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Biomaterials Functionalisation with Polyphenols and Characterisation

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INTRODUCTION

Polyphenols constitute an interesting class of biomolecules, which are known still the Antique Greece, but are methodically studied only in the last years. Extractable from numerous types of plants, they are becoming more and more important in scientific world because of a series of interesting properties, which could be exploited for different applications: antioxidant, antibacterial and anti-inflammatory properties are only the main. Because of that, a regular polyphenol intake is recommanded in the daily diet; anyway, the most part of the assumed polyphenols contained in food is difficultly adsorbed at the intestinal level.

Concerning clinical applications, the study of polyphenols have been deepened to evaluate the possibility to include these biomolecules in medical devices, from the micro- to the macro-scale: bypassing the intestinal tract, polyphenols could be significantly more bioavailable, locally exercising their properties. In fact, they could be encapsulated in nanoparticles to be released *in vivo* for localised therapies or grafted on macroscopic devices to locally exercise their beneficial properties from the surface of an implant. Various applications have been hypothesised: anti-cancer therapies, anti-inflammatory treatments and tissue regeneration.

Functionalisation techniques are still studied to optimise the process in function of the considered biomaterial, but are methodically studied in the last years.

In the recent years, the modalities of extraction of phenolic compounds have been optimised in function of the subclass at which they appertain and the plant from which they are extracted.

The aim of this work is to optimise biomaterials to be applied to musculoskeletal implants, treating them with polyphenols. In the current research, a mixture of lyophilised polyphenols extracted from red grape (type Barbera) is utilised to functionalised different biomaterials: hydroxyapatite, Ti6Al4V – previously chemically treated to obtain a porous and bioactive surface – and two types of bioglass have been considered and functionalised with polyphenols: different processes have been tested, comparing the effects of the different imposed parameters. The functionalisation process has been optimised and the functionalised surfaces of each tested material have been characterised, evaluating the quantity of grafted polyphenols, their redox and scavenging activity when grafted and eventual alterations of their behaviour after functionalisation. Parallelly, the eventual variations of the characteristics of the biomaterials after functionalisation treatment have been evaluated. Different techniques have been exploited: FTIR-ATR spectroscopy, UV spectroscopy, fluorescence microscopy, zeta potential, DPPH test, Folin and Ciocalteu test and pH evaluation. To better characterise the polyphenol-biomaterial system, a test of release in water has been implemented. The samples have also been sterilised and newly characterised after sterilisation, to evaluate the possibility of a future industrial production for clinical applications.

CHAPTER 1
POLYPHENOLS: GENERALITIES, PROPERTIES AND CHARACTERIZATION

Polyphenols are a heterogeneous group of molecules which can be found in a great variety of plants, sometimes also in high concentrations. They contribute to give the characteristic pigmentation of the various plants and have a long series of proprieties from which plants themselves but, as described in this chapter, not only them, benefit. They in fact, thanks to their colour, attract insects to increase the probability the plant will be fecundated; they protect plants from ultra-violet (UV) radiations and other risks, such as bacteria and parasites. But these molecules are also fundamental for their antioxidant activity, that can be noted in different cases. For example, they help the digestion of proteins in poultry, pigs and human beings and grass in ruminants[1].

Already in Ancient Greece and in Ancient Rome the beneficial effects of polyphenols – also if they were not known as molecules, were appreciated. In Greece, during Olympic Games, champions received an amphora which contained olive oil, a substance that – now it is assured, is very rich of these molecules: they used to strew it on their bodies to tract benefit from it, both for the skin and for internal tissues. In Rome instead a more accurate work was done. Romans classified oils on the basis of the maturation of olives and of the final use: the most esteemed was *ex albis* oil, obtained from green olives, and the oil of the worst quality was *caducum* oil, extracted from the olives that had already fallen on the ground, intended for slaves[2].

Otherwise, only at the end of the XIX century the presence of polyphenols in olive oil was known: the Italian chemist Francesco Canzoneri studied olives and the leaves of their plant in his laboratory in Bari, one of the firsts chemical laboratories in Italy. His goal was the characterization of this type of oil on the basis of minor components as polyphenols are. He understood that polyphenols are very concentrate in extra virgin olive oil: their concentration can variate from 100 to 1500 mg for each kg of olive oil[3].

In the last years polyphenols are becoming more and more important in our society because of the more attention we pay to health and prevention: polyphenols, as reported previously, have in fact a significant antioxidant activity that is maintained also after their extraction from plants and they are so capable to contrast various types of cancer, cardiovascular diseases and different dermal disorders[4].

1.1 Polyphenols classification

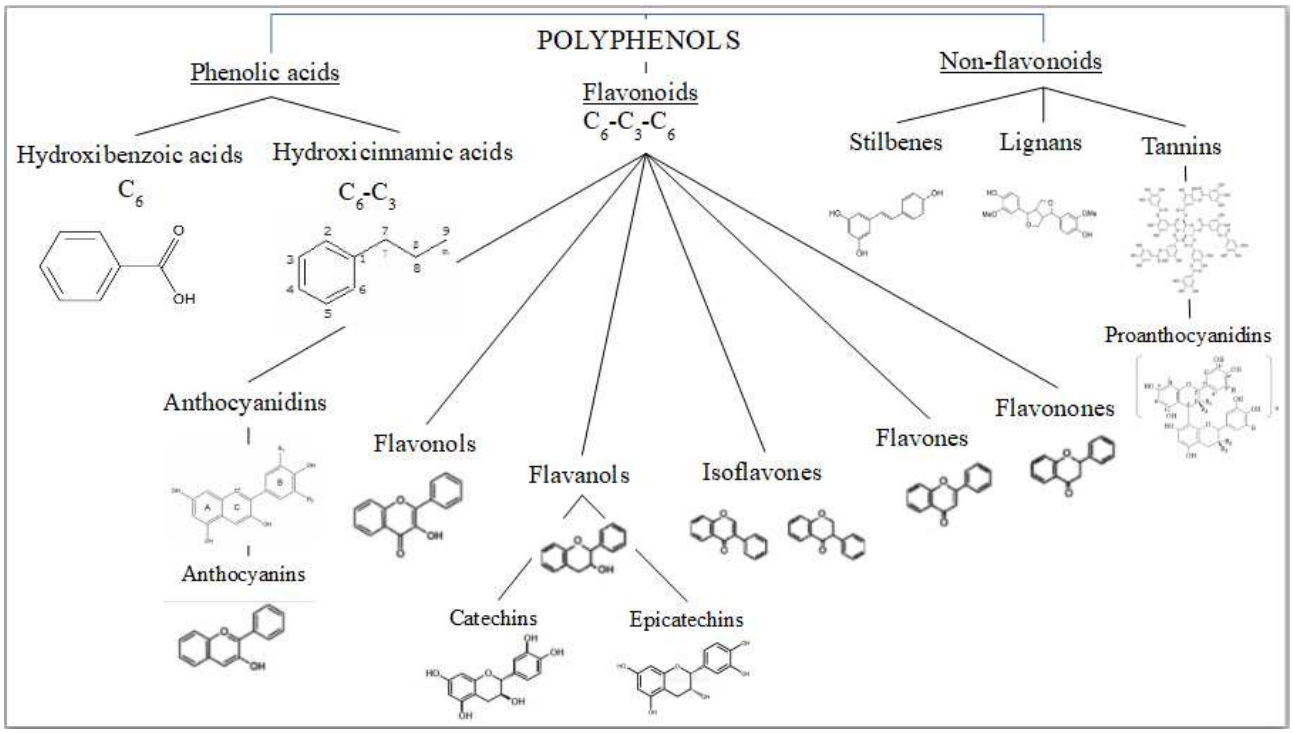

Figure 1.1 Polyphenols classification[5]

Polyphenols comprise a diverse range of molecules: more than 8000 polyphenols have been identified. In the upward scheme (Figure 1.1), it is possible to appreciate the great variety of these categories of molecules[6].
All the types of polyphenols have in common a hydroxyl-substituted benzene ring to which different groups or other substituents are bonded; different types of polyphenols have also a different number of rings which are bonded to one another in different ways: in fact polyphenols are classified into different groups on the basis of these feature, as described in this paragraph.
Polyphenols can be classified in three principal categories: phenolic acids, flavonoids and non-flavonoid compounds.[7]Polyphenols belonging to all the classes can be found in their native forms – this type is called aglycone, or, most commonly, as their glycosyl derivatives. Also some types of carbohydrates molecules can be present in the structure of some polyphenols: the most common carbohydrates that take part to the chemical structure of polyphenols are rhamnose, glucose, galactose and arabinose[8].

Phenolic acids are the first class of polyphenols we find in nature, where they are very common. They are characterised by different chemical structures and biodisponibility. This first class of polyphenols has been quite critical to classify: not all the scientists have considered them strictly as polyphenols because of their monomeric nature and thought that they were only a basic molecule that can contribute to form polyphenols only if chemically bonded in more complex structures[9]. Anyway in the last years the scientific world agrees quite unanimously to consider phenolic acids as a polyphenols class properly said.
Phenolic acids are generally divided into two main groups, hydroxibenzoic acids and hydroxicinnamic acids[10].

Hydroxibenzoic acids are extracted from red grape, generally from its skin. They are appreciated for their anti-inflammatory and anti-carcinogenic properties. Hydroxibenzoic acids are characterised by a basic cyclic structure, constituted by a single benzoic ring with different substituents: one or more phenolic and methoxyl groups and a carboxyl group directly bonded to the ring. It is so correct to say that the benzoic ring at which is bonded carboxyl group – a structure called benzoic acid, is the basic structure of this category of molecules; adding the possible different substituents, it is possible to obtain hydroxibenzoic acids11. Benzoic acid structure is reported in Figure 1.2.

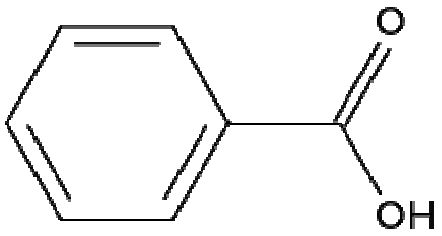

Figure 1.2 Benzoic acid[12]

Hydroxicinnamic acids are instead compounds composed by a benzoic ring at which is bonded a chain constituted by three carbon atoms: this structure is also referred as C_6-C_3. The main source of these molecules is red grape and they are extracted from its skin as the precedent compounds, but in this case especially from vacuoles[13]. The concentration of hydroxicinnamic acids decreases in the grape during its maturation, but overall we note its increasing correlated with the increasing of the dimensions of the fruit itself14. Other sources of hydroxicinnamic acids are coffee, cereals and different types of vegetables[15,16].

Flavonoids are the most important class of polyphenols because they are the most common polyphenols present in nature and for their numerous and various subclasses in which they can be divided. Also if each subclass is different from the others for chemical structure and properties, each type of flavonoids has in common the basic structure. It is formed by a diphenilpropane skeleton (Figure 1.3), a structure also known as C_6-C_3-C_6.It is constituted by three aromatic rings (A, B, C): A and C rings are bonded together, while B and C rings are connected by a three carbon atoms chain. C ring is a pyran ring, that is a heterocyclic ring in which we find an oxygen atom17. The different subclasses of flavonoids are due to the presence of different substituents, the structure of heterocyclic C ring, the way through B ring is connected to C ring, the oxidation levels of the molecule, the biosynthetic origin and the different possible mechanisms of glycosilation and hydroxilation[18,19].

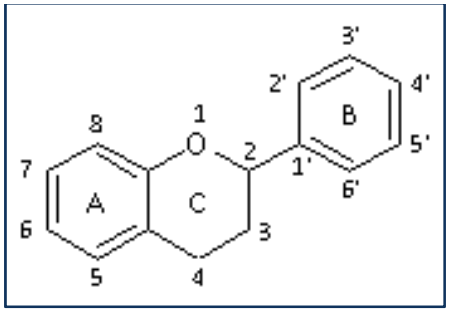

Figure 1.3 Diphenilpropane skeleton[20]

During biosynthesis in plants, the central basic pathway is conserved and depends on the species of the plant. Biosynthesis is modified by a group of enzymes (isomerase, reductase and hydroxylase): the action of these enzymes let flavonoids be very different each other at the end of this process and so they can be divided into different classes. Some flavonoids are both intermediary compounds during biosynthesis and final products present in plants, such as flavonones; others, such as anthocyanins, flavones and flavonols, are instead only present in nature as final products. In nature, flavonoids are compounds associated with carbohydrates in conjugated forms, called glycosides. They are often hydroxylated in positions 3, 5, 7, 3′, 4′ and 5′. It is possible that some of hydroxyl groups are modified during biosynthesis (for example methylated, acetylated or sulfated). These changes brought to the molecule structure often alter solubility, reactivity and stability of the flavonoid. The most part of flavonoids can be extracted from fruit as berries, cherries, grape, apples and citrons, from vegetables as broccoli and onions, but also from other sources such as tea.[21]

The different types of flavonoids are six: antocyanidins, flavonols, flavanols, isoflavones, flavones and flavonones.

Antocyanidins, extracted from the skin and the seeds of grape, from berries and cherries, are fundamental in the pigmentation of the fruits: the colour expressed is pH dependent and changes from red at low pHs, to blue-purple at neutral pHs, to yellow in alkaline environments[22]. They are usually glycosylated: these structures are called antocyanins or antocyans. The sugar is normally bonded to C ring in the third position[23].

Flavonols can be extracted from different types of vegetables, such as onions, broccoli and capers, and fruits, such as cherries, berries, grape and apples, but also from tea and cocoa[24,25]. They are researched for their antioxidant and anti-inflammatory properties and for anti-carcinogenic factor of which this kind of molecules are rich. They are characterised by a double bond in the C ring between the second and the third positions; in C ring there is also an oxygen atom in the fourth position[26].

Flavanols are present in skin and seeds of different fruits, such as grape, berries – especially blackberries and apples, but it is possible to find them also in cocoa, beer, green tea and red wine. They are fundamental in cardiovascular applications for their capability to reduce arteries pressure and their anti-inflammatory properties targeted especially to these tissues. Concerning their chemical structure, in the C ring there is a hydroxyl group in the third position: for this reason, they are also known as flavan-3-ols. They are capable to aggregate in oligomers and polymers, called proantocyanidins, that exist in two different configurations: with trans- configuration they are called catechins, with cis- configuration they are called epicatechins[27]. These two stereoisomers have a similar behaviour, preventing and contrasting cancer[28,29]. Anyway, it was noted that epicatechins are easier to absorb by intestine during digestion; catechins have instead a lower bioavailability, also if their efficacy on human health is significant. Only few catechins, such as theaflavins – dimeric catechins extracted from green tea – are not absorbable[30,31].

Isoflavones are exclusively extractable from soy and from other plants as *Iridaceae, Leguminosae* and *Faboideae.* They are weak estrogen agents and have chemopreventive effects[32]. They are characterised from a particular bond between the B and the C ring present in the third position of of the B ring. They are structurally and chemically similar to estrogens: so it is demonstrated the estrogen action in health applications[33].

Flavones are present not only in the already cited fruits and vegetables, but they are more diffuse, also in seaweeds and lichens. Their properties are quite different and not all are already known, but surely they are fundamental in plants and flowers pigmentation, giving them a characterizing yellow

colour[34]. Chemically, they present characterizing differences in all the rings, A, B and C rings. A ring has a hydroxyl group in the fifth position, it is hydroxylated in the seventh position and glycosylation is visible in both the positions. C ring has a double bond between the second and third positions and a ketone group is present in the fourth position. Finally, B ring presents hydroxylation in its 3' and 4' position[35].
The last flavonoids subclass to analyse is the flavanones subclass. They are really diffused in vegetable world – but not only, and they are fundamental in human applications because they are the most stable polyphenols if exposed to light[36]. Their stability is explained by the saturation of C ring and the double bond between its second and third positions. They are being studied for their anti-carcinogenic properties[37].

The third and last category of polyphenols is the class of non-flavonoids. They are more different each other and present variable structures that can broadly include different monomers and chemical groups. They are divided into three subclasses: stilbenes, lignans and tannins[38].
Stilbenes can be defined as hydrocarbons, where, in correspondence of the two carbon atoms connected by a double bond, an ethane is substituted by two phenyl groups. They can be extracted in the grape stem and is very present in red wine. The most important molecule appertaining to this subclass is resveratrol: it is very diffuse and present in significant quantities in plants. It has two possible configurations: cis- and trans-; the second one is preferred for human application because of its important bioactivity. Stilbenes have interesting properties applicable to health field, such as antioxidant and chemotherapeutic properties. They can be utilized especially in cardiac applications, for their cardioprotective effects[39].
Lignans are formed by two subunits, precisely phenylpropane subunits, which are bonded through lateral chains: their structure is quite similar to the one we find in hydroxicinnamic acids[40]. They are usually extracted from integral cereals and from seeds, such as linen and sesame seeds[41]. Their anti-carcinogenic properties are studied: this effect is particularly visible in prevention of estrogen-dependent tumours, such as breast tumour, very diffused in the last years in middle-aged women. Lignans are also studied for prevention of type II diabetes[42].
Tannins are extracted from the cortex of different types of trees: oaks, chestnuts and firs. They contribute to pigmentation of plants, vegetables and fruits in which they are present. They have not particular applications in human health field, but they can extract salts throwing down them from heavy metals[43,44]. There are two types of tannins: hydrolysable tannins and condensed tannins. The firsts are constituted by a polyol – the most common in nature is D-glucose, and, as their name suggests, are hydrolysable: it means that, in contact with water molecules, they react and different internal bonds are broken, and so it conduces to a degradation of the tannin. Condensed tannins instead cannot be hydrolyzed because there have not hydrolysable groups in the main chain of the molecule: they in fact are formed by oligomeric or polymeric subunits that have a flavonoid nature and these subunits are connected by C-C bonds; condensed tannins are also known as protoantocyanidins[45].

1.2 Polyphenols photo-oxidation and thermal stability

Some studies were carried in Romania in 2013 to demonstrate the relative photo-oxidation stability of polyphenols, if exposed to UV rays. An extract of polyphenols – precisely, gallic acid, catechin and vinillic acid, as extracted from plants, was exposed to UV-C rays for 8 hours complexly and

degradation was observed. It was noted that catechin had a faster degradation, while gallic and vinillic acids are more stable, as it is possible to see from the following graphics (Figure 1.4).

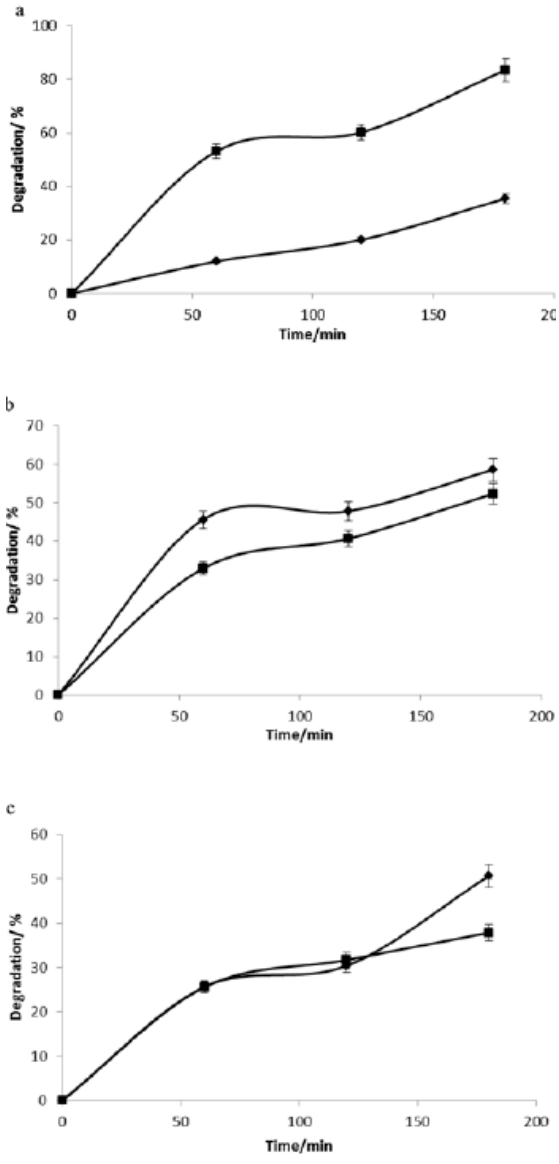

Figure 1.4 Polyphenols behaviour during UV exposition:

a. Catechin
b. Gallic acid
c. Vinillic acid

For each graphic, two curves are plotted: the polyphenols solution to analyse (indicated with a rhombus) and the standard, constituted by an aqueous compounds solution (indicated with a square).[46]

Catechin in fact is the most degraded molecule at the end of the UV exposition: after three hour of irradiation, its volume has decreased of about the 80%, while gallic and vinillic acids only of 60%. This is due to the presence of ascorbic acid in the first molecule, that accelerates the degradation process. Generally, it is possible to say that polyphenols have a quite good resistance to UV and the environment in which they are is not particularly correlated to the degradation of these molecules: the eventual degradation or denaturation in food is not frequent, and so the looseness of properties.
Another property that was analysed in these studies was thermal stability: the same solution of polyphenols was treated with high temperatures (60°C and 100°C were reached), and the behaviour of the molecules was evaluated as time-dependent. Similarly to the precedent case, catechin is the less resistant molecule: it degrades instantly 20% at 60°C and 32% at 80°C, while the two acids have an analogue behaviour, degrading 15% at 60°C and 25% at 100°C; after a longer exposition (4 hours), the level of degradation of the three different polyphenols is instead more homogeneous inside the solution[47].
An important difference to evidence is the different behaviour between flavonoid and non-flavonoid compounds: the firsts are more resistant to external agents, such as light and temperature, than the second ones. In fact the observed degradation is faster in non-flavonoids polyphenols; particularly, confronting the two classes of polyphenols, it is possible to note that the presence of hydrolysed tannins increases with the duration of the treatment, while the concentration of anthocyanins does not present significant variations[48].

