogenic bacteria strain by provoking irreversible permeability changes in cell membrane, causing cells to lose the ability to maintain cytoplasm macromolecules, and binding to DNA to inhibit cellular functions.[907,908]

CHAPTER 8

LACCASE OXIDATION

8.1 Laccase immobilization

The supports chosen for this study are Multi Walled Carbon Nanotubes (MWCNTs), which unlike in Single Walled Carbon Nanotube (SWCNTs), show less surface area but are cheaper, easy available and with a better dispersion in aqueous solutions. As already described, the driving force which lead the laccase (Lac) physical adsorption on carrier is the interaction between the enzyme's hydrophobic regions and MWCNTs, mainly through electrostatic interactions.

The carrier were prepared for three different catalyst:

- Catalyst 1: MWCNTs + Laccase
- Catalyst 2: MWCNTs + Laccase + PDDA + Laccase
- Catalyst 3: Oxidized MWCNTs + PDDA + Laccase

MWCNTs show a very low dispersion in aqueous solution, therefore they were treated with ultra-sonication for 2,5 hours, increasing their dispersion and their surface area available for the enzyme interaction.

Poly(diallyldimethylammonium chloride) (PDDA) is a particularly stable immobilization agent used to form a homogeneous charged layer above the surface of the carrier by the layer-by-layer (LbL) technique. PDDA is a charged polyelectrolyte: the cationic groups on PDDA are quaternary ammoniums (**Figure 8.1**), meaning they are much less susceptible to oxidation. The polymer backbone of PDDA is derived from allylic groups and contain no peptide bonds. The layer of positively charged PDDA interacts electrostatically by the presence of negative charges on MWCNT-ox (catalyst III) or negative charge of enzyme already immobilized (catalyst II). The MWCNTs-ox, conversely, are more soluble in aqueous solutions because oxidation process add charged functional groups (carboxylic and hydroxyls group) on nanotube surface. The oxidation protocol provides treatment with 3:1 mixture of

concentrated sulphuric acid and nitric acid (sulphonitric solutions) in ultra-sonication bath for 4h at 25 °C. The effectiveness of the immobilization procedure was investigated in terms of Immobilization Yield and Activity Yield by the analysis of the residual enzymatic activity in the waste waters after the reaction with the support.

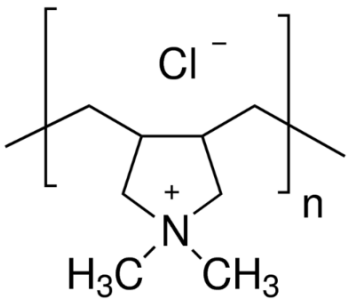


Figure 8.1. Poly(diallyldimethylammonium chloride)

8.2 MWCNTs-Laccase

The preparation of catalyst I consisted in the direct coating of the enzyme on the surface of MWCNTs (**Figure 8.2**). The enzyme immobilization occurred by orbital shaking for 2,5h at 200rpm in Na-acetate buffer 0.1M pH5 with free Lac and MWCNTs in the previously optimized 1:5 ratio (lac:MWCNTs).[909]

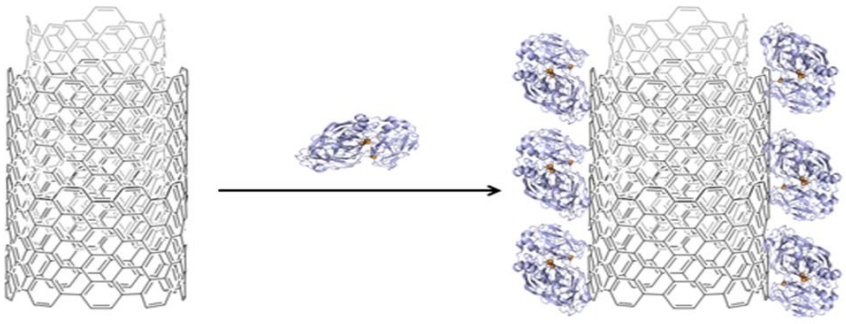


Figure 8.2 Laccase immobilization on MWCNTs

8.3 MWCNTs-Laccase-PDDA-Laccase

The catalyst I has been used to prepare catalyst II by a two step procedure: the first step was the coating of the polyelectrolyte charged layer of PDDA on catalyst I (**Figure 8.3**), followed (second step) by the immobilization of a second layer of enzyme. PDDA was added with 2:1 ratio (2.0 mg of PDDA each 1mg of catalyst I) in saline solution (NaCl 0.5M pH7) containing catalyst I (**Figure 8.4**). The mixture was sonicated to increase the solubility and centrifuged many times to remove NaCl surnatant thought milliQ water.

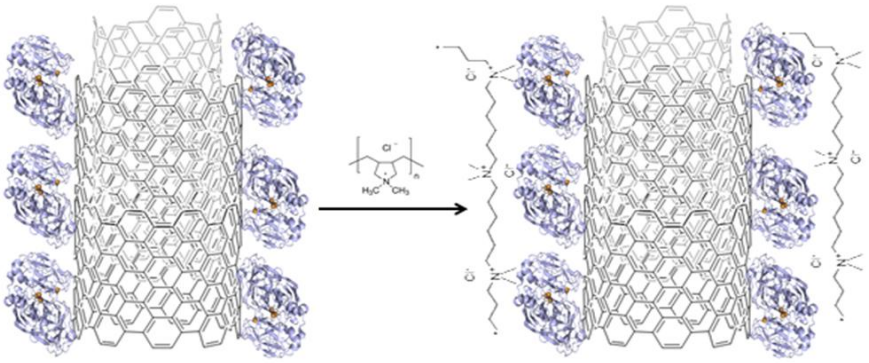


Figure 8.3. Deposition of PDDA on immobilized Laccase.

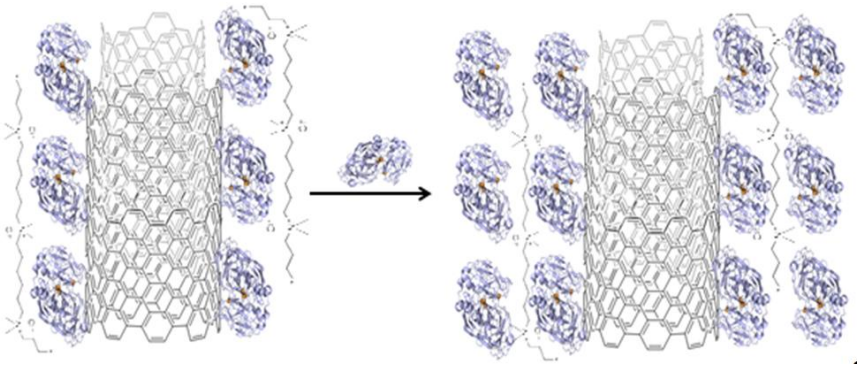


Figure 8.4. Addiction of free laccase on immobilized PDDA.

After eliminating the salt, an appropriate amount of enzyme was added to maintaining the same proportions as first enzyme immobilization (i.e. ratio 1:5). The enzyme immobilization occurred in Na-acetate buffer pH5 0.1M by orbital shaking for 2,5h.

8.4 Oxidized MWCNTs-PDDA-Laccase

The MWCNTs underwent an oxidation process which added charged functional groups (carboxylic and hydroxyls group) on nanotube surface. The oxidation protocol provides treatment with 3:1 mixture of concentrated sulphuric acid and nitric acid (sulphonitric solutions) in ultra-sonication bath for 4h at 25 °C (**Figure 8.5**).[910]

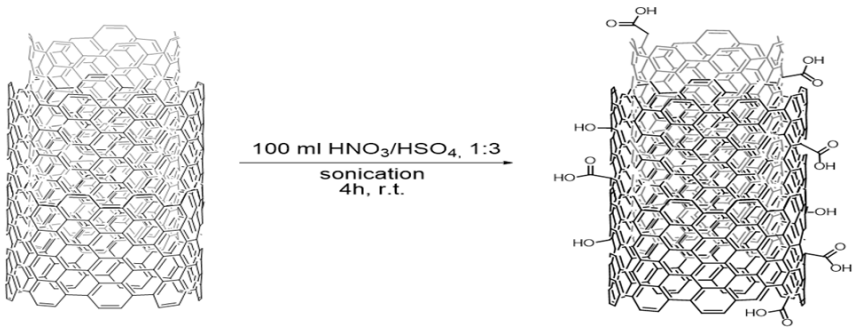


Figure 8.5. Oxidation of MWCNTs

Several centrifuge/sonication cycle of acid solution containing MWCNTs-ox were done for removing the excess of the acid until a neutral pH of solution was obtained using H20 milliQ in each cycle. Subsequently a PDDA layer was added on MWCNTs-ox in the 1:2 ratio (2mg of PDDA each 1mg of MWCNTs-ox) (**Figure 8.6**).

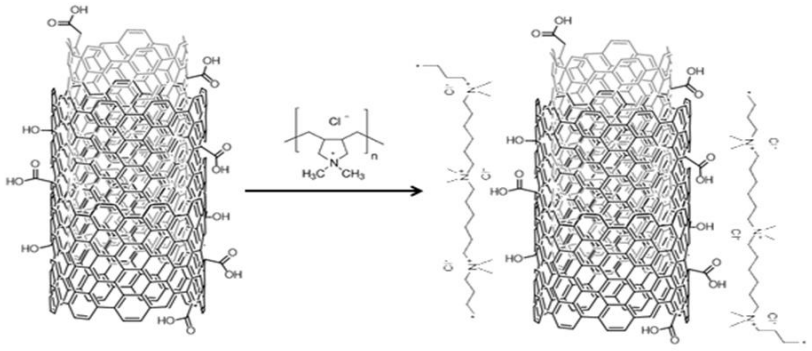


Figure 8.6. Layer of PDDA deposit on oxidized MWCNTs

In the last step an appropriate amount of the enzyme was added to maintain the same proportions as first enzyme immobilization (i.e. ratio 1:5) (**Figure 8.7**).

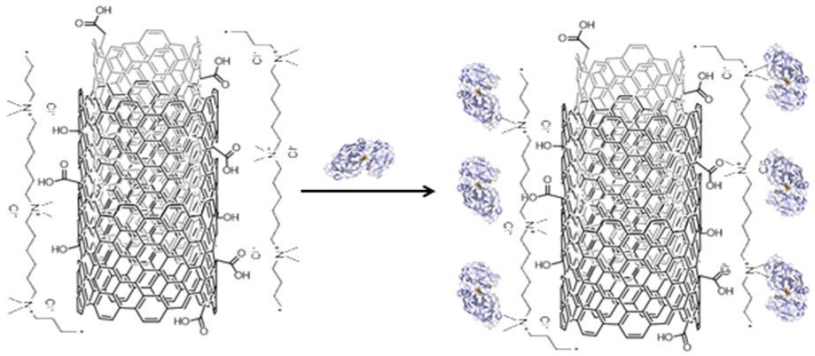


Figure 8.7. Enzyme immobilization on PDDA layer

The enzyme immobilization occurred in Na-acetate buffer pH5 0.1M by orbital shaking for 2,5h. Data for the activity, activity yield and immobilization yield for catalysts I-III are reported in **Table8.1** The activity of Lac and catalysts I-III was determined pectrophometrically using ABTS as a substrate (one activity unit U was defined as the amount of enzyme that oxidized 1 μm ABTS/min). As reported in **Table 8.1**, catalysts II and III showed a value of activity (U/mg) comparable to that of Laccase (**Table 8.1**, entry 1 versus entries 3 and 4). On the other hand, in accordance with the literature,[911] the activity significantly decreased in the case of catalyst I (**Table 8.1**, entry 1 versus entry 2). Catalysts II and III showed higher values of activity yield and immobilization yield than I, Catalyst II being the best system.

Entry	Catalyst	Activity (U/mg)	Imm. Yield[a] (%)	Activity Yield[b] (%)
1	Lac	5.9	-	-
2	**I**	0.28 (0.3)	10	18
3	**II**	1.96	65	64
4	**III**	1.38	55	51

[a] Calculated as in Eq. (1). [b] Calculated as in Eq. (2). [c] Data reported in (J. H. Park et al. 2012),.

Table 8.1. Activity, activity yield and immobilization yield for catalysts I, II and III.

8.4 Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM)

Morphology and size of MWCNTs in the steps of preparation of the different catalysts are studied by combining scanning electron microscopy (SEM) and atomic force microscopy (AFM) analyses. SEM observing were carried out using a Zeiss LEO 1530 apparatus equipped with a field emission electron gun, while tapping mode AFM measurements are performed in air with a Digital D5000, equipped with Nanoscope IV controller, using commercial silicon nitride cantilevers (cantilever resonance frequency range 51-94 kHz for ‘‘soft’’ organic samples).

The analyses were performed on well-dispersed and separated MWCNTs supported on a silicon substrate (Si). All representative samples were dispersed in milliQ water, in order to prevent the enzyme denaturation. Solutions were whirled (by a Vortex) in milliQ water, for 5 min at room temperature (r.t.), and a drop of each solution was taken and deposited onto silicon (Si) substrates, heated at 50 °C to quickly evaporate the water.

First SEM observing were done to identify individual dispersed nanotubes and then, through a mapping of Si substrate, we detect same nanotubes with the AFM. Figure 4.2.1 shows SEM images and corresponding AFM images of selected areas of the different samples.

A long nanotube and more shorter tangled nanotubes of catalyst I (MWCNTs/Laccase) are shown in **Figure** (a and b). Nanotubes are well dispersed but also shorter than pristine MWCNTs, as reported in a previous work. Probably MWCNTs are more fragile after the laccase immobilization, and the vortex or the sonication process breaks the nanotubes easily.

A single nanotube of catalyst II (MWCNTs/Laccase/PDDA/Laccase) is shown in **Figure** (c and d). Nanotubes were easily dispersed but also broken by the stirring process. All nanotubes seem like rigid rods.

A MWCNTs tangle of catalyst III is reported in **Figure 8.8** (**e** and **f**). In this case it was more difficult to disperse and identify a single nanotube and those identified appear very tangled. The interaction between nanotubes is much more intense than the catalysts I and II; a simple process of stirring is not enough to separate the functionalized MWCNTs . In the **Figure 8.8**

(**f**) is shown as the AFM tip, during the scanning, drags the compact tangle of nanotubes that appear as white spots at higher height.

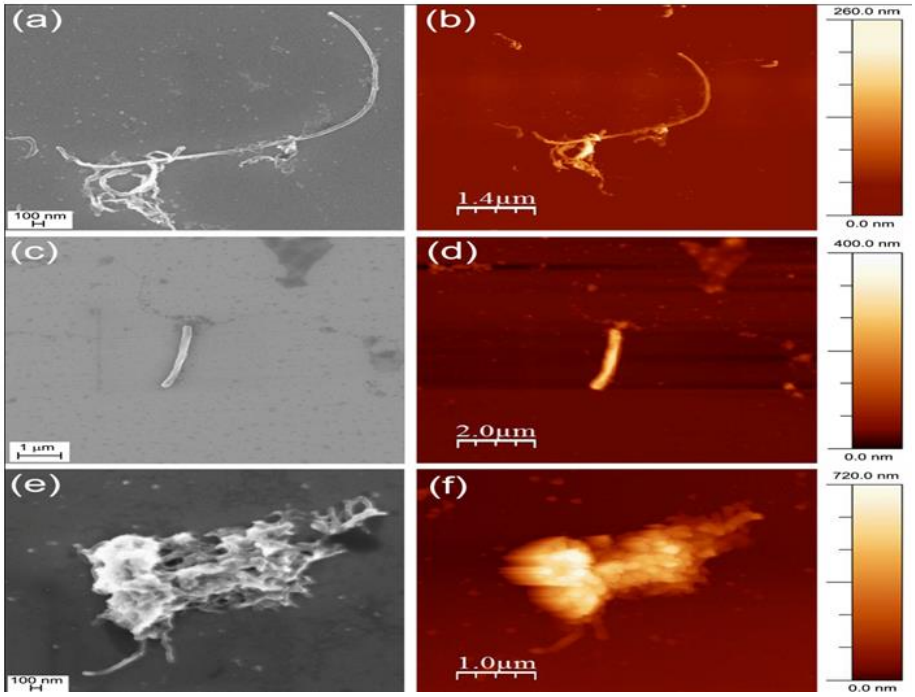


Figure 8.8. SEM and AFM images of MWCNTs-Laccase catalyst I (a and b), MWCNTs-Laccase-PDDA-Laccase catalyst II (c and d), Oxidized MWCNTs-PDDA-Laccase catalyst III (e and f) deposition onto Si substrate.

Figure 8.9 reports high resolution AFM images (2D, 3D) of individual MWCNTs and related image profiles for selected directions (lines in **Figure 8.9 c**, **f** and **i**). The maximal height (h) and the width at half-height (d) have been evaluated by the profile analysis and the width has been estimated at half-height and not at the bottom, in order to reduce errors resulting from to effects of tip convolution. Line profiles are shown with the same x-scale to better show differences in the width. h and d values are shown in the **Table 8.2** and compared with values of pristine MWCNTs and Oxidized MWCNTS/PDDA, as reported in our previous work .

Images of **Figure 8.9 a** and **b** are details of catalyst I of **Figure 8.9 b**. The nanotube surface is smooth, the maximum height (h) and the width at half height (d) (**Table.**), are 47.1 ± 0.5 and 55 ± 2 nm, respectively. These values are similar to that pristine MWCNTs confirming the low value of the immobilization yield previously reported.

High-resolution AFM images of catalyst II are reported in **Figure 8.9 d** and **e**, **h** and **d** were 302.2 ± 0.5 and 270 ± 2 nm, respectively (**Table 8.2**) and the surface has different structure and roughness. Width and height are six times larger than the pristine MWCNTs, confirming that more external shells cover the nanotube. In addition shells are very compact and make the nanotube's structure more rigid than the pristine MWCNT.

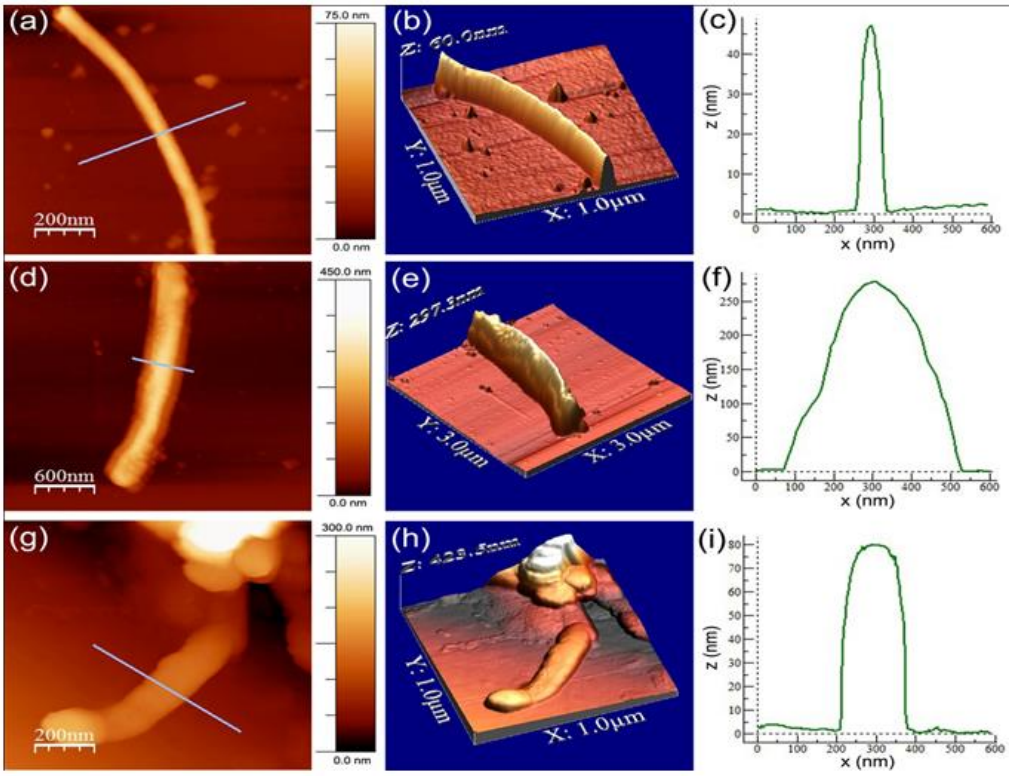


Figure 8.9. High resolution AFM images bidimensional, tridimensional and image profiles of catalyst I (a, b and c), of catalyst II (d, e and f) and of catalyst III (g,h and i).

Catalyst	h±Δh (nm)	d±Δd (nm)
MWCNT	39.0 ± 0.5	53 ± 2
I	47.1 ± 0.5	55 ± 2
II	302.2 ± 0.5	270 ± 2
Ox-MWCNT-PDDA	60.5 ± 0.5	90 ± 2
III	80.0 ± 0.5	153 ± 2

Table 8.2. Maximal height (h) and width at half height (d) of catalyst I (MWCNT-Laccase), II (MWCNT-Laccase-PDDA-Laccase), III (Oxidized MWCNT-PDDA-Laccase), pristine MWCNTs and Oxidized MWCNTs-PDDA evaluated by a profile analysis of AFM images.

The high-resolution AFM analysis of catalyst III (**Figure 8.9** g and h) shows a smooth surface, and height and width of 80.0 ± 0.5 and 153 ± 2 nm, respectively (**Table 8.2**). The doubled height to respect to pristine MWCNTs, suggests that the external shells are present. The ratio 1:2 (height/width) suggests that a large interaction with the substrate is present as well as between the nanotubes, confirmed by the presence of tangles of catalyst III. Then the CNT surface oxidation and the presence of PDDA produce a stronger bond with enzymes than the un-functionalized MWCNTs.

8.5 Kinetic parameters of immobilized Laccase

The enzyme activity was expressed in units defined as the amount of enzyme oxidizing 1 μmol of ABTS min-1. Data are reported in **Table 8.3**. The apparent Km value of native Lac was estimated to be 0.16 mM, and the corresponding Vmax value was 2050 U/mg.

Entry	Catalyst	K_m (mM)	V_{max}[a] (U/mg)	V_{max}/K_m[b]
1	Lac	0.16	2050	12812
2	I	0.11	247	2245
3	II	0.12	1482	12350
4	III	0.14	1044	7457

[a] Vmax was defined as ΔAbs (min μgenzyme)$^{-1}$. [b] Vmax/Km was defined as ΔAbs (min μg_{enzyme} mM)$^{-1}$. Each experiment was conducted in triplicate. Average errors in kinetic parameters were ± 2-4% for Km and ±1-3% for Vmax.

Table 8.3. Kinetic parameters of free Laccase (Lac) and catalyst I, II and III.

The catalysts I-III showed values of Km (mM) comparable to that of Lac (Free Laccase) (**Table**, entry 1 versus entries 2-4). On the other hand, irrespective to procedure used for immobilization, Vmax and Vmax/Km decreased for supported laccases, indicating a reduction of the catalytic efficiency probably due to mass transfer limitations.[912,913] A similar behaviour was previously observed during the immobilization of laccase on the epoxy resin Eupergit

C250L.The decrease in efficiency is however much less evident in the case of catalysts II and III that maintain values of the kinetic parameters the same to those of the native enzyme.

8.6 Effect of pH on Laccase activity

The pH/activity curves related to catalysts I-III are shown in **Figure 8.10** The activity was determined using ABTS as substrate in the range of pH 2.0–6.0 (citrate-phosphate buffer for pHs 2.0–6.0). The catalysts I-III showed a similar pH activity profile, with a maximum of activity in the range between 2.8 and 4. As a general trend, the catalysts II and III retained slightly more activity than catalyst I, suggesting the beneficial effect exerted by the polyelectrolyte coating on the maintenance of the active enzyme conformation. The Immobilization of enzymes on charged supports can leads to displacement of the pH-activity profile to either alkaline or acidic regions.[914,915] In the present study, catalysts I-III and Lac show a similar activity profile.