1.3 Antioxidant properties

1.3.1 Mechanisms

One of the most important properties of polyphenols is the antioxidant activity: antioxidant molecules interact with free radicals and oxygen species and are able to scavenge them, inactivating

them. Free radicals are any species of molecules containing unpaired electrons; for this reason, they are very reactive and could oxidise other molecules, often denaturing or anyway damaging them. For human beings, they are significantly dangerous because human body is a particularly aggressive environment and oxidation damages cells and tissues: peroxidation of lipids and cellular membranes, mutations, damages to proteins and nucleic acids. To limit these negative effects, antioxidants are fundamental in our diet. Oxidant species are produced by mitochondria because of the cell metabolism, but we can introduce them in our organism also with alimentation. Immune system usually inhibits itself the pro-oxidant action of these products, but assuming antioxidant factors, such as polyphenols, through food can be fundamental[49,50].
There are three different mechanisms through which polyphenols can act as antioxidants, as illustrated in Figure 1.5.

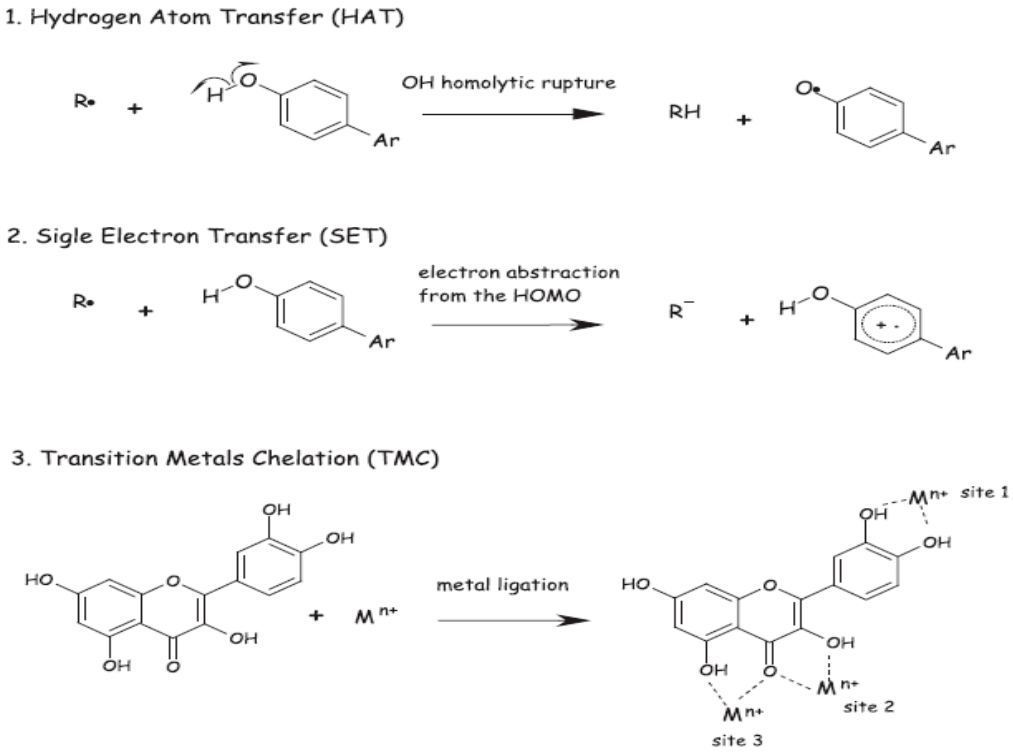

Figure 1.5 The three polyphenols anti-oxidation mechanisms[51]

The first mechanism, called Hydrogen Atom Transfer (HAT), the radical R° is inactivated by the antioxidant ArOH – the polyphenol characterized by an aromatic structure and an exposed hydroxyl group, which reacts with R°, transferring a hydrogen atom to it, through a homolytic rupture of the OH bond. The overall reaction is here reported:

$$ArOH + R^{\circ} \rightarrow ArO^{\circ} + RH$$

The products of this reaction are the inactivated radical (RH) and the oxidized radical (ArO°). ArO°, even it is a radical species, is nevertheless less reactive because previously stabilized; in fact, the odd electron originated by R° inactivating reactions can be spread over the entire molecule: this situation stabilizes this radical, letting it be less reactive than the other radical R°.

The second mechanism, called Single Electron Transfer (SET), is characterized by the donation of a single electron to the radical R°, following this reaction:

$$ArOH + R^{\circ} \rightarrow ArOH^{+\circ} + R^{-}$$

R^- is energetically stable, even if electrically charged and so $ArOH^{+\circ}$, that, even if a radical, similarly to the precedent case, is not particularly reactive, so unable to damage living tissues.
The third and last antioxidant mechanism is Transition Metal Chelation (TMC): transition metal ions can be chelated – as it will be described; it means that, through an interaction with polyphenols and ions themselves, the biomolecules are capable to form stable complexed compounds and inactivate the reactive species[52]. This mechanism is described in the next paragraph.

1.3.2 Iron chelation

Transition metals, such as iron *in primis*, can often cause generation of free radicals in living organisms. In fact, iron exists in two different forms: ferric ion, Fe^{3+}, and ferrous ion, Fe^{2+}. The first one is the most stable and we can consider it as biologically inactive. Otherwise, it is possible that in certain conditions – especially pH-dependent, ferric ion is reduced to ferrous ion: this phenomenon, more relevant at extreme environment pH values, produces hydroxyl radicals or superoxide anions, capable to negatively interact with the organism, peroxiding lipids, damaging DNA and conducting to mutagenic effects. Chelating agents inactivate or, at least, inhibit metal ions bonding with them, limiting the just described phenomenon. Polyphenols are important chelating agents, capable to contrast hydroxyl radicals release at physiological pH. This property is due to the presence of ortho-dihydroxy polyphenols, containing catechol or galloyl group. At pH=7.4 they can form complexes with ferrous ion, inactivating it and not permitting to damage living tissues[53,54]. It is interesting to evaluate the pH-dependent efficiency of chelation: during the electron transfer reaction, the ratio of iron and polyphenol-based ligand is 1:1; then, when pH increases, the ratio becomes 2:1. It is due to the increasing of the overall rate of polyphenols consumption caused by the sensitivity to alkaline pHs. On the other side, a study demonstrated how catechin can chelate iron in human stomach, in a condition of very low pH (in a range from 1 to 3), thanks to the numerous sites with which iron is capable to be bound[55,56]. It was observed that, at low pH conditions, 26 types of flavonoids, especially flavonols and flavanols, can cause iron reduction, with a following potentiation of hydroxyl radical production. Only two flavonoids – 7-hydroxyflavone and hesperetin – exercise a dose-dependent influence on inhibition of hydroxyl radical production. Then, it has noted that at high concentrations the antioxidant action loses effect, becoming more and more inefficient and, with concentration increasing, these molecules can have a pro-oxidant activity, that can be dangerous if not controlled. The critical concentration is defined through the flavonoids to iron concentration ratio: pro-oxidant activity starts when the ratio is higher than 1:1[57].

Another significant aspect of iron chelation is that some polyphenols belonging to the phenolic acids class can have a synergic behaviour. While they chelate Fe^{2+} ions, they facilitate the reduction of Fe^{3+} ions to obtain other ferrous ions to chelate. Furthermore, the carboxylate group and catechol substitution instead of galloyl moiety facilitate the ferrous ion oxidation – and so its inactivation, with a higher efficiency. Nevertheless, the behaviour of polyphenols is not the same for each type of molecule appertaining to this class: caffeic acid and protocatechuic acid are capable to accelerate Fe^{2+} autoxidation with a particular efficacy, because they act at very low concentrations, almost

lower than 1% of the initial amount of ferrous ion; villanic, 3- and 4-hydroxybenzoic acids instead inhibit ferrous ion autoxidation; monophenolic acids are not capable to reduce ferric ions. However, it is possible to assert that generally polyphenols, especially phenolic acids, are characterized by a ferroxidase-like activity that increases the efficiency of the complex phenomenon of iron chelation[58].

1.3.3 Anti-oxidation structure-dependence

As said before, polyphenols have antioxidant effects, so they can interact with free radicals and are able to scavenge them. From in vitro tests conducted by Yilmaz and Toledo in 2004, we know they neutralize radicals of hydroxyl, peroxyl, superoxide, nitric oxide and DPPH – for the last cited molecule, this property is utilised for specific analysis that will be described successively. The polyphenols that have this behaviour are especially phenolic acids and flavonoids, particularly flavanols and anthocyanidins: they are present in grape, especially in its skin, seed and pulp[59].

Flavonoids are one of the most important polyphenols for antioxidant properties: they are anti-inflammatory, anti-allergic, anti-thrombotic, anti-ischemic and anti-carcinogenic. They can interact with enzymes, deactivating them and inhibiting their activity[60]. For example, they are capable to inhibit nitric oxide synthase, a specific enzyme that produced nitric oxide: it is an extremely dangerous compound because it is itself a radical, but it generates peroxynitrite species, that are other radicals[61]. Flavonoids constitute also an important help against metallosis, especially they protect human body from iron-induced free radical reactions. Otherwise, the situation is more critical. It is important to pay attention to their structure before using a determinate flavonoid molecule for medicine applications: antioxidant activity is structure-dependent and a different chemical bond can transform the molecule from antioxidant to pro-oxidant. Flavones, isoflavones and flavonones are antioxidant, but in presence of the ion Cu^{2+} they have a pro-oxidant activity. In the following figure (Figure 1.6), where F refers to a general flavonoid, the series of Cu^{2+}-induced reactions are reported: it is possible to see the formation of a free radical at the fifth step and the damage of the substrate at the last step.

$$2Cu^{2+} + F\text{-}OH \rightarrow 2Cu^{+} + F{=}O + H^{+}$$

$$Cu^{2+} + O_2 \rightarrow CuO_2^{+}$$

$$CuO_2^{+} + F\text{-}OH \rightarrow Cu^{+} + F{=}O + HO^{-}$$

$$CuO_2^{+} + Cu^{+} + 2H^{+} \rightarrow 2Cu^{2+} + H_2O_2$$

$$\text{target-}Cu^{+}\text{complex} + H_2O_2 \rightarrow \text{target-}Cu^{2+}\text{-}HO^{\bullet} + OH^{-}$$

$$\text{target-}Cu^{2+}\text{-}HO^{\bullet} \rightarrow \text{damaged target} + Cu^{2+}$$

Figure 1.6 Polyphenols Cu^{2+} ions-induced pro-oxidant activity[62]

The pro-oxidant activity is initiated by the ion Cu^{2+} itself with a first reaction of reduction, which successively propagates through the flavonoid structure and forms a reactive complex. Aggressive molecules are produced in the last steps: hydrogen peroxide (H_2O_2), formed in the fourth step,

significantly contribute to the damaging activity of the final free radical complex, obtained in the fifth reaction[63].
Similarly, the same flavonoid molecule can have different behaviours on the basis of the number of hydroxyl substitutions: a single hydroxyl substitution has no effects on this aspect, but it is known that higher the number of –OH groups, more important antioxidant effects. Instead the O-methylation of –OH groups deactivate both antioxidant and pro-oxidant activities[64].
Another important characteristic in the flavonoids structure is unsaturation in the C ring that allows electron delocalization across the molecule for stabilization of the aryloxyl radical: it confers a more evident antioxidant property[65].
There is another molecule conferring antioxidant properties when applied: it is gallic acid (GA), also known as 3,4,5-trihydroxybenzoic acid. It has four different conformations, as demonstrated in the following figure (Figure 1.7).

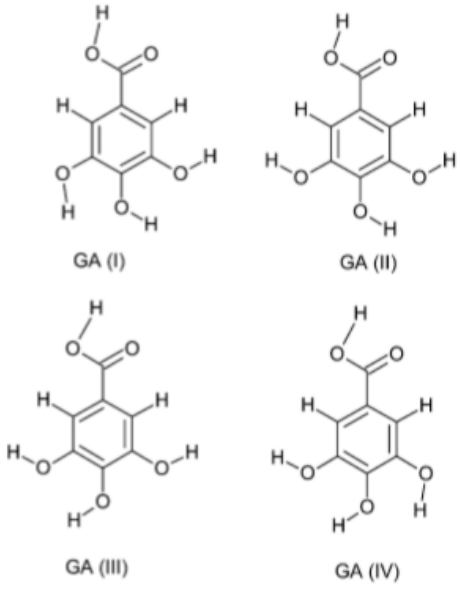

Figure 1.7 Gallic acid: four conformations[66]

It is the simplest polyphenol, appertaining to the phenolic acids class. It is basilar in protection against oxidative cell damage, especially caused by radicals, such as hydroxyl, superoxide and peroxyl groups, and non-radical oxidant molecules, such as hydrogen peroxide and hypochlorous acid. So, gallic acid can scavenge free radicals, as numerous polyphenols, but its characterizing property is the capability to induce apoptosis in cancer cells through a signalling pathway. GA in fact, similarly to flavonoids, can behave also as a pro-oxidant molecule, in particular situations, such as if in contact with diseased cells, that are characterized by an altered physiology. Other beneficial demonstrated effects are:

- anti-inflammatory effect: GA can inhibit pro-oxidant enzymes activity, such as myeloperoxidase, that produces tyrosyl radicals, and can also scavenge free radicals, with a synergic action;
- antimicrobial effect: GA has an important activity against different types of bacteria, such as *F. fusiformis, Fusarium semitectum* and *Helicobacter pylori* and fungi, protecting plants *in primis*, but also human organism, if introduced with food or medicines;

- antiviral effect: gallic acid plays an important role in inhibition of human immunodeficiency virus type 1 – better known as HIV-1, of different types of herpes and of against RNA viruses; the strategy applied by GA is the inhibition of virus replication;
- anti-allergic effect: GA can inhibit the production of histamine, a molecule released from human body that presents an immediate hypersensitivity;
- neuroprotective effect: neurodegenerative diseases, such as Parkinson's and Alzheimer's ones, are due to an important dopamine oxidation that conduces to dopamine denaturation; GA is fundamental in neuroprotection thanks to its antioxidant capacity, but also to hydrophobicity; as demonstrated from *in vivo* and animal experiments, hydrophobicity is essential because it let GA be transported to the reaction site with more efficacy;
- nephroprotective and hepatoprotective effect: in this case, gallic acid is capable to restore the activity of endogenous antioxidants, while inhibits sodium fluoride, lindane and ferric nitriloacetic acid, particularly damaging substances for human organism.

So, gallic acid is used in phytochemical and pharmaceutical sector and it is studied for its beneficial effects on tumours[67].

1.4 Polyphenols in human diet: diseases prevention

In the last years, thanks to increasing of wellness, the quality and length of life, population is always paying more and more attention to the quality of lifestyle. The most part of people is very sensitive to the quality of food they eat every day and to prevention of diseases. Science contributes studying various molecules which could improve people's health, characterising natural ones and creating synthetic ones, to utilise in food industry and pharmaceutical sector.

Different synthetic molecules with antioxidant effect have being created, but often they are carcinogenic. So, research is focusing on finding antioxidant molecules in nature to apply in human health, to replace synthetic ones and so eliminate their collateral effects[68].

For their fundamental and beneficial properties, polyphenols have to be present in our daily food in a quite high quantity, around 1 g per day. Their assumption is very important for human health because these molecules are more and more concentrated in food – about 10-100 times more concentrated, than other antioxidants present in our diet, such as vitamin C, vitamin E and carotenoids. We can assume them eating various types of fruit and vegetables and drinking fruit juices, tea, coffee, but also red wine; other sources of polyphenols are chocolate, cereals and dry legumes, very recommended food. Different scientific experiments, both in vitro and with animals, have been done to verify these properties: even if the quantity of polyphenols given to animals was higher than the quantity we usually assume every day, it is proved that polyphenols have basilar effects on prevention of diseases correlated to stress oxidation. Another important aspect of the behaviour of these molecules is their capacity to improve the status of different oxidative stress biomarkers in human body[69]. They can be found in blood and urine, from which they are extract and analysed[70]. The meaningfulness of these biomarkers is already unsure and using them as predictors of the risk to have a certain disease is perhaps not scientifically significant; different studies are trying to prove the meaningfulness of biomarkers for the most diffused and dangerous diseases in our society: cardiovascular and neurodegenerative diseases and cancers. For cardiovascular diseases the phenomenon has been already proved: polyphenols can improve human health status, if assumed with food, and the presence of biomarkers correlated to cardiovascular risk is significant[71].

Polyphenols assumed through food are generally in glycosylated forms and present sugar residues conjugated to a hydroxyl group or an aromatic ring. For this reason, the efficiency of absorption during the digestion process is not particularly high. In fact, only aglycones can be absorbed in the stomach, while the most part of dietary polyphenols is metabolised in small intestine and colon though a diffusive process. So, aglycones are the best absorbed polyphenols, especially the aglycones of some isoflavones; anyway, also gallic acid, catechins, epicatechins, flavanones and quercetin glucosides are quite well-absorbed, while anthocyanins, proanthocyanidins and galloylated tea catechins not. It is possible in fact to find a high concentration of the last cited molecules in urine 24 hours after polyphenols assumption[72,73]. Also gut microbiota are involved in the process of absorption: different species and genera of microbiota can induce specific reactions on polyphenols to facilitate their metabolism carried by human body. There are families of microbiota having specific genes capable to code for precise enzymes that induce biotransformations of polyphenols and consequently their absorption. The individual richness of these types of polyphenols influences the absorption efficiency[74].
Bioavailability of dietary polyphenols are so due to different factors: the molecular structure of the single polyphenol, the intestinal environment, the presence of other substances or other molecules such as enzymes, the typology of microbiota involved in digestion. Anyway, because the mechanism of absorption in intestine is generally passive and follows a diffusive law, it is possible that in not healthy people intestinal absorption is not sufficient. Nanoincapsulation – as it will be descripted in the next paragraphs – has been thought as a new strategy to increase its efficiency in therapeutic field, to let polyphenols release more efficient and permit the eventual reaching of a therapy-targeted tissue[75].

1.4.1 Polyphenols and cancer

Cancer is one of the most fatal diseases in the last years, especially in occidental world, because of the longevity of population and economical and social conditions characterizing this epoch. On the basis of a study conducted in 2018 by Iarc – International Agency for Cancer Research, 18.1 million were the new diagnosis all over the world in 2018 and 9.6 million the deceases in the same year. The most fatal tumours were, in order of severity, lung, colon, stomach, liver and breast tumour. In this situation, prevention is fundamental and different studies have been started to try to take front to these important diseases[76].
A research conducted in 2019 demonstrates that colorectal cancer (CRC) can be prevented by polyphenols, especially flavonoids, thanks to their antioxidant and anti-inflammatory properties. They in fact can exercise these properties *in situ*, during their degradation by the colonic microflora, facilitated by the unstable nature of their metabolites. As enounced in the precedent paragraphs, flavonoids are more stable at low pH, while in alkaline conditions – such as in the intestine, they degrade more easily. Moreover, their life in the intestine is quite long thanks to the low capacity to be absorbed: this fact increases the efficacy of the action of these molecules. The most active flavonoids metabolite that was investigated is 2,4,6-trihydroxybenzoic acid (2,4,6-THBA), capable to inhibit cancer cell proliferation. Through *in vitro* and *in silico* tests, it was demonstrated that 2,4,6-THBA, dose-dependently, can inhibit Cyclin Dependent Kinase (CDK) and different amino acids are involved in this reactions. DNA expression of a monocarboxylic acid transporter (SLC5A8) and the presence of inhibitory proteins are fundamental in inhibiting cancer cells proliferation: the metabolite in fact inhibits the proliferation of the cells expressing SLC5A8 gene. So, through a specific molecular pathway, 2,4,6-THBA participates to prevention of colorectal cancer[77].

Concerning breast cancer, instead, polyphenols play an important role as antioxidants, but they interact also with other pathways, especially with receptor signalling. In particular, they can act as anti-oxidant themselves or increase anti-oxidant genes expression, they can block pro-inflammatory cytokines, kinases and transcription cancer factors and, similarly to the colorectal case, they inhibit cancer cells proliferation, especially contrasting chronic inflammation. Finally, polyphenols interact with estrogen receptor and tyrosine kinase receptor, altering their pathways and so induce cancer cells apoptosis. It has been demonstrated that a rich-polyphenols diet can contrast the breast cancer formation, but it also can be an optimal treatment to associate to the pharmaceutical therapy, to increase its efficacy[78].

Another severe type of cancer is skin cancer. It is not as fatal as the before cited ones, but it has an important incidence on population because it is due to UV radiation exposition. UV, divided into UV-A (320-400 nm) and UV-B (290-320 nm) radiation, can be dangerous to human skin: in particular UV-B has a minor spectrum – a minor range of wavelengths, is more energetic and, if absorbed by skin, can alter DNA and so cause chronic inflammation, deregulation of cellular signaling pathways and photocarcinogenesis, through an important generation of reactive oxygen species. There are two forms of skin cancer: nonmelanoma skin cancer and melanoma. As demonstrated by a study conducted in 2018, both the tumours can be prevented: tea extracted polyphenols are one of the most influencing type of phytochemicals capable to inhibit skin cancer, appreciated for their low toxicity, low cost and efficiency in prevention. These polyphenols are extracted from *Camellia sinensis*, a plant appertaining to the *Theaceae* family. They can repair eventual DNA damages and contrast tumours formation through their antioxidant properties, their capacity to interact with signalling pathways and to induce cancer cells apoptosis. Different modality of release of tea polyphenols are being studied to optimize the beneficial effects[79].