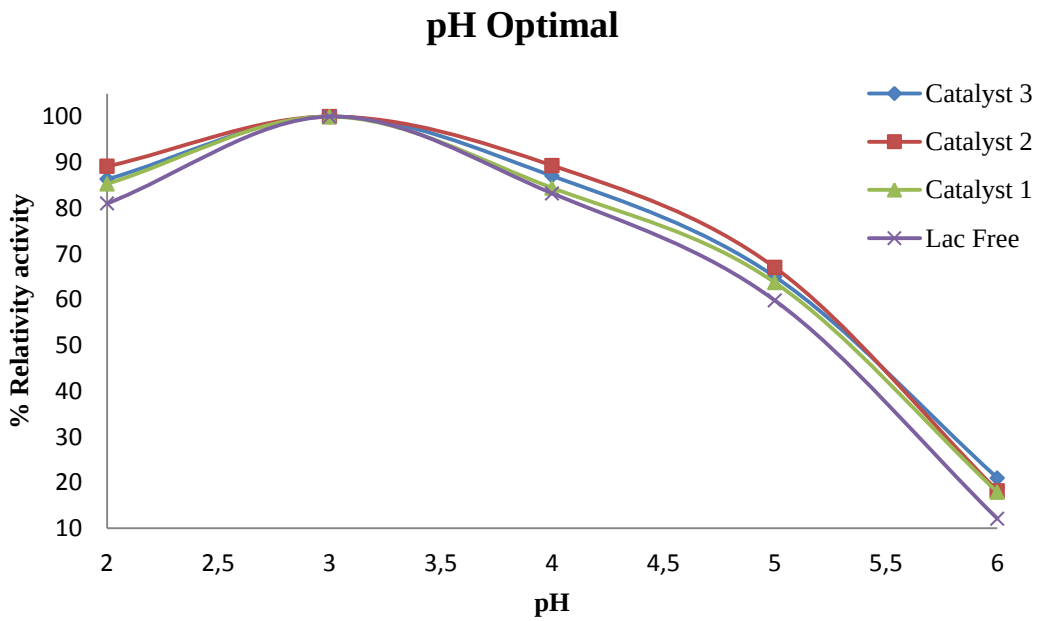


Figure 8.10. Optimal pH of free (Lac) and immobilized laccase (Catalyst I, II and III). Laccase activity was determined using ABTS as substrate in the range of pH 2.0–6.0. Results are the mean of triplicate assays.

8.6 Catalytic behaviour of Laccase-MWCNTs and Laccase-MWCNTs-PDDA

The catalytic activity of the laccase-MWCNTs and the laccase-MWCNTs-PDDA systems entrapped onto the SPE surface was tested in the presence of a diffusional electron mediator, ABTS. The process catalyzed by laccase in the presence of ABTS was monitored by recording the electrochemical signal that the redox compound generates onto the electrode surface operating in oxygen-containing atmosphere. Laccase molecules at the surface of the electrode were oxidized by O_2 and then re-reduced by the substrates, acting as electron donors for the oxidized form of the enzyme. These products can be reduced at the electrode surface, giving a reduction current which is proportional to the substrate concentration.[916] The bioelectrochemical behavior of the two laccase based systems modified electrodes in the presence of 0.5 mM ABTS was characterized by cyclic voltammetry, as reported in **Figure 8.11 A** and **B** for the two systems studied. Slow-scan rate voltammograms were recorded in ABTS solutions with both a bare screen printed electrode (**Figure 8.11 A-B** curves a) and a Laccase-MWCNTs-screen printed electrode (**Figure 8.11 A-B** curve b) and a laccase-MWCNTS-PDDA-screen printed electrode (**Figure 8.11 B** curve b), respectively. In both cases, the CVs was characterized by the decrease of the anodic process due to the catalytic oxidation of the mediator, and a significant enlargment of the cathodic process related to the reduction of the oxidized form of the mediator generated by the enzymatic reaction. As a consequence the CV shape changed from diffusion control peaks to a catalytic curve. This indicates the electrocatalytic reduction of oxygen and demonstrated that the bio-electrocatalytic recycling of the substrate proceeds efficiently between the electrode and the immobilized laccase. By comparing the reduction currents of **Figure 8.12 A and B** it is possible to note that the reduction current is definitely higher for the laccase-MWCNTs-PDDA system, again indicating that the presence of the polymer effectively enhances the enzyme efficiency.

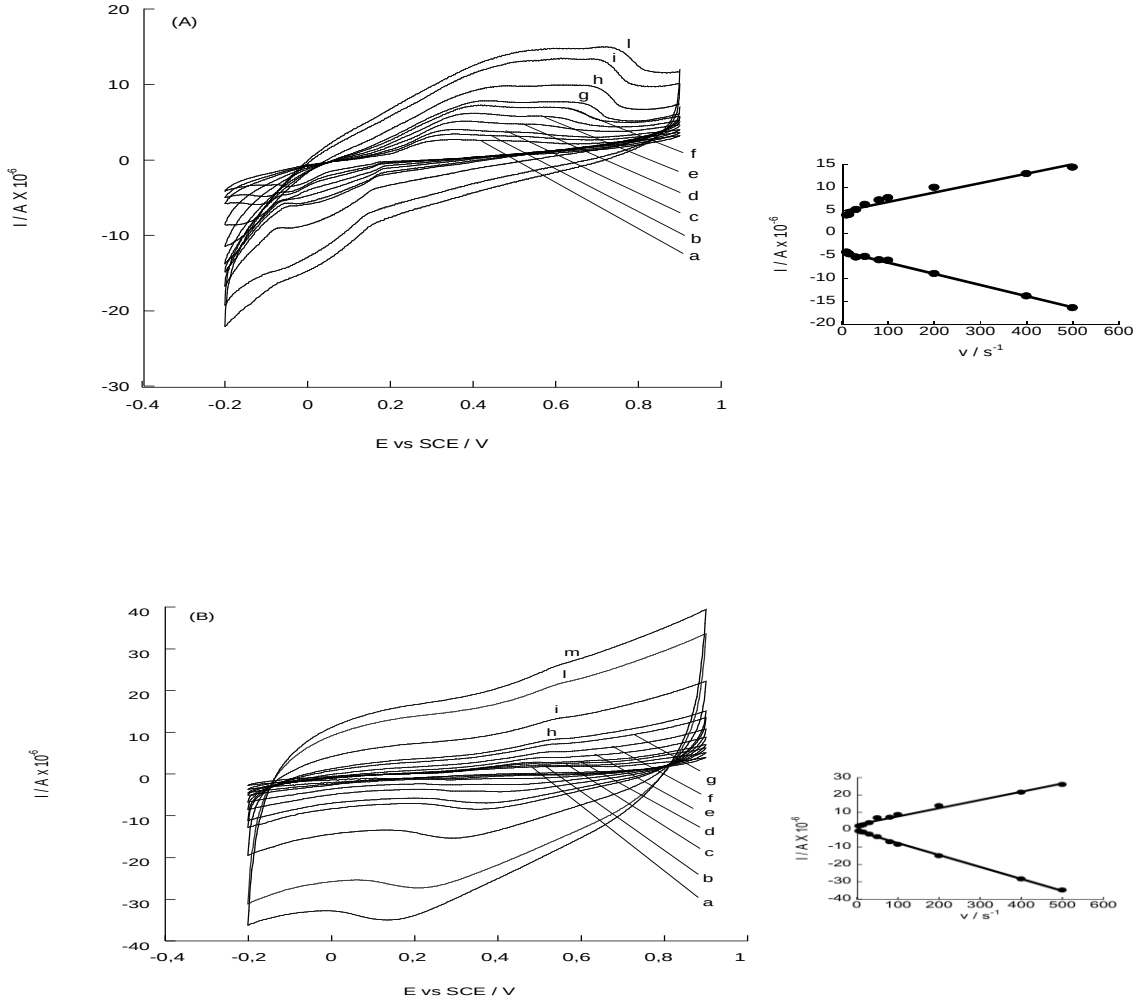


Figure 8.11. Cyclic voltammograms of the Laccase-MWCNTs (A) and Laccase-MWCNTs-PDDA (B) at different scan rates: 2 (a); 5 (b); 10 (c); 15 (d); 30 (e); 50 (f); 80 (g); 100 (h); 200 (i); 400 (l); 500 (m) mV s-1. The insets represent the variation of anodic and cathodic peak intensities of the first peak couple as a function of scan rate.

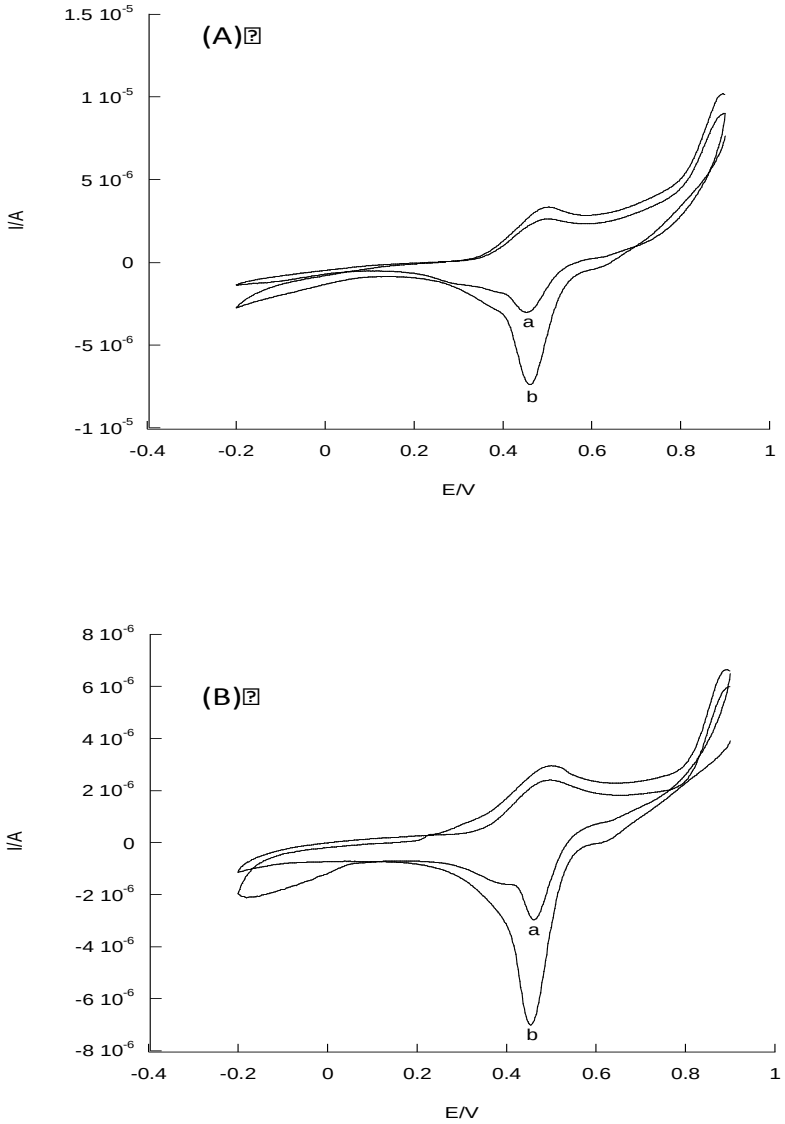


Figure 8.12. Cyclic voltammograms of 0.5 mM ABTS in absence (curves a) and in presence (curves b) of laccase-MWCNTs (A) or laccase-MWCNTs-PDDA (B). Measurements carried out in 0.1 M B-R buffer, pH=5 at a scan rate of 5 mV s-1.

8.7 Storage stability

The measurement of the residual activity as a function of time was performed in order to evaluate the effect of MWCNTs on the stability of laccase under storage. The activity of native laccase and catalysts I-III was evaluated by storing the sample in sodium acetate buffer 0.1M, pH 5 at 4 °C for 30 days. At different times (up to 30 days), aliquots were taken and the activity was determined by the previously described ABTS method, followed the warming of

the solution at 25°C. In **Figure 8.13**, the enzyme activity is expressed as a relative percentage of the activity at time zero. With the only exception of the first two days, the catalysts I-III were significantly more stable than laccase, the stabilizing effect being more pronounced by the increasing of time (maximum Δvalue after 30 days). As a general trend, the catalysts II-III were more stable than I, suggesting a benign role of the PDDA coating on the stability of laccase. On the other hand, the similar pattern of storage stability observed for catalysts II and III suggests that the oxidation state of CNTs (that is pristine MWCNTs versus ox-MWCNTs) is not a relevant parameter for the stability of Laccase.

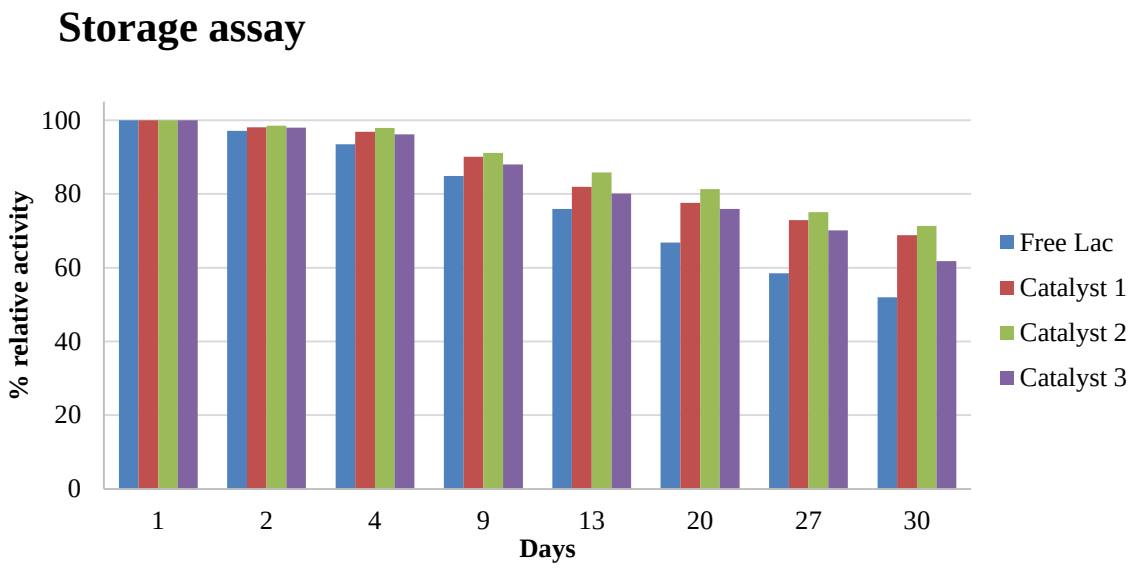


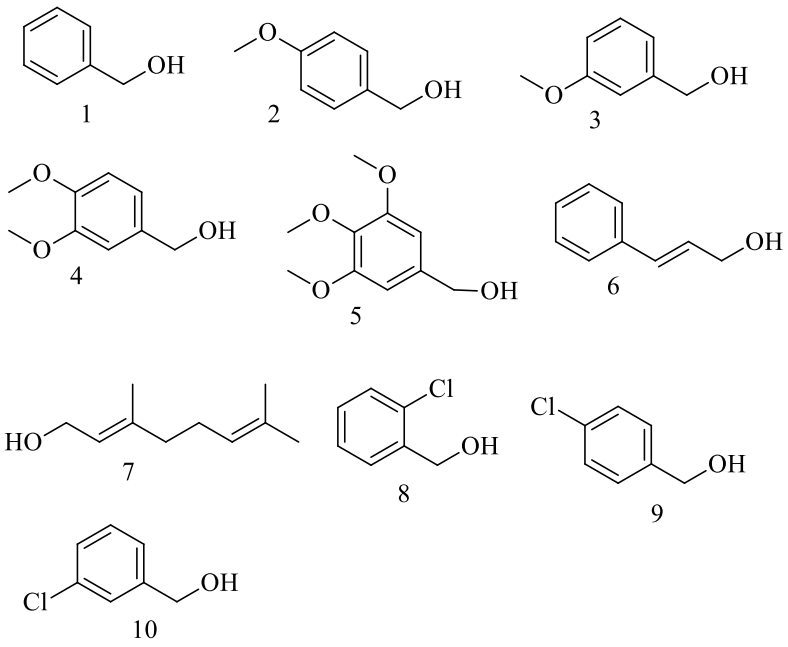
Figure 8.13. Storage stability of free (Lac) and immobilized laccase (Catalyst I,II and III) at 4°C in Na-acetate buffer 0.1 M, pH 5.0. Data are the means of three experiments. Standard deviations of data were less than 5%.

8.8 Alcohol oxidations by catalysts

The oxidation of alcohols to corresponding aldehydes is a key process in organic chemistry. Usually, traditional chemical methods shows a limited selectivity, affording mixtures of aldehydes and carboxylic acids in experimental conditions that are not environmental friendly On the other hand, laccases are able to perform this oxidation with high selectivity in the presence of mediators, such as 2,2,6,6-tetramethyl-1-piperidinyloxy free radical (TEMPO; E^0= 0.75 V), and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS; E^0=0.5 V). TEMPO and ABTS show different reaction mechanisms. ABTS follows the electron-transfer

(ET) process, while TEMPO undergoes the one-electron transfer mechanism to form the oxoammonium ion. Recently, we reported that Lac from *Trametes versicolor,* immobilized on the epoxy resin Eupergit C-250L and coated by the LbL procedure, was able to selectively oxidize alcohols to corresponding aldehydes, TEMPO being the most efficient mediator (M. Guazzaroni, et al. 1987 – 1996). On the basis of these data, with the aim to evaluate the catalytic relevance of catalysts I-III, a large panel of aromatic and aliphatic alcohols was oxidized, including benzyl alcohol **1**, 4-methoxybenzyl alcohol **2**, 3-methoxybenzyl alcohol **3**, 3,4-dimethoxybenzyl alcohol **4**, 3,4,5-trimethoxybenzyl alcohol **5**, cinnamyl alcohol **6**, geraniol **7**, 2-chlorobenzyl alcohol **8**, 4-cholorobenzyl alcohol **9** and 3-cholorobenzyl alcohol **10**. Note that the value of the redox potential decreases by increasing the number of methoxyl groups in the aromatic alcohols. The reaction was carried out by treating the appropriate alcohol (20 mM) with catalysts I-III (0.65 U) and mediators (TEMPO and ABTS, 6 mM) in 0.1 M Na-acetate buffer pH 5 (1.0 ml) at 25°C in presence of O_2. The work-up of the reactions was performed after 16hr for the more reactive TEMPO, and after 24 hr in the case of ABTS. In the absence of the mediator we did not observe any oxidation, probably because none of the substrates **1-10** are natural targets for laccase. Irrespective to catalyst used, the oxidation of alcohols **1-7** in the presence of TEMPO was more efficient that with ABTS, leading to selective formation of aldehydes **11-17** in quantitative conversion of substrate and yield (>98%), and in a lower reaction time (**Table**, entries 1-28). Catalysts II and III showed a very similar reactivity, and in some cases slightly higher, than that of Lac and catalyst I, indicating that the presence of the coating resulted in no appreciable kinetic barrier to the diffusion of the substrate at the catalytic-site (see for example, **Table**, entries 1 and 2 versus entries 3 and 4). The very high efficiency of TEMPO suggested a mechanism that did not depend on the redox features of the substrate. It is known that the active form of TEMPO is the oxoammonium ion formed by oxidation with laccase. This intermediate oxidized the alcohol to aldehyde producing the corresponding hydroxylamine, that can be regenerated by a comproportionation with the oxoammonium cations. Furthermore, the catalysts I-III showed a better performance than those based on the immobilization of laccase on Eupergit C 250L, producing aldehydes in higher yield even when the reactions were carried out using a significantly smaller amount of enzyme (i.e., 0.65 U for catalysts I-III compared to 10 U in the case of Eupergit C 250L). The oxidation of chlorinated alcohols **8-10** requires a more detailed discussion. As general trend, the reaction performed with TEMPO yielded mixtures of aldehydes **18**, **20** and **22**, and carboxylic acids **19**, **21** and **23**, respectively, in a different ratio depended on the reaction time (**Scheme**, **Table**, entries 1-18). The formation of

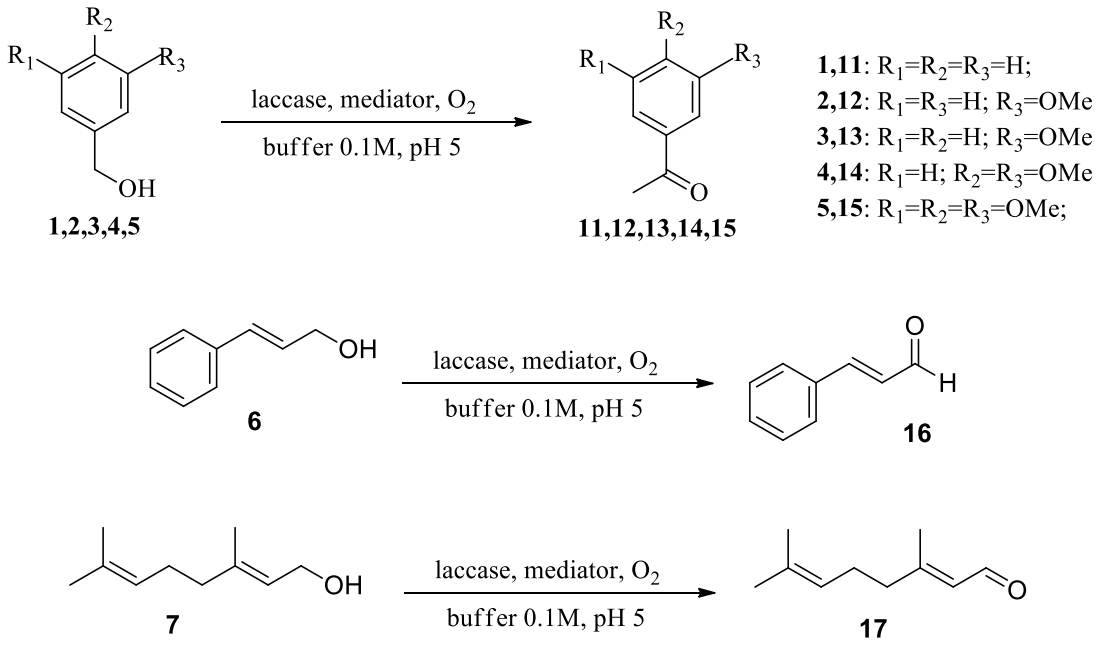
carboxylic acids was also observed with the immobilized laccase on Eupergit C 250L, and it is probably due to stereo-electronic effects exerted by the chlorine substituent on the reactivity of theoxoammonium ion intermediate of TEMPO. Noteworthy, at difference of Eupergit C 250L, the complete selectivity for the oxidation to aldehydes was obtained with catalysts I-III for short reaction times (see for example: **Table 8.4** , entry 8 versus entry 10 for catalyst I, and entry 11 versus entry 12 for catalyst II). Each catalyst was involved in oxidation of alcohols of different types, in order to understand and compare its efficiency. Alcohols which were used in oxidation reactions were benzyl alcohol **1**, 4-methoxybenzyl alcohol **2**, 3-methoxybenzyl alcohol **3**, 3,4-dimethoxybenzyl alcohol **4**, 3,4,5-trimethoxybenzyl alcohol **5**, cinnamyl alcohol **6**, geraniol **7**, 2-chlorobenzyl alcohol **8**, 4-chlorobenzyl alcohol **9** and 3-chlorobenzylalcohol **10**. The mediator is necessary because none of these substrates is a natural target of the Laccase; were used two different mediator (TEMPO and Abts) for some substrates, in order to compare the reactivity. Briefly, alcohol (20 mM), appropriate Laccase (10 U) and mediator (6 mM) were placed in 0.1 M Na-acetate buffer pH 5.0 (1.0 mL) in vigorous stirring at room temperature.



Scheme 8.1. Alcohols oxidized by free and immobilized Laccase.

The oxidation performed by catalysts converted each alcohol (**1-10**) in aldehyde (**11-20**), regardless of the mediator used and in some cases oxidation has went beyond producing acids.

The reactions were performed at a pH value different from the optimum measured previously using ABTS: it was understood running identical reactions but with different pH (3-5) and controlling the product amount. Interestingly the reaction carried out with the buffer at pH 5 showed a higher yield compared to that performed at pH 3.



Scheme 8.2. Oxidation of alcohols 1-7.

Yields were determined by GC-MS analysis using acetophenone as internal standard

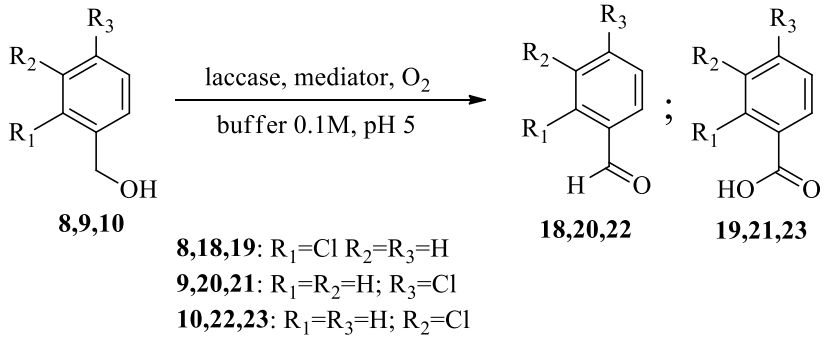
Entry	Substrate	Catalyst	Mediator	Time	Product	Yield %
1	1	Lac	TEMPO(ABTS)	16	11	96(4)
2	1	I	TEMPO(ABTS)	16	11	>99(2)
3	1	II	TEMPO	16	11	71
4	2	Lac	TEMPO(ABTS)	16	12	>99(18)
5	2	I	TEMPO	16	12	>99
6	2	II	TEMPO	16	12	>99
7	2	III	TEMPO	16	12	>99
8	3	Lac	TEMPO(ABTS)	16	13	>99(13)
9	3	I	TEMPO	16	13	>99

10	3	II	TEMPO	16	13	>99
11	4	Lac	TEMPO(ABTS)	16	14	>99
12	4	I	TEMPO	16	14	>99
13	4	II	TEMPO	16	14	>99
14	5	Lac	TEMPO(ABTS)	16	14	>99(38)
15	5	I	TEMPO(ABTS)	16	15	>99(28)
16	5	II	TEMPO	16	15	>99
17	5	III	TEMPO	16	15	>99
18	6	Lac	TEMPO(ABTS)	16	16	97(6)
19	6	I	TEMPO	16	16	>99
20	6	II	TEMPO	16	16	>99
21	7	Lac	TEMPO(ABTS)	3	17	93(4)
22	7	I	TEMPO(ABTS)	5	17	>99(53)
23	7	II	TEMPO	5	17	>99

[a] **1** benzyl alcohol; **2** 4-metoxybenzyl alcohol; **3** 3-metoxybenzyl alcohol; **4** 3,4-dimethoxybenzyl alcohol; **5** 3,4,5-trimethoxybenzyl alcohol; **6** cynnamyl alcohol; **7** geraniol. [b] **I** MWCNTs/Lac; **II** MWCNTs/Lac/PDDA/Lac; **III** ox-MWCNTs/PDDA/Lac. [c] TEMPO 2,2,6,6-tetramethyl-1-piperidinyloxy free radical; ABTS 2,2'-azino-bis(3-ethylbezthiazoline-6-sulphonic acid).

Table 8.4. Oxidation of alcohols 1-7.

The series of benzylalcohol confirmed the trend in accordance with the data described by R. Saladino et al.[917] yield were directly proportional to the number of methoxy groups of benzylalcohol substrate.



8,9,10

18,20,22

19,21,23

8,18,19: R_1=Cl R_2=R_3=H
9,20,21: R_1=R_2=H; R_3=Cl
10,22,23: R_1=R_3=H; R_2=Cl

Scheme 8.3. Oxidation of alcohols 8-10.

Entry	Substrate	Catalyst	Mediator	Time	Product	Yield
1	8	Lac	TEMPO	3	18(19)	25(25)
2	8	I	TEMPO	16	18	>99
3	8	II	TEMPO	2	18(8)	36(64)
4	8	II	TEMPO	5	18	>99
5	9	Lac	TEMPO	3	20(21)	66(33)
6	9	I	TEMPO	16	20	>99
7	9	II	TEMPO	2	20(9)	67(32)
8	9	II	TEMPO	5	20	>99
9	9	II	TEMPO	16	20(21)	32(68)
10	10	Lac	TEMPO	3	22(23)	13(85)
11	10	I	TEMPO	16	22	>99
12	10	I	TEMPO	2	22(23)[10]	87(1)[11]
13	10	I	TEMPO	5	22(23)	63(37)
14	10	II	TEMPO	16	22(23)	51(48)
15	10	II	TEMPO	5	22	>99
16	10	II	TEMPO	2	22(10)	97(3)
17	10	III	TEMPO	5	22	>99

[a] All reactions were carried out with TEMPO. **1** benzyl alcohol; **2** 4-metoxybenzyl alcohol; **3** 3-metoxybenzyl alcohol; **4** 3,4-dimethoxybenzyl alcohol; **5** 3,4,5-trimethoxybenzyl alcohol; **6** cynnamyl alcohol; **7** geraniol. [b] **I** MWCNTs/Lac; **II** MWCNTs/Lac/PDDA/Lac; **III** ox-MWCNTs/PDDA/Lac. [c] **8** 2-chlorobenzylalcohol; **9** 4-chlorobenzylalcohol; **10** 3-Chlorobenzylalcohol; **18** 2 -chlorobenzaldeyde; **19** 2-chlorobenzoic acid; **20** 4-chlorobenzaldeyde; **21** 4-chlorobenzoic acid; **22** 3-chlorobenzaldeyde; **23** 3-chlorobenzoic acid.

Table. Oxidation of alcohols 8-10.

Overall the reactivity values with the different mediators were of accordance with data reported by Guazzaroni et al.: in fact, the best efficiency of TEMPO and the lower reactivity of ABTS were confirmed.

8.9 Enzyme recycle

To evaluate the reusability of catalysts I-III, we studied the oxidation of 4-methoxybenzyl alcohol 2 as a selected example. Compound 2 (20 mM) was oxidized with catalysts I-III (0.65 U) and TEMPO (6.0 mM) under the previously reported experimental conditions. After 10 h, the catalyst was recovered by centrifugation, washed, and reused with fresh substrate and TEMPO. **Figure 8.14** shows the conversion of 2 depending on the recycle number. As a general trend, the catalysts II and III retained a reactivity higher than of catalyst I, showing c.a. 50% conversion of substrate after 6 runs, catalyst II being the most stable system (52% conversion). These results further confirmed the cability of the LbL procedure to create a specific microenvironment ables to protect the enzyme from denaturing agents,[918] even in the presence of oxidative mediators, such as TEMPO and ABTS.

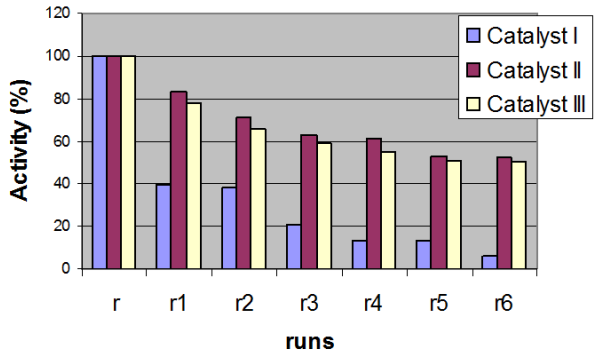


Figure 8.14. Conversion of substrate depending on the recycle number.

EXPERIMENTAL SECTION

CHAPTER 9

PHENOLS LOW-MEDIUM MOLECULAR WEIGHT

9.1 Extraction

1 mg of sample was extracted with petroleum ether in soxhlet for 6 hours, this extraction removes mainly the lipid component. The pellet was extracted again with dichloromethane in soxhlet for 6 hours. In the last stage the pellet was extracted with methanol 80% in soxhlet for 6 hours. When necessary (that is high amount of sugars in the sample), the extractive from methanol 80% was evaporated under reduced pressure, diluted in EtOAC and washed with water. All the extractives (petroleum ether, dichloromethane, methanol 80% and, when present, ethyl acetate) were evaporated under reduced pressure and high vacuum, and then weighed for the subsequent analysis of quantification.[919,920,921,922,923]

9.2 Characterization GC-MS and quantification

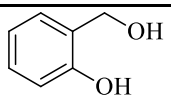
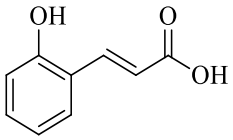
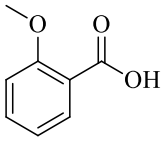
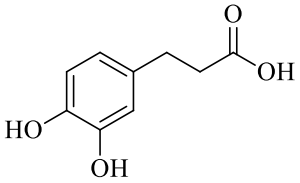
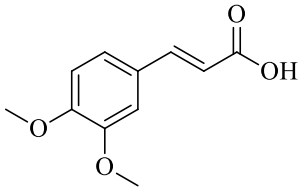
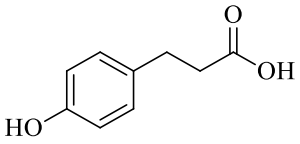
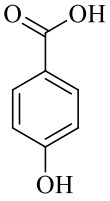
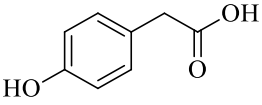
9.2.1 Derivatization - Silylation[924]

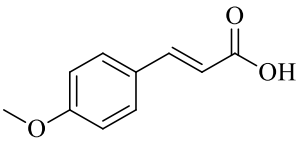
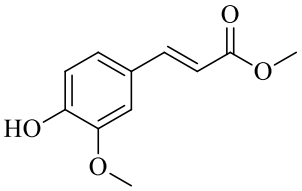
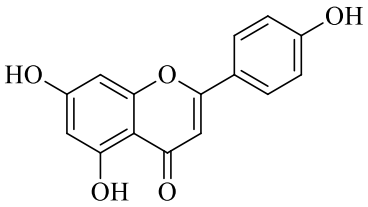
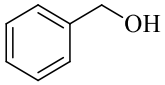
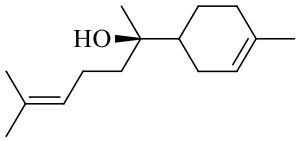
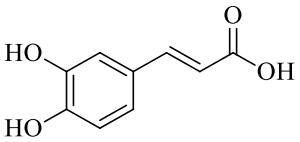
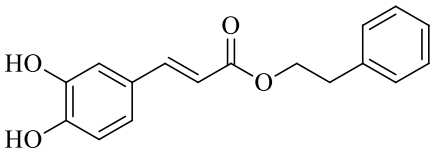
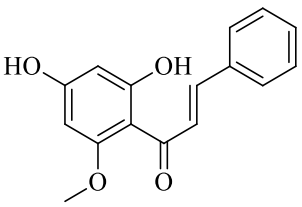
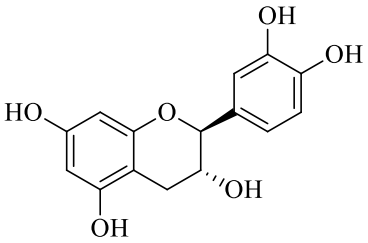
Samples

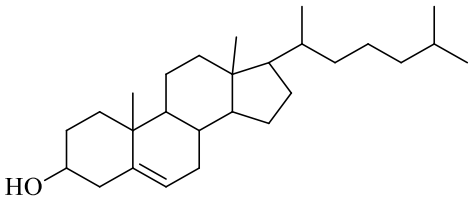
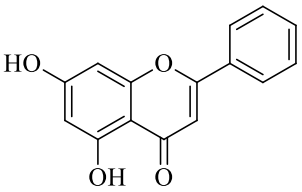
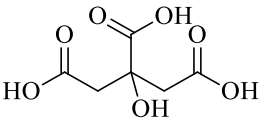
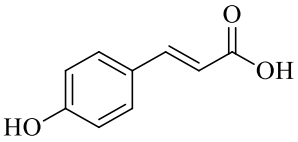
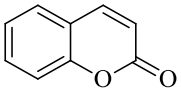
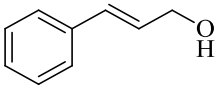
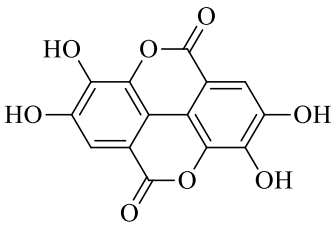
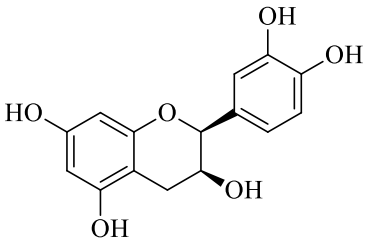
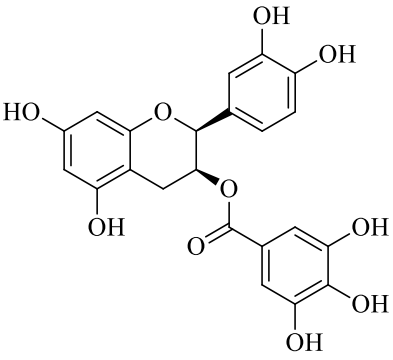
10 mg of dry extractive were dissolved in 100 µl of anhydrous pyridine and N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% of Trimethylchlorosilane (TMCS). At the mixture was added 0.001 mmol (0.25 mg) of oleic acid as internal standard. The reaction was conducted at 90°C for 1 hours under nitrogen pressure.

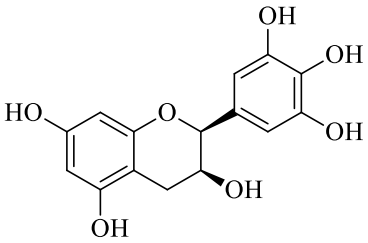
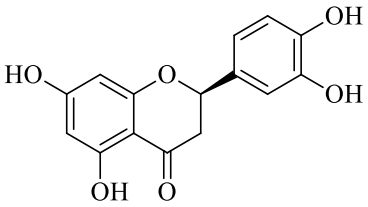
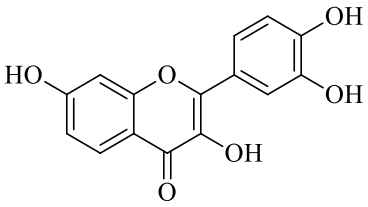
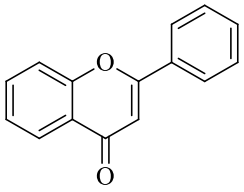
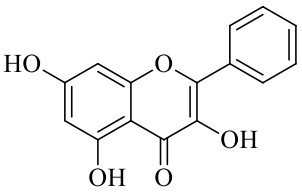
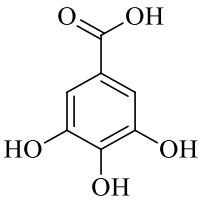
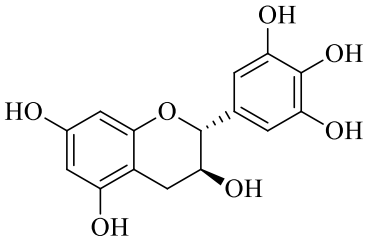
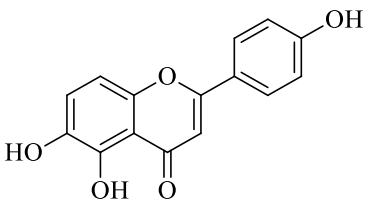
Standards

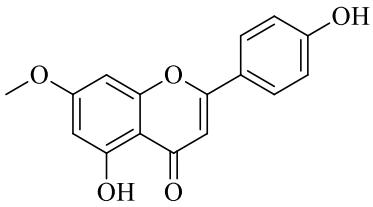
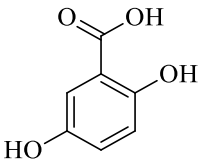
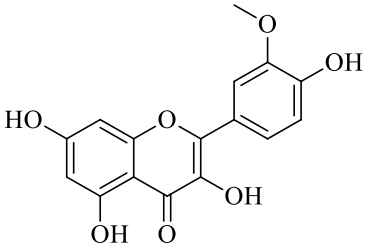
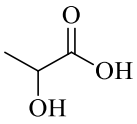
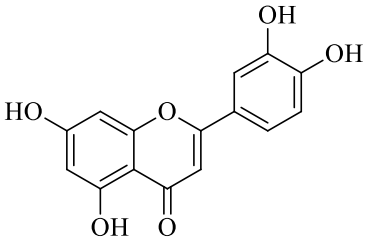
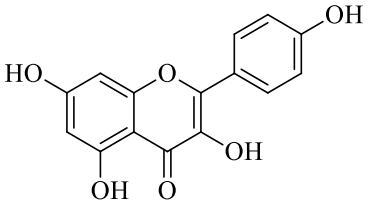
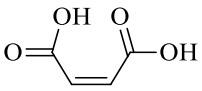
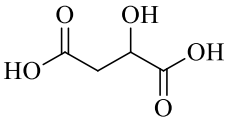
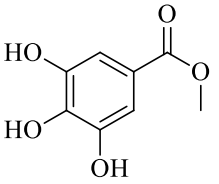
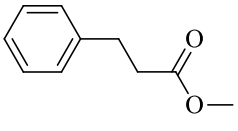
When available, commercially standards of appropriate phenols were used to determine the conversion factor necessary for quantitative determinations. In this case, 5 mg of standard sample was dissolved in 100 µl of pyridine anhydrous and N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% of Trimethylchlorosilane (TMCS). At the mixture was added 0.002 mmol (0.5 mg) of oleic acid. The reaction was performed at 90°C for 1 hours under nitrogen pressure. The standards analysed are reported in in **Table 9.1**.

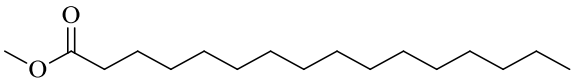
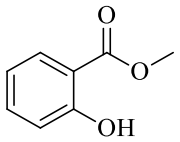
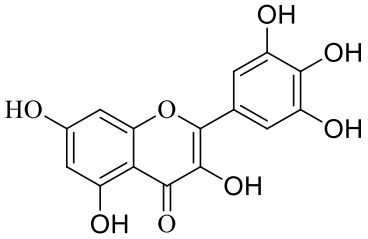
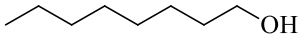
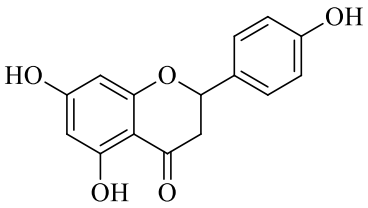
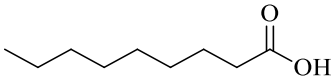
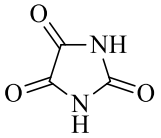
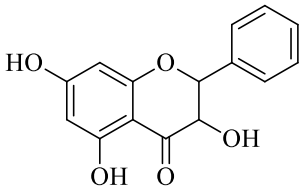
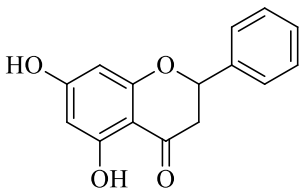
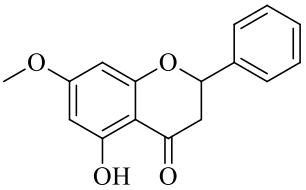
Entry	Standards	Structure
1	2-hydroxybenzyl alcohol	
2	2-hydroxycinnamic acid	
3	2-methoxybenzoic acid	
4	3,4-dihydroxyhydrocinnamic acid	
5	3,4-dimethoxycinnamic acid	
6	3-(4-hydroxyphenyl) propionic acid	
7	4-hydroxybenzoic acid	
8	4-hydroxyphenylacetic acid	

9	4-methoxycinnamic acid	
10	Acrilate	
11	Apigenin	
12	Benzyl alcohol	
13	Bisabolol	
14	Caffeic acid	
15	Caffeic acid phenethyl ester	
16	Cardamonin	
17	(+)-catechin	

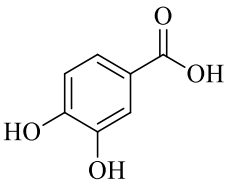
18	Cholesterol	
19	Chrysin	
20	Citric acid	
21	Coumaric acid	
22	Coumarin	
23	Cynnamyl alcohol	
24	Ellagic acid	
25	(-)-epicatechin	
26	(-)-epicatechin gallate	

27	(-)-epigallocatechin	
28	Eriodictyol	
29	Fisetin	
30	Flavone	
31	Galangin	
32	Gallic acid	
33	(-)-gallocatechin	
34	Genistein	

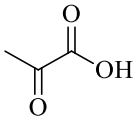
35	Genkwanin	
36	Gentisic acid	
37	Isorhamnetin	
38	Lactic acid	
39	Luteolin	
40	Kaempferol	
41	Maleic acid	
42	Malic acid	
43	Methyl-3,4,5-trihydroxybenzoate	
44	Methyl cinnamate	

45	Methyl palmitate	
46	Methyl salicylate	
47	Myricetin	
48	Octyl alcohol	
49	Naringenin	
50	Nonanoic acid	
51	Parabanic acid	
52	Pinobanksin	
53	Pinocembrin	
54	Pinostrobin	

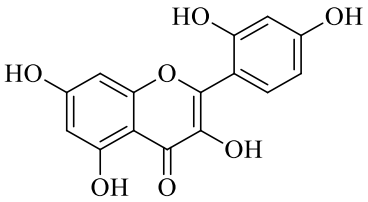
55 Protocatechuic acid



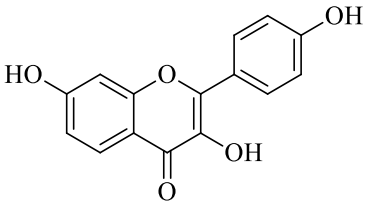
56 Pyruvic acid



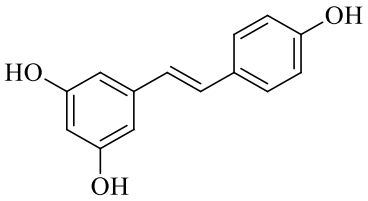
57 Quercetin



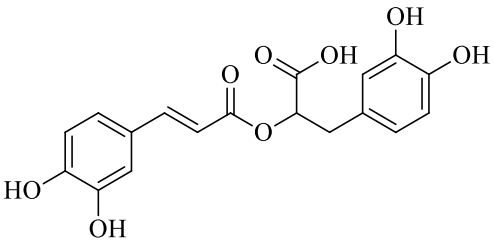
58 Resokaempferol



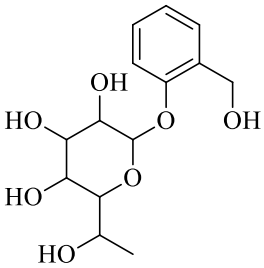
59 Resveratrol



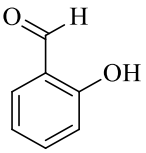
60 Rosmarinic acid

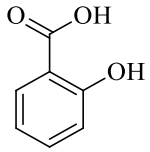
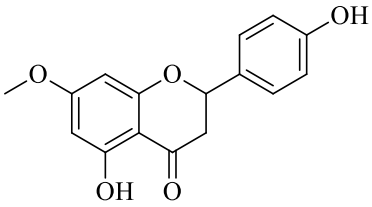
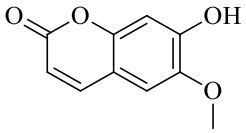
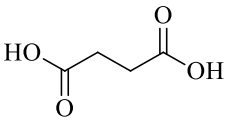
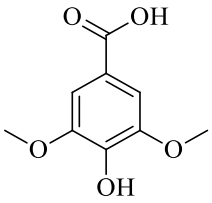
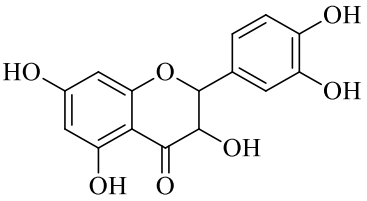
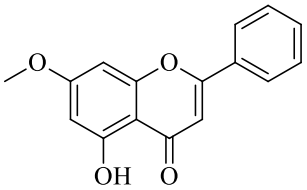
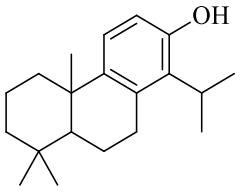
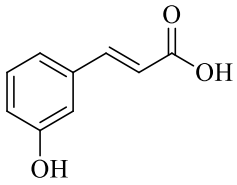


61 Salicin



62 Salicylaldehyde



63	Salicylic acid	
64	Sakuranetin	
65	Scopoletin	
66	Succinic acid	
67	Syringic acid	
68	Taxifolin	
69	Tectochrysin	
70	Totarol	
71	*trans*-3-hydroxycinnamic acid	

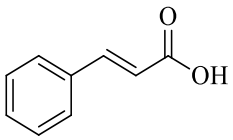
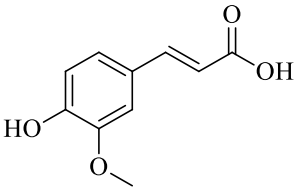
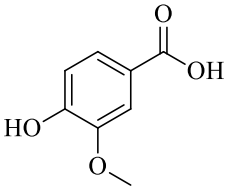
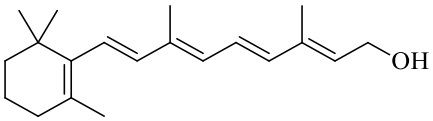
72	*trans*-cinnamic acid	
73	*trans*-ferulic acid	
74	Vanillic acid	
75	Vitamin A alcohol	

Table 9.1. Standars

9.2.2 Gas Chromatography-Mass Spectrometry (GC-MS)

The sample trimethylsilylated was subject to GC-MS analyses on a Varian 450 GC 320 MS (Agilent COMBO). The instrument was equipped with a VF-5MS CP8944 Varian silica capillary column (30 m 0.25 mm I.D.). The carrier gas was helium at flow-rate 1 ml min^{-1}. The column temperature was programmed from 140°C (held 2 min) at 5°C $min^{-1}$to 300°C which was maintained for 25 min. The injector temperature was 250°C. Mass spectra was recorder in the range 50-1000 m/z. [925]

9.2.3 Construction library

The fragmentation spectra of the standard samples trimethylsilylated were included in the library for their use on the characterization of samples, the chromatograms of standards were reported in **Figures** .