One of the most promising frontiers in cancer therapy is nanotechnology. It is the study of particles characterized by a diameter equal or minor than 100 nm, called for this reason nanoparticles. They have a high area-to-volume ratio: thanks to this property, they have a significant surface activity that can change physical and biological properties at the nanoscale. It is possible to produce different types of nanoparticles: liposomes, micelles, microspheres, metal- and polymer-based nanoparticles; they are often treated to bond or adsorb molecules – such as drugs or, in this case, polyphenols, and release them in the desired way in the desired sites. One of the most used materials to product nanoparticles is poly(lactic-co-glycolic acid) (PLGA), characterized by a high biocompatibility and capable to resist in human organism thanks to its relative hydrophobicity, but also biodegradable, with non-toxic degradation products. Molecules delivery via nanoformulations – as it will be more diffusively described in Chapter 3 – has the advantage to be more precise reaching the diseased tissue, limiting the quantity of drug in non-target sites and so ideally eliminate collateral effects, also thanks to the selectivity of this technology. So, after the therapy, the diseased tissue returns healthy, the other organs – in a first approximation, are not targeted by the particles and the degradation products are eliminated by liver and kidneys, limiting the invasiveness of the treatment[80,81].

1.4.2 Polyphenols and cardiovascular diseases

Cardiovascular diseases are the first cause of death all over the world. It is age-dependent and it is caused by an unhealthy lifestyle, with a higher incidence on patients genetically predisposed, especially men. There are different risk factors contributing to the insurgence of these pathologies, first of all hypertension. Hypertension is an age-dependent risk, often caused also by unhealthy diet and genetic factors, that can be the main cause of different cardiovascular diseases: atrial fibrillation

(AF) is the most common type of cardiac electrical rhythm disturbance, whose incidence can be increased by the presence of hypertension, valvular heart disease, coronary artery disease and diabetes. This problem can be mitigated by dietary practices, assuming fruit and vegetables containing numerous phytochemicals that interfere with different pathways conducting to this disturbance, inhibiting the development of AF. The most relevant polyphenols contrasting cardiovascular diseases are extracted from grape. Characterised by a significant antioxidant, anti-inflammatory, anti-ageing and anti-microbial activities, grape polyphenols have an important role in cardioprotection, improving vascular health and reducing the risk of hypertension[82]. Polyphenols inhibit hypertension phenomena, especially anthocyanins from wine and grape skin, that contrast this situation inducing vasorelaxation. But they have also an important role in reducing hyperlipemia and oxidative stress, capable to conduct the patient to an atherosclerotic state. In fact, a regular consumption of red grape juice with high concentration of polyphenols can decrease the concentration of cholesterol in blood and increasing antioxidant capacity of plasma.Then, polyphenols can reduce platelet adhesion and aggregation, limiting stenotic situations, and contrast the formation of superoxide anions, dangerous to cardiac cells[83].

Another important cause of cardiovascular diseases is obesity. Thanks to the capability to interact with adipocytes, polyphenols are also used in obesity treatments. Obesity is a severe problem in our society principally caused by unhealthy diet and physical inactivity, that can constitute a social problem, but, first of all, can conduct to other worse diseases, such as cardiac alterations and diabetes mellitus. Polyphenols are able to contrast hyperglycemia and hyperinsulinemica in diabetics, controlling the level of these two parameters. Resveratrol, in particular, can inhibit adipocytes glucose utilisation and, in this way, can be fundamental to find efficient and non-invasive therapies for patients affected by diabetes and obesity. This molecule, acting as an antioxidant, interact with α-glucosidase, inhibiting its activity, and pancreatic lipase, increasing its effect. Moreover, resveratrol can counteract insulin and insulin-like lipogenic agents effects, inhibiting glucose transport in mature adipocytes and the incorporation of glucose itself in those cells[84].

1.4.3 Polyphenols and nervous system pathologies

Thanks to their antioxidant properties, polyphenols have an important role in preventing numerous pathologies related with brain and nervous system, such as stroke, brain dysfunction and neurodegenerative diseases[85].

A study conducted in 2005 on human SH-SY5Y – a cell line used as a neuronal model, demonstrated how polyphenols, especially gallic acid and its derivates, can scavenge free radicals and inhibit cellular apoptosis due to auto-oxidation. This activity is caused both by the antioxidant properties and the hydrophobicity characterising polyphenols. Gallic acid, one of the simplest polyphenols can in fact cross the cellular membrane and easily introduce inside the cell, where it can exercise its antioxidant properties[86].

Polyphenols are fundamental in contrasting cerebrovascular pathologies. Their epigenetic-protective effects were tested *in vitro* in 2018, simulating brain ischaemia through a cell-based model: primary cultures of cortical neurons of mice were treated with polyphenols and successively deprived of oxygen. It was observed that polyphenols interact with enzymes pathways, reducing stress condition in the treated cells. It is important to evidence that the utilised concentration of polyphenols in these tests is compatible with the levels reached in human brain after an oral dose, suggesting that *in vivo* the behaviour of these molecules can be analogue[87].

Stroke is a very fatal disease related to oxidative stress that yearly causes about the 30% of the global deaths. Excluding the non-modifiable risk factors, such as age, gender, ethnicity and family history, the most common causes of strokes are a sedentary lifestyle, an unhealthy diet and consummation of alcohol and tobacco, that produce a high oxidative stress and cell damage. Specifically, stroke is due to the interruption of the blood flow in a zone of the brain and the consequent absence of oxygen, basilar for brain cells, that immediately conduces to the death of the interested neurons; on the basis of the time of oxygen deprivation and the extension of the brain zone interested by ischaemia, the damage can be minor or severe and often it can conduct to death.
As demonstrated by the study illustrated before, polyphenols have an important role in stroke prevention, if assumed in daily alimentation. They in fact contrast the inflammatory cytokines, reducing them and letting them being less reactive, so less damaging for brain; exercising their antioxidant properties, then, these molecules inactivate free radicals and decrease peroxidation processes, also increasing the activity of antioxidant enzymes. Finally, polyphenols contrast the apoptotic process, interacting with cellular and molecular pathways (Figure 1.8).

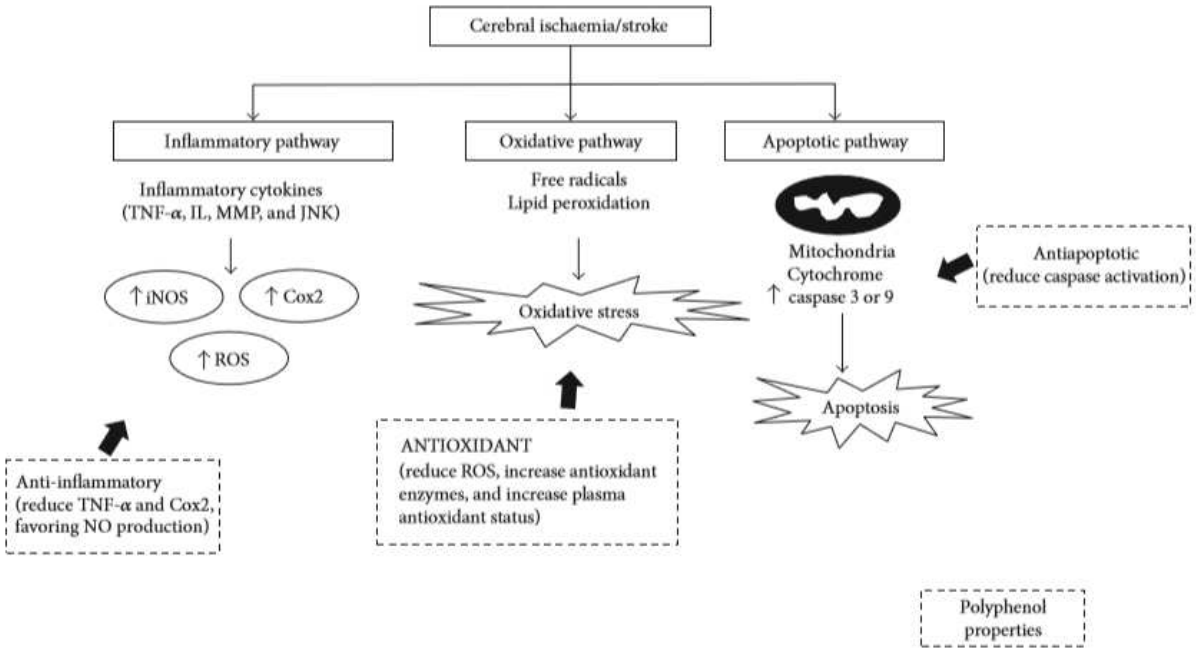

Figure 1.8 Signalling pathways and polyphenols interactions in stroke prevention[88]

The main polyphenols able to contrast stroke are quercitin – a flavonoid extracted from green tea that exercises an anti-hypertensive effect, resveratrol – capable to decrease apoptosis and mitochondrial lipid peroxidation, antocyanins – extracted from blueberries, with anti-atherogenic and anti-inflammatory properties, and hydroxytyrosol and oleuropein – from olive oil, contrasting atherogenesis and platelet hyperactivity[89].
Polyphenols are also important in contrasting and preventing neurodegenerative pathologies, such as Parkinson's and Alzheimer's. Oxidative stress is the main cause of these pathologies and often it is age-related. In particular, Alzheimer disease is caused by the role played by amyloid-β peptide, forming senile plaques, that in a casual agglomerate of proteins that is toxic to neurons because causing apoptosis and mitochondrial dysfunction. The presence of oxidation products such as hydrogen peroxide and reactive oxygen species characterises this pathology and conducts to cellular death. Curcumin, resveratrol, rosmarinic acid and epigallocatechin are the principal polyphenols

capable to interact with the processes conducting to neurodegeneration. They scavenge free radicals and increase the activity of endogenous cellular defences pathways, modulating signal transduction cascades and gene expression[90].

1.4.4 Polyphenols and their effects on bone

Two pathologies of the bone tissue that are not fatal, but particularly invalidating are osteoporosis and arthritis. The percentage of patients with these diseases is increasing in the last years because of their age-relation. Resveratrol is the main polyphenol utilised to contrast these diseases because of its anti-ageing properties. Osteoporosis is due to bone resorption. This pathology in fact is caused by the alteration of the correct equilibrium between osteoblasts and osteoclasts: osteoclasts have a more intense activity and osteoblasts cannot repair the just created damage, conducting to bone resorption. Osteoporosis is particularly present in women, incremented by the declining estrogen during menopause. Resveratrol has an estrogenic activity that, with its anti-inflammatory, antioxidant and proliferative properties, can positively influence bone metabolism[91]. In fact this molecule influences the differentiation of osteoclasts progenitors – RAW 264.7 cells, causing also apoptosis in certain cases, through inhibition of reactive oxygen species production. RANKL is a specific ligand capable to induce osteoclasts differentiation when bond to its receptor RANK, present on the surface of osteoclasts progenitors; resveratrol interacts with RANKL, inhibiting its activity and so the maturation of RAW 264.7 cells[92]. Moreover, resveratrol can influence the gene expression of osteogenic markers: the presence of resveratrol in the bone tissue can induce the mRNA production of morphogenetic proteins, osteopontin, bone sialoprotein, osteoprotegrin and RANKL, that are all factors determining bone repair activity[93].

Also another polyphenol was studied for its beneficial effects on bone repair, epigallocatechin-3-gallate (EGCG), that presented an important influence on osteogenic differentiation. It was noted that this effect is dose-dependent: in fact, at low concentration ($\leq$ 10 μM) EGCG can stimulate bone to self repair influencing progenitor cells to differentiate in osteoblasts, while, at higher concentrations, this effect decreases until it is not relevant[94].

The other important bone disease is arthritis. There are different types of arthritis, such as acute rheumatism and gout, but one of the most diffuse is rheumatoid arthritis (RA), a quite diffused age-related disease that, analogously to osteoporosis, involves aged people, especially women. It is characterised by a progressive destruction of joints and involves both bone and cartilages. This pathology is more frequent in patients that are genetically susceptible to abnormal immune responses and have been exposed to specific environmental factors, so it was hypothesised that arthritis can be influenced by oxidative processes[95]. Polyphenols, thanks to their important antioxidant, anti-inflammatory and immunomodulant properties, are thought to have an important role in contrasting the infiltration of lymphocytes and the severe synovitis that characterise rheumatoid arthritis. These molecules are able to interfere with the specific pathways that conduct to this pathology, decreasing reactive oxygen species and free radicals levels, the production of cytokines and RA biomarkers and interfering with enzymes activity. The most important polyphenols in this contest are curcumin, EGCG and hydroxytyrosol[96]. Anyway, also polyphenols extracted from cranberries and from grape have an important effect. The regular and long-term consumption of these fruits can significantly decrease the inflammatory processes due to the disease: antibodies are present in minor quantities and the symptoms are less invalidating, while the secretion of inflammatory cytokines and the response of monocytes and macrophages are inhibited[97,98].

1.4.5 Polyphenols in inflammation and infections treatment

A relatively diffused problem is an altered inflammatory response that often causes chronic immune-mediated inflammatory diseases (IMID), such as psoriasis and systemic lupus erythematosus. These diseases affect 5-8% of occidental population and are caused by a synergic action of different factors: genetic, epigenetic and environmental. Also obesity is one of the main causes of chronic inflammation: adipose tissue expansion generate an inflammation state in which adipokines are producted; these molecules participate to alter immunitary system. The polyphenols contained in extra-virgin olive oil have an important role in reducing inflammation risks, thanks to their antioxidant, anti-inflammatory and immunomodulatory properties. They act at different levels, interacting both with molecular pathways and with systemic regulation. The most important molecules are hydroxytyrosol, tyrosol and oleuropein, but also flavonoids and lignans. They can decrease cytokines levels, such as inflammatory markers and tumour necrosis factor, acting both in blood and in adipose tissue[99].

Another recent study conducted in Spain has evaluated the possibility to carry polyphenols directly on chitosan to treat colon inflammation and diseases, such as Crohn's disease and ulcerative colitis. Chitosan nanoparticles were prepared and resvetrol was carried in them, varying the resvetrol-chitosan ratio and temperature to optimize the process. Successively, this first system was compared with a hydrogel created from these nanoparticles. The hydrogel was obtained through an ionic gelling procedure: chitosan nanoparticles containing resvetrol were put in a tricarballyc acid soution which let chitosan gel. It was noted that the hydrogel system has a better resvetrol release. After 48 hours, only 29-34% of the initial resvetrol quantity was released: it demonstrated that there is a more continuous release during different days. Instead, the simple nanoparticles presented a final cumulative release of 79-81%: it means that there is a burst release, while the next days the encapsulated resvetrol is released in non-significant quantities[100]. The effect of resvetrol is prolonged carrying it in the hydrogel, from which a metabolizable quantity is released, while with the nanoparticles an excessive quantity is expelled and the organism cannot absorb it all and, in this way, a significant part of the encapsulated polyphenols is lost.

A simpler method to conjugate polyphenols and chitosan to contrast infection is grafting: it is possible to graft polyphenols to a chitosan substrate to let them be exposed and, also if a covalent bond does not permit release, exercise their beneficial properties. Using a film constituted by N,O-carboxymethyl chitosan (NOCC) as a substrate, gallic acid, caffeic acid and ferulic acid can be grafted to it through a free radical mediated grafting method. Free radicals can be formed adding acetic acid to the substrate and applying an oxygen free nitrogen gas flow on it. Grafting is possible thanks to the ability of polyphenols to scavenge free radicals, in this case through the reaction between amine of chitosan and carboxyl groups of polyphenols, from which a covalent bond is formed. Differently from the other two illustrated methods, in the current one there is not a polyphenols release, but these molecules maintain anyway their properties[101].

1.5 Polyphenols reaction to sterilization

Because of the clinical applications of polyphenols, sterilization is fundamental to prevent bacteria adhesion and infections. Furthermore, sterilization is also applied in food industry to disinfect food itself and to preserve it for a longer time without altering nutrition parameters. Sterilization can modify chemical and physical properties of molecules and so it is very important to study the effects this process can have on polyphenols before its application[102].

Different studies have been conducted to verify the maintenance of the polyphenols properties and eventual variations on their behaviour: the total polyphenols content and their antioxidant activity

were evaluated in various plants before and after sterilization. Sterilization is usually effectuated through γ-irradiation, that is an efficient method to eliminate contaminations[103]. These radiations are able to destruct pathogenic micro-organisms, such as bacteria, but they also comport significant collateral effects. First of all, they can introduce dangerous free radicals that could induce oxidation phenomena in human organism and, if polyphenols are present, they can be damaged and loss efficiency or be denatured. So, different researches are conducted to optimise the sterilising procedures, varying exposure time, irradiation dose and eventual presence of organic compounds or solvents[104].

This sterilisation method has a double effect: analysing two researches conducted respectively in 2007 and 2014, confronting the quantity of polyphenols in almonds before and after γ-irradiation, a decreasing of the total polyphenols content was appreciated in the second category. Anyway, it is interesting to evidence also that the antioxidant activity of the residue molecules is amplified by γ radiations: the antioxidant power measured in irradiated almonds is higher than in non-irradiated ones. It can be an important advantage because in this way polyphenols can contrast the pro-oxidant activity triggered by sterilization[105,106]. There is also another significant factor influencing these results: the eventual storage of the food before the analysis. It is noted that non-stored almonds have the just described behaviour after irradiation; instead, in stored irradiated almonds the total phenolic content yield is the same of non-irradiated almonds, but it can also increase. This phenomenon is related to specific doses of radiations: in fact, for higher doses (10 kGy), the yield of total phenolic content is the same before and after irradiation, but flavonoids content increases[107]. Also the antioxidant activity is dose-dependent: for lower doses (≤ 2 kGy), the antioxidant activity increases, while for higher doses (from 10 to 50 kGy) the anti-radical activity is reduced, still it reaches the values assumed before irradiation[108,109]. It was finally demonstrated that 50 kGy are sufficient to completely denature polyphenols[110]. Anyway, this threshold is largely higher than the standard value of intensity of γ radiation to sterilise biomedical devices, imposed to be 25 kGy111.

Moreover, while evaluating the criticality of γ irradiation on total phenolic content and antioxidant activity, also the eventual presence of additives inserted in packaging of stored polyphenols has to be considered. While the dose-related effects are the same, the behaviour of polyphenols conserved in polythene and polypropylene packs is not, presenting different value of concentration of polyphenols – in polythene packs the concentration is higher. Anyway, eventual damages to the biomolecules triggered by the pack are solved by the significant antioxidant properties of polyphenols amplified by irradiation[112]. The higher power of antioxidant activity after irradiation and storage can be explained by the fact that γ irradiation can enhance the synthesis of total phenolic compound, thanks to the activity of an enzyme called phenylalanine ammonia-lyase (PAL). In *Citrus clementina*, PAL significantly contributes to biosynthesis of different polyphenols, hesperidin *in primis*, and the storage time is fundamental to its activity because a longer storage time lets the reaction be[113].

Various studies have also been conducted to evaluate decontamination properties of sterilized polyphenols. The importance of these properties is very high because food, before to be commercialised, has to be purified and decontaminated. In a study conducted in 2017, *Aloysia citrodora*, an aromatic plant used in alimentation industry, was contaminated with two different mycotoxins. After sterilization with γ radiations, the effects on polyphenols were the just described: without storing, the total polyphenols content is lower, but the antioxidant activity increases. Anyway, there are not significant effects on mycotoxins, the content of which decreases only of a small percentage, appertaining to a range going from 4.9 to 9.6% of the initial quantity, absolutely

not sufficient to obtain safe food. The observed effect is due to the low intensity of γ radiations, going from 1 to 10 kGy, and the partial denaturation of polyphenols[114].
Another efficient method to sterilize biological materials is autoclave. It is used to eliminate natural microbiota and pathogenic microorganisms from food without utilising antibiotics, that have severe collateral effects. Autoclave-sterilization is based on high temperature treatments: polyphenols are quite sensitive to temperature variations. Anyway, according to a study conducted on *Jatropha dioica* and *Opuntia oligacantha* in 2018, the concentration of total phenols and flavonoids increased almost double the initial quantity before sterilization. In fact some flavonoids are stable at high temperature and it can increase their solubility: this phenomenon can so cause an increase of flavonoids content. The antioxidant activity instead was always the same before and after sterilization, although the polyphenols content have increased, because some of antioxidant compounds – such as nitrogen compounds, carotenoids or ascorbic acid, were lost during the process. Finally, another important effects of the treatment is the complete elimination of the bacteria and the maintenance of antimicrobial properties of polyphenols also after autoclave-sterilization[115].

1.6 Polyphenols toxicity

Also if polyphenols have beneficial properties, they are dose-dependent. A study conducted in 2015 in Canada demonstrated that for certain doses there are cytotoxic effects, especially for EGCG extracted from green tea. For concentrations higher that 100 μM, it has negative effects on hepatocytes, with reactive oxygen species formation. This phenomenon is due to the significant decreasing of mitochondrial membrane potential – a real collapse, induced by these molecules and to their hydrophobicity[116]. In fact, at toxic doses, EGCG can induce oxidative stress *in vivo* because of the formation of two cysteine conjugates. High doses of green tea – and so an excessive consumption of polyphenols, can induce hepatotoxicity and gastrointestinal irritation; in some cases, during pregnancy, they can provoke leukemia risks for the fetus[117].
The dose-dependent cytotoxicity of polyphenols, potentially dangerous for healthy tissues, can be used to treat cancer cells, inducing in this way their apoptosis, without utilising invasive drugs or radiations[118]. A study conducted in 2018 demonstrated that the exposure of cancer cells to critical concentrations of polyphenols induces oxidative stress able to inhibit cellular adhesion, which produce a cytotoxic or, at least, cytostatic effect on the cellular culture119. Another more specific researches successively evaluated *in vitro* glioblastoma cells behaviour when treated for 6 days with different concentrations of EGCG. A significant difference was evident between cancer cells treated with low concentration (100 nM) and high concentration (500 μM) of this polyphenol. While the first cell cultures were not significantly affected and proliferated over the entire time of observation, the second ones presented numerous autophagic vacuoles and high concentration of ROS: it means that apoptosis and autophagy in glioblastoma cells were significant phenomena induced by critical concentrations of EGCG120.
Significant cytotoxicity assays have also been performed on osteosarcoma cells (U2OS), previously extracted from human bone. These cancerous cells have been cultured on bioglasses functionalised with polyphenols for 7 days. It was observed that oxygen and nitrogen reactive species (RONS) were stimulated to be produced by the contact of cancerous cells with polyphenols. In this way, permanent DNA damages were ported, causing a significant decrease of cellular viability, lower at each time step of the test. Exploiting human fetal pre-osteoblasts (hFOB) as control, it was demonstrated how the cytotoxicity exerted by polyphenols is selective: while these biomolecules are toxic toward U2SOS cultures, promoting the just cited pro-oxidant activity causing DNA

damage, on the other side they have an anti-inflammatory action toward hFOB cells, able to carry on their peculiar anti-oxidant effect[121].