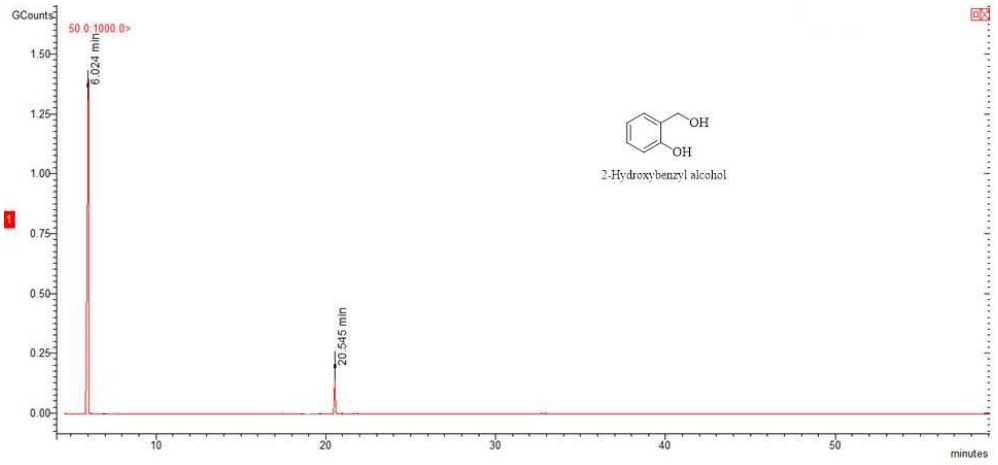


Figure 9.1. Chromatogram of 2-hydroxybenzyl alcohol trimethylsilylated.

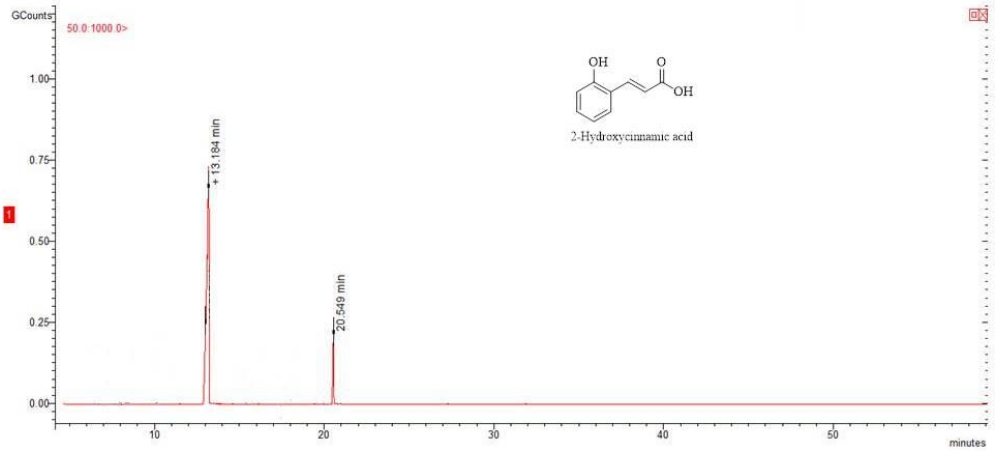


Figure 9.2. Chromatogram of 2-hydroxycinnamic acid trimethylsilylated.

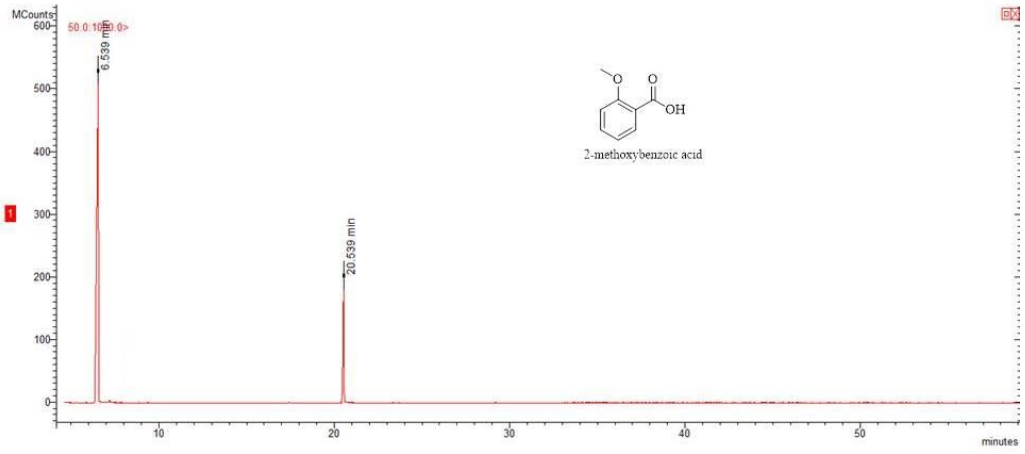


Figure 9.3. Chromatogram of 2-methoxybenzoic acid trimethylsilylated.

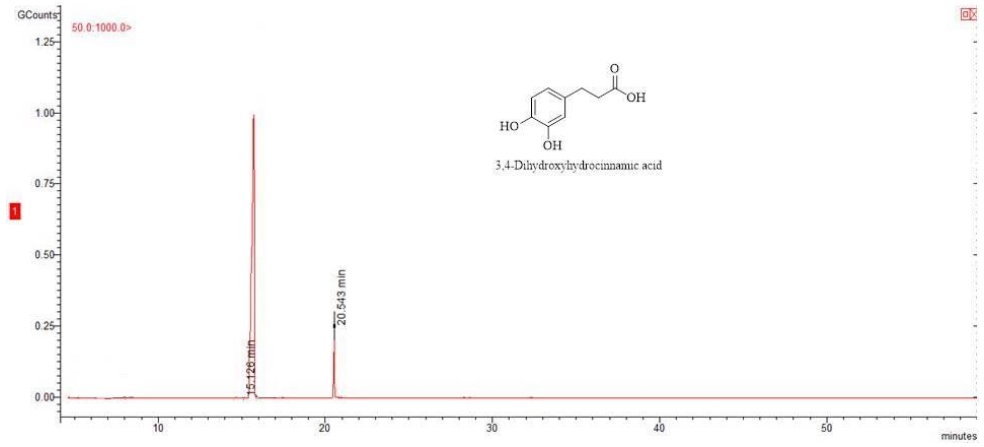


Figure 9.4. Chromatogram of 3,4-dihydroxyhydrocinnamic acid trimethylsilylated.

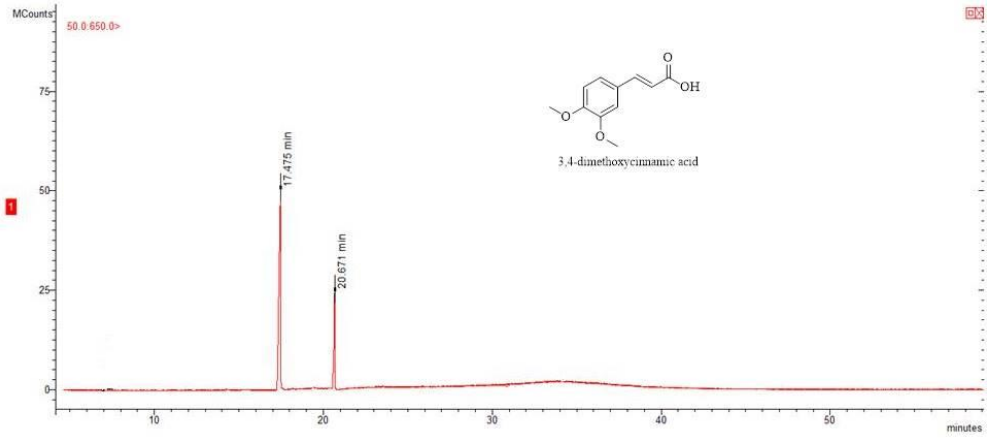


Figure 9.5. Chromatogram of 3,4-dimethoxycinnamic acid trimethylsilylated.

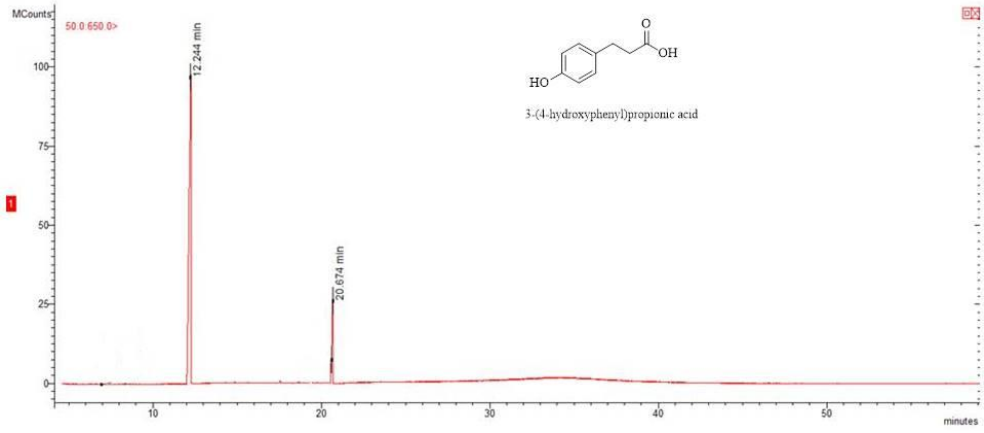


Figure 9.6. Chromatogram of 3-(4-hydroxyphenyl)propionic acid trimethylsilylated.

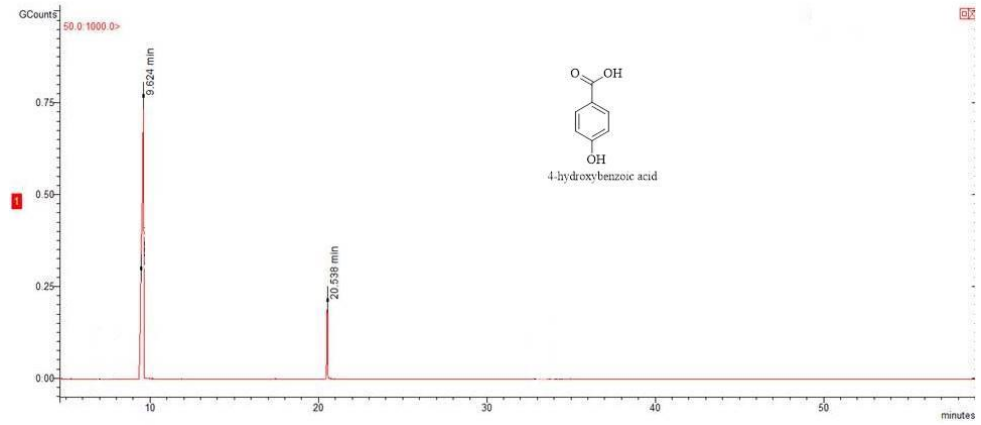


Figure 9.7. Chromatogram of 4-hydroxybenzoic acid trimethylsilylated.

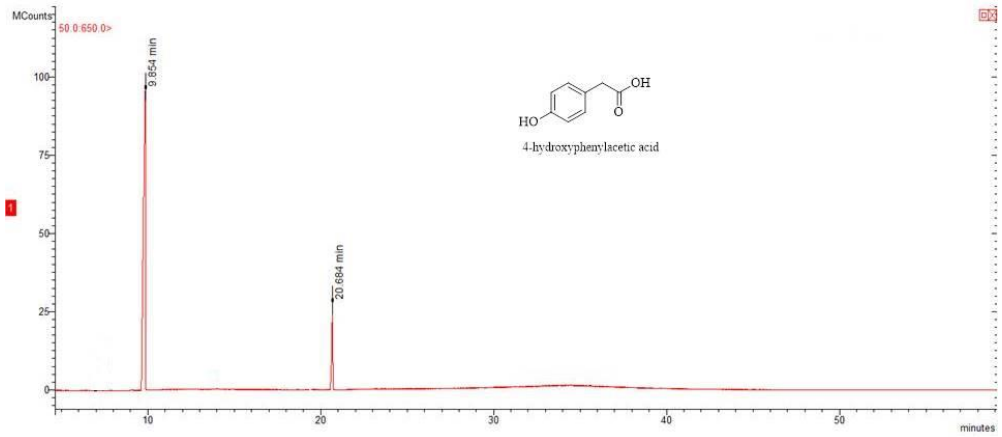


Figure 9.8. Chromatogram of 4-hydroxyphenylacetic acid trimethylsilylated.

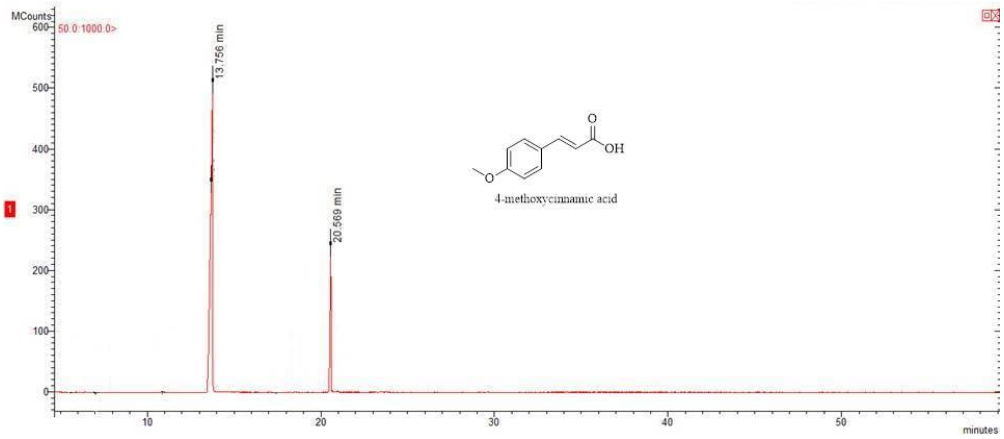


Figure 9.9. Chromatogram of 4-methoxycinnamic acid trimethylsilylated.

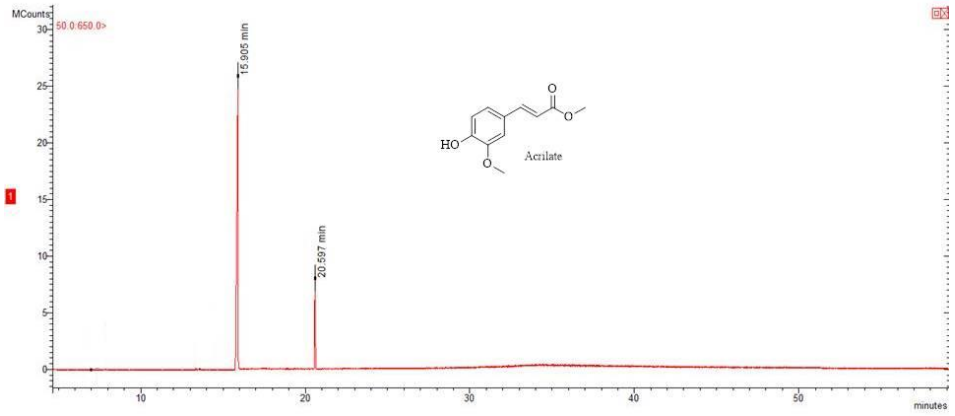


Figure 9.10. Chromatogram of Acrilate trimethylsilylated.

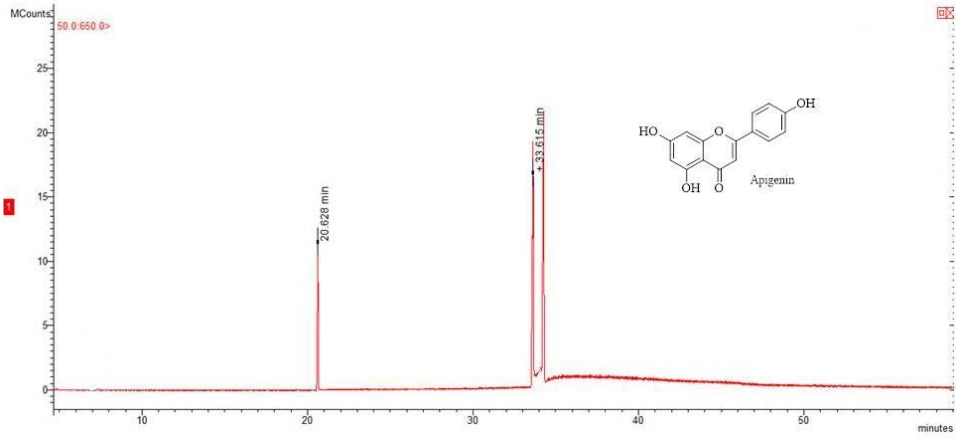


Figure 9.11. Chromatogram of Apigenin trimethylsilylated.

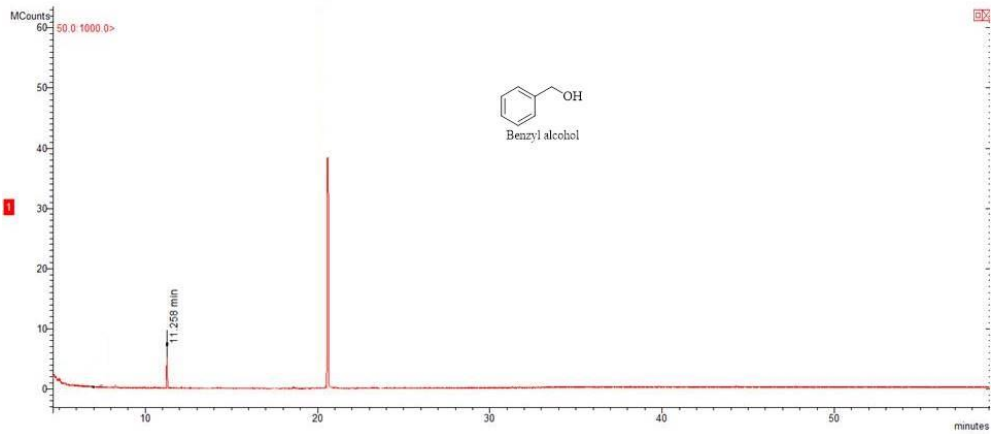


Figure 9.12. Chromatogram of Benzyl alcohol trimethylsilylated.

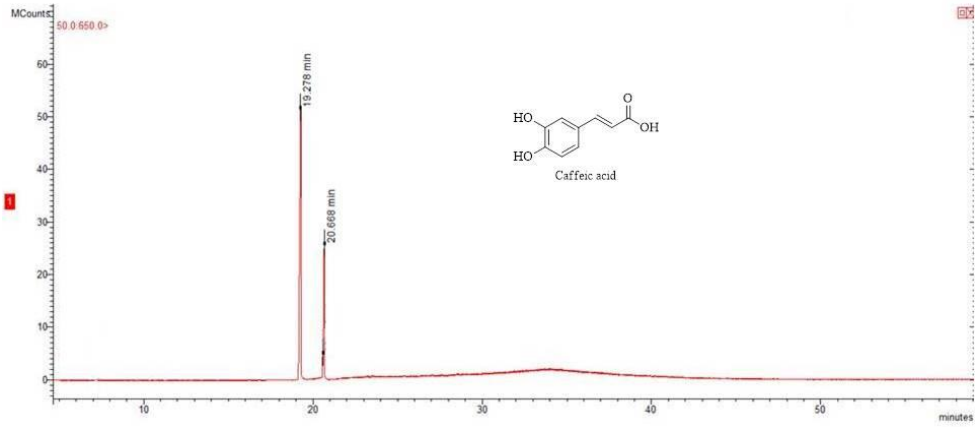


Figure 9.12. Chromatogram of Caffeic acid trimethylsilylated.

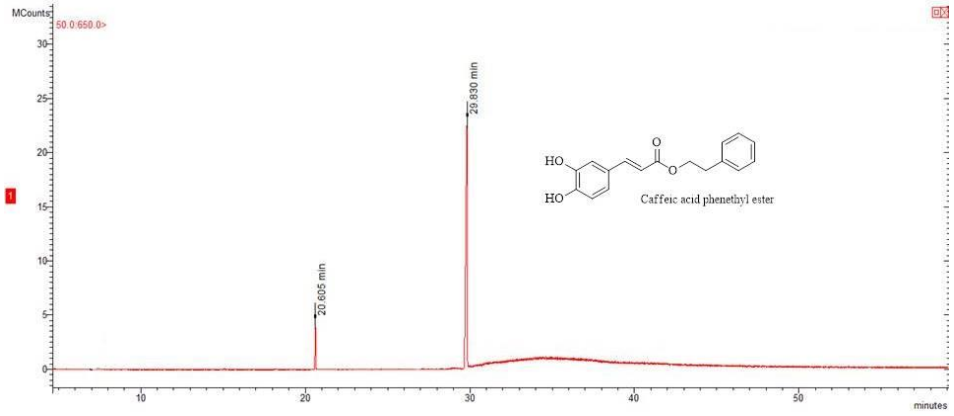


Figure 9.13 Chromatogram of Caffeic acid phenethyl ester trimethylsilylated.

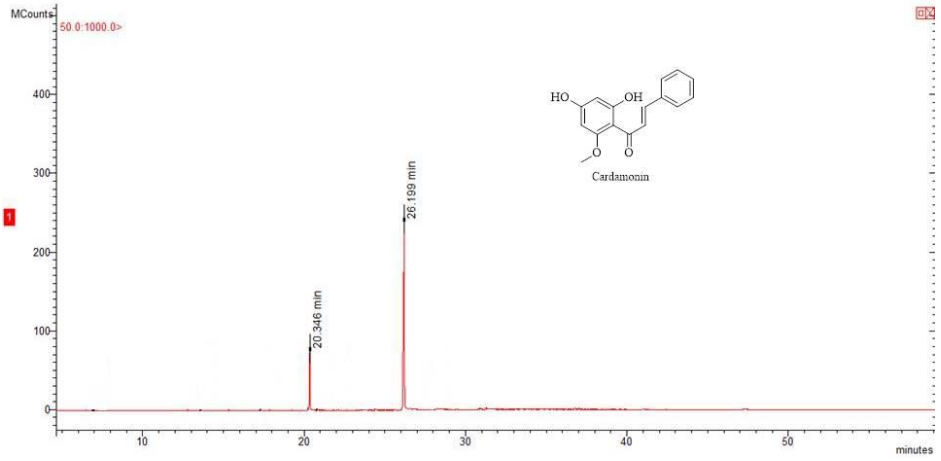


Figure 9.14. Chromatogram of Cardamonin trimethylsilylated.

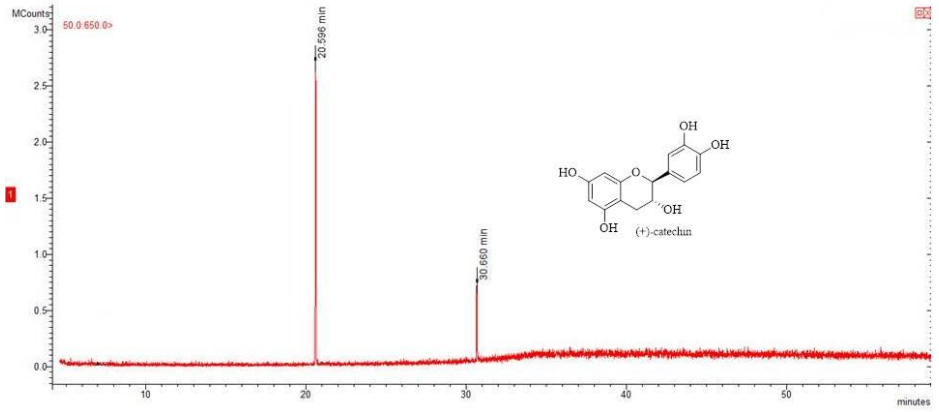


Figure 9.15. Chromatogram of (+)-Catechin trimethylsilylated.

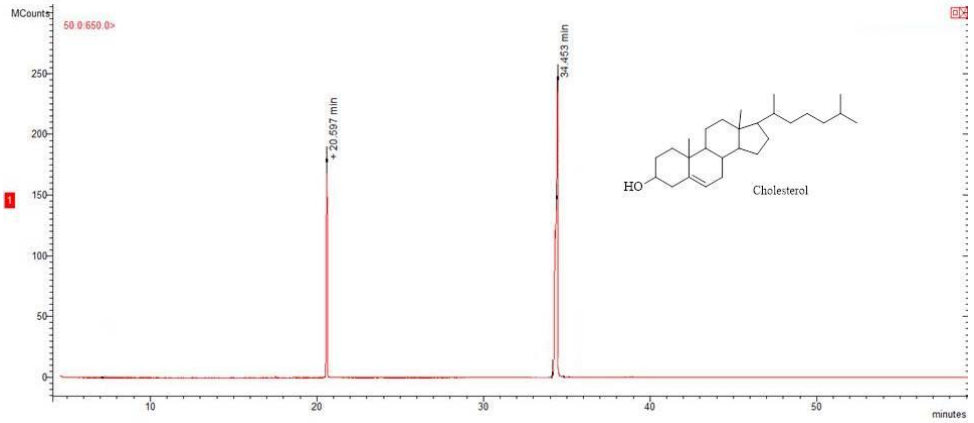


Figure 9.16. Chromatogram of Cholesterol trimethylsilylated.

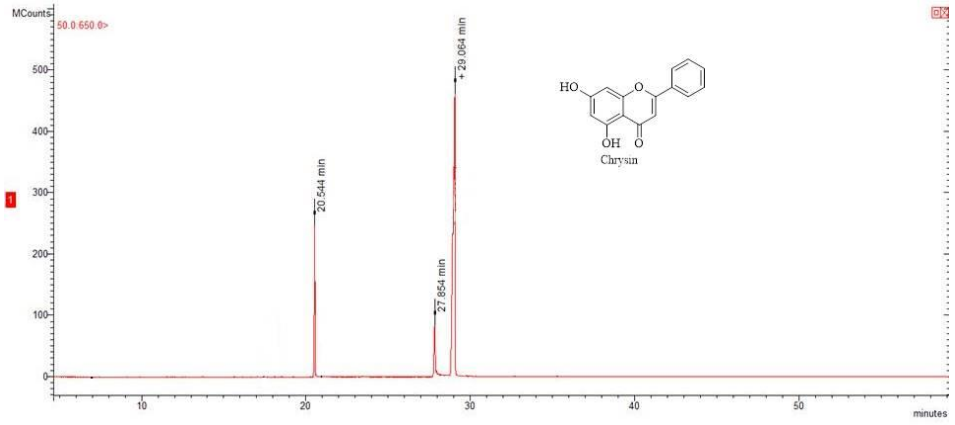


Figure 9.17. Chromatogram of Chrysin trimethylsilylated.

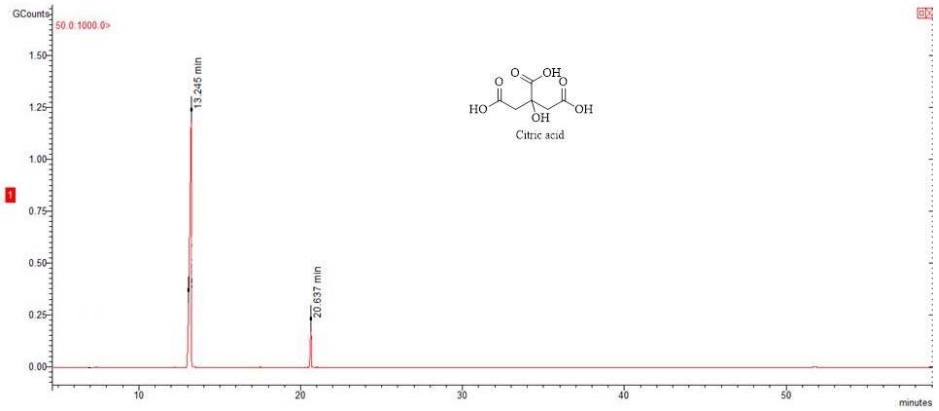


Figure 9.18. Chromatogram of Citric acid trimethylsilylated.

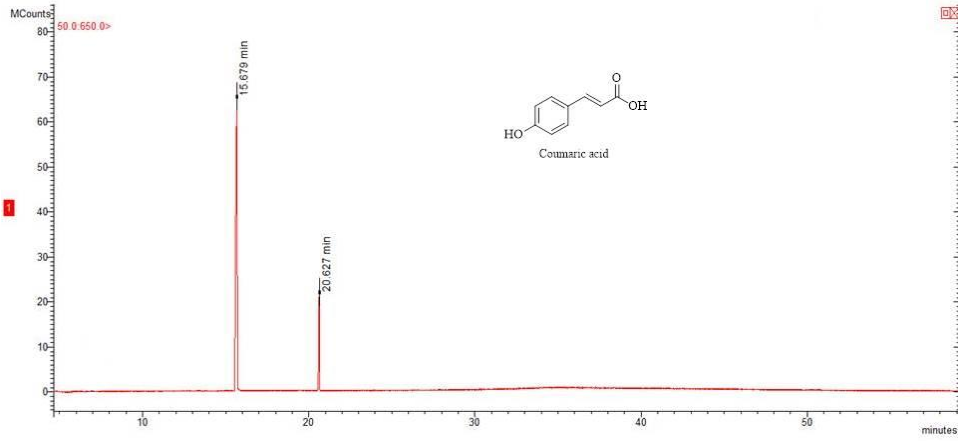


Figure 9.19. Chromatogram of Coumaric acid trimethylsilylated.

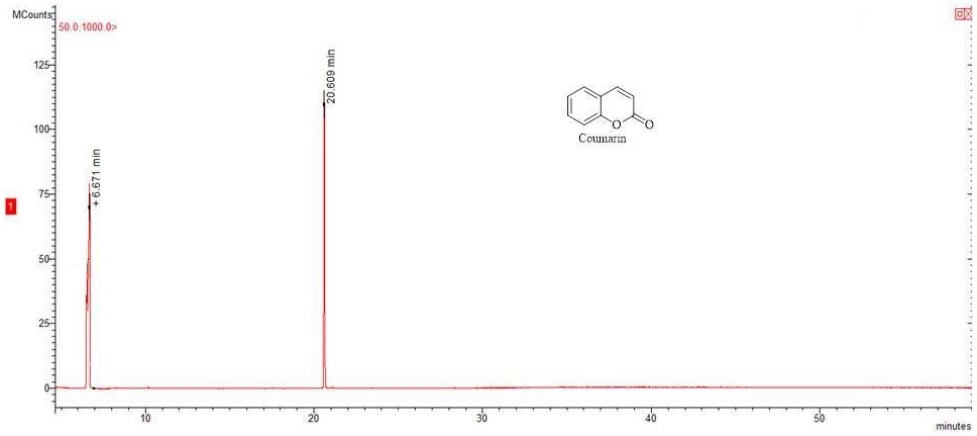


Figure 9.20. Chromatogram of Coumarin trimethylsilylated.

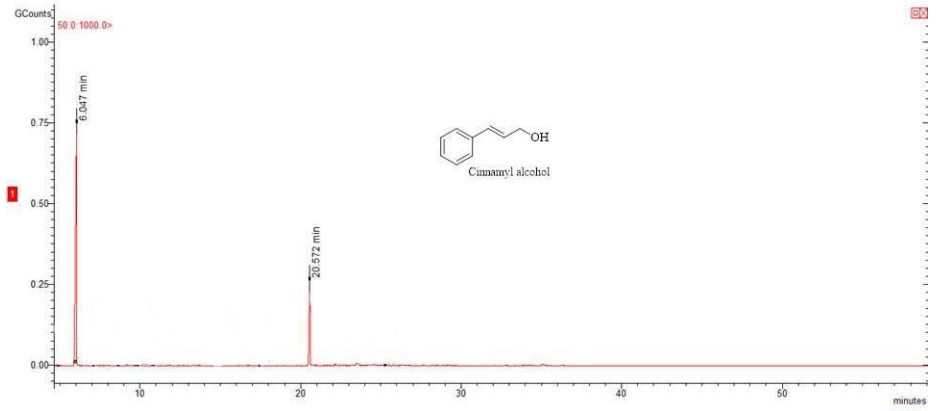


Figure 9.21. Chromatogram of Cinnamyl alcohol trimethylsilylated.

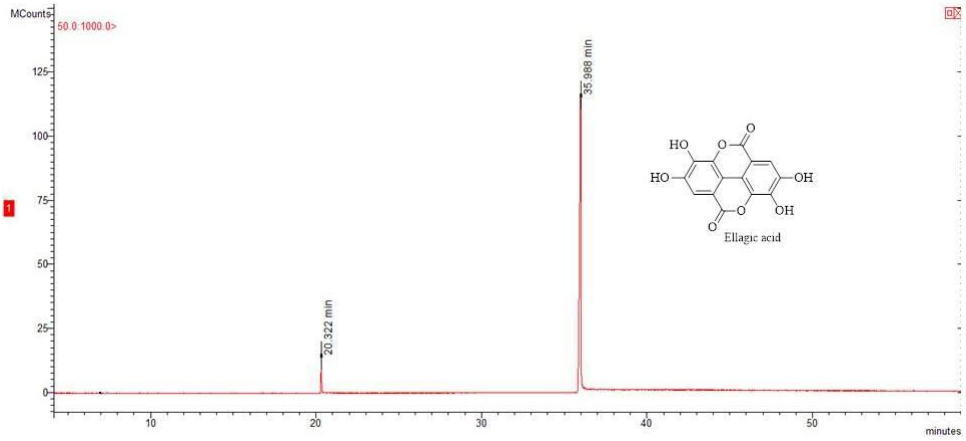


Figure 9.22. Chromatogram of Ellagic acid trimethylsilylated.

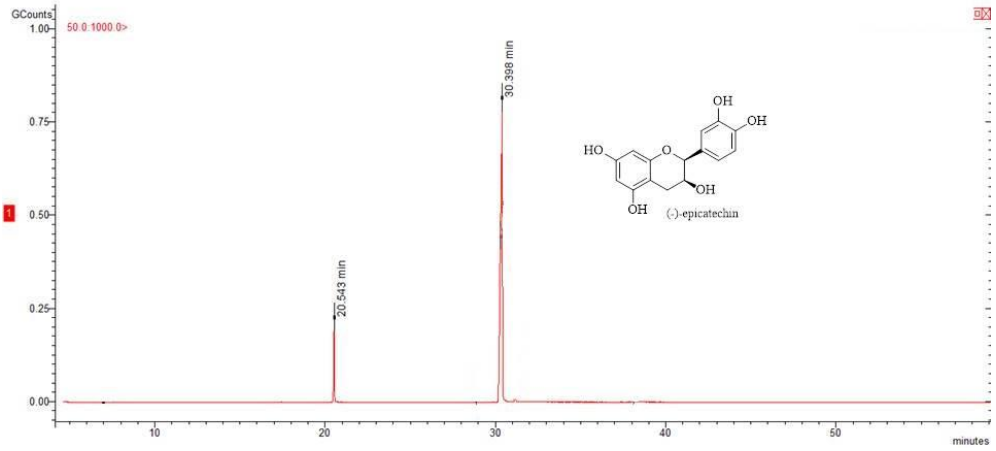


Figure 9.23. Chromatogram of (-)-epicatechin trimethylsilylated.

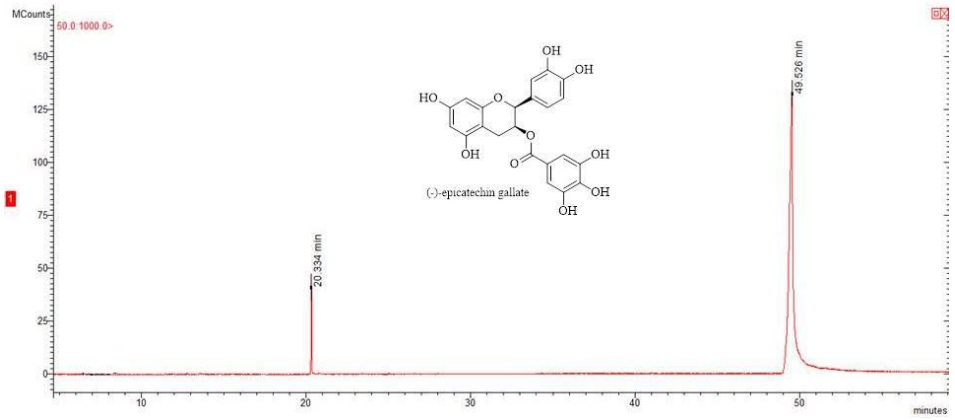


Figure 9.24. Chromatogram of (-)-epicatechin gallate trimethylsilylated.

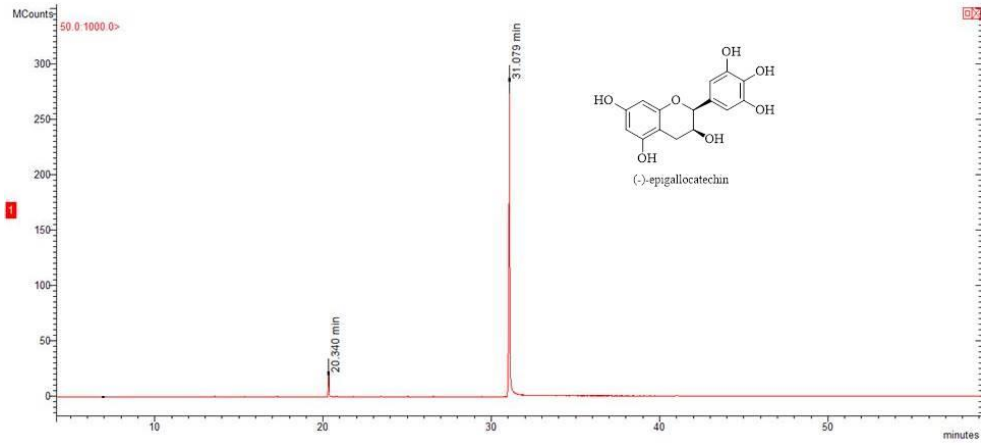


Figure 9.25. Chromatogram of (-)-epigallocatechin trimethylsilylated.

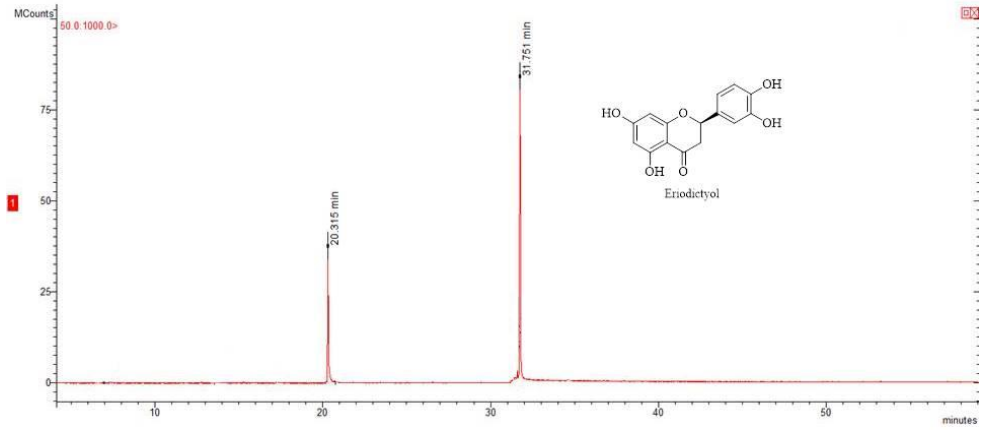


Figure 9.26. Chromatogram of Eriodictyol trimethylsilylated.

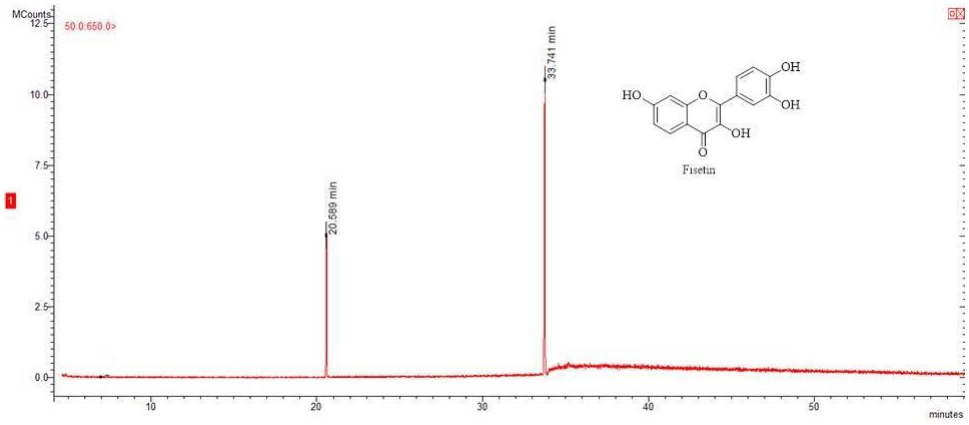


Figure 9.27. Chromatogram of Fisetin trimethylsilylated.

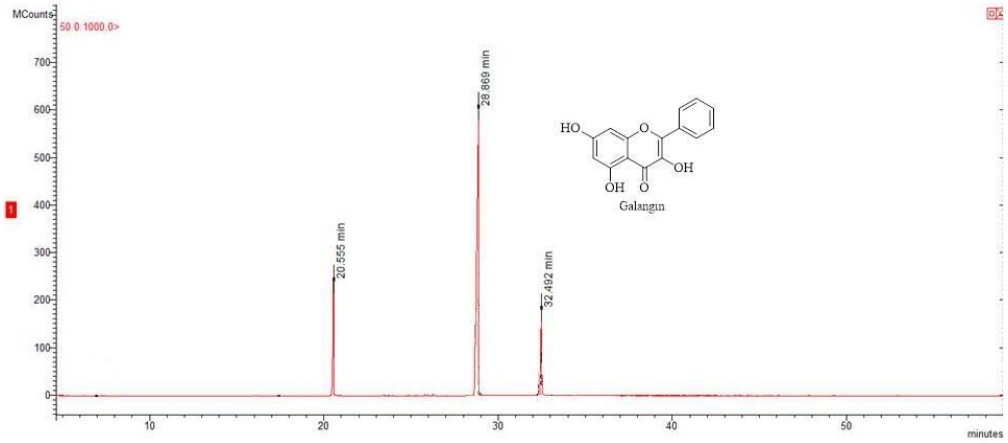


Figure 9.28. Chromatogram of Galangin trimethylsilylated.

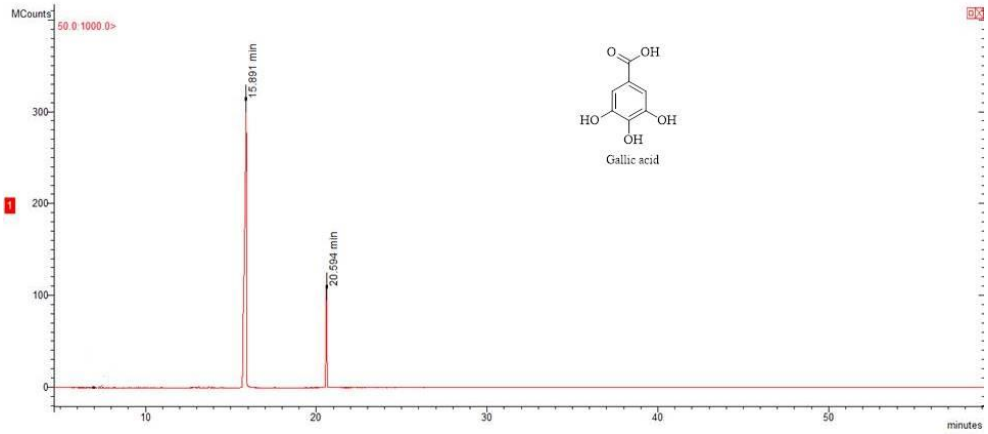


Figure 9.29. Chromatogram of Gallic acid trimethylsilylated.

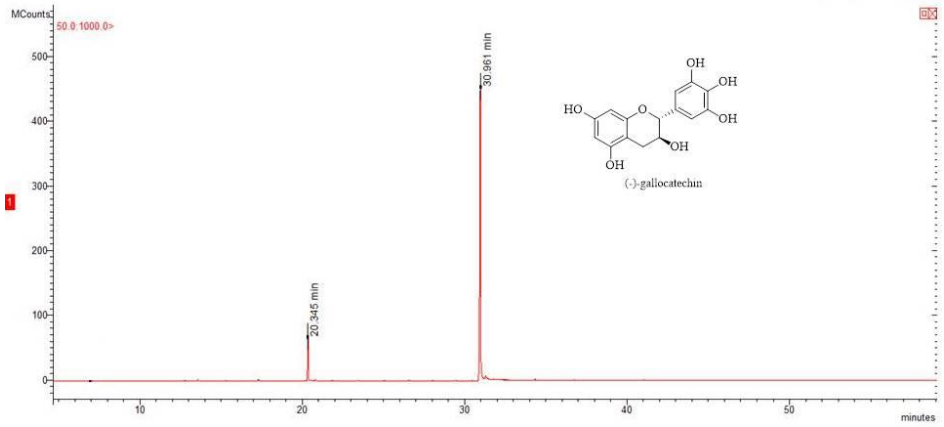


Figure 9.30. Chromatogram of (-)-gallocatechin trimethylsilylated.

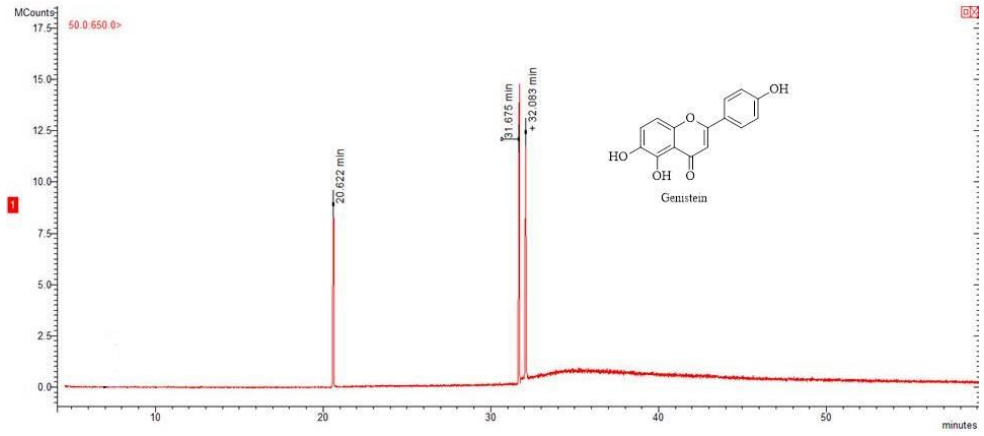


Figure 9.31. Chromatogram of Genistein trimethylsilylated.

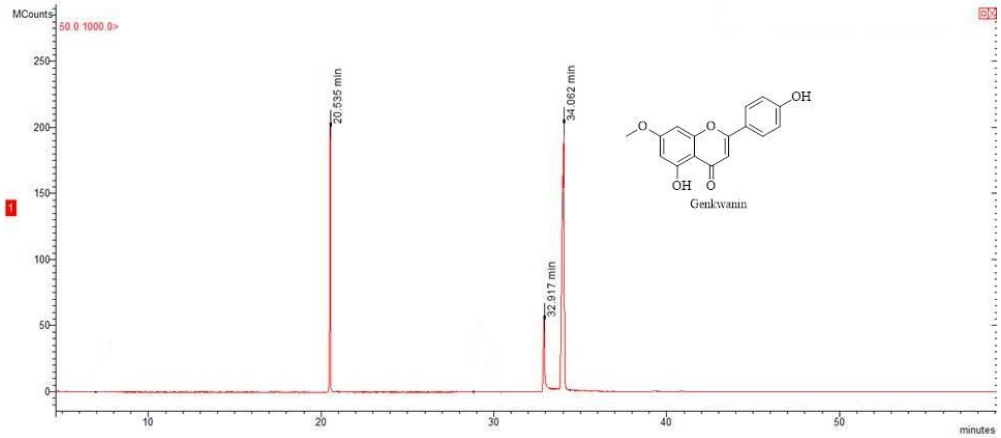


Figure 9.32. Chromatogram of Genkwanin trimethylsilylated.