1.7 Polyphenols characterization

The first measurement to characterise a polyphenolic solution is the evaluation of pH. This is a basilar evaluation, but fundamental to study and characterise a substance. Polyphenols have acidic pH – around 4, and eventual variations signify variations in composition and concentration of the solution itself.

Anyway, the main technique to analyse polyphenols is spectroscopy, that gives a more complete profile of the analysed solution. There are various types and variations of this technique, based on absorbance and transmittance of radiations of the analysed sample. One of the most utilised techniques is Folin-Ciocalteu (F&C) assay, used to determine the quantity of total phenolic compounds in a semi-quantitative way: it can be a disadvantage because it is not a real quantitative measurement, but it is very important to understand the behaviour of the analysed molecules at the first steps of the research[122]. A second type of spectroscopy is Inductively Coupled Plasma Mass Spectrometry (ICP-MS), also used to determine the concentration of polyphenols in the analysed solution. With ICP-MS it is possible to determine more precisely than with F&C assays the concentration and the elemental composition of a sample[123].

In fact, spectroscopy is more convenient than other more invasive analysing techniques that require chemical treatments of the sample, such as solubilisation or extraction. Especially ultraviolet-visible (UV-vis) spectroscopy is very used in laboratories. Polyphenols have the important property of fluorescence: this phenomenon can be profited to analyse these compounds in a simple and efficient way[124].

Another important technique utilised to separate and analyse polyphenols is High Performance Liquid Chromatography (HPCL). To apply this, it is necessary to use a reversed-phase C_{18} column, a UV-vis diode array detector and a binary solvent system, containing acidified water and a polar organic solvent. HPCL is efficient and simple to apply, but has the disadvantage to require chemical treatments of the polyphenols sample before the effective analysis[125].

In the following paragraphs the main analysing techniques applied to study polyphenols are illustrated.

1.7.1 pH

Measurement of pH is fundamental to investigate the properties of a polyphenols solution. Normally, polyphenols have a low pH, around 3-4, that gives acid characteristics to the environment in which they are. Anyway, variations of pH can determine variations on macroscopic properties, such as the colour of the solution, and on molecular properties, such as anti-oxidation[126]. In fact, at low pH, they maintain their characterizing properties: they behave as antioxidant molecules as described in the precedent paragraphs and are also capable to stimulate cells proliferation. However, the excessive presence of oxygen and transition metal ions can modify these properties: pH increases, becoming alkaline, and this phenomenon can cause auto-oxidation of polyphenols themselves. Furthermore, alkaline pH has a double negative effect: it can decrease the capability of polyphenols to scavenge free radicals and contrast oxidant species – especially H_2O_2, while stimulates the production of the same species from the plant extracts themselves[127].

So, it is basilar to know in which type of environment polyphenols will work: on the basis of the value of pH, they can have opposite behaviours that can be applied for different applications. During the first macroscopic analysis, it is possible to foresee the value of pH of a solution,

controlling its colour: acid polyphenols solutions are characterised by a lighter colour, with a well-defined red-wine nuance; alkaline polyphenols solutions are instead of darker colour, that can be defined as dark purple[128].

1.7.2 Spectroscopy

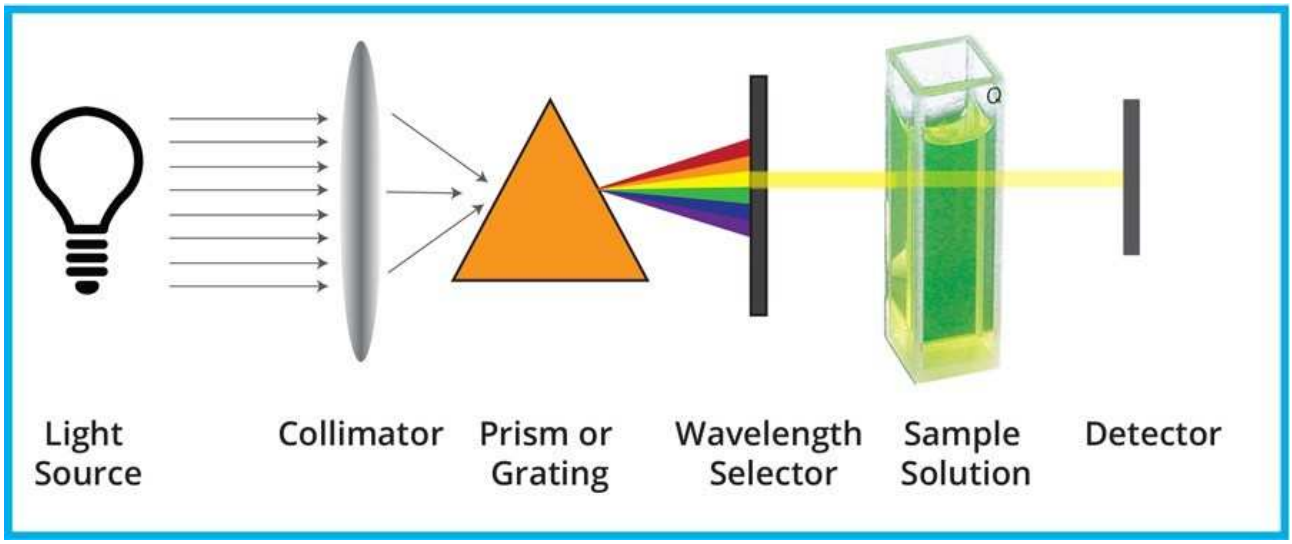

Figure 1.10 Block diagram of spectroscopy[129]

Spectroscopy is based on the analysis of a radiation that goes through the sample. Light is emitted by a light source and a determined wavelength is selected thanks to a collimator, a prism and a wavelength selector; crossing the sample, the radiation is partially absorbed: the transmitted light is studied and correlated with the physical dimension to measure (Figure 1.10). Light is an electromagnetic radiation, composed by oscillating magnetic and electric fields, that are able to transfer energy through space. Energy propagates in space as a wave, with a characteristic wavelength for each radiation. There are different forms of electromagnetic radiation, such as X-rays, visible, infra-red and radio waves[130]. Analogously, as mentioned before, various types of spectroscopy exist, but the more used one for polyphenols analysis is UV-vis spectroscopy, that is also applied in the context of other measurements, such as F&C test and DPPH test[131].
UV-vis spectroscopy is based on the visible absorption spectrum. This phenomenon is caused by the radiation that, when hits the sample, gives it energy: this energy raises an electron from its molecular orbital to another with higher energy, emitting a difference of energy ΔE[132]:

$$\Delta E = h\nu$$

where $h=6.626 \times 10^{-34}$ Js is the Planck constant and ν is the oscillating frequency of the electromagnetic field[133].
The absorbance of a specie at a determined wavelength λ is found through the Lambert-Beer relation:

$$A = \ln(I_0/I) = \varepsilon dc$$

where, as illustrated in Figure 1.11, I_0 is the intensity of the incident radiation and I is the intensity of the radiation going out from the sample; ε is the molar absorption coefficient of the specie for the wavelength λ, d is the thickness of the sample crossed by the radiation (called also optical path) and c is the molar concentration of the specie[134].

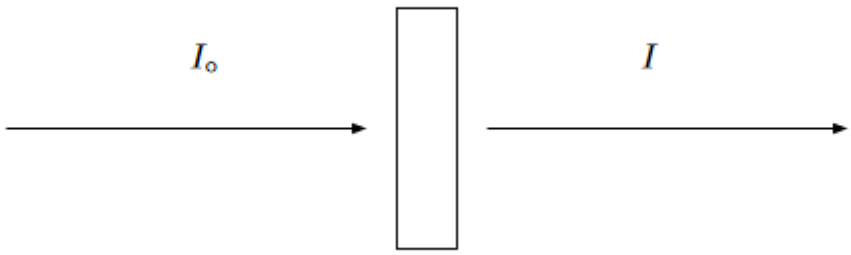

Figure 1.11 Absorbance phenomenon[135]

Spectroscopy is fundamental in polyphenols analysis, to evaluate the total content and concentration of this molecule[136]. It is necessary to correlate the absorbance measured by the instrument with the concentration of polyphenols solution through a linear relation, determined by the calibration of the instrument itself, that has this form:

$$A=ac+b$$

where A is the absorbance, c the molar concentration and a and b the two coefficient determined by the instrument during the calibration process. This relation is called calibration curve[137].
The first application of spectroscopy is Folin-Ciocalteu method. It was proposed by the two scientists in 1927 and was based on a treatment of the polyphenols solution to analyse with the homonymous reagent, that induces a different intensity of blue that is proportional to the concentration of total polyphenols present in the solution. The reaction is a redox reaction: the F&C reagent is an oxidant, while polyphenols, as described in paragraph 1.3, behave as antioxidants, inhibiting the effect of the reagent, reacting more with the increasing of the quantity of molecules. So, higher the concentration of polyphenols in the analysed solution, higher their antioxidant capacity, higher the quantity of the blue product to quantify through spectroscopy. To measure this after the reaction between the solution and the F&C reagent, the product is analysed through spectroscopy, in photometric modality, with the wavelength corresponding to the colour blue (λ=760 nm): higher the value of absorbance, higher the concentration of total polyphenols, quantified by the calibration curve. In the case of polyphenols analysis, the calibration is done using gallic acid: so, the results are expressed as Gallic Acid Equivalent (GAE). It means that the concentration of total polyphenols obtained as the result of the measure is the equivalent as if the polyphenols present in the solution were only gallic acid[138].
Another important application of spectroscopy is DPPH test. It is used to evaluate the antioxidant power of polyphenols. 1,1-diphenyl-2-picrylhydrazyl (DPPH) is a radical specie that, if inserted in a polyphenols solution, can be scavenged and deactivated by these molecules: evaluating the quantity of scavenged DPPH, it is possible to estimate the concentration of polyphenols through their antioxidant activity139. So, when DPPH is added to a polyphenols solution, it confers it a dark

colour, precisely a very dark purple; anyway, reacting with polyphenols, it is bonded to them and this phenomenon makes the solution of a lighter purple because of the de-solubilisation of the radical. Lighter the nuance of purple characterising the solution, higher the antioxidant power, corresponding to a higher concentration of polyphenols. This is measured through spectroscopy in photometric modality, irradiating the sample with λ=515 nm, corresponding to the wavelength of purple. To quantify this phenomenon, the absorbance of the sample at this wavelength is evaluated: lower the absorbance, higher the antioxidant power of the solution[140,141].
Another type of spectroscopy, as cited before, is ICP-MS. It is the most sensitive type of atomic spectroscopy, capable to find single chemical elements[142]. It is a relatively young technique because it was commercially introduced in 1983. This instrument has the advantage to combine a high-temperature ICP source with a mass spectrometer: the first converts the atoms present is the analysed sample in ions, while the second detects them[143]. In Figure 1.12 the block diagram of ICP-MS is reported.

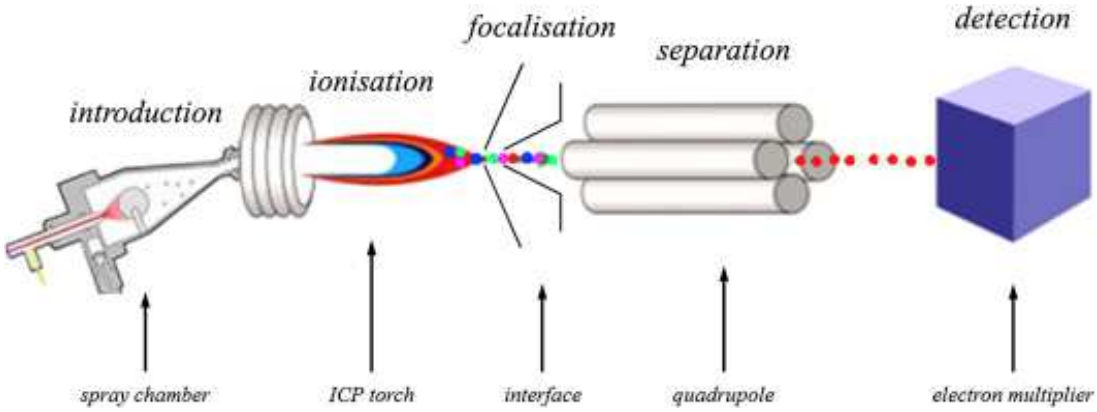

Figure 1.12 Block diagram of ICP-MS[144]

The basic scheme of the machine is the following. The first block is the spray chamber: the sample is usually introduced into the instrument as an aerosol; successively, it crosses the ICP torch that transforms the nebulised sample in gas and finally in plasma at a very low pressure. Then, the ions of the plasma are focused and separated in the quadrupole mass filter by their mass-to-charge ratio. Finally, the ions reach the detector and the signal is generated. The output is a spectrum in which a single value of the mass-to-charge ratio corresponds to a precise chemical element and the highness of the peak is proportional to the quantity of this element in the sample[145].
Applying ICP-MS technique to analyse polyphenols, it is possible to note a significant presence of nitrogen, phosphorus, potassium, sodium, magnesium, chlorine and sulphurous. The percentage of them varies in function of the type of fruit and vegetable from which they had been extracted. For example, sulphurous is more abundant in polyphenols derived from apples, while nitrogen quite absent in them; vice-versa in artichokes polyphenols, in which nitrogen is in major quantities than sulphourus[146,147].

1.7.2 HPLC

HPCL is another technique used to separate and quantify polyphenols and is particularly efficient for fruits analysis. In Figure 1.13 the structure of the chromatograph is reported.

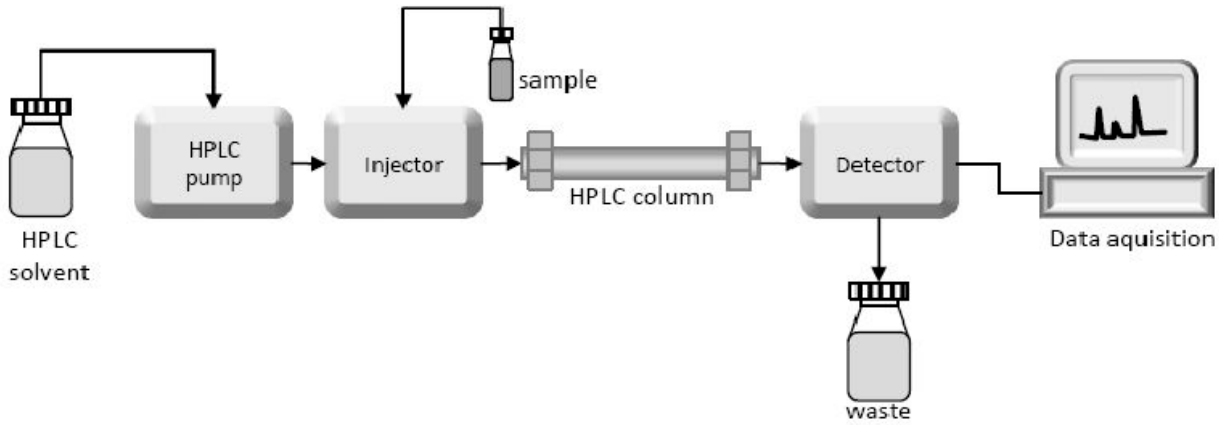

Figure 1.13 Block diagram of HPLC[148]

The elements of the instrument are a reversed-phase (RP) C_{18} column that is the analytical tool in which the molecules are separated, a detector that can be a diode array detector or a spectrometer and the HPLC solvent, composed by acidified water and a polar organic solvent. The HPLC solvent is introduced in the instrument by a pump, while the liquid sample is introduced by an injector. The two liquids are mixed and cross the column, in which the separation of the molecules to analyse happen, by their molecular mass[149]. In the column there is a stationary phase and a mobile phase, that lets the sample flow through the column itself. Sample components can differently interact with mobile phase: stronger is the interaction with the stationary phase, longer is the permanence time inside the column. So, in this case, the various polyphenols are separated and successively detected by their molecular mass[150]. This technique can be applied to analyse a juice extracted from a fruit or a vegetable containing polyphenols, or of a piece of the same fruit or vegetable. An example of the application of ICP-MS is the identification of polyphenols contained in pomegranate (*Punica granatum L.*) husk. It is possible to identify in it 50 different polyphenols, 35 tannins and 15 flavonoids, that, basing on the dimensions of their molecules and their mass-to-charge ratios, are separated to be detected[151].

The just described HPCL is the reverse-phase HPCL, in which the stationary phase is non-polar, while the sample – constituting the mobile phase – is hydrophilic: more the sample component is polar, before it goes out the column; and vice-versa. There are other different types of this technique, such as Normal Phase, Size-Exclusion and Ion-Exchange HPLC, but they are rarely used for polyphenols applications[152].

1.7.3 Zeta Potential

The charge at a solid-liquid interface as an electrochemical phenomenon was studied for the first time in the XIX century by different scientists, such as Reuss, Quincke, Helmholtz and Smoluchowski, who elaborate a theory based on the presence of a double layer of charge at the interface that generate an electro-kinetic potential. It is important to study this potential as one of

the first steps after a treatment to analyse the surface changes, that can determine electrostatic interaction, to evaluate the functionality of the surface, the interaction of dissolved compounds and the adsorption processes[153]. In Figure 1.14 the electrochemical phenomenon at a solid-liquid interface is illustrated.

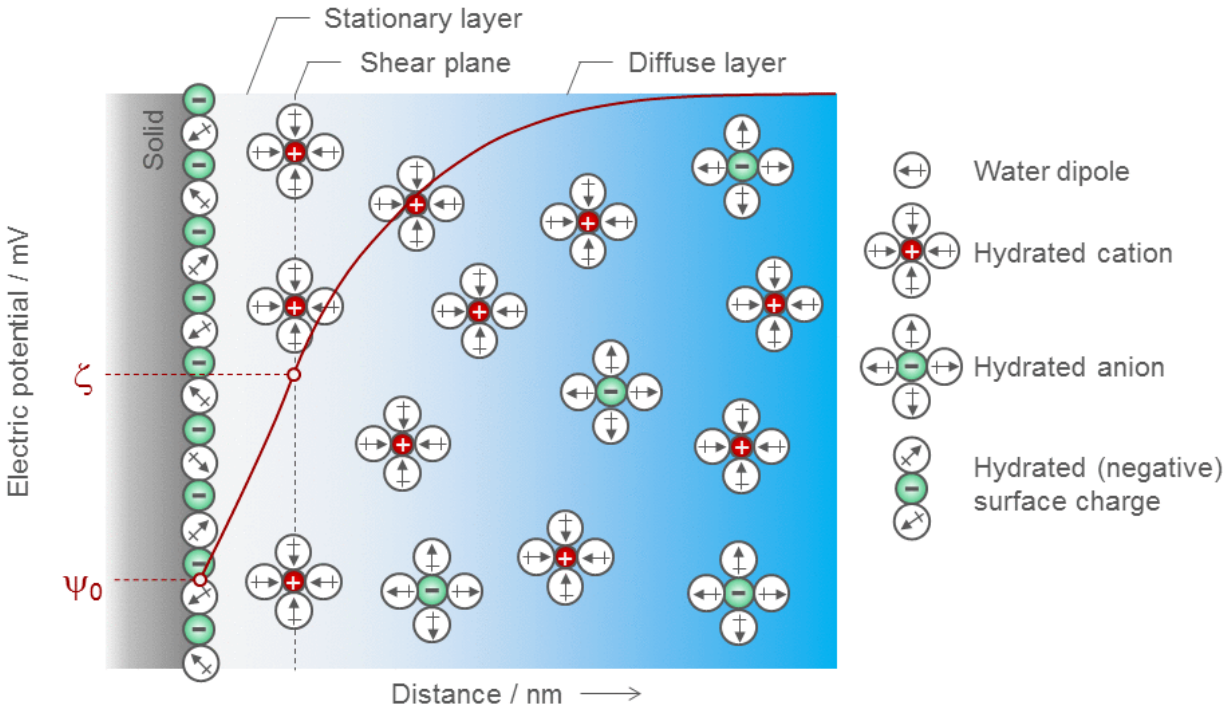

Figure 1.14 Electrochemical double layer of solid-liquid interface[154]

When a solid surface comes in contact with an aqueous medium, at the interface a charge migration happen and a surface potential (ψ_0) is generated because of weak interactions: functional groups of solid material are exposed and ions are adsorbed from the solution[155]. This charge, to minimise the energy of the system, has to be compensated: in this way, a double layer is formed, with the opposite charge that the surface has:

- a stationary layer near the solid surface, also called Stern layer;
- a diffuse layer, some nanometres more distant, but always at the interface, also called Guy-Chapman layer.

In this model, these two layers are divided by a plane, called shear or slipping plane: in this point zeta potential is measured[156]. This plane indicates the location of the slipping of the moving liquid phase relatively to the stationary liquid phase during the electrokinetic measurement: the potential here measured – called zeta (ζ) potential by Perrin and Freundlich, is an interfacial property that depends on both the surface charge and the properties of the liquid[157].

This measurement can be done using a specific instrument with four possible techniques: streaming potential, electrophoresis, electro-osmosis and sedimentation potential. The first is the most common and is implemented by a water flow that induces charges on the surface of the solid sample, as illustrated in Figure 1.15.