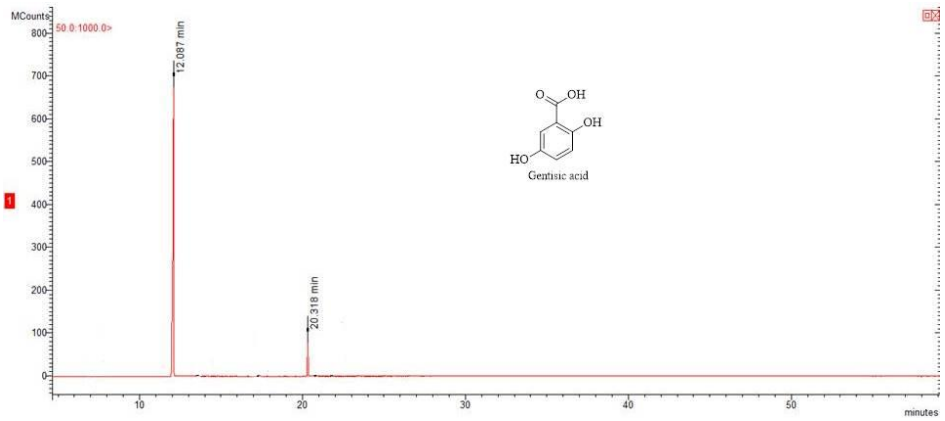


Figure 9.33. Chromatogram of Gentisic acid trimethylsilylated.

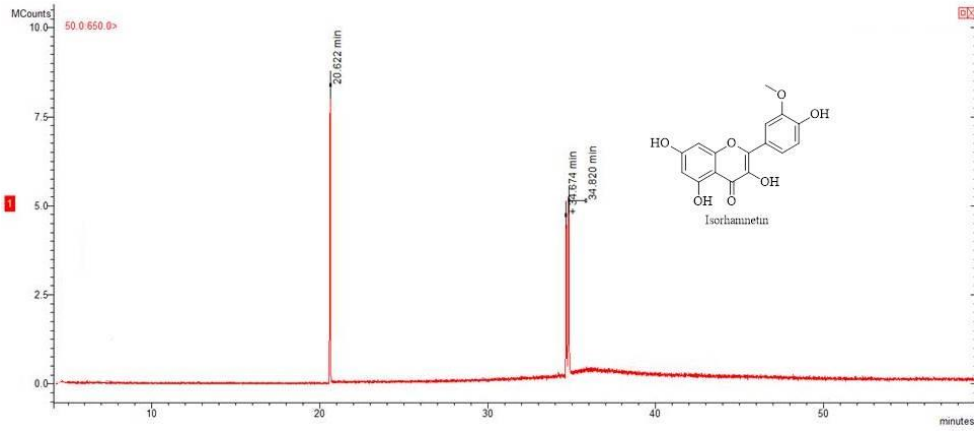


Figure 9.34. Chromatogram of Isorhamnetin trimethylsilylated.

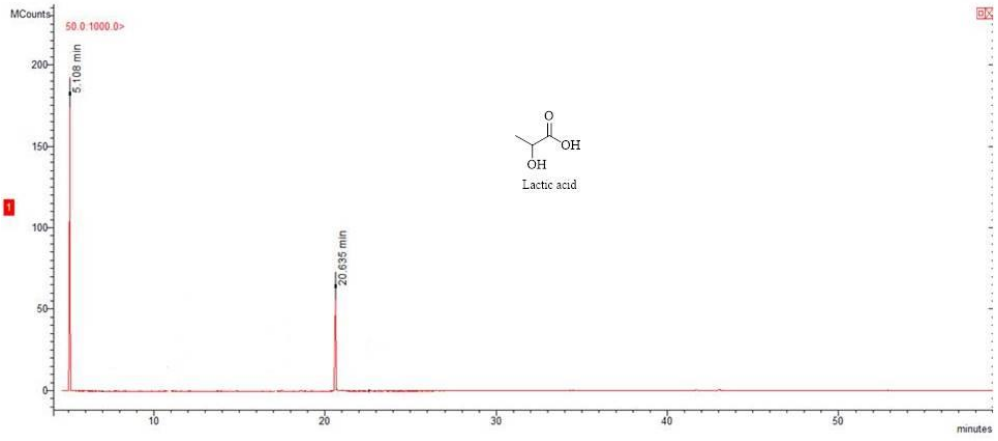


Figure 9.35. Chromatogram of Lactic acid trimethylsilylated.

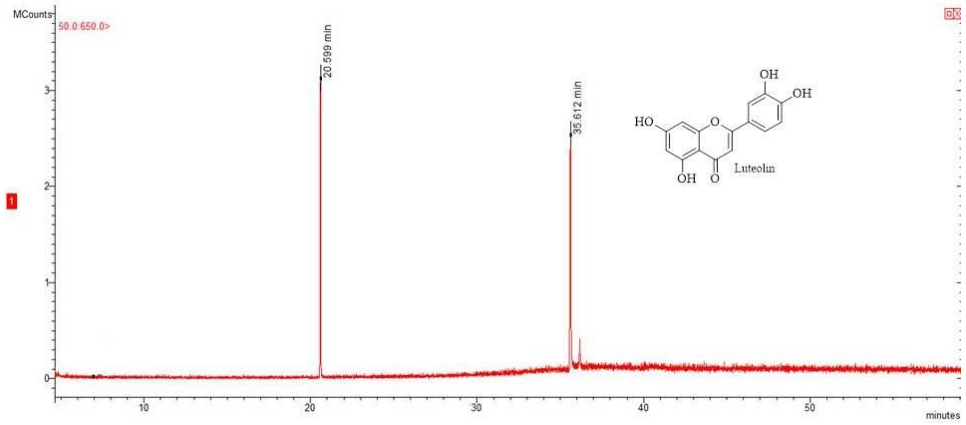


Figure 9.36. Chromatogram of Luteolin trimethylsilylated.

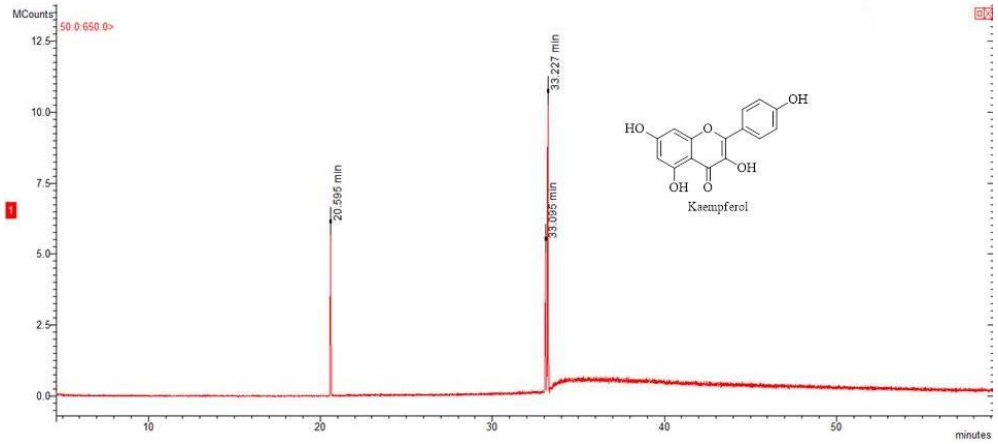


Figure 9.37. Chromatogram of Kaempferol trimethylsilylated.

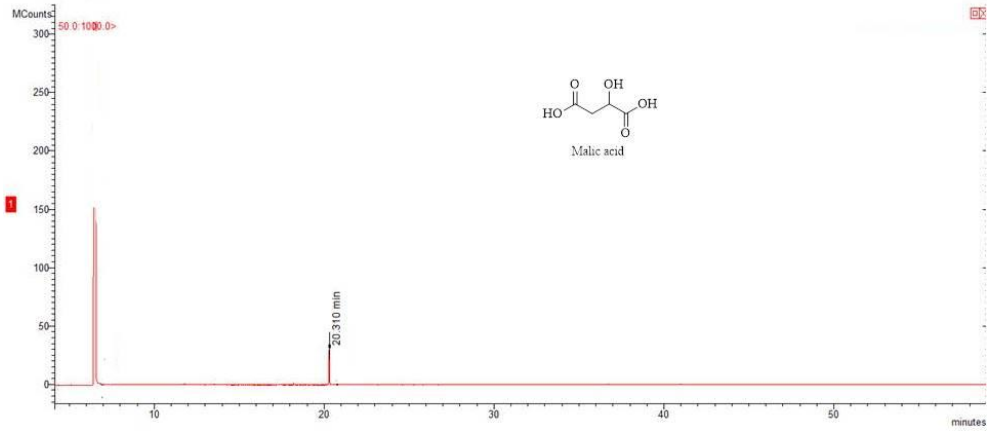


Figure 9.38. Chromatogram of Malic acid trimethylsilylated.

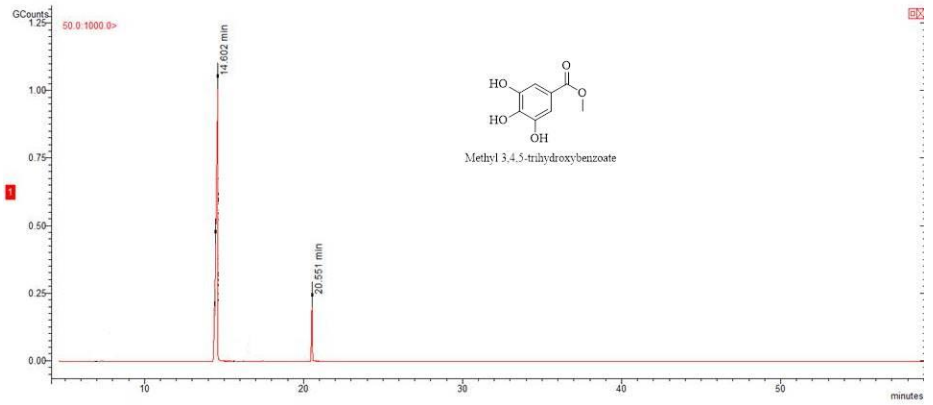


Figure 9.39. Chromatogram of Methyl 3,4,5-trihydroxybenzoate trimethylsilylated.

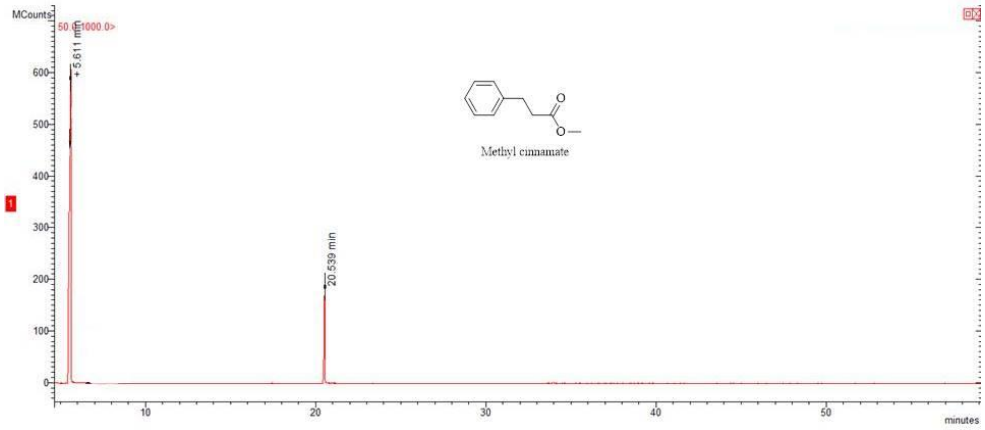


Figure 9.40. Chromatogram of Methyl cinnamate trimethylsilylated.

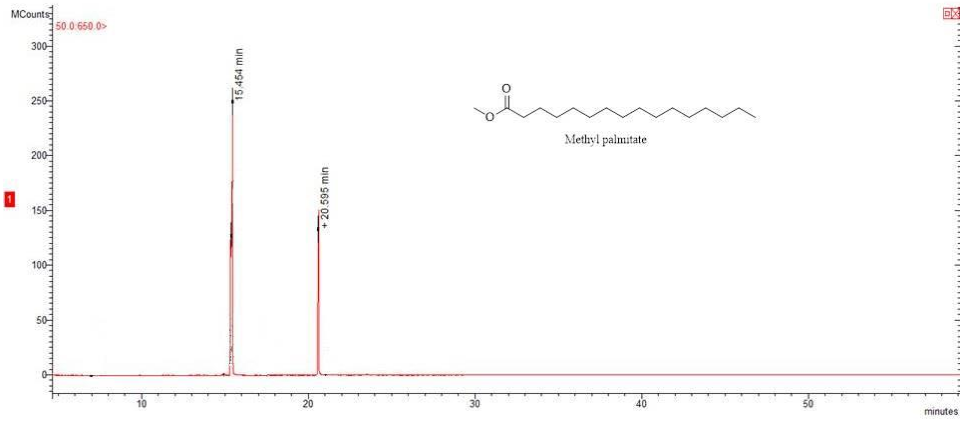


Figure 9.41. Chromatogram of Methyl palmitate trimethylsilylated.

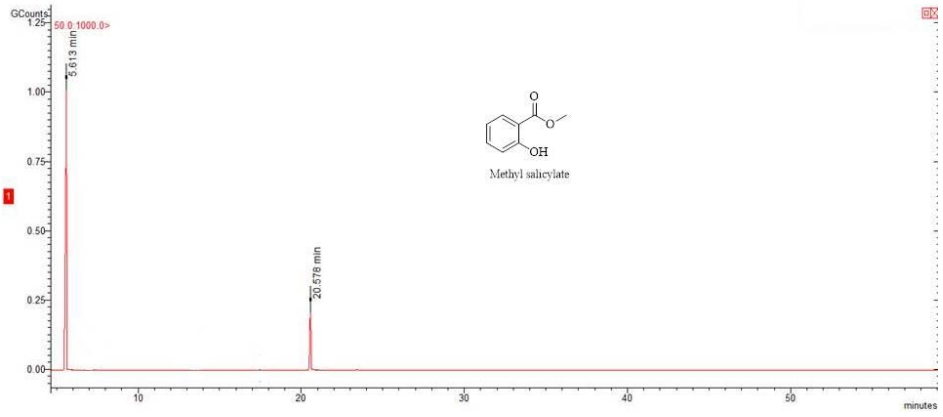


Figure 9.42. Chromatogram of Methyl salicylate trimethylsilylated.

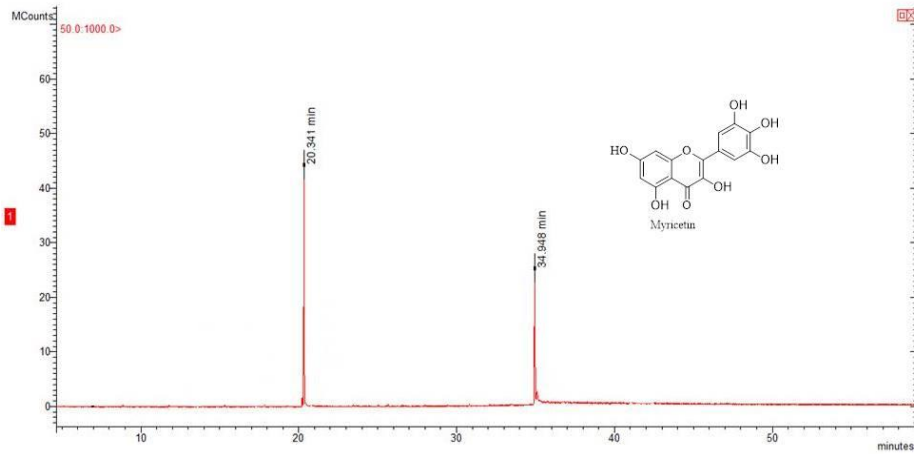


Figure 9.43. Chromatogram of Myricetin trimethylsilylated.

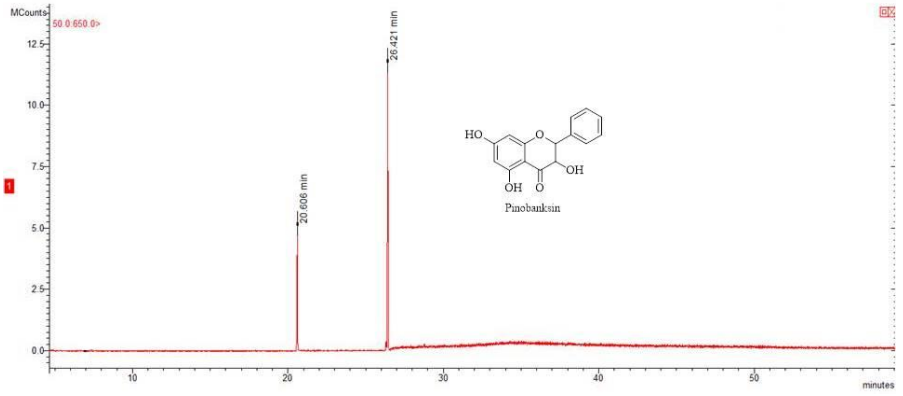


Figure 9.44. Chromatogram of Pinostrobin trimethylsilylated.

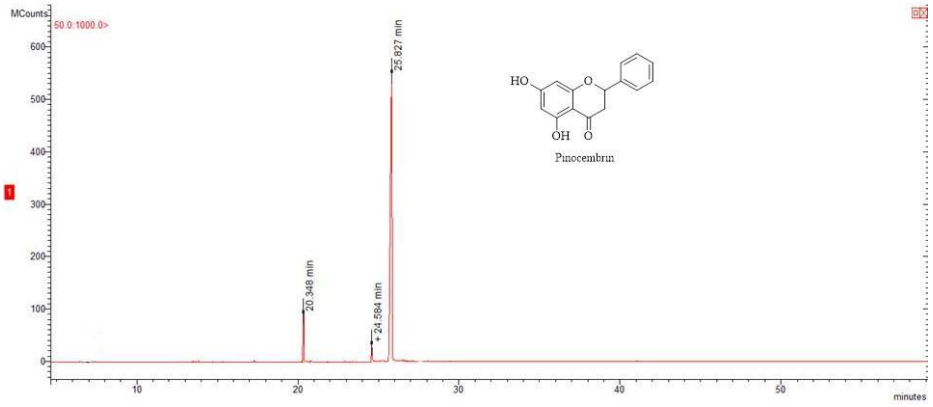


Figure 9.45. Chromatogram of Pinocembrin trimethylsilylated.

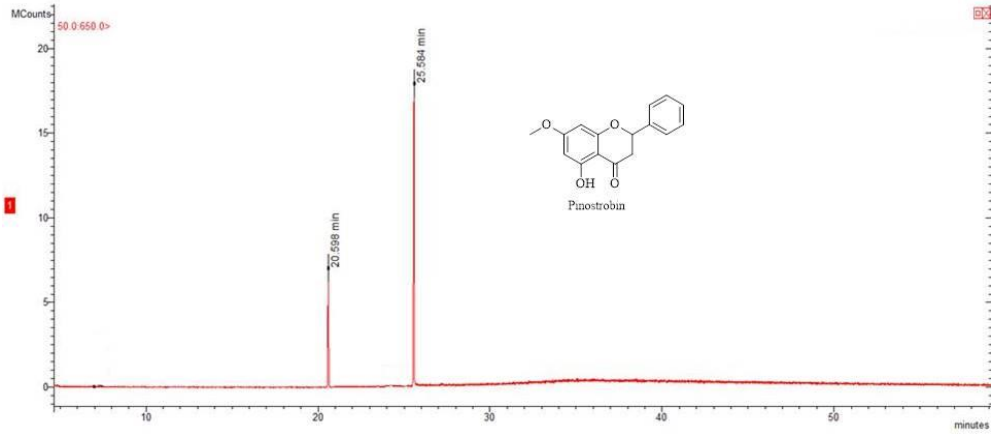


Figure 9.46. Chromatogram of Pinostrobin trimethylsilylated.

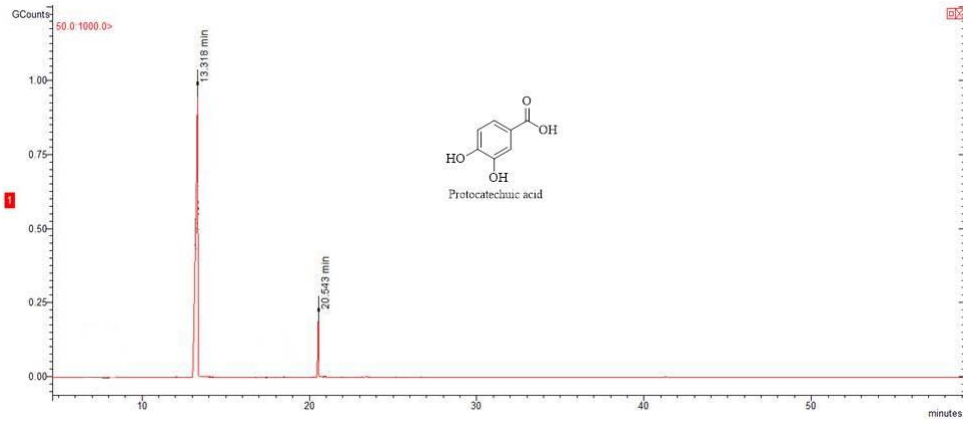


Figure 9.47. Chromatogram of Protocatechuic acid trimethylsilylated.

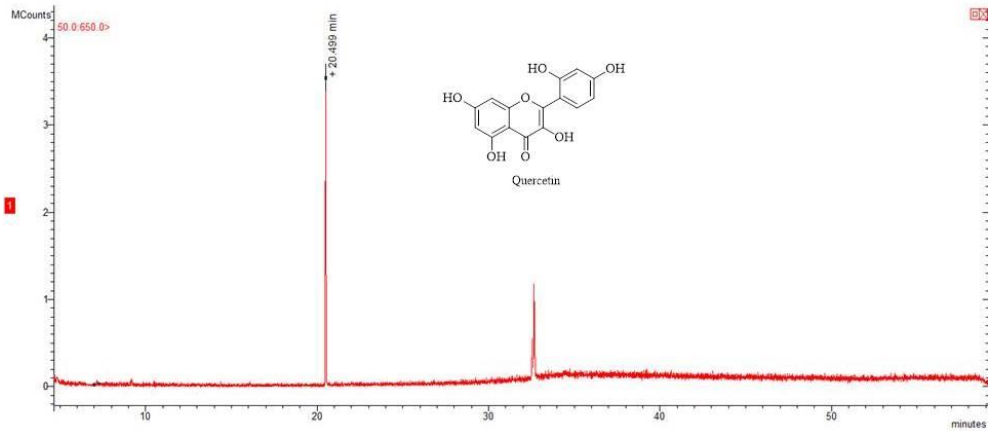


Figure 9.48. Chromatogram of Quercetin trimethylsilylated.

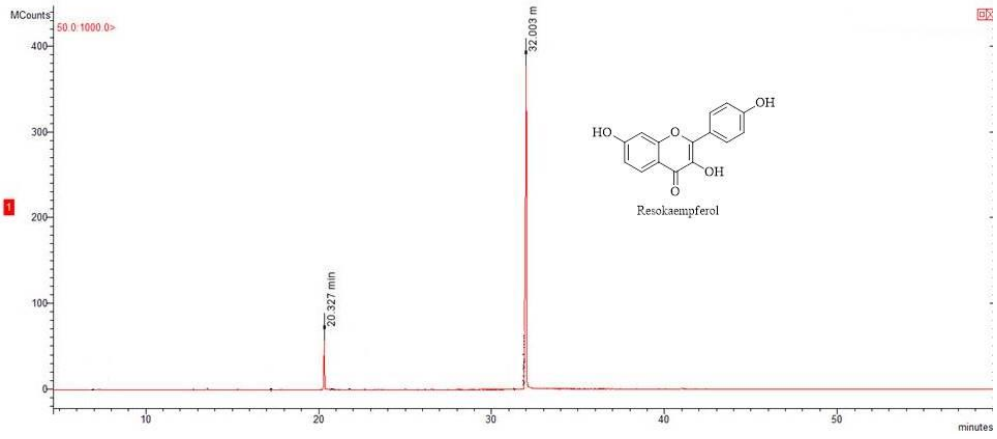


Figure 9.49. Chromatogram of Resokaempferol trimethylsilylated.

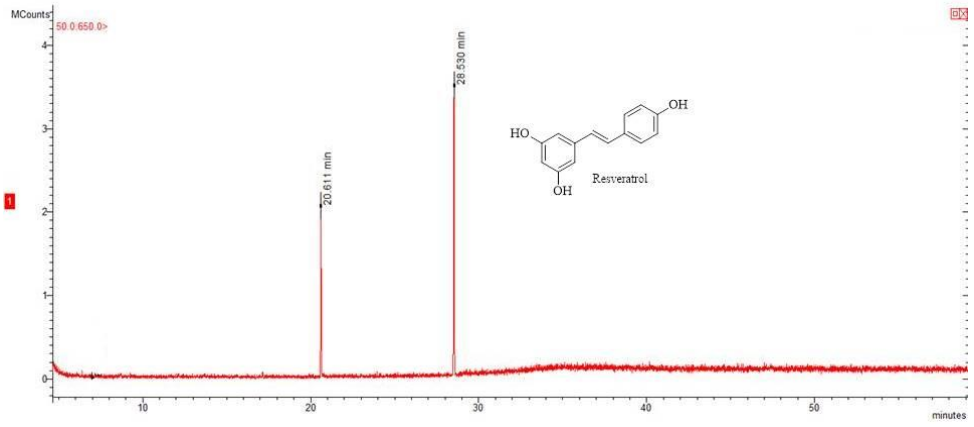


Figure 9.50. Chromatogram of Resveratrol trimethylsilylated.

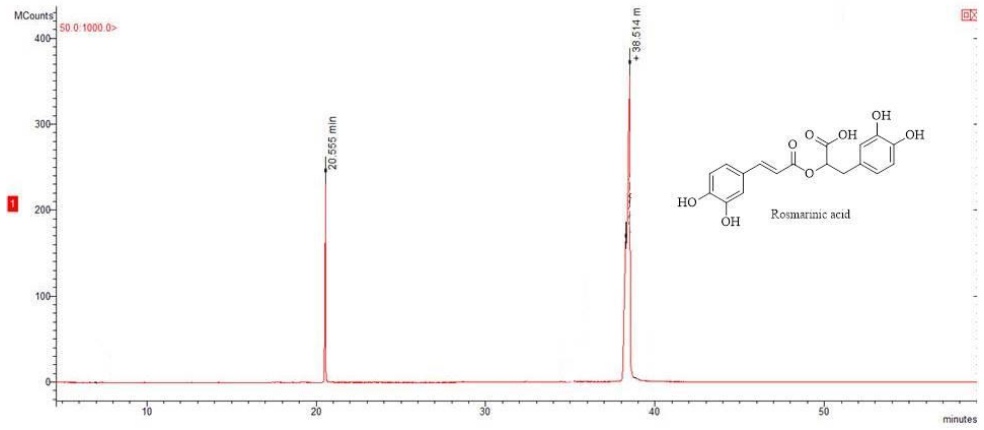


Figure 9.51. Chromatogram of Rosmarinic acid trimethylsilylated.

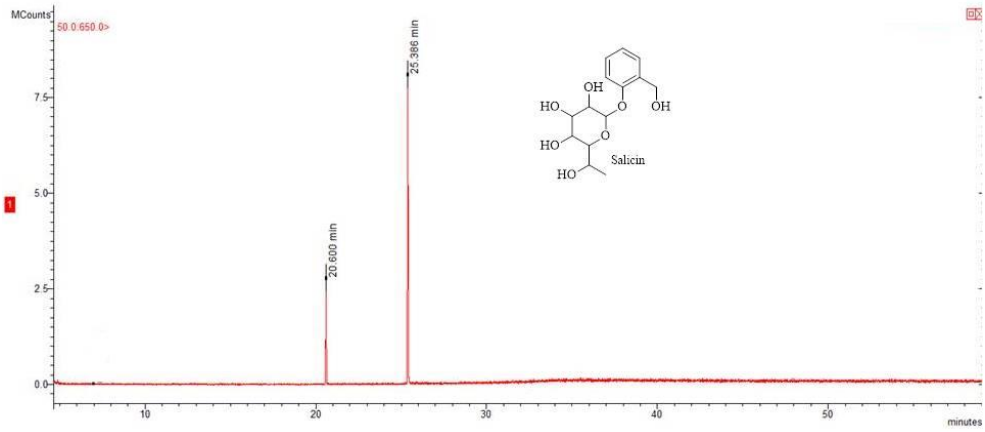


Figure 9.52. Chromatogram of Salicin trimethylsilylated.

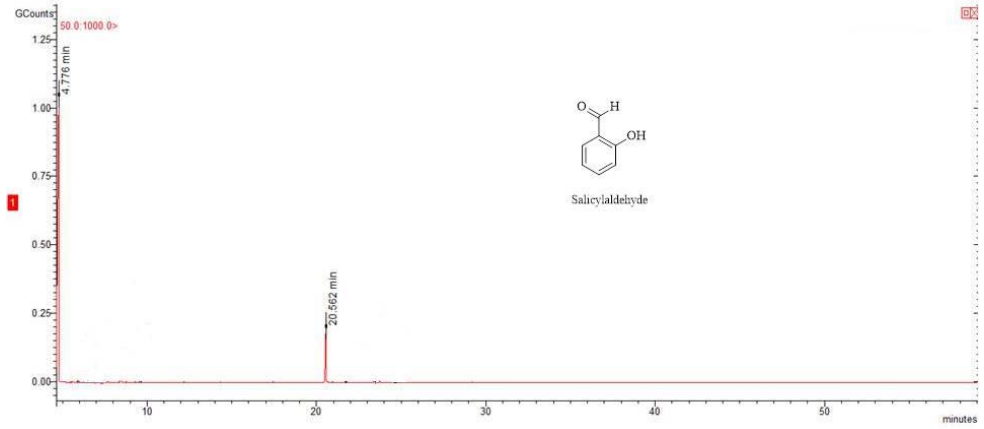


Figure 9.53. Chromatogram of Salicylaldehyde trimethylsilylated.

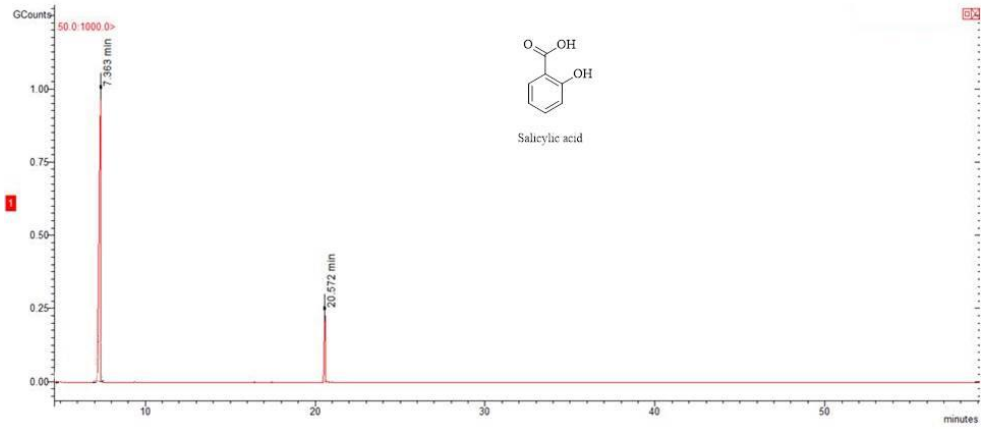


Figure 9.54. Chromatogram of Salicylic acid trimethylsilylated.

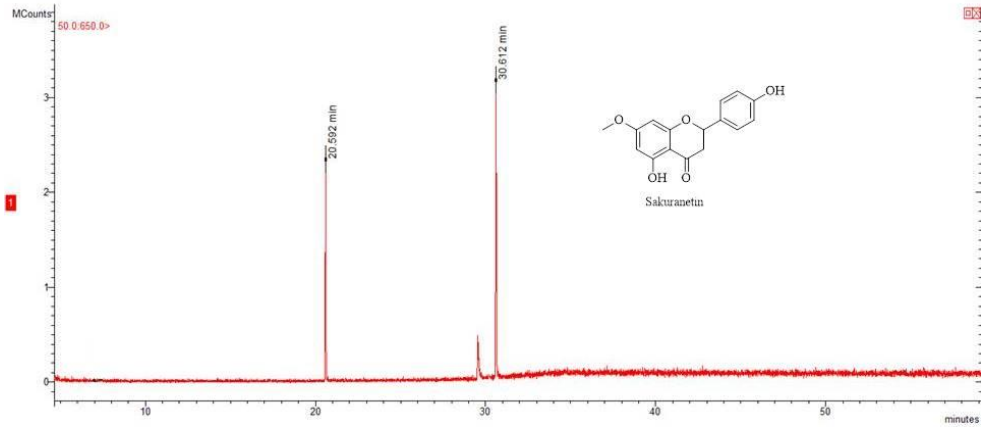


Figure 9.55. Chromatogram of Sakuranetin trimethylsilylated.

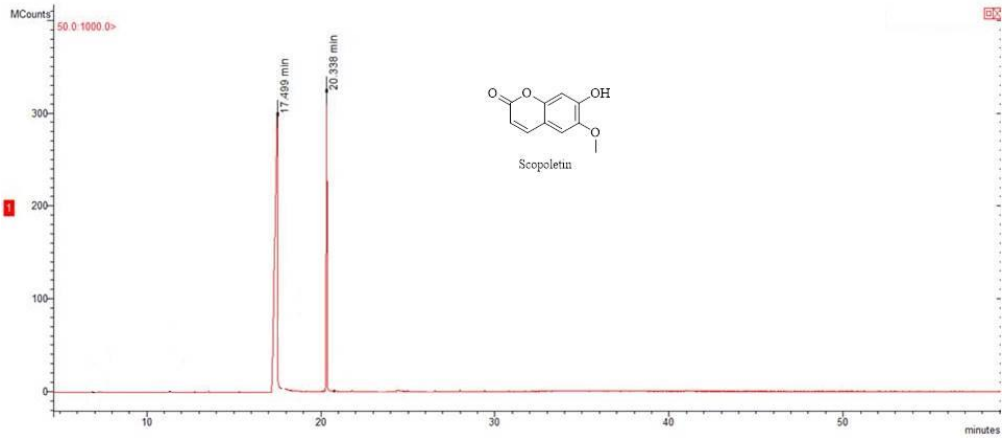


Figure 9.56. Chromatogram of Scopoletin trimethylsilylated.

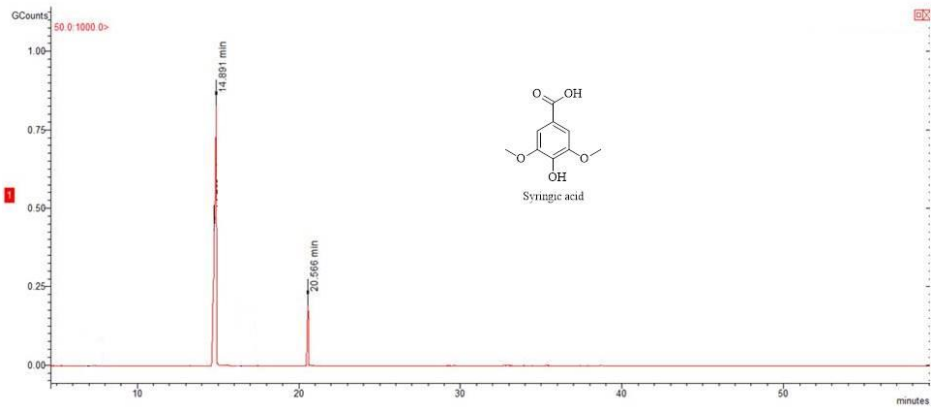


Figure 9.57. Chromatogram of Syringic acid trimethylsilylated.

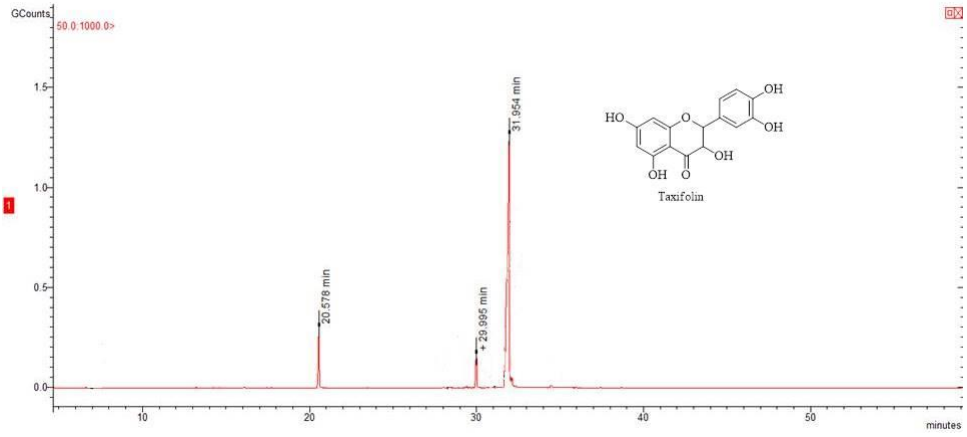


Figure 9.58. Chromatogram of Taxifolin trimethylsilylated.

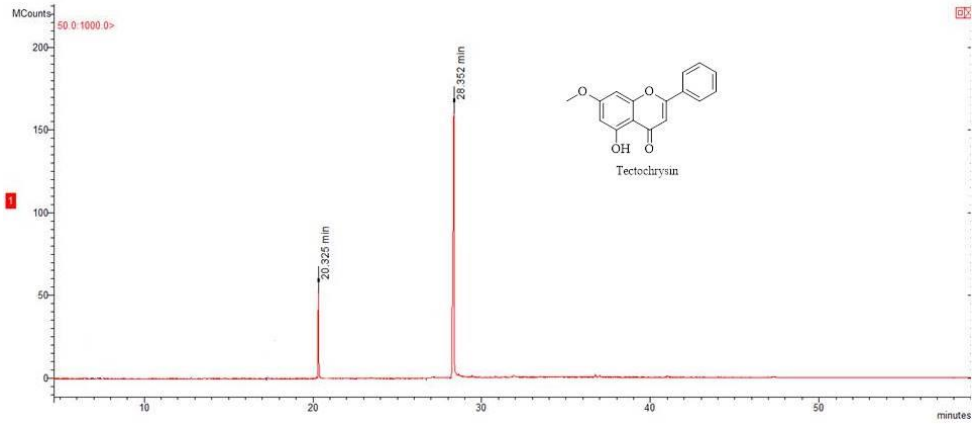


Figure 9.59. Chromatogram of Tectochrysin trimethylsilylated.

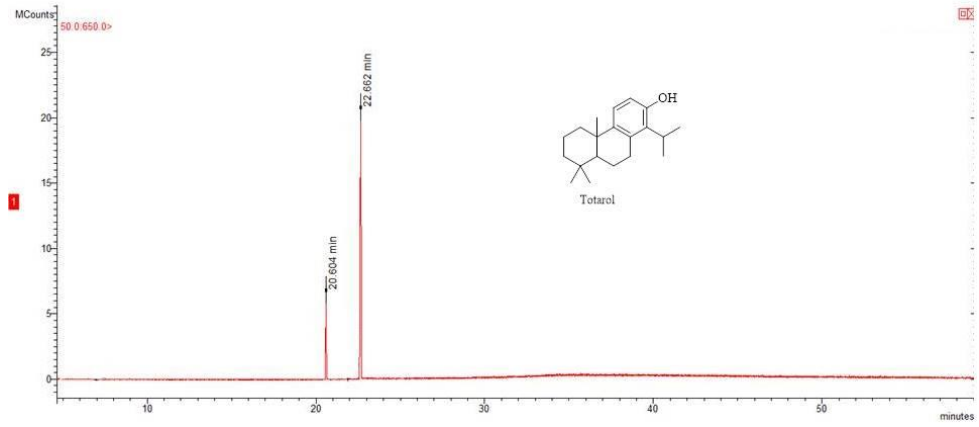


Figure 9.60. Chromatogram of Totarol trimethylsilylated.

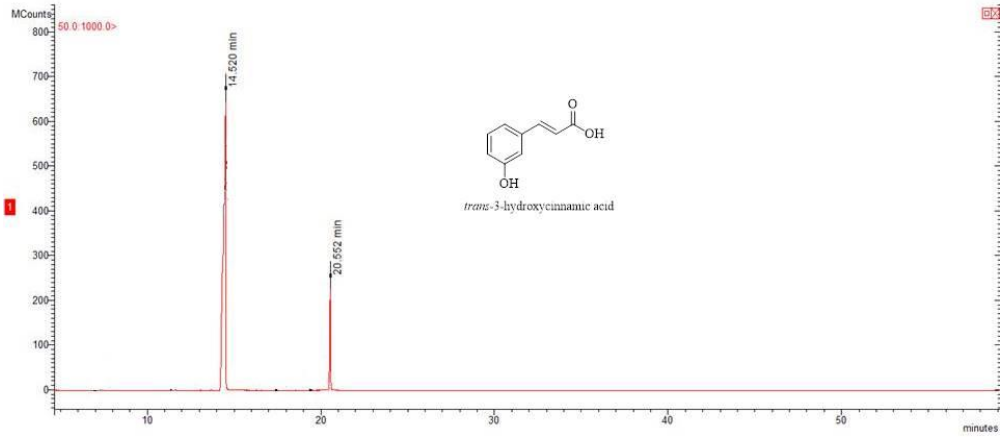


Figure 9.61. Chromatogram of *trans*-3-hydroxycinnamic acid trimethylsilylated.

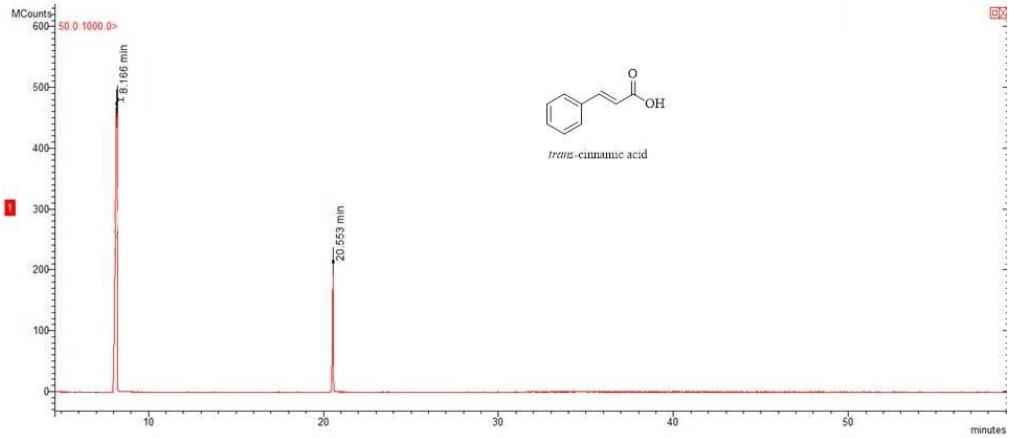


Figure 9.62. Chromatogram of *trans*-cinnamic acid trimethylsilylated.

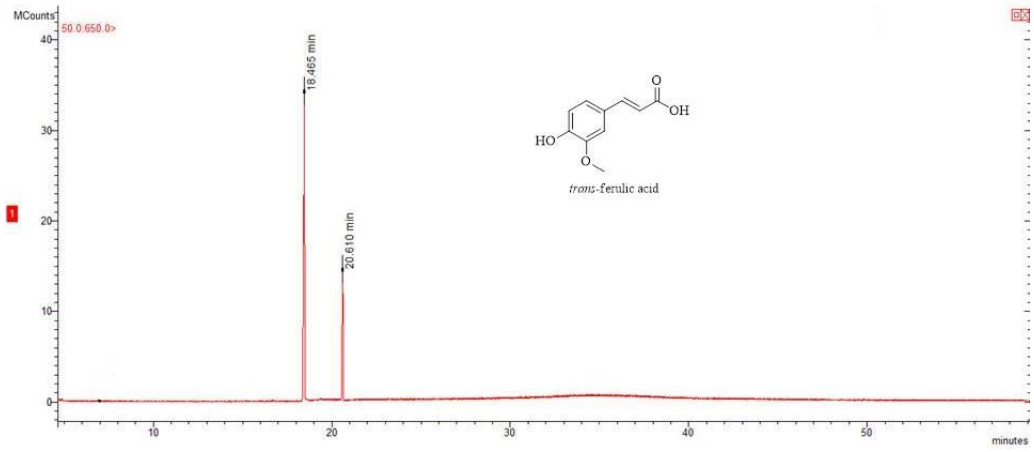


Figure 9.63. Chromatogram of *trans*-ferulic acid trimethylsilylated.

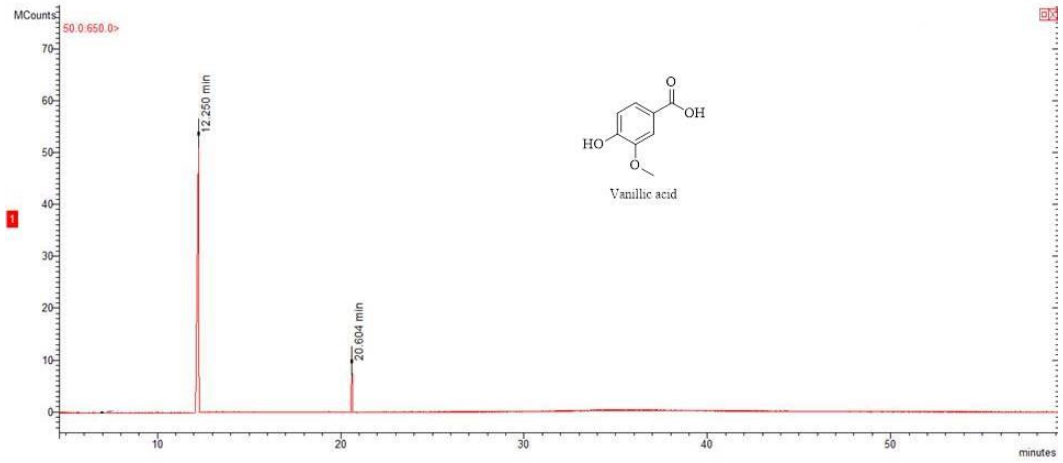


Figure 9.64. Chromatogram of Vanillic acid trimethylsilylated.

9.2.4 Sample: characterization and quantification

The characterization was realized by comparison of fragmentation spectrums of samples with standards content in commercial libraries and a library specially built.

The quantitative analysis was realized by the method of internal standard taking into account application of the conversion factor for the different compounds analysed.