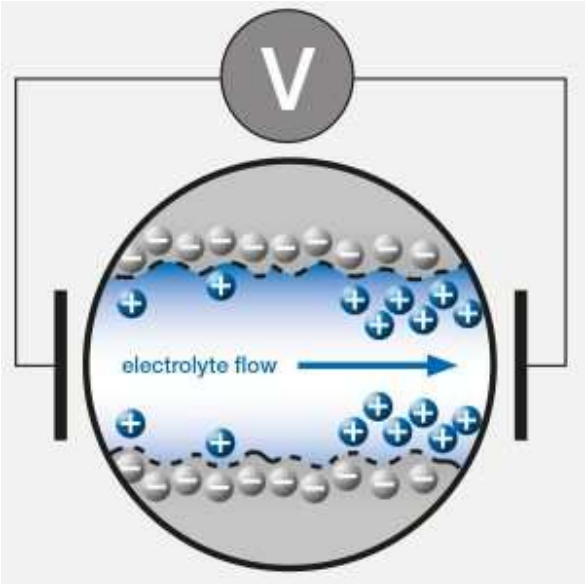

Figure 1.15 Zeta potential instrument simplified mechanism[158]

The zeta potential is measured by the instrument and calculated through the following equation:

$$\zeta = \frac{dI_{str}}{d\Delta p} \times \frac{\eta}{\varepsilon \times \varepsilon_0} \times \frac{L}{A}$$

Figure 1.16 Zeta potential equation[159]

where:

- dl/dp is the slope of streaming current versus differential pressure;
- η is the electrolyte viscosity;
- ε is the dielectric coefficient of the electrolyte;
- ε_0 is the permittivity;
- L is the length of the streaming channel;
- A is the cross-section of the streaming channel[160].

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CHAPTER 2
POLYPHENOLS POLYMERIZATION

2.1 Degree of polymerisation

Polyphenols are – as described in the precedent chapter – widely distributed in nature, especially in plants and there are different classes and various types of these molecules. Molecular masses of polyphenols have a particularly distribute range, going from 100 to 10000 Da. Low-molecular-mass polyphenols are often present as glycosides or organic acid esters, but it is possible convert them in a free aglycone form through chemical treatments as enzymatic digestion in food processing or alcoholysis and solvent extraction in laboratories. Generally, these compounds are quite hydrophobic and not highly water-soluble. High-molecular-mass polyphenols are instead very polar molecules: in fact, with increasing of degree of polymerisation, hydrophobicity decreases, letting these compounds become very hydrophilic and, consequently, water-soluble. This second category is perhaps the most important for human applications because hydrophily lets these molecules be more processable by human body and, in this way, be fundamental antioxidants capable to inhibit the activity of different enzymes thanks to non-specific binding ability[1].

Another significant aspect of degree of polymerisation is the influence it has on bioavailability and toxicity on human cells. The mean of the degree of polymerisation of polyphenols can vary in a range going from 3.0 to 13.4 and it can be related also to the content of polyphenols in solutions. Lower degrees can be obtained because of the hydrophobicity of certain polyphenols. Also oxidation can have an important role limiting polymerisation: especially high polymeric procyanidins are very sensitive to oxygen and this fact can decrease the speed of polymerisation. Further, a high polymerisation can induce a lower polyphenols content, perhaps because of the desorption of polyphenols in presence of dissolved cell wall polysaccharides during extraction process[2].

A study conducted in New Zealand in 2011 on proanthocyanidins extracted from different types of tea confirmed that in these plants there is a high distribution of molecular masses and, comparing the degree of polymerisation of different molecules appertaining to different classes of polyphenols, demonstrated that this fact – correlated to degree of polymerisation – influences the properties of the molecules[3]. An application on human health of low-molecular-mass polyphenols is prevention of obesity and diabetes. These molecules, characterised by a low degree of polymerisation, can interact with digestive enzymes, inhibiting their activity and precipitating them, behaving as biodrugs. Proanthocyanidins, containing a low number of catechin and epigallocatechin units, can exercise this kind of activity against lipases, amylases and proteases. This, thanks to their small dimensions: in fact, these small oligomers can enter inside the enzymes and bind them in the active site forming specific interactions to inhibit their activity, while other higher-molecular-mass polymers could not[4]. On the other side, high-molecular-mass polyphenols, especially procyanidins characterised by a higher degree of polymerisation, are capable to interact with different proteins simultaneously forming a significant number of cross-link. This phenomenon limits the precipitation of proteins that characterises instead the interaction between these biomolecules and polyphenols[5].

2.2 Mechanisms of polymerisation

Polyphenols can polymerise in different ways. In nature, polymerisation is a spontaneous process, through which the free energy contained in molecules is minimised. There are also different methods used to artificially synthesise polyphenols starting from phenolic monomers or small phenolic compounds; they are based on letting the molecule become unstable and, using its instability, letting it react with other unstable molecules to minimise the total energy of the system[6,7].

2.2.1 Spontaneous phenolic compounds polymerisation

It was observed that polymerisation can be spontaneous in acidic environments. For example, in red wine, characterised by a very low pH (pH < 2) and by the presence of acetaldehyde, its constituting polyphenols – the main polyphenols in red wine are (-)-epicatechin (Ec) and malvidin-3-O-glucoside(Mv3G), can react with acetaldehyde to have a polymerisation process. It is possible thanks to the low pH, that causes the formation of a positive charge carried by the red flavylium cation. As illustrated in Figure 2.1, Mv3G exists in three possible different forms. The principal, which ispresent in extremely acid environments (pH<2),is red flavylium cation ($Mv3G_F$). It could be modified through a nucleophilic attack of water at position 2 or 4 of the pyrylium nucleous to obtain a new form, the colorless hemiketal ($Mv3G_{OH}$); if, instead, deprotonation has place, quinonoid bases are produced ($Mv3G_B$)[8].

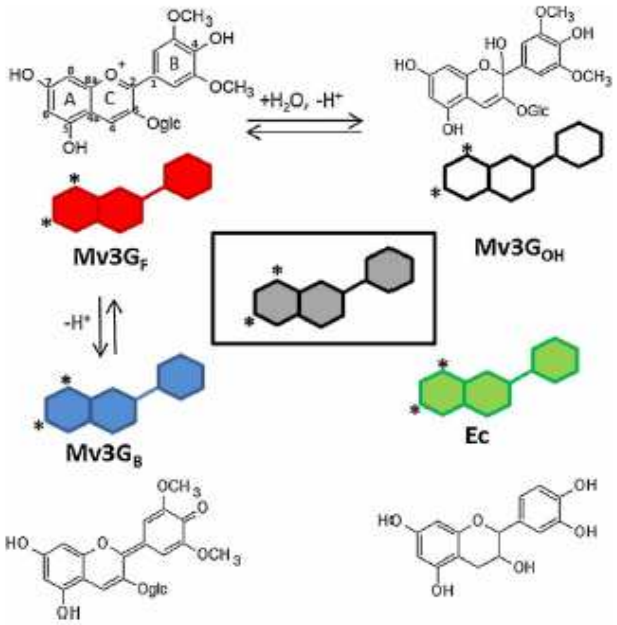

Figure 2.1 Initial transformations of polyphenols before polymerisation[9]

The cited nucleophilic attack of water to polyphenols, possible in low-pH environments,could conduct to different transformations of acetaldehyde, which terminates with its protonation. These reactions so permit another successive one, in which protonated acetaldehyde and nucleophilic carbons (C-6 and C-8) react to produce an ethyl-linked dimer. The process finishes with a

cycloaddition: flavylium cation can undergo the cycloaddition at C-4 and 5-OH with the just formed dimer[10].

Another polymerisation process is tannins condensation. Condensed tannins, also known as proanthocyanidins, can have different degrees of polymerisation, going from 2-10, to give oligomers, to values higher than 10, to give polymers. Oligomers and polymers are present in nature in two different forms: B-type and A-type proanthocyanidins. B-type ones have monomeric catechins units (flavan-3-ol monomers) linked each other through C-C bonds (mainly C4-C8 and C4-C6), while A-type ones, less common in nature, have a C2-O7 ether bond in addition to a C4-C8 bond[11,12]. In the Figure 2.2 the condensation mechanisms are reported. It is possible to see that there are two main units involved in this process: the nucleophilic catechin obtained by an isomerisation of an epicatechin and the electrophilic C-4 carbocation obtained by a cleavage process. These two units react to have a condensation through which a proanthocyanidin is formed, constituted by three monomers; in this first step a trimer is so formed, but, iterating this process, it is possible to obtain a more complex polymer[13].

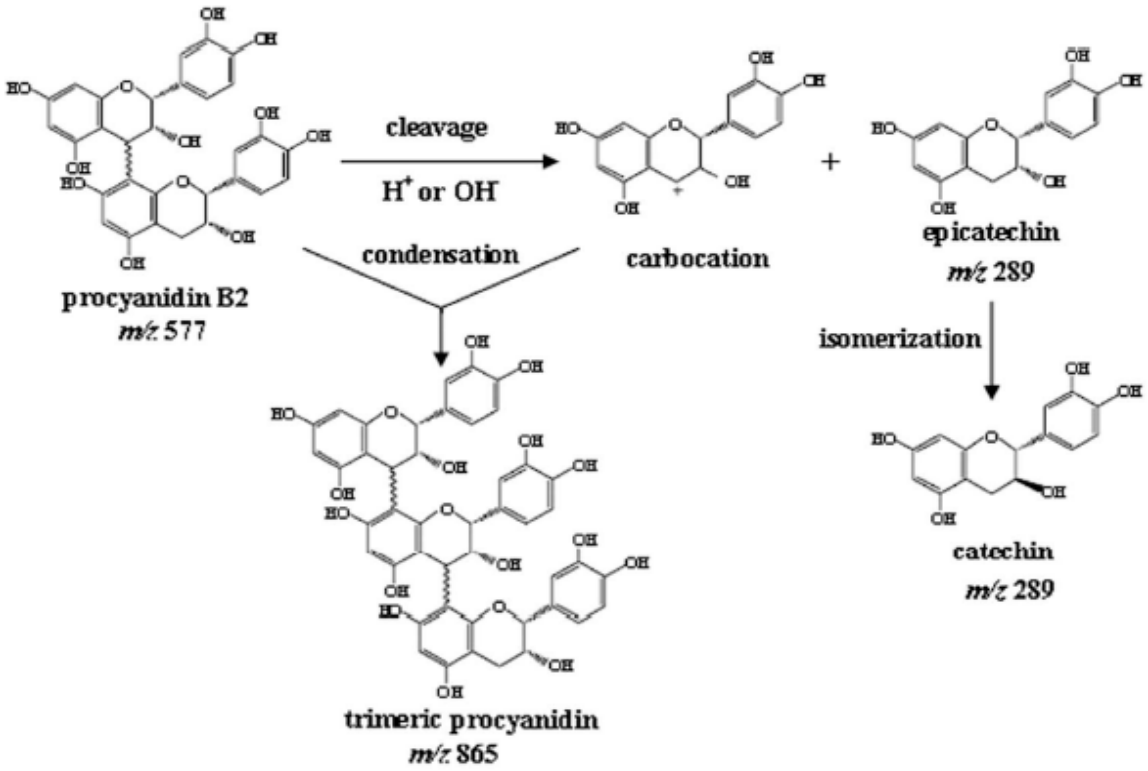

Figure 2.2 Scheme of proanthocyanidins auto-condensation mechanism[14]

These reactions have place inside the plants, precisely in cells. It is interesting to see how the biosynthesis of condensed tannins starts inside a vesicle in a cell, that successively fuses with the central vacuole. Then, this structure goes to the surface of the cell, in the endoplasmic reticulum, remaining in the cytosol; here the (-)-epicatechin is transformed into epicatechin 3'-O-glucoside by an enzyme, called glycosyltransferase[15]. This enzyme is capable to catalyse the transferring of saccharide groups from a chemical species called "glycosyl donor" to another, called "glycosyl acceptor", generally nucleophilic[16]. In this case, it transfers a saccharide group from the cellular environment to the (-)-epicatechin, to obtain epicatechin 3'-O-glucoside, a molecule that behaves as

a substrate capable to activate the transporter of a multidrug and toxic compound extrusion (MATE), a vacuolar species that has to be eliminated after the reaction. At the end, condensation of (-)-epicatechin and epicatechin 3'-O-glucoside has place, thanks to different enzymes, such as tyrosinase. *In vitro*, it is possible to substitute these enzymes with an enzyme-like gene, named TT10, capable to behave as laccase, inducing an oxidative polymerisation[17].

2.2.2 In vitro polyphenols synthesis: artificial polymerisation

The first method to polymerise polyphenols in laboratory foresees the usage of toxic substances, such as bases and organic solvents. The most used catalysts capable to induce polymerisation during extraction are NaOH, Na_2CO_3, $NaHCO_3$, Na_2SO_3 and, at the end of the process, other toxic compounds are released; the most common is formaldehyde. There are strict laws, determining the upper thresholds for the concentration of the different substances, under which security is guaranteed[18].

Anyway, the toxicity of these substances is significant and, so, other more efficient and secure techniques to obtain polyphenols were studied. In this way, the process of polymerisation can now produce more pure molecules, without dangerous impurities due to the catalysts used in polymerisation reactions, that, also after purification precesses, could remain in low percentages in the so treated polyphenols and could be assumed by people through food, with eventual collateral effects[19,20].

2.2.2.1 Photo-catalysis

To eliminate toxic chemical substances from the process of polymerisation, a new technique was thought. It is probably the simplest method to synthesise polymers starting from catechin units and is based on irradiation with UV-visible light. This process is called photo-catalysis. It is necessary to work in an alkaline or neutral aqueous environment because alkaline pH induces the solution to be unstable and so more reactive: in fact, polyphenols are stable for low pH (pH=3-4) and, for this range of pH, it is more difficult to have some reactions. This high-pH catechins solution is then irradiated with visible light, characterised by a wavelength appertaining to a range going from 400 to 800 nm. Blue light is precisely the wavelength which catechins are more sensitive at: analogously to high pH, blue light makes catechins unstable and so more reactive. Through the synergy of these two attaches, catechins start a rearranging process to minimise the energy of the system and polymerise. It is possible to note molecular mass increasing, while catechins are interacting each other to form proanthocyanidins; the antioxidant power of the polymerised molecules does not vary significantly during this process, maintaining the initial antioxidant properties[21].

2.2.2.2 Enzymatic polymerisation

Polymerisation can also be catalysed by biomolecules, such as enzymes. Enzymes can play an important role in polyphenols polymerisation. There are two types of enzymes: oxidative or hydrolytic ones[22]. This solution was thought to synthesise polyphenols in alternative to other treatments in which toxic substances such as formaldehyde were used[23].

Fungal, plant and bacteria enzymes can induce an oxidative polymerisation in polyphenols. In particular, the recombinant catalase-peroxidase HPI extracted from *Escherichia coli* was tested in a study conducted in Italy in 2018 in order to product (-)-epicatechin oligomers. Analogously to the process induced by metal oxides, this enzyme can oxidise epicatechin substrate, that becomes reactive and so can polymerise. In this way, it is possible to obtain oligomers containing still 7 units

of epicatechin. The properties of the obtained oligomers are very interesting: they are capable to inhibit tumour cell growth, as demonstrated different tests conducted *in vitro*, that is very promising for future applications on human health[24].

Other enzymes applied to phenols polymerisation are soybean peroxidase and horseradish peroxidase, associated to a treatment with room-temperature ionic liquids (RTILs), to have an oxidative process. Through them, it is possible to obtain synthetic polyphenols and copolymers with an important variety and different degrees of polymerisation. Instead of a pure water solution, in which low-molecular-mass phenolic compounds are not particularly soluble and can precipitate as monomers or as small oligomeric compound (dimers and trimers), it is preferred to use RTILs. This innovative solution, containing 1-butyl-3-methylimidazolium tetrafluoroborateorganic and 1-butyl-3-methylpyridinium tetrafluoroborate as solvents added to water, capable to create a non-aqueous environment in which polyphenols are more soluble and so, not precipitating, can better interact with enzymes[25].

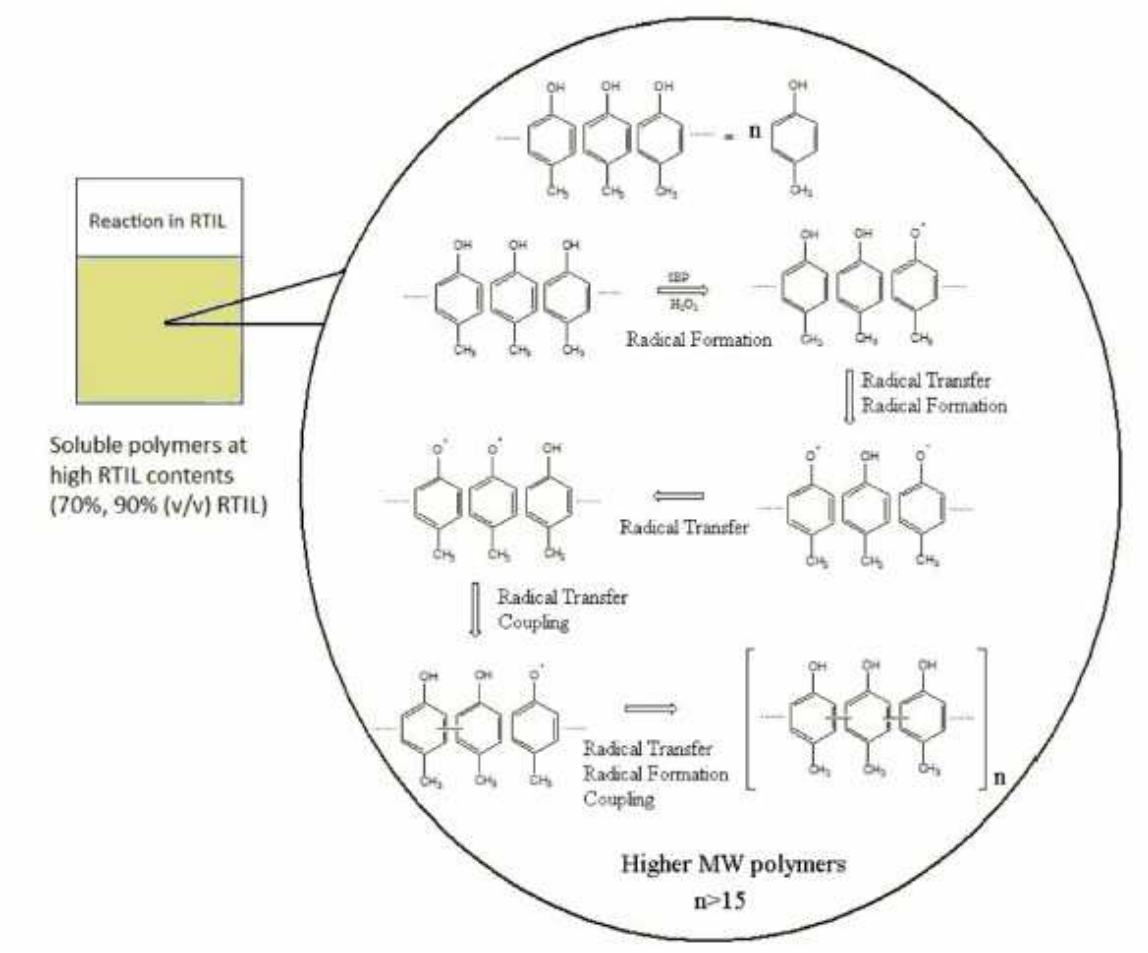

Figure 2.3 Polyphenols polymerisation mechanism in RTILs environment[26]

Through this technique, illustrated in Figure 2.3, it is possible to obtain very thermostable phenolic polymers and copolymers characterised by a quite elevate degree of polymerisation and a molecular mass ranging from 1200 to 4100 Dalton[27].

2.2.2.3 Short-range order metal oxides induced polymerisation

Short-range order (SRO) metal oxides can be other catalytic agents, capable to induce an oxidative polymerisation of catechins. These compounds implement a new innovative technique that is studied at the moment. Mn(IV)-, Fe(III)- and Al-oxides, usually found in acid soils, are the most common molecules influencing the abiotic transformation of catechins, thanks to their oxidising power and their behaviour as electron acceptors. To implement this kind of polymerisation, it is necessary to work in a low-pH environment: also if polyphenols are more stable in these conditions, the oxides are more efficient and so, thanks to a more efficient oxidation, it is possible to obtain a more efficient polymerisation. When catechins are in contact with Mn(IV)-, Fe(III)- and Al-oxides, oxidation is induced: in this way, catechins are activated and become reactive, capable to interact each other and polymerise, releasing CO_2 in proportion with the obtained degree of polymerisation. Anyway, the catalytic power of metal oxides is not completely known and it is object of different researches at the moment[28].

2.3 Polyphenols separation

Ones obtained polymerised polyphenols, it is possible to treat them to separate them basing on molecular mass or degree of polymerisation to have more homogeneous groups, ideally separating every single polyphenols class from the others. The most used technique to do that is chromatography, with its numerous variants, generally utilising the degree of polymerisation as a discriminant, while other chromatographic techniques are based on size-exclusion, normal-phase or counter-current[29,30]. Also mass spectrometry is an important technique used to characterise the molecular mass distribution of a polyphenolic extract: fast-atom bombardment mass spectrometry (FABMS), liquid secondary ion mass spectrometry (LSIMS), field desorption mass spectrometry (FDMS), electro-spray ionisation mass spectrometry (ESIMS) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) are the most utilised techniques[31].

2.3.1 Chromatography

Chromatography is perhaps the simplest technique to separate polyphenols. These molecules are anyway present in nature in the two already described forms (glycosides and free aglycones) and they are distributed in an important range of molecular mass (100-10000 Da). Regarding low-molecular-mass, aglycones are easier to analyse: in fact, before analysing polypheols, they are often converted in this form through an alcoholysis process, based on solvent extraction or enzymes activity. Regarding instead high-molecular-mass polyphenols, especially tannins, their identification is more difficult because of the great quantity of isomers of a single type of molecule and their significant variety; for this class of polyphenols, mass spectrometry is preferred to characterise the specific molecules[32].

High-performance-liquid-chromatography (HPLC), as already described in Chapter 1, is a diffuse method to separate and analyse molecules, based on their sizes and molecular masses. A study carried in Germany in 2015 analysed proanthocyanidins extracted from Hawthorn leaves and flowers (*Crataegus* spp., Rosaceae). Polyphenols were extracted through a water solution of acetone, that also separated the molecules having different degrees of polymerisation (from 2 to 10) in nine clusters. In Figure 2.4 the result of the HPLC analysis of the extract is reported, in which the

numbers refer to the degree of polymerisation of the molecules (from 2 to 10) and the letter "n" to superior degrees of polymerisation, that are not considered in this study[33].

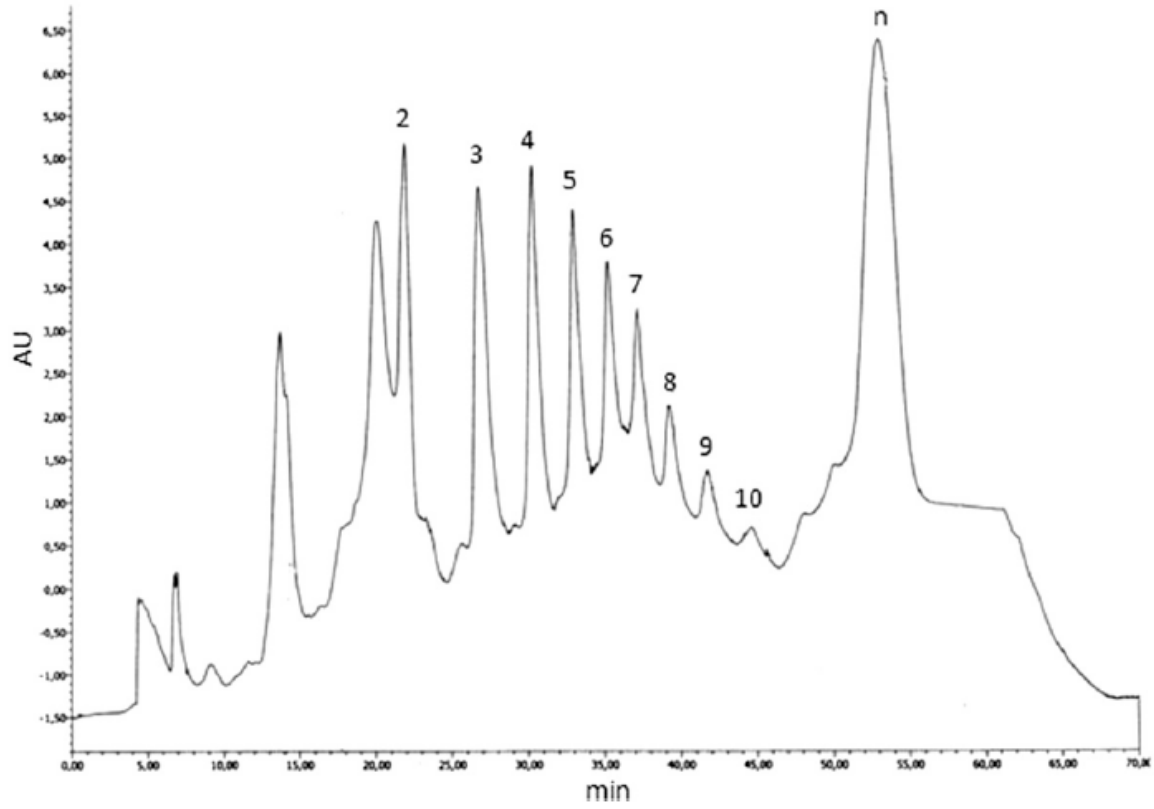

Figure 2.4 HPLC output analysing polyphenols extracted from Hawthorn leaves and flowers[34]

It is possible to see how the polyphenols characterised by higher molecular masses are detected by the instrument (UV detection, wavelength of 280 nm) after a longer time: molecules having 2 as degree of polymerisation are detected after 15-20 minutes, while the ones having 10 as degree of polymerisation after 45 minutes. Peaks are well distinguishable, especially the ones characterising the low-degree-of-polymerisation molecules[35].

HPLC is a technique based on size-exclusion. There are two methods: normal phase and reversed phase size-exclusion chromatography[36].

Normal phase separation, using degree of polymerisation as the discriminant, is based on the differences of the analysed molecules in polarity. The polarity of polyphenols is directly proportional to its molecular mass: increasing the polarity, also molecular mass increases. With this technique, it is possible to observe how the low-molecular-mass polyphenols go out of the silica column of the instrument containing the elution (a gradient of polar organic solutes and water) before the high-molecular-mass ones. This is explained by the major affinity of the high-molecular-mass polyphenols with the polar chemical species inside the column and the strong interaction with the silica stationary phase: their retention lets the smaller molecules going out of the instrument in a shorter time; then, the interactions between big polyphenols and the elution decrease and so also the last retained molecules can be detected by the instrument. Separating monomers from oligomers having their degree of polymerisation in a range going from 2 to 5, a chromatogram is obtained, as

the one reported in Figure 2.5. It is possible to see the different peaks corresponding to the different degrees of polymerisation, with lower times of retention for lower degrees of polymerisation[37].

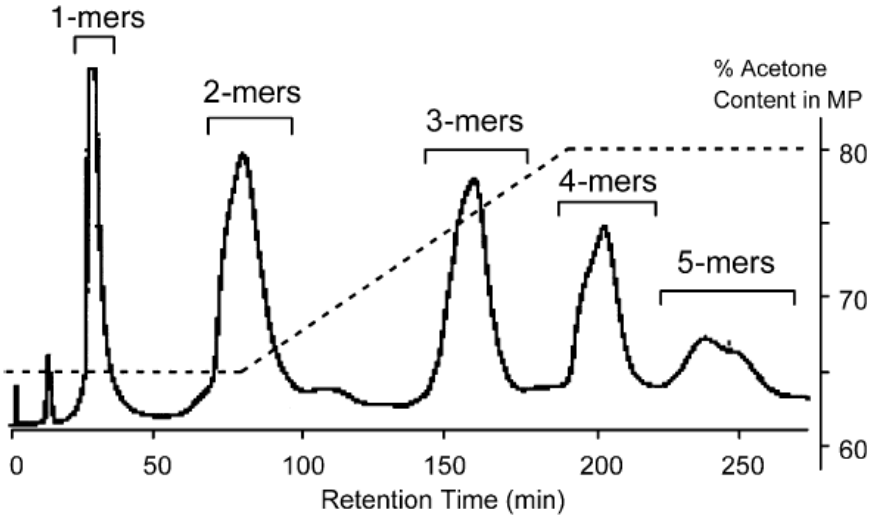

Figure 2.5 Normal phase size-exclusion chromatography output[38]

The other size-exclusion technique is the reversed phase chromatography. In this method, the column does not contain polar species, but hydrophobic ones; it is composed also by a gel matrix, that contributes to retain the analysed molecules. In this way, high-molecular-mass polyphenols, characterised by a higher degree of polymerisation, are hydrophilic: they are repelled by the chemical species contained by the column. These molecules are so expelled first, while the gel contributes with a low mechanical retention, not sufficient to interrupt the polyphenols flowing and expelling process. The smaller polyphenols instead, characterised by a low molecular mass and a low degree of polymerisation, that are generally hydrophobic, better interact with the chemical species inside the column and better infiltrate between the cross-links of the gel and are capable to permeate it; so they are detected by the instrument after a longer time[39,40]. In the Figure 2.6 it is possible to see the chromatogram given by reversed-phase HPLC in two different scales, in which the peaks corresponding to the higher degree of polymerisation polyphenols are first detected by the instrument; the last peak is the one corresponding to the monomer[41].

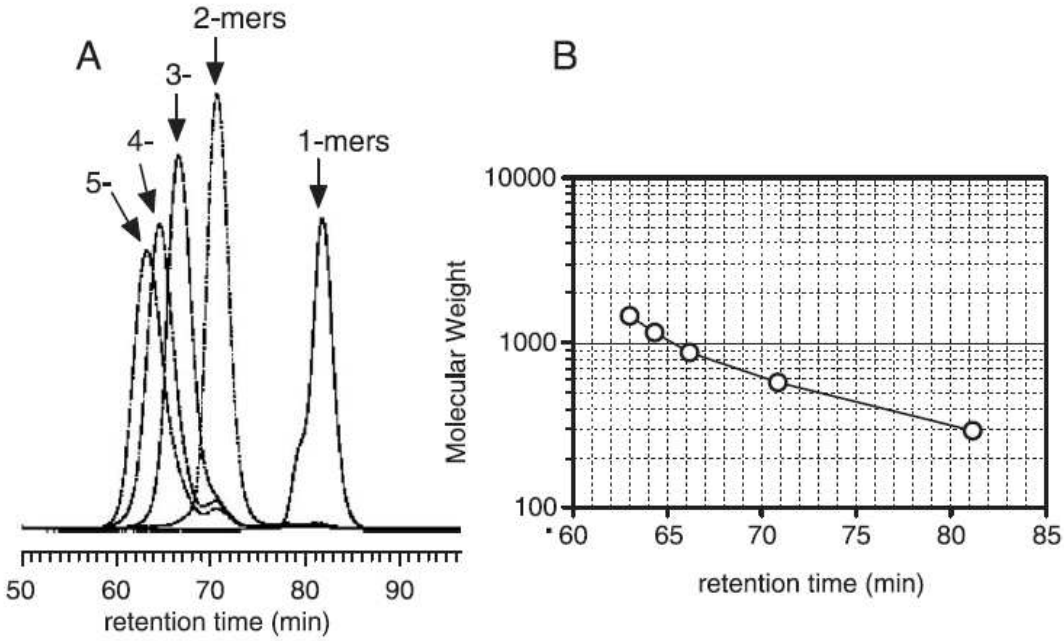

Figure 2.6 Reversed phase size-exclusion chromatography output.
(A) Linear scale, (B) logarithmic scale[42]

In addition to HPLC, another frequently used type of chromatography is counter-current chromatography (CCC). This technique operates under gentle conditions, to isolate polyphenols without damaging them. CCC, thanks to the absence of a stationary phase inside the column, also guarantee an advantage that HPLC not always does: there are not losses of polyphenols during separation and all the biomolecules that were put in samples are recovered at the end of the process. While with HPLC a small percentage of analysed molecules are absorbed in the column, with CCC, having a liquid phase as a stationary phase, this risk is not, through a simple pumping out this phase. There are two modality of usage of this technique. The first – rarely used – permits the separation of the molecules through a double rotation of the device, that generates a fluctuating force field; this field lets the less dense phase move to the superior extremity of the column, while forces the denser one to go to the other extremity. The other modality instead uses the above cited stationary liquid phase, with which the column is filled, and the mobile phase, lighter, that in pumped from an extremity to the other[43,44].
In Figure 2.7 a chromatogram obtained through CCC technique is reported, in which it is possible to see the different peaks corresponding to different degrees of polymerisation: F1 refers to monomers, the peaks going from F2 to F6 to oligomers having the respective degree of polymerisation, F7 to generic polymers[45].

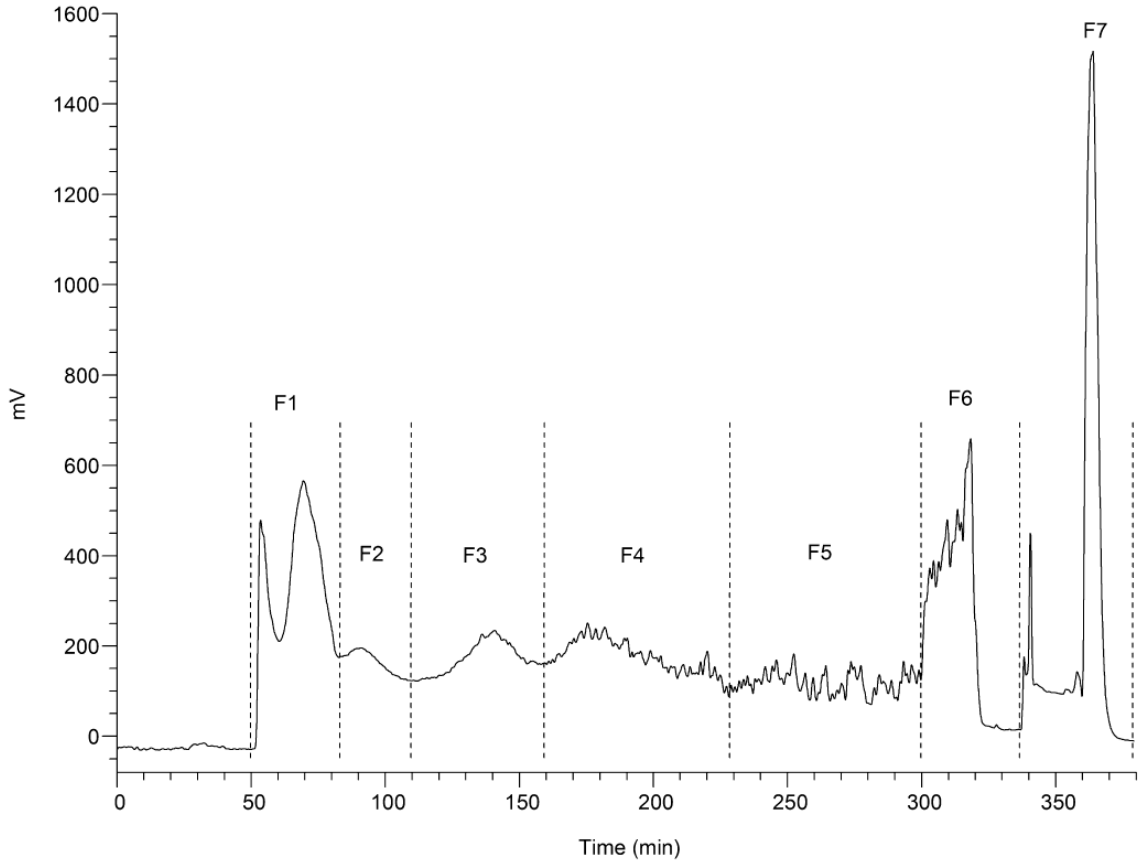

Figure 2.7 Counter Current Chromatography output[46]

2.3.2 Mass Spectrometry

Mass spectrometry includes – as cited before – different techniques. The most utilised to analyse polyphenols is MALDI-TOF MS, capable to give in output an estimation of the molecular mass distribution of a heterogeneous mixture of polyphenols. Mass spectroscopy is based on the detection of gas phase ions, both positive and negative, whose separation uses mass-to-charge ratio (m/z) as the discriminant. To prepare the samples, is so necessary to make an ionisation. There are two ionisation methods: the first is the electron impact, the other is the chemical ionisation, before which vaporisation of the sample is fundamental. Because of the low volatility of high-molecular-mass polyphenols, it is possible to use a matrix-assisted laser desorption (MALDI), that, for this reason, has become very frequent in polyphenols analysis[47].

To prepare the sample, using a laser pulse, it is necessary to give it much energy (about 10^6 W/cm^2) for a very short time interval (about a few ns), so the kinetic energy is not dissipated by the molecules, but thanks to this accumulated energy, the sample is blasted away from the target area and its various components, differencing in the time of flight (TOF), reach a collector on which they are detected. In the figure 2.8 a spectrogram obtained through this technique is reported[48].

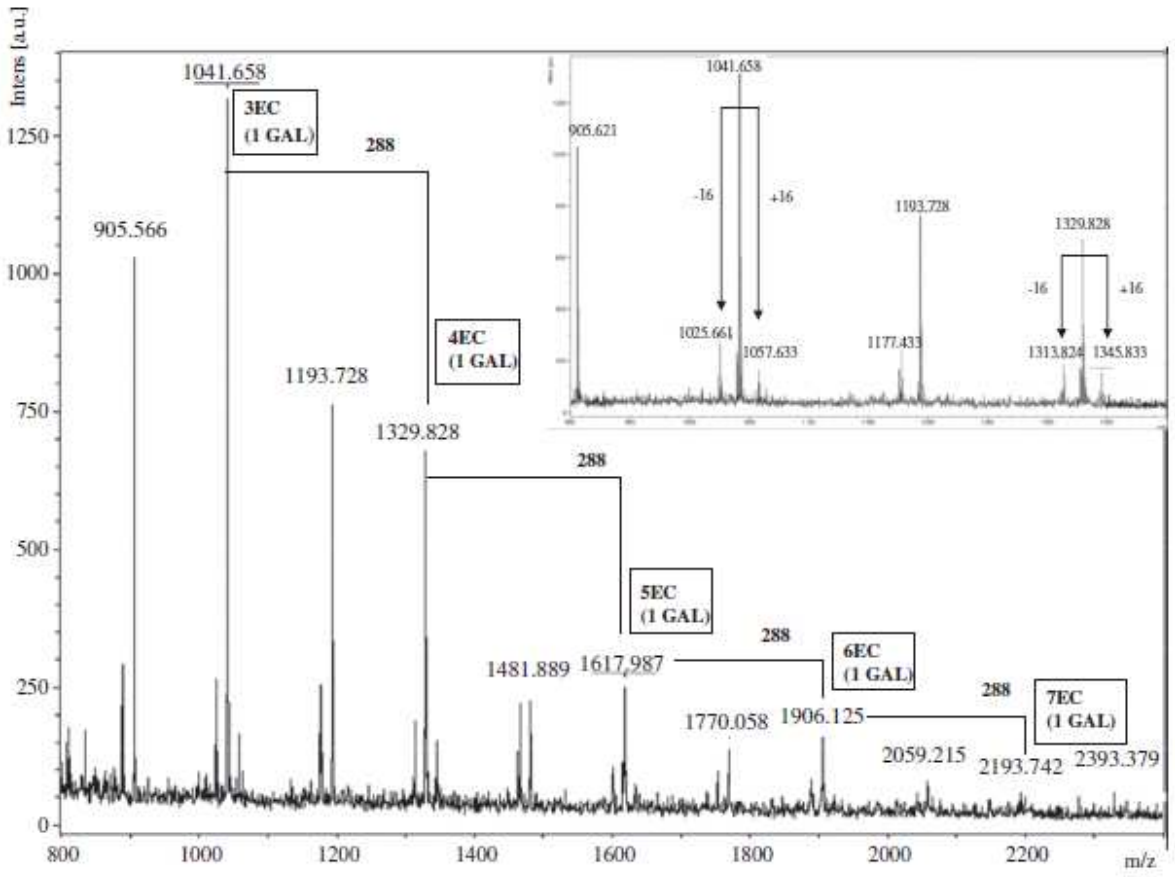

Figure 2.8 MALDI-TOF MS output[49]

Every peak corresponds to a different chemical specie, precisely to a different polyphenol, which differs from the others for its m/z ratio. These results are obtained from the analysis of the polyphenols contained in almond blanch water, in which it is possible to distinguish the various components; the peaks characterising a certain extract can be different varying the cationising agents used for the previous ionisation[50].

2.3.3 An alternative: computational tools

A study conducted in 2016 in USA developed an advanced computational tool capable to analyse polyphenols – precisely proanthocyanidins – starting from the data obtained by ultra-high-performance liquid chromatography-diode array detection-high resolution accurate mass-mass spectrometry (UHPLC-DAD-HRAM-MS). Through this software it is possible to identify proanthocyanidins and quantify them, individuating chromatographic peaks and evaluating them. Using a deconvolution process, isotopic pattern of A-type and B-type are distinguished, multi-charged proanthocyanidins ions are recognized and so the structure of these molecules can faithfully predicted. Through an external calibration curve, also the concentrations can be evaluated: the concentration of the single species, of the total proanthocyanidins and of the groups of proanthocyanidins characterised by different degrees of polymerisation. This software was tested on different polyphenols extracts, deriving from different sources: fruits, seeds and cocoa[51].

2.4 Polyphenols degradation

Polyphenols are quite stable compounds, but, under certain conditions, can degrade through different mechanisms. The main degradation processes are photo-oxidation, thermal denaturation and degradation after γ irradiation[52,53,54].

Photo-oxidation is possible applying UV-irradiation or visible light irradiation to polyphenolic compounds for a sufficient time interval. As previously said, photo-irradiation can also be utilised to initiate polymerisation process: in this case, irradiation conditions are significantly less aggressive than the ones applied to degrade the molecules (lower power of the ray applied for a shorter time interval)[55, 56]. The power necessary to induce degradation in polyphenols is in the order of 10 W/m^2, but also 6-7 W/m^2 are sufficient to damage the bonds of the chain present in the molecules. After 29 days in which polyphenols extracts are irradiated for 16 hours per day under these conditions, it is possible to see a significant decrease of the polyphenols content, that, at the end of the period of observation, has decreased approximately of the 50% or even more[57].

Another technique to degrade polyphenols consists in applying a thermal source to alter their kinetic energy providing heat[58]. Heating polyphenols extracts in vacuum to reach a temperature around 750 °C, it is possible to induce pyrolysis. First, at a temperature that can vary for the different polyphenols, but always higher than 70 °C, there is a reaction of decarboxylation, in which CO_2 is produced; this phenomenon is reported in Figure 2.9[59].