9.3 Analysis Glycosides

9.3.1 HPLC conditions

Chromatographic analysis were performed with an Agilent 1100 LC/MSD VL system (G1946C) (Agilent Technologies, Palo Alto, CA) constituted by a vacuum solvent degassing unit, a binary high-pressure gradient pump, an 1100 series UV detector, and an 1100 MSD model VL benchtop mass spectrometer. Separation was performed on a Beckman Coulter Ultrasphere ODS C-18 column (4.6 x 150 mm, 5µm particle size), maintained at room temperature. A gradient of formic acid in water (0.5%, v/v %, eluent A) and formic acid in acetonitrile (eluent B) was applied at a flow rate of 0.4 mL min-1; 0 min: 5 B% (v/v %), 20 min: 25 B% (v/v %), 24 min: 90 B% (v/v %), 26 min: 100 B% (v/v %), 28 min: 5 B% (v/v %). UV-spectra were recorded at 280 nm, Injection volume was 20µL. The Agilent 1100 series mass spectra detection (MSD) single-quadrupole instrument was equipped with the orthogonal spray API-ES (Agilent Technologies, Palo Alto, CA). Nitrogen was used as nebulizing and drying gas. The pressure of the nebulizing gas, the flow of the drying gas, the capillary voltage, the fragmentor voltage, and the vaporization temperature were set at 40 psi, 9 L/min-1, 3000 V, 94 V or 150 V, and 350°C, respectively. UV detection was monitored at 280 nm. The LC-ESI-MS determination was performed by operating the MSD in the negative ion mode. Spectra were acquired over the scan range m/z 100-800 using a step size of 0.1 u.[926]

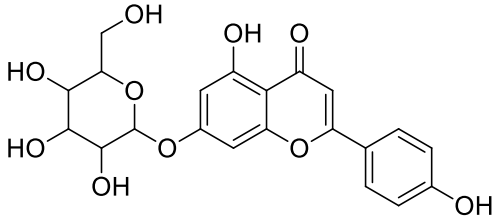
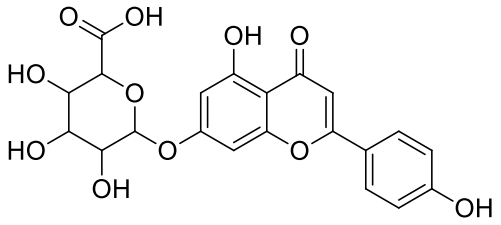
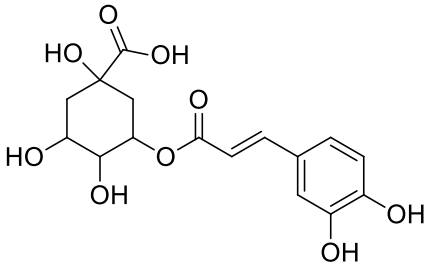
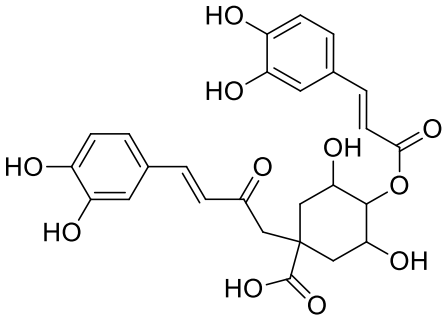
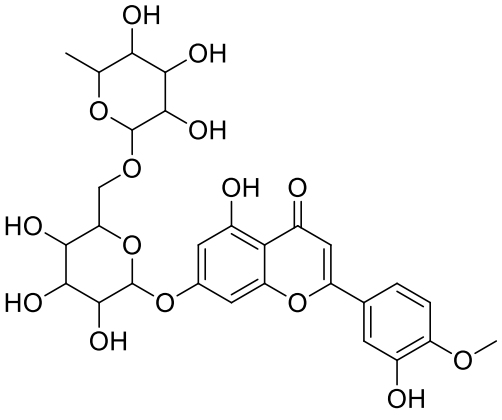
9.3.2 Sample: characterization and quantification

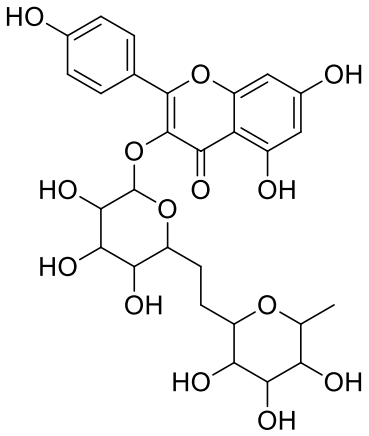
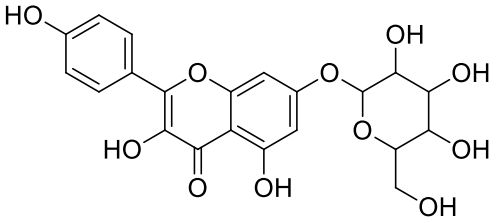
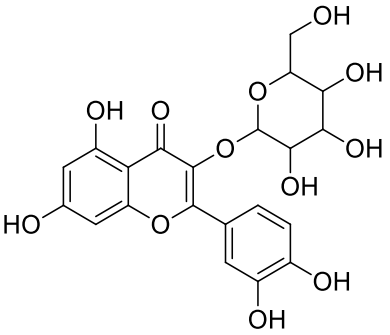
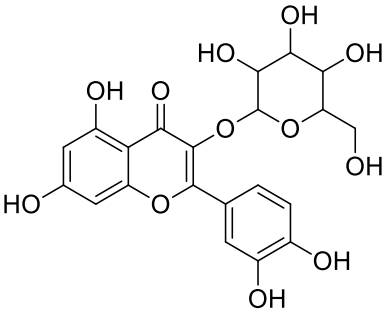
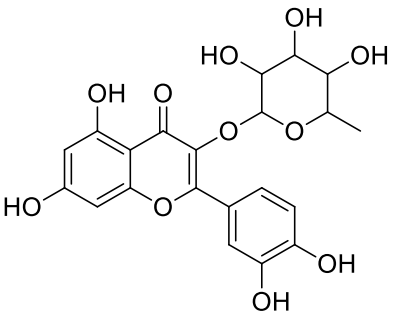
Analysis was conduced on the methanol 80% extract and a solution of 10 mg/ml was prepared and it was injected.

Assignation, qualitaty and quantitaty, was conduced by the calibrate curve method and the method of internal standard, in addiction for the confirmation of compound was used the the addition method.

The standards analyzed were reported in **Table 9.2**.

Entry	Standards	Structure

76	Apigenin-7-O-glucoside	
77	Apigenin-7-O-β-D-glucuronide	
78	Chlorogenic acid	
79	Cynarin	
80	Hesperidin	

81	Kaempferol-3-O-rutinoside	
82	Kaempferol-7-O-glucoside	
83	Quercetin-3-β -D-glucose	
84	Quercetin-3-D-galactoside (hyperoside)	
85	Quercitrin	

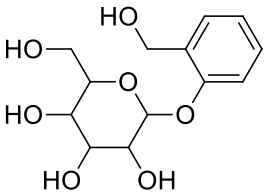
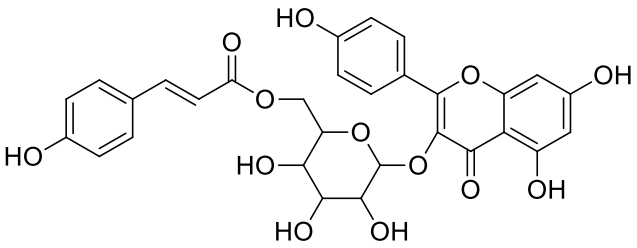
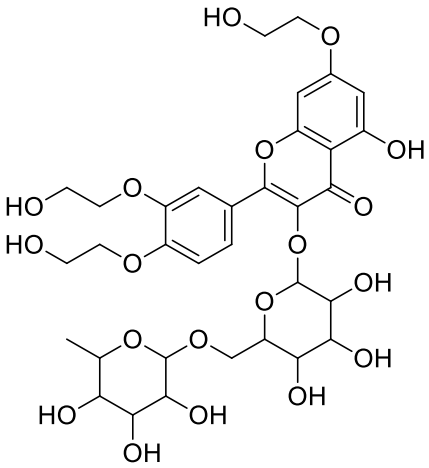
86	Salicin	
87	Tiliroside	
88	Troxerutin	

Table 9.2. Standards

CHAPTER 10

OLIGOPHENOLS AND POLYPHENOLS HIGH MOLECULAR WEIGHT

10.1 Lignin

10.1.1 Extraction

10 gr of sample was extracted with petroleum ether in soxhlet for 6 hours, this extraction removes mainly the lipid component. The pellet was extracted with acetone in soxhlet for 24 hours. The extracted pulp is then refluxed under nitrogen atmosphere with 0.1 M HCl in dioxane in 1:9 for 2 hours. The solution were cooled and filtered through a coarse-sintered glass Buchner funnel packed with a Celite filter aid to remove fines. The mixture was then neutralized by saturated sodium bicarbonate (pH value of 5.0-5.5) and concentrated at 35°C under reduced pressure to approximately 10% of the original volume. Deionized water was added and the mixture was concentrated again under reduced pressure to remove traces of *p*-dioxane. The resultant aqueous lignin solution was transferred to a beaker, diluited with deionized water, acidified to pH of 2.0-2.5, and transferred in centrifuge bottles. Finally, water was added to supernatant to wash the lignin. The lignin was freeze-dried and weighted.[927]

10.1.2 ^{31}P-NMR characterization

Phosphitylation procedure

A solvent mixture composed of pyridine and deuterated chloroform in a 1.6:1 v/v ratio was prepared under anhydrous conditions. A solution was then prepared by utilizing the above preparation; chromium(III) acetylacetonate (5.0 mg/mL) and cholesterol (0.1 M) served as relaxation reagent and internal standard, respectively. 20 mg of dry lignin was accurately weighed into a 1 mL volumetric flask. The sample was then dissolved in 0.5 ml of the above solvent mixture. The 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (0.100 ml) was then added, followed by the internal standard and the relaxation reagent solution (0.100 ml

each). Finally, the solution was made up to the 1 mL mark with more solvent mixture. The flask was tightly closed, and the mixture was stirred for 2 hours at ambient temperature.[928,929]

^{31}P-NMR spectroscopy analysis

The ^{31}P NMR spectra were recorded on a Bruker 400 MHz spectrometer using a published methods to obtain a good resolution of spectra, a total of 500 scans were acquired for any sample. The maximum standard deviation of the reported data was 2 E-2 mmol/gr, while the maximum standard error was 1 E-2 mmol/gr.

10.2 Tannins

10.2.1 Extraction

10 gr sample were defatted by extraction with petroleum ether in soxhlet for 6 hours. The dried sample was then extracted with acetone 70% in soxhlet for 6 hours. The extractive was evaporated under reduced pressure and then freeze-dried. 200 mg of the dried extractive was purified on a column of Sephadex LH-20 equilibrated with methanol 80%. It was dissolved in an appropriate aliquot of methanol 80%, loaded on the column and eluted with 80% methanol. The fractions were monitored at 280 nm through Varian UV-Visible Spectrophotometer Cary 50 Scan until absorbance was <0.005 nm. Then, the column was eluted with acetone 50% and the fraction were monitored at 400 nm until absorbance was < 0.005 nm. The collected tannins fractions eluted with acetone 50% were evaporated under reduce pressure and freeze-dried.[930]

10.2.2 ^{31}P-NMR characterization

Phosphitylation procedure and ^{31}P-NMR spectroscopy analysis were the same used for the analysis of lignin.[931,932]

10.3 Melanin

10.3.1 Extraction and purification[933,934]

10 gr of sample were extracted with petroleum ether in soxhlet for 6 hours, this extraction removes mainly the lipid component. The sample was homogenizated in 1 M KOH solution under magnetic stirring and reflux for 3 hours in oil bath, maintaining an atmosphere of nitrogen at all times during the refluxing. The solution was filtered through two layers of Whatman's paper #1 using a vacuum pump. At the solution was added concentrated HCl to the dark filtrate until pH 2, stirring the mixture with a magnetic bar. The mixture was centrifuged at 6000 rpm per 20 minutes to recover the pellet. The supernatant was discarded and the pellet was washed with distillated water until the supernatant was colourless.

At the pellet was extracted adding 7 M HCl in a ballon under magnetic stirring to reflux for 2 hours in an oil bath, maintaining an atmosphere of nitrogen. The solution was centrifuged at 6000 rpm for 20 minutes. The pellet was washed with 0.01 M HCl and when the supernatant was colourless, the pellet was washed with distilled water until the pH of supernatant was 7. The pellet was re-dissolved in 1 M KOH followed by the addition of 1-butanol and dichloromethane, the mixture was stirred for 30 minutes at room temperature under nitrogen atmosphere. A the end, the mixture was centrifuged at 6000 rpm per 20 minute and the pellet was blended with 1 M KOH, 1-butanol and dichloromethane. The procedure was repeated for 3 times. The solution was acidified at pH2 by adding concentrated HCl under stirring with a megnitic bar. The mixture was centrifuged at 6000 rpm for 20 minutes, the supernatant was removed and the pellet was washed with distilled water until the supernatant was clear and its pH was 7.

10.3.2 Identification

Solubility assay

5 mg of the melanin sample was solubilized in 1 ml of: H_2O, 7 M HCl and 1 M KOH. The melanin was soluble in 1 M KOH but not in H_2O and 7 M HCl.

UV-visible spectroscopy

The melanin was dissolved in a 1M KOH solution to a final concentration of 0.01 mg/ml and scanned the UV-visible spectra in a Varian UV-Visible Spectrophotometer Cary 50 Scan. The spectra were recorded in the wavelength range of 190 to 700 nm and 1M KOH was used as blank.

CHAPTER 11

BIOCATALYSIS AND LACCASE

11.1 Biocatalyst preparation

11.1.1 Laccese preparation and activity assay

Free laccase (powder form) was mixed in sodium acetate buffer 0.1M, pH 5.0 and stored at 6 C°. This mother solution was used to determine the concentration and enzyme activity. Protein concentration was measured spectrophotometrically at 595 nm according to Bradford assay, using BSA as standard. Free and immobilized laccase activity assay was determined spectrophotometrically using ABTS as substrate. An appropriate amount of free or immobilized enzyme in sodium-acetate buffer was added to ABTS solution (5 mM) in sodium-acetate buffer, the mixture and the initial rate was immediately measured as the increase in optical density at 436 nm at a certain frequency (5s) at 25°C for 10 min.[935] One activity unit was defined as the amount of enzyme that oxidized 1 μmol ABTS/min.

$$\mathbf{U/ml} = \frac{\Delta Abs \times V_t \times fd}{\varepsilon \times V_e}$$

V_t : Total volume
fd: diluition factor
V_e: enzyme volume

The immobilization yield was calculated as the difference between the laccase unit (U) in the started enzyme solution and that recovered at the end of the immobilization procedure.

11.1.2 MWCNTs oxidation

MWCNTs (50mg) were suspended in 100mL of concentrated H2S04/HNO3 (3:1 v/v) and the mixture was sonicated in ultrasonication bath for 4 hours. The acid mixture was diluted with 100milliQ water and centrifuged for 20 minutes at 6000rpm. The acid supernatant was discarded and the nanotubes were transferred into 15mL falcon test tube with milliQ water. The mixture was sonicated for 20 mintues and then centrifuged again. The ultrasonication/centrifugation step was repeated until the centrifugation step was no effective and the pH of the solution was about neutral. The mixture was lyophilized.

11.1.3 MWCNTs laccase immobilization (catalyst I)

The MWCNTs (1.0 mg) were added to Na-acetate buffer (10 mL, pH 5) and sonicated for 30 minutes at 25°C. The mixture was centrifuged at 13000 rpm for 10 minutes and the supernatant was removed. The MWCNTs were added to sodium acetate buffer (0.5 mL, pH 5) containing laccase (Lac, 0.2 mg, 69 U mg-1). The mixture was incubated for 2.5 h at 25°C with orbital shaking. At the end of the coupling period, the mixture was centrifuged at 13000 rpm for 10 minutes and the supernatant was removed. The MWCNTs/Lac system was conserved at 4°C without any further purification. The amount in milligrams and units of coupled Lac were calculated by difference between the amount units loaded and those recovered in the washings, by conventional Bradford and activity ABTS assay.

11.1.4 MWCNTs laccase immobilization with Layer-by-Layer method (catalyst II)

The MWCNTs immobilized with laccase (1.0 mg) was suspended in a mixture of 0.5 M NaCl solution (1.0 mL, pH 5) in the presence of poly(allyl dimethyl ammonium chloride) (PDDA) (2.0 mg) and sonicated for 5 minutes, then mixture was incubated for 2.5 h at room temperature with orbital shaking. At the end, the mixture was centrifuged at 13000 rpm for 10 minutes, the supernatant was removed and the solid residue (that is the MWCNTs/Lac/PDDA intermediate) was washed with milliQ water to remove residual PDDA. The MWCNTs/Lac/PDDA intermediate was added to sodium acetate buffer pH 5 (0.5 ml) containing laccase (Lac 0.2 mg, 69 U mg-1). The mixture was incubated for 2.5 h at 25°C with orbital shaking. At the end of the coupling period, the mixture was centrifuged at 13000 rpm for 10 minutes and the supernatant was removed. The multilayer MWCNTs/Lac/PDDA

system was conserved at 4°C without any further purification. The amount in milligrams and units of coupled laccase were calculated by difference between the amount units loaded and those recovered in the washings by conventional Bradford and activity ABTS assay.

11.1.5 Oxidized MWCNTs laccase immobilization (catalyst III)

The oxidized MWCNTs (ox-MWCNTs) were recovered by lyophilization. Successively, ox-MWCNTs were suspended in a mixture of 0.5M NaCl pH 7 (1.0 ml) and poly(diallydimethylammonium chloride) (PDDA) (2.0 mg), sonicated for 5 minutes and incubated for 2.5 h at 25°C with orbital shaking. Finally, the mixture was centrifuged at 13000 rpm for 10 minutes, the supernatant was removed and the residue washed with milliQ water to remove the residual PDDA. The ox-MWCNTs/PDDA intermediate was then added to Na- acetate buffer (0.5 mL; pH 5) and laccase (0.2 mg, 69 U mg-1). The mixture was incubated for 2.5 h at room temperature with orbital shaking. At the end of the coupling period, the mixture was centrifuged at 13000 rpm for 10 minutes and the supernatant was removed. The ox-MWCNTs/PDDA/Lac system was conserved at 4°C without any further purification. The amount and units of coupled laccase were calculated by difference between the amount units loaded and that recovered in the washings by conventional Bradford and activity ABTS assay.

11.2 Biocatalysts assays and structural characterization

11.2.1 Activity and kinetic assay laccase

The activity and kinetic parameters (Km, Vmax and Kcat) of free and immobilized laccase was measured spectrophotometrically with ABTS as substrate in Na-acetate buffer at 25 °C. The initial rate of the enzyme reaction was determined from the linear increase of the absorbance at 436 nm. Kinetic parameters were determined by measuring enzyme activity at different concentrations of ABTS (0.1mM - 5.0mM) and plotting data to a double reciprocal plot Lineweaver-Burk plot.

11.2.2 Assays of pH and temperature stability

The activity of free and immobilized laccase was measured over the pH range from 2 to 6, using ABTS as substrate and Citrate-phosfate as buffer. Similarly the thermo-stability assay was done at a certain temperature range (30-80 °C), using ABTS as substrate, and sodium acetate as a buffer. In both assays measurements were carried with the spectrophotometer readings at 436 nm each minute. The activity of free and immobilized laccase was measured over the pH range from 2 to 6, using ABTS as substrate and Citrate-phosfate as buffer. Similarly the thermo-stability assay was done at a certain temperature range (30-80 °C), using ABTS as substrate, and sodium acetate as a buffer. In both assays measurements were carried with the spectrophotometer readings at 436 nm each minute.

11.2.3 Storage stability assay

Laccase storage stability was measured incubating three different catalyst in Na-acetate buffer 0.1 M, pH 5.0 at +6°C. At regular time intervals was measured enzyme activity by the ABTS method described above. For each sample, laccase activity was expressed as relative percentage activity respect to that at time zero.

11.2.4 Electrochemical characterization

Cyclic voltammetry experiments were performed using an Autolab electrochemical system (Eco Chemie, Utrecht, TheNetherlands) equipped with PGSTAT-12 and GPES software (Eco Chemie, Utrecht, The Netherlands). A multi-walled carbon nanotubes (ref. 110MWCNT) (MWCNTs) modified screen printed electrodes (SPE) and an edge connector (ref. DRP-DSC) were purchased from DropSens (Oviedo, Spain). The experiments were performed in a conventional thermostatted three-electrode cell, with a MWCNTs working electrode with a diameter of 3 mm, a platinum counter electrode and a saturated calomel electrode (SCE) as reference electrode.

Laccase modified electrode: preparation and electrochemical characterization 1.3 mg of a powder of laccase-MWCNTs and laccase-MWCNTs-PDDA were mixed with Nafion and 5 μL aliquot of this solution was dropped onto MWCNTs SPE surface and kept at 25°C for 2 h. Unless otherwise specifically defined, all electrochemical experiments were anaerobic, and solutions were kept under N_2 flow for 1 h before experiments. For aerobic experiments,

compressed air was bubbled through the solution for at least 10 min before experiments. Cyclic voltammetry experiments were performed using an Autolab electrochemical system (Eco Chemie, Utrecht, TheNetherlands) equipped with PGSTAT-12 and GPES software (Eco Chemie, Utrecht, The Netherlands). A multi-walled carbon nanotubes (ref. 110MWCNT) (MWCNTs) modified screen printed electrodes (SPE) and an edge connector (ref. DRP-DSC) were purchased from DropSens (Oviedo, Spain). The experiments were performed in a conventional thermostatted three-electrode cell, with a MWCNTs working electrode with a diameter of 3 mm, a platinum counter electrode and a saturated calomel electrode (SCE) as reference electrode.

Laccase modified electrode: preparation and electrochemical characterization 1.3 mg of a powder of laccase-MWCNTs and laccase-MWCNTs-PDDA were mixed with Nafion and 5 μL aliquot of this solution was dropped onto MWCNTs SPE surface and kept at 25°C for 2 h. Unless otherwise specifically defined, all electrochemical experiments were anaerobic, and solutions were kept under N2 flow for 1 h before experiments. For aerobic experiments, compressed air was bubbled through the solution for at least 10 min before experiments.

11.2.5 SEM and AFM characterization of catalysts

Surface morphology of the samples has been studied by scanning electron microscopy (SEM) analysis, making use of a Zeiss, LEO 1530 apparatus equipped with a field emission electron gun, while atomic force microscopy (AFM) through a Digital Dimension D5000 instrument with Nanoscope IV controller, using commercial silicon tips (frequency range 51-94 kHz) scanned by means of a Veeco Nanoman closed loop XY head. For the SEM and AFM analysis different solutions of the samples were prepared. In particular, MWCNT and ox-MWCNTs/PDDA samples were dispersed in pure ethanol, while catalysts I in milliQ water, in order to prevent the enzyme denaturation. All the solutions were sonicated for 5 min at room temperature and a drop of each solution was taken and deposited onto silicon substrates (Si). At the end, the obtained samples deposited on Si were subjected to annealing at 50 °C to quickly evaporate the solvent (ethanol or water).

11.3 Alcohol oxidation reactions

11.3.1 Alcohols oxidation[936]

A large panel of alcohols was oxidized, including benzyl alcohol 1, 4-methoxybenzyl alcohol 2, 3-methoxybenzyl alcohol 3, 3,4-dimethoxybenzyl alcohol 4, 3,4,5-trimethoxybenzyl alcohol 5, cinnamyl alcohol 6, geraniol 7, 2-chlorobenzyl alcohol 8, 4-cholorobenzyl alcohol 9 and 3-cholorobenzyl alcohol 10. The oxidations were carried out using the following conditions: alcohol (20 mM), Lac and catalysts I-III (0.65 U) and the mediator (TEMPO or ABTS; 6.0 mM) were placed in 0.1 M Na-acetate buffer pH 5 (1.0 ml) in vigorous stirring at room temperature in presence of O2. Reactions were monitored by thin layer chromatography (TLC). After the disappearance of the substrate, the reaction mixture was extracted with EtOAc (10 ml x2). The organic extracts were treated with a saturated solution of NaCl and dried over anhydrous Na2SO4, then filtered and concentrated under vacuum to yield the crude. When necessary, the purification was performed by flash-chromatography. In the case of immobilized enzyme, the catalysts I-III were recovered by filtration and the solution were subjected to the same work up described above.

11.3.2 Identification and characterization of products

GC-MS analyses were performed on a Varian 450GC-320 MS apparatus using a SPB column (25 m x 0.25 mm and 0.25 mm film thickness) and an isothermal temperature profile of 70°C for 2 min, followed by a 10°C min-1 temperature gradient to 280°C for 25 min. The injector temperature was 280°C. Chromatography-grade helium was used as the carrier gas with a flow of 1 mL/min-1. Mass spectra were recorded using a Varian 300 MS/MS with an electron beam of 70 eV. Acetophenone was used as internal standard. 1H and 13C NMR spectra were recorded on a Bruker (400 MHz) spectrometer. Mass spectra and NMR analyses for all products are in supplementary material.

11.3.3 Enzyme recycling

Catalysts I-III were recycled as follows: 4-methoxybenzyl alcohol (20 mM), immobilized laccases (0.65 U), and TEMPO (6 mM) were placed in Na-acetate buffer 0.1 M pH5 (1.0 ml). After 16 h, the catalyst was recovered by centrifugation and reused with fresh TEMPO (6.0

mM) and substrate (20 mM). For each run, the reaction mixture was extracted with EtOAc and the products analysed by GC-MS.

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