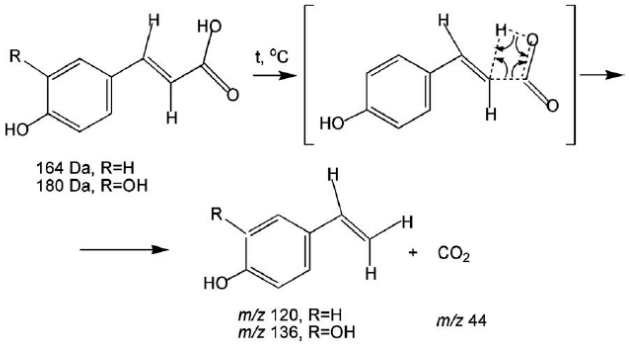

Figure 2.9 Decarboxylation of a polyphenol (coumaric acid)[60]

Other polyphenols, such as cinnamic acid, instead, are more stable and there is not decarboxylation around 70 °C, but the molecule remains unvaried until 200 °C is reached, then sublimates. To let thermally stable molecules be decarboxylated, it is necessary to treat the molecule, such as bonding it to a silica surface: so, also this type of molecule can be decarboxylated without incurring in sublimation. The reasons of this phenomenon are still debated; two main causes were proposed: the first explains this decarboxylation as a process induced by the formation of carboxylate anion, while the second as a syn-elimination reaction[61].

Anyway, for both the types of polyphenols – more or less thermally stable, pyrolysis is possible thanks to a hydrogen transfer from an electron-donor to an electron-acceptor, with the formation of a carbanion (Figure 2.10). Precisely, the electron-acceptor substituent (in this case, the hydroxyl group) can accelerate decarboxylation, stabilising the carbanion intermediate by delocalisation of its negative charge. In this way, the intra-molecular hydrogen transfer is facilitated upon syn-elimination and CO_2 is produced and expelled (Figure 2.9, 2.10)[62].

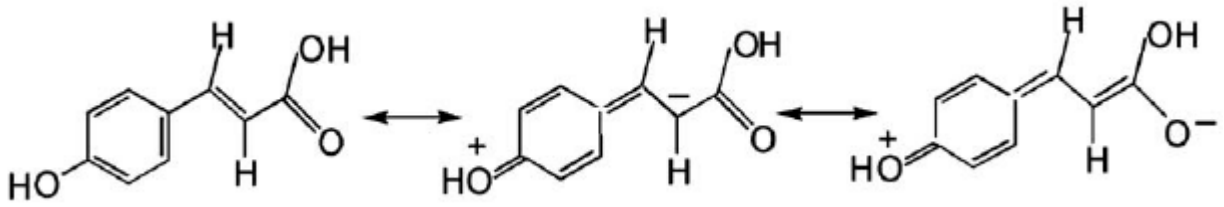

Figure 2.10 Formation of carbanion and hydrogen transfer in polyphenol pyrolysis[63]

The last polyphenols degradation technique is through γ irradiation. As discussed in Chapter 1, these radiations are utilised in sterilisation process; in this application, the eventual degradation of the biomolecules is an undesired collateral effect to limit varying the time of exposure and the intensity of the radiation. Anyway, γ irradiation can be also utilised as a technique to degrade polyphenols, using a precise dose, higher than a critical threshold. By different analysis, it was ascertained that 50 kGy constitutethe critical threshold necessary to completely denature polyphenols[64].

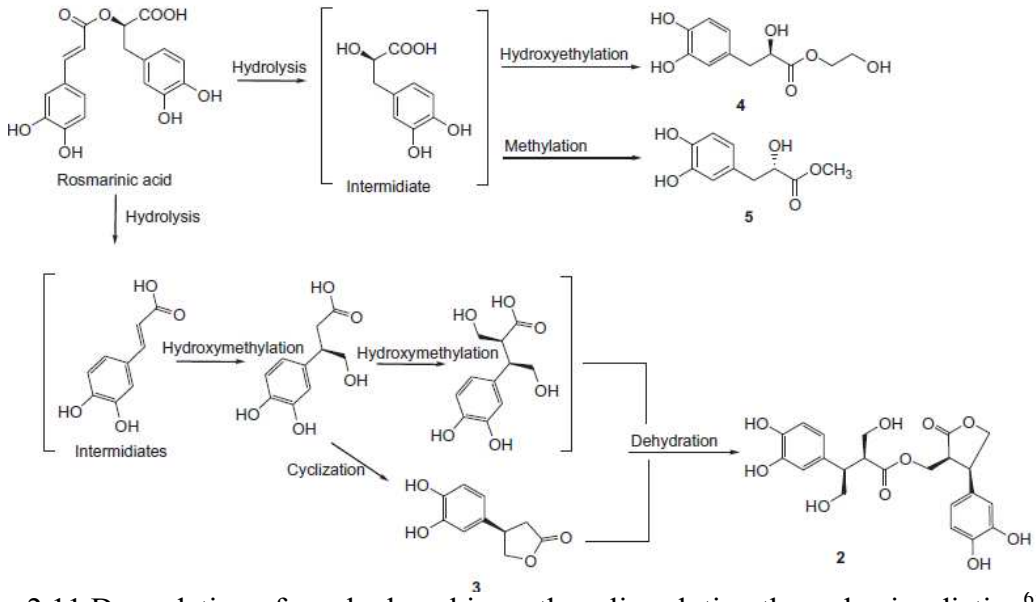

Figure 2.11 Degradation of a polyphenol in methanolic solution through γ irradiation[65]

In figure 2.11 a typical pathway of polyphenol degradation is reported, in which rosmarinic acid has reported as an example. The polyphenol, put in a methanolic solution, is irradiated. The first reaction is hydrolysis[66]. During this hydrolytic process, due to the synergy of the aggressive solution and the irradiation, also called alcoholic radiolysis, a bond is broken and, in this way, the molecule is divided into two different intermediates. Other successive reactions involve respectively both the intermediates: cyclization, dehydration, hydroxyethylation and hydroxymethylation. At the end of the entire process, it is possible to see two molecules having lower molecular masses and another chemical specie having both hexagonal and pentagonal cycles[67,68].

2.4.1 Beneficial effects of polyphenols irradiation

An interesting effect of irradiation is the capacity of vegetables and fruits containing polyphenols to increase their content and modify gene expression to increase polyphenols production. It is possible

to irradiate them with both UV and visible light, obtaining analogue results: short-term UV C irradiation stimulates biosynthetic pathways in plants, while irradiation with visible blue light has the same effect through a long-term treatment. In this way, it is possible to synthesise different types of polyphenols: flavan-3-ols and their derivates, oligomeric compounds, galloyled derivates and acylated derivates. Two experiments were carried to study this phenomenon and demonstrate this beneficial effect[69,70].

Studying the effect of visible light irradiation on fruits containing polyphenols, it was demonstrated that, stimulating raspberry fruits and leaves with different wavelengths, there were different behaviours given by the plant. In fact, it was observed that, irradiating raspberries with white light (wavelength going from 400 to 700 nm) for seven days the polyphenols content significantly decreases and this phenomenon is highly temperature-dependent: decreasing the temperature, the total polyphenols content also decreases during the treatment[71,72]. Irradiating instead with blue light (wavelength of 480 nm), the total polyphenols content significantly increases and this process is not temperature-dependent; through HPLC it is possible to individuate the different molecules: flavan-3-ols derivates, (+)-catechins, (-)-epicatechins, procyanidins and proanthocyanidins particularly increase their content. With further analysis, such as semi-quantitative real time polymerase chain reaction (RT-PCR), the gene expression of chalcone synthase is also increased: this enzyme is so produced in higher quantities and this stimulates the necessary pathways that increase the total polyphenols production[73].

To increase the bioactive phytochemical content in fruits it is also possible to irradiate with UV light. It was demonstrated that preharvest UV C – radiations having a wavelength going from 100 to 280 nm – is particularly efficient to increase polyphenols content (25-75 % increase), especially cyanidin, quercetin and ellagic acid, both in glucoside form and in aglycone one[74,75]. These radiation are capable to induce this effect only if lower than a precise threshold of energy density: polyphenols content increases only for irradiation with a dose lower than 15 kJ/m^2, while no effect is observed irradiating with higher energy doses. Analogously to visible light irradiations, significant effects on gene expression are observed, particularly on flavonoid pathway structural genes. In Figure 2.12 it is possible to appreciate the differences in two different polyphenols content (cyanidin-3-glucoside and quercetin-3-glucoside) before and after strawberries UV C irradiation with three different doses: low, 9.6 kJ/m^2; middle, 15 kJ/m^2; high, 29.6 kJ/m^2. As introduced before, with doses equal or lower than the threshold of 15 kJ/m^2, the increase is significant, especially for the middle dose. For higher doses, there are no modifications, while for cyanidins, a small decrease can be observed, due to the partial degradation induced by the aggressive radiations[76].

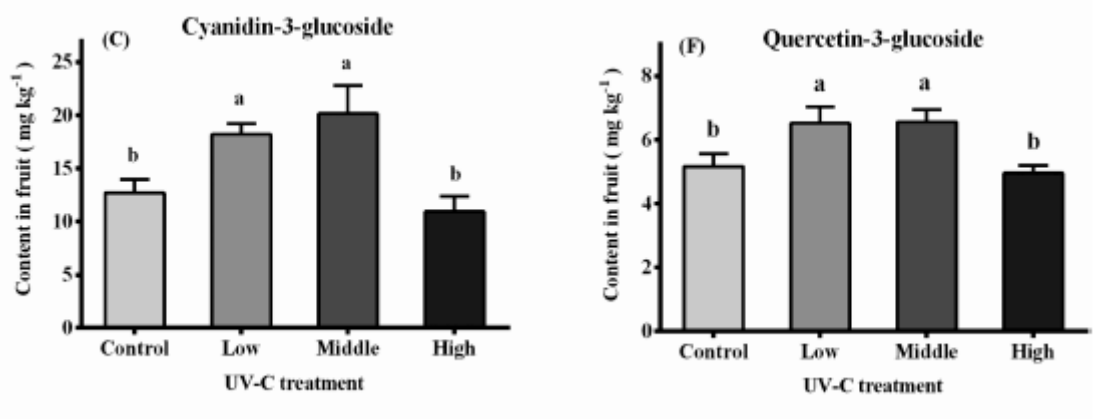

Figure 2.12 Polyphenols content after UV C irradiation[77]

2.4.2 Polyphenols fermentation

Fermentation is a recurrent process that can be spontaneous or can be conducted and controlled in laboratory. It is especially applied to cocoa beans and tea leaves in food treatment and alimentary industry, but there are also recent researches in which fermentation is studied to optimise the content of bioactive compounds, such as polyphenols[78,79,80].

Cocoa spontaneous fermentation, also called solid-state fermentation (SSF), is a process that can go on for a few days, precisely from two to five days; the period of fermentation varies on the basis of the type of cocoa that is involved. It is divided into two phases and is induced by an initialising microbiological attack in a small amount of free water. Involved micro-organisms are generally yeasts, acetic acid bacteria (AAB) and lactic acid bacteria (LAB)[81,82]. The first step of this process is performed at lower temperature, going from 25 to 45 °C, and involves sugar transformations in the pulp that surrounds the bean. During the second phase, instead, that has place inside the bean itself, higher temperatures are reached – from 42 to 52 °C – and the reactions are hydrolytic reactions that generate the characteristic aroma precursors, which are typical of the various types of cocoa[83]. In the first step, yeasts are the main involved micro-organisms: they degrade the pulp, consuming the present sugars, citric acid and the available oxygen. Yeasts also secrete pectinases, specific enzymes capable to degrade pectin, generating an anoxic micro-environment because of the high oxygen consuming and, at the end of this reaction, ethanol is produced and released in the micro-environment[84]. The anoxic conditions, fundamental to let LAB start to work in an optimised environment, are the initial conditions of the second step of the fermentation process. LAB produce lactic acid utilising the available ethanol just formed. After the reintroduction of oxygen in the environment, AAB intervene, letting the residual ethanol and the lactic acid react to produce acetic acid, a volatile specie that is dispersed through a particularly exothermic reaction, for which the temperature can increase, generally reaching 50 °C, with the possibility to have higher peaks of 60 °C. These reactions permit the formation of the cited aroma flavour precursors, thanks to the high temperatures, that induce the breakdown of pectin and other proteins – especially albumin and vicilin – into peptides and amino acids and the decrease of the polyphenols content[85].

Tea fermentation is another interesting procedure originally applied by antique cultures in China and Japan to obtain different types of tea, such as black and oolong tea. In this process, the enzymes contained in the fresh leaves of the tea plants catalyse reactions to chemically alter the various constituents. *Camellia sinensis* is the most utilised tea and its leaves can be worked to obtain four cathegories of tea: unfermented, that is the well-known green tea; fermented, that is the black tea; semi-fermented, that is the oolong tea; post-fermented, that can be pu-ehr or dark tea. The last type of tea is becoming more and more studied because of its particularly beneficial properties. The fundamental difference between the post-fermented tea and the others is the different fermentation process: while the first three teas are obtained by the simple enzymatic fermentation, this tea is instead obtained through a microbial fermentation at high temperatures, that can be aerobic or anaerobic. This fermentation is analogue to the one applied to cocoa beans, but with a significant difference. During the fermentation process, it is possible to observe the decrease of catechins content, due to the microbial activity, during which enzymes are produced and auto-oxidation is promoted; anyway, the total polyphenols content significantly increases. This is explained by the polymerising effect of the initial degradation of the catechins, for which they are induced to react together to minimise the free energy of the system. In Figure 2.13 it is possible to see a spectrogram in which the different peaks characterising the polyphenols produced during the post-fermentation process are evidenced; these polyphenols are totally absent in unfermented tea[86].

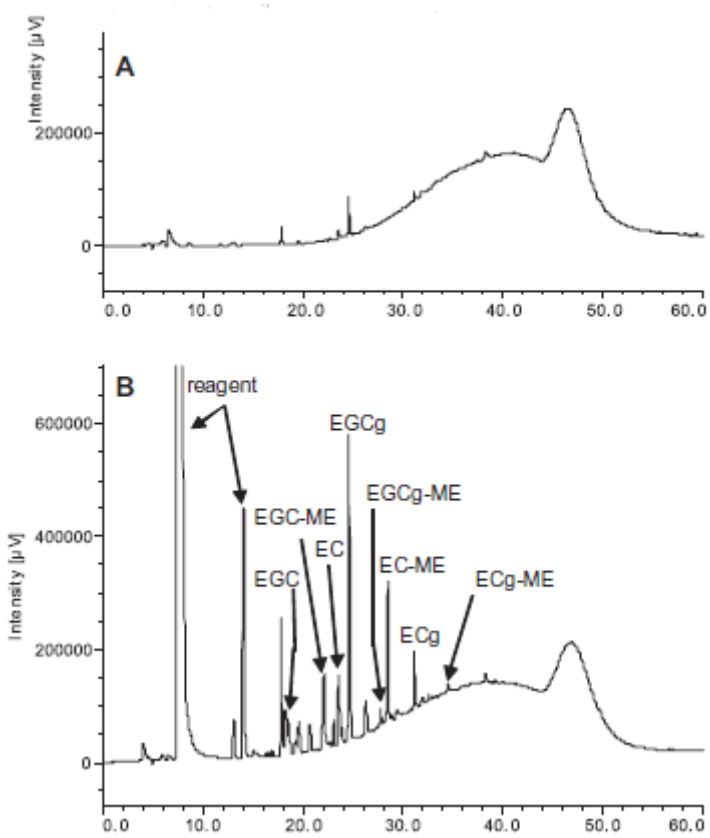

Figure 2.13 Comparison between unfermented and post-fermented *Camellia sinensis*[87]

Recent studies conducted on broccoli puree and bread wheat doughs have demonstrated – and confirmed – the behaviour of polyphenols and other antioxidant compounds, such as carotenoids and glucosinolates, for which the intervention of LAB on their biochemistry can be significantly beneficial, inducing biotransformations capable to increase their content and, consequently, to increase the beneficial effects on human health when assumed[88,89]. During an experiment conducted on bread wheat doughs, it was also noted the difference on the final products of the biotransformations varying the strain of the LAB involved in the fermentation: *Lactobacillus fermentum*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum* and *Lactobacillus brevis*. *Lactobacillus plantarum* is the most efficient bacterium in increasing the phenolic acids content (the increasing rate is included in an interval going from the 60% to 100%) and, consequently, the antioxidant capacity. It is due to the synergic effects of the microbial conversion and the endogenous activity of the enzymes produced by the bacteria[90].

2.4.3 Polyphenols extraction

Polyphenols extraction techniques are various, based on electrical, mechanical and – recently – also biological principles. Anyway, polyphenols extraction is quite critical: it is possible to degrade the molecules during the process because of the imposed aggressive conditions[91,92].

The electrical techniques are based on high-voltage electrical discharge or on pulsed electric fields[93,94].

The objective of the high-voltage electrical discharge (HVED) technique is the damage and the disruption of the cells structure of the plant – or of its parts, such as seeds, peel, pulp or leaves – from which it is possible to extract intracellular components, such as polyphenols. It is based on the phenomenon of the electrical breakdown in water, obtainable through the electrochemical system illustrated in Figure 2.14[95].

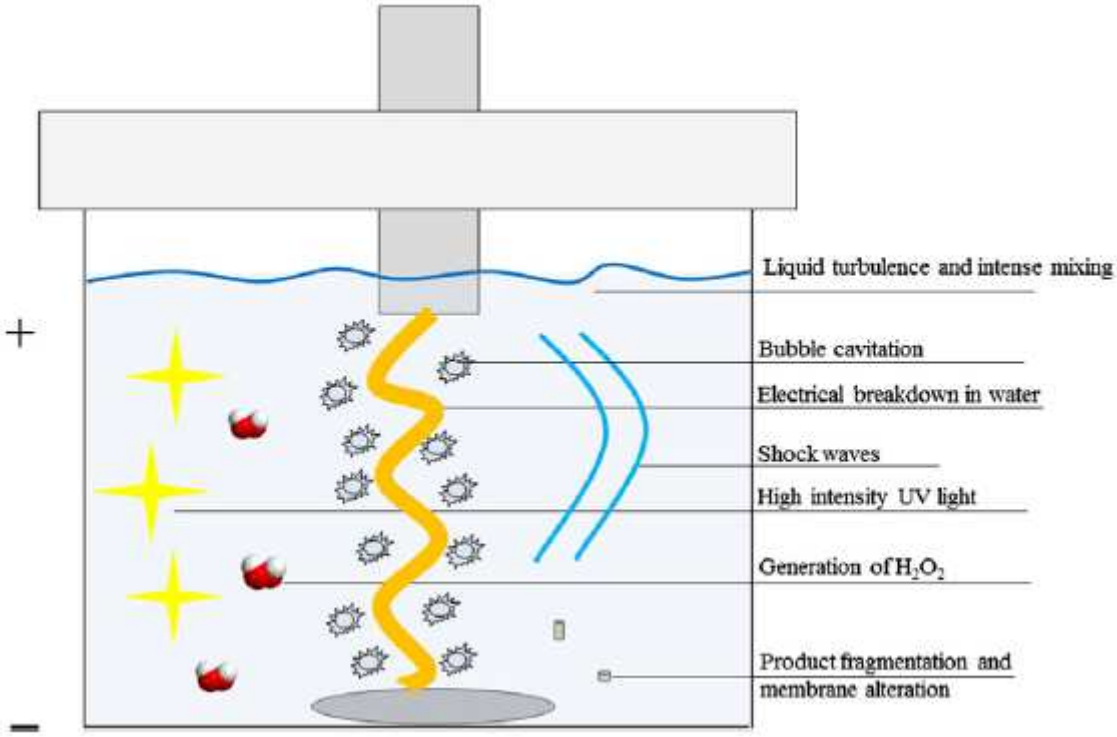

Figure 2.14 The HVED electrochemical system[96]

The system includes two electrodes, a cathode (negative charged) and an anode (positive charged), by which a high voltage is imposed, capable to induce a liquid ionisation thanks to the propagation of an intense stream of electron from the anode to the cathode and, consequently, an electrical breakdown. This phenomenon leads to the liquid turbulence and mixing, the emission of high-intensity UV radiations, the production of hydrogen peroxide and, finally, the generation of shock waves and bubble cavitation. During the breakdown, an electric arc intervenes, working in a synergetic way with the shock waves and the bubble cavitation, to obtain the desired mechanical effect on the plant, ergo the fragmentation of its seeds, thanks which polyphenols can be extracted[97]. This technique can also be used in synergy with a biological treatment: enzymatic hydrolysis can be involved to polyphenols extraction. In this way, the extraction is optimised applying HVED prior to enzymatic hydrolysis, to first obtain a significant mechanical effect, capable to physically damage the cells of the plant from which polyphenols have to be extracted, and, successively, let the enzymes catalyse the necessary reactions. The enzymes involved in this process are arabanase, β-glucanase, cellulase, hemicellulase and xylanase, united in a multi-enzyme complex, which hydrolyses the tissues of the plant to facilitate the liberation of the biomolecules. Increasing the energy of the HVED treatment to hundreds of kJ/kg, the successive enzyme action is more efficient: the obtained particles have in fact a smaller diameter and the diffusion kinetic of the polyphenols is

improved, while the cellular disintegration index – evaluating the membrane damaging phenomenon – increases (Figure 2.15)[98].

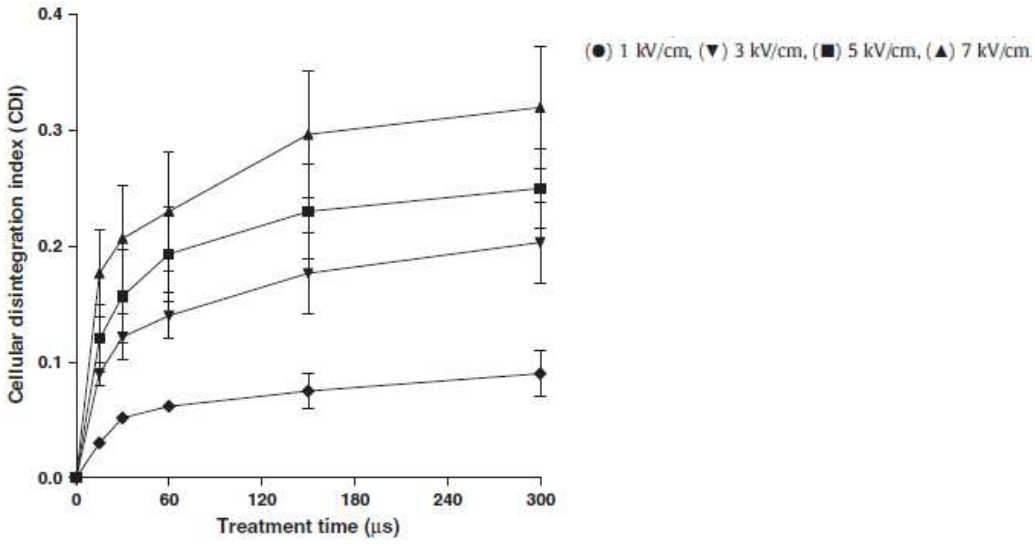

Figure 2.15 Influence of intensity of the electric field on cell disintegration[99]

Anyway, the high values of energy have a collateral effect: the aggressivity of the treatment can be the cause of the production of hydroxyl radicals, atomic hydrogen and ozone, that could oxidise polyphenols and start their degradation process, especially at high pH[100]. In particular, hydroxyl radical reacts with phenol, formed by the UV light produced by the discharge, to obtain other oxidant species, such as phenoxyl radical, that contributes to damage the polyphenols to be extracted[101].

To ulteriorly optimise the described process to obtain a purer extract, it is also possible to add a last step at this extraction technique, after the HVED and the enzymatic treatments: a final delignification through alkaline hydrolysis, using NaOH solutions that, after the necessary time to obtain the desired effect (about 4 hours at 50 °C), are neutralised adding HCl. It is an efficient way to increase the polyphenols extraction. In fact, after their liberation thanks to the electromechanical effect of HVED and the chemical action of enzymes, there are residual polyphenols covalently linked through ester bonds to polysaccharides and to lignin components that are present in the environment. Through this last step, NaOH interacts with the ester bonds hydrolysing them to liberate polyphenols and to let these biomolecules be accessed[102].

The other most utilised extraction technique is based on pulsed electric fields (PEF). The objective of PEF is provoking electroporation of cell membranes to let polyphenols be more efficiently extracted. This technique can be applied to different parts of the plant, similarly to HVED, but also to whole fruits, such as entire apples, oranges, lemons and blueberries. To implement this treatment, it is necessary to generate an intense electric field, applying to the fruit a voltage going from a few kV/cm to 10 kV/cm – values that vary basing on the type of fruit or part of it from which polyphenols have to be extracted. In Figure 2.16 the PEF treatment chamber is illustrated. It is composed by two electrodes, a cathode and an anode, through which the high voltage is imposed; the behaviour of the system is analogue to the one utilised for HVED. Generally, the number of pulses imposed to the fruit is in the order to the hundreds and each pulse is long a few μs[103].

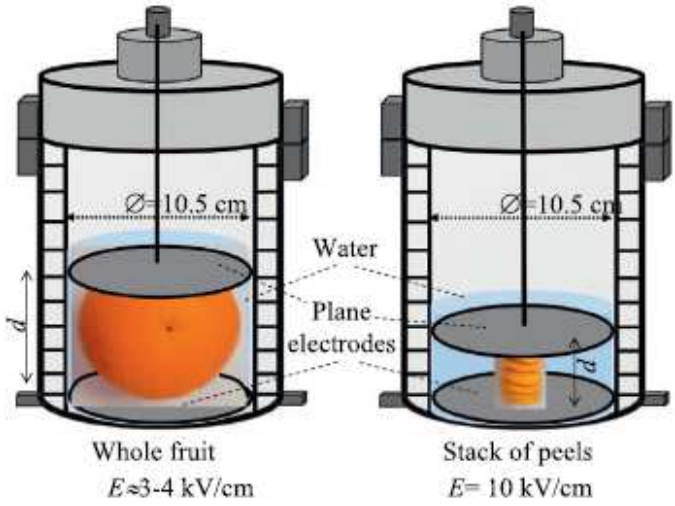

Figure 2.16 PEF treatment chamber[104]

It is possible to use PEF as a pretreatment before pressing the fruits to reduce the energy consumed during the entire extraction process and to increase the total polyphenol extraction yield to 159% and the antioxidant activity of the extract to 192%[105]. This is an interesting environmentally friendly solution to reduce extraction times without using organic solvents that are potentially toxic, such as hexane, acetone and methanol[106]. In fact, the permeabilisation of the cytoplasmatic membranes induced by PEF facilitates the release of intracellular compounds, such as polyphenols, especially during the successive pressing treatment. The final polyphenols content of the extracts significantly increases, but also the variety of polyphenols: different molecules appertaining to different classes can be so found in the obtained extract[107]. Anyway, PEF is a pH-dependent process: it is in fact efficient for low pH (pH<7), in which polyphenols are chemically stable, while, for high pH (around 11) there are no differences between the extracts deriving from PEF-pretreated and non-pretreated fruits[108].

A new innovative extraction technique has been studied in the last years: it is the polyphenols extraction through magnetic microspheres. They are constituted by a magnetic core in Fe_3O_4, covered by a tannic acid (TA) coating. The coating is possible thanks to the capability of TA to copolymerise preferentially on the magnetic surfaces of the microspheres with other two compounds utilised to let the reaction be: N-vinyl-2-pyrrolidone (NVP) and N-isopropylacrylamide (NIPAM). In Figure 2.17 the section of a magnetic microsphere is reported, illustrating the layers deposed on the magnetic core, and a zoom on the chemical interactions between TA, NVP and NIPAM is evidenced[109].

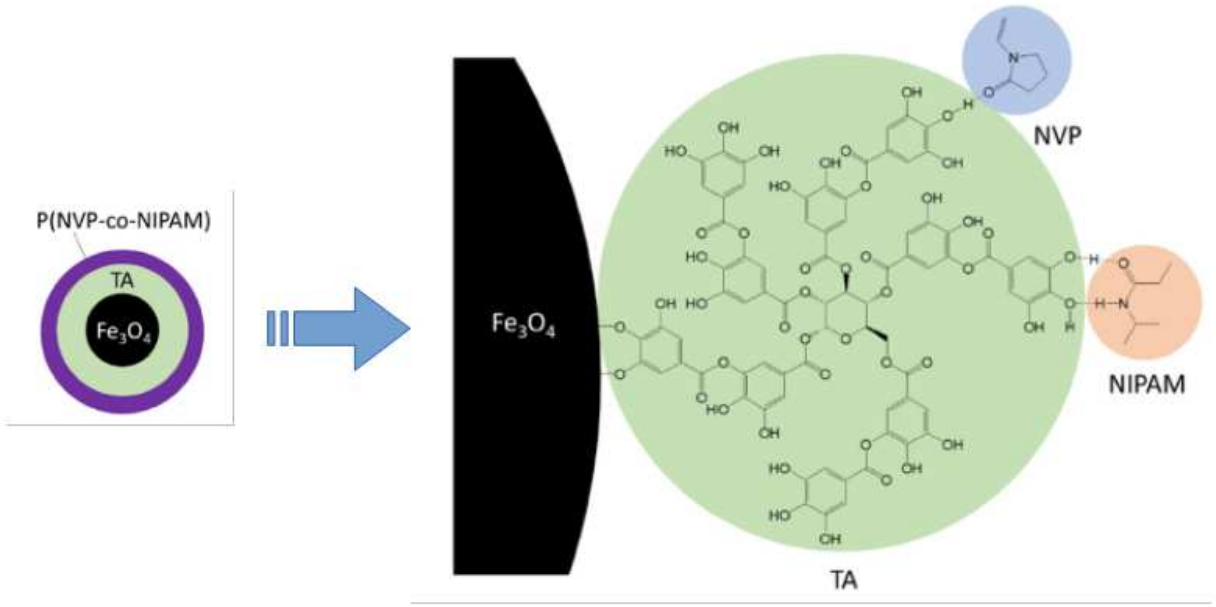

Figure 2.17 TA interactions with NVP and NIPAM inside the magnetic microsphere[110]

Magnetic microspheres are a smart technology because, maintaining superparamagnetic properties and not loosing magnetisation after their coating, they are capable to participate to polyphenols extraction: the coated microspheres demonstrate in fact outstanding absorption ability to typical polyphenols, following the Langmuir absorption model. In fact, NVP participates to polyphenols absorption with TA, while TA also produces multilayered films with NVP and NIPAM, forming hydrogen-bonding interactions that permit the copolymerisation and, so, the coating of the microspheres[111].

Also if various techniques have been studied, there are some polyphenols that cannot be extracted, both in laboratory and during the digestion process: they are called non-extractable polyphenols (NEPPs). Anyway, these biomolecules can be utilised thanks to their capability to maintain their properties without be extracted. Although NEPPs assumed by food cannot be released during the digestion process – during the mastication, the acidic attach of the stomach nor by the action of the digestive enzymes in intestine – because of the impossibility to be extracted with hydrophilic solvents, they have the capability to positively influence the gastrointestinal and the cardiovascular health. A recent study conducted on dried persimmon fruit (*Diospyros kaki*) has demonstrated an interesting *in vitro* bile acid-binding capacity, that may influence the reduction of cholesterol inside the organism[112].

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CHAPTER 3
BIOMATERIALS FUNCTIONALISATION WITH POLYPHENOLS

3.1 Functionalisation with polyphenols: chemical and physiological implications

Thanks to their properties and their numerous benefits, polyphenols can be applied in biomaterials surfaces functionalisation to improve biocompatibility and to reduce the negative impact that an external body can have that is implanted in the human tissues environment, as a medical device[1]. As diffusively described in the precedent chapters, they are in fact able to absorb UV radiation, scavenge free radicals, chelate metal ions, but they also interact with human metabolism mechanisms, taking part of cells activity: human health is particularly well influenced by the consumption of these biomolecules through food and beverages[2]. Polyphenols have so attracted the attention of the scientific world in the last years to try to find new ways to apply them, not only in human diet, but also in clinics, for medical applications. One of these possible applications is multifunctional biomaterials surfaces coating, finalised to optimise the implant of the biomaterial itself[3].

Biomaterials implantation in human body can be quite critical because of the difficulty of control the third body reaction. It is an aggressive reaction triggered by human body itself, for which the implanted material is covered by macrophages and successively by fibroblasts, that isolate it from the surrounding environment, constructing a structure called fibrotic capsule, whose target is limiting every type of exchange between the human tissues and the potentially dangerous material. Fibrotic capsule limits also the efficacy of the medical device. Anyway, it is possible that the biomaterial implantation induces a chronic inflammation, too, for different causes, such as ions release or formation of debris. This is a worse situation, for which the biomaterial must be quickly removed[4].

Polyphenols, if present on the biomaterial surface, are able to influence the phenomena that have place in the first moments after biomaterial implantation (from few seconds to different days), increasing the hydrophilicity of the surface, while working as antioxidants, reducing third body reaction and, consequently, permitting a better integration of the biomaterial in human body, fundamental to optimise its efficacy[5,6]. Another important aspect of polyphenols coatings is that the biomolecules film does not modify the surface roughness, with a total absence of variations of surface morphology: it is fundamental because it permits to obtain the desired surface morphology before the functionalisation treatment, generally applied to the medical device as one of the last steps of its production[7]. Anyway, functionalising a surface, a criticality is introduced: a new interface is created. Instead of a single interface (biomaterial-environment one), there are two: biomaterial-polyphenols and polyphenols-environment interfaces. It is necessary to consider this phenomenon while studying the behaviour of polyphenols-functionalised biomaterials: different reactions with different kinetics could have place because of the new interfaces formation and the chemical properties of the bound biomolecules[8,9].

Functionalisation with polyphenols has been studied to be applied to different substrates, thanks to the versatility of the polyphenols themselves: they can easily form films on polymers, glasses, metals and native-oxides, after a sufficient time of incubation (generally few hours). It was observed that gold (Au) and titanium dioxide (TiO_2) are significantly affine to polyphenols solutions, so it is possible to see deposits of tannic acid (TA) and pyrogallol (PG) on their surfaces, whose thickness varies in function of the time of incubation (the thickness of polyphenols film is in the order of dozens of nanometres). Also stainless steel, often used in orthopaedics, and

polytetrafluoroethylene (PTFE), diffused in soft tissue surgery, are possible substrates to easily polyphenols-coat[10]. There are two main categories in which polyphenols-treated biomaterials can be divided. The first class of substrates release polyphenols in the surrounding environment through diffusive processes, letting them reach the tissues around the implant. The biomaterials appertaining to the second class instead do not release the molecules because of stronger bonds: polyphenols, in this case, are bound to the surface through covalent bonds or incorporated or strictly adsorbed to the first atom layers of the biomaterial[11].

Polyphenols coatings are possible thanks to oxidation process: oxidation reactions and fermentation are responsible to high-molecular-mass molecules, less soluble, that so precipitate on the biomaterial surface. This process is facilitated in a mildly alkaline environment (pH around 7.8) in presence of available dissolved oxygen; it is a favourable situation, because very similar to the one that characterises human body environment, so the coatings are in stable conditions also after the implant. Polyphenols coatings maintain the properties of the pure molecules before the treatment. They can efficiently scavenge radical and non-radical reactive oxygen species (ROS), both the ones that are present in the environment around the implant and the intracellular ones, which significantly decrease, comparing it to the substrates without any polyphenols coatings. It means that the cellular response can be modulated by the presence of the polyphenols film, able to work for an intracellular protective effect and, consequently, to limit acute inflammatory response and third body reaction after biomaterial implantation[12].

Another not negligible aspect to consider is the antimicrobial properties of polyphenols coatings, that is maintained after the treatment. Antibacterial activity considers both positive and negative gram, while antifungal and antiviral properties are also possible to see. Polyphenols coatings inhibit the adhesion of bacteria thanks to their antifouling properties, but, if a microbe would adhere to the surface anyway, they are able to destruct it through the synergy of three different mechanisms utilised to contrast biofilm development: first, the environment deprivation of iron ions, fundamental to bacteria to grow; then, the interaction with intracellular enzymes that inhibit bacteria metabolism; finally, the ability to destruct the cellular membrane, through the alteration of protein-to-lipid ratio. In this way, a particularly efficiency is developed to contrast microbial adhesion and proliferation to implanted biomaterials, limiting infections risks, a very dangerous situation that could be after surgery, which, if it becomes chronic, could lead also to fatal consequences[13].

3.2 Functionalisation techniques

Because of the well-known beneficial properties carried by polyphenols through biomaterials functionalisation, surface chemistry has been studied to optimise different functionalisation techniques which could maintain both polyphenols and biomaterials properties after treatment. The synergy of the characteristics of the two elements composing the new interface has to optimise the medical device behaviour after its implantation. Different techniques have been proposed to functionalise biomaterials surfaces: physical adsorption, electrostatic interactions and covalent grafting are three possible methods[14].

3.2.1 Physical adsorption

Adsorption of macromolecules, as polyphenols are, is one of the simplest technique to functionalise a surface[15]. It was observed that polyphenols interestingly interact with amphiphilic lipids, binding to their hydrophilic heads – this phenomenon is called adsorption – while they penetrate into the hydrophobic chains – this is absorption. This ability is due to a high number of hydrogen bonds at the interface: phenolic hydroxyl groups behave as hydrogen bond donors and the oxygen atoms

appertaining to the amphiphilic lipids as hydrogen bond acceptors. This phenomenon can be exploited to coat a surface having amphiphilic lipids-like characteristic or, to obtain a more efficient adsorption, a membrane with analogue properties[16].

Anyway, not all the materials have the necessary affinity to polyphenols to be efficiently functionalised. So, to optimise polyphenols adsorption, it is possible to treat the target surface with specific precursors, such as allylamine, acrylic acid and ethanol, to induce the exposition of certain groups, whose reactivity and affinity to the macromolecules permit a better adsorption: the presence of amine, carboxyl and hydroxyl groups, induced by the previous treatment, in fact are able to activate the biomaterial increasing the efficacy of the polyphenols-treatment[17]. Substrates can also be activated by biomolecules, such as proteins. A recent study has demonstrated that, through a physical adsorption process, it is possible to coat carbon with milk proteins to increase the affinity of the material to polyphenols; in this way, activated carbon is obtained, which is able to physically adsorb polyphenols with a higher efficacy. Activated carbon is characterised by high adsorption performance, surface area and a particular versatility, through which it is possible to adsorb a significant range of polyphenols with efficiency, while the antioxidant activity of polyphenols themselves is maintained[18].

Polyphenols physical adsorption can also be applied to biodegradable materials, even in association with other functionalisations. In recent studies, poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) was previously functionalised with the cell-adhesion-inductive amino acidic sequence Arg-Gly-Asp (RGD) and, successively, treated with epigallocatechin gallate (EGCG) to permit its physical adsorption. A sufficient stability between EGCG and PLL-g-PEG chains and RGD sequences was possible thanks to numerous hydrogen bonds. The efficiency of the system was confirmed by an *in vivo* cells adhesion and a significant antioxidant activity[19].

An interesting application of functionalisation through physical adsorption is the treatment of a suspension of cell wall material with polyphenols extracted from apples. The adsorption of procyanidins on the material is possible immersing it in a solution of procyanidins. Increasing the initial degree of polymerisation, the concentration, the molecular weight, the percentage of galloylation and the presence of (+)-catechin, the number of bound procyanidins increases itself, with a consequent increasing of the efficiency of the treatment. Adsorption is possible thanks to the presence of specific polysaccharides, such as β-cyclodextrin, characterised by a hydrophobic cavity and a hydrophilic outer side: while the polyphenol is physically incorporated into the internal structure of β-cyclodextrin, hydrogen bonds and hydrophobic interactions are formed to have a stable complex[20].

3.2.2 Electrostatic interactions

It is possible to functionalise biomaterials exploiting electrostatic interactions, to not immobilise polyphenols on the substrate, but to let them be released when implanted in human body, that is an aqueous environment rich of ions and electrolytes, able to alter the equilibrium of the coating[21,22].

Electrostatic interaction are often used to functionalise biomimetic membranes. Physical adsorption plays an important role in this application, but electrostatic interactions are also fundamental to guarantee the sufficient stability at the interface between polyphenols and substrate. Biomimetic membranes, constituted by amphiphilic lipids and characterised by a bilayer structure like in nature, can easily interact with polyphenols to be coated by them and to be influenced in their molecular structure by their presence. In fact, it was demonstrated that flavonoids and non-flavonoids have opposite effects on the membrane structure, as successively described[23,24].

So, it was confirmed that there are two types of interactions between polyphenols and membranes: hydrogen bonds and hydrophobic interactions. The combination of them permits the formation of a stable interface, thanks to the synergy of more mechanisms[25]. First, the interbilayer fluid space is three times reduced thanks to the amphipathic nature of polyphenols: it lets a better distribution into the bilayer region possible, and the sufficient longness of the molecules through which it is possible to span the interbilayer interface permits to polyphenols behave as cross-linkers[26]. Analysing specifically chemical aspects, a phenyl ring bearing a hydroxyl group plays a fundamental role in hydrogen bond interactions, behaving both as hydrogen bond donor and as an acceptor, increasing in this way the possible interactions inside the polyphenols-membrane system; this behaviour is promoted in presence of gallate moieties, especially if characterised by cis configuration: it permits also to maintain polyphenols bioactivity with a major efficiency. Furthermore, hydrophobic interactions are due to the planar aromatic nucleus of phenol, that induces Van der Waals interactions through π stacking. The synergy of these phenomena, applied to membrane functionalisation with flavonoids, such as gallocatechin and epigallocatechin-gallate, induces membrane aggregation, decreasing its fluidity and increasing its viscosity[27]. On the other side, the functionalisation with non-flavonoid compounds, such as resveratrol, induces opposite effects: phase separation and formation of liquid-ordered domains causes a significant decreasing of viscosity, increasing instead fluctuation effects and fluidity[28].

3.2.3 Covalent grafting

Functionalisation through physical adsorption and electrostatic interaction permits polyphenols coatings, also if characterised by weaker interfaces, and the possibility of polyphenols release. Covalent bonds are instead stronger and, through them, it is possible to stably anchor biomolecules to a reactive – or previously activated – surface and immobilise them, without the possibility of any release, because of the higher stability of the bonds between polyphenols and the substrate[29].

A typical application of functionalisation through covalent bonds is biodegradable material treatments. Polycaprolactone (PCL) is a common biodegradable polymer often utilised in tissue engineering, appertaining to the family of esters (Figure 3.1)[30].

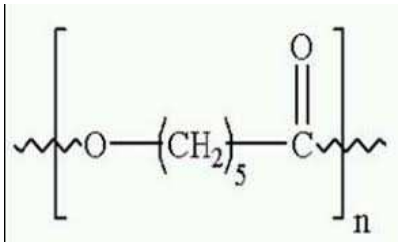


Figure 3.1 PCL molecular structure[31]

It is possible to covalently bind polyphenols to PCL surface to increase its biocompatibility and eventually induce a specific answer in human body after implantation, such as osteoconduction. First, it is necessary to activate the substrate, to let it react with the biomolecules. To do that, vapour phase grafting technique and UV irradiation are utilised: UV radiations induce a controlled degradation of the synthetic polymer to increase its superficial reactivity, while, through vapour phase grafting, it is possible to treat it with acrylic acid (AA) to permit the next functionalisation with polyphenols. Polyphenols – in this specific application resveratrol (RSV) has been chosen – are bound to the activated surface through a condensation, during which two hydroxyl groups – the first appertaining to AA, the second to RSV – react to obtain a covalent bond and a molecule of

water (Figure 3.2). There are two configurations through which RSV can be bound to the surface: the first (Figure 3.2 (A)) is more energetically advantageous, so it is more probable to find at the end of this treatment, while the second (Figure 3.2(B)) is less likely because of the steric hindrance of the substituted phenyl group. So, in this way, it is possible to obtain a functionalised material through hydrolysable covalent bonds; polyphenols are not released thanks to the intrinsic stability of the linkage, but the tissues surrounding the implant can be influenced by them by contact or during PCL degradation process[32].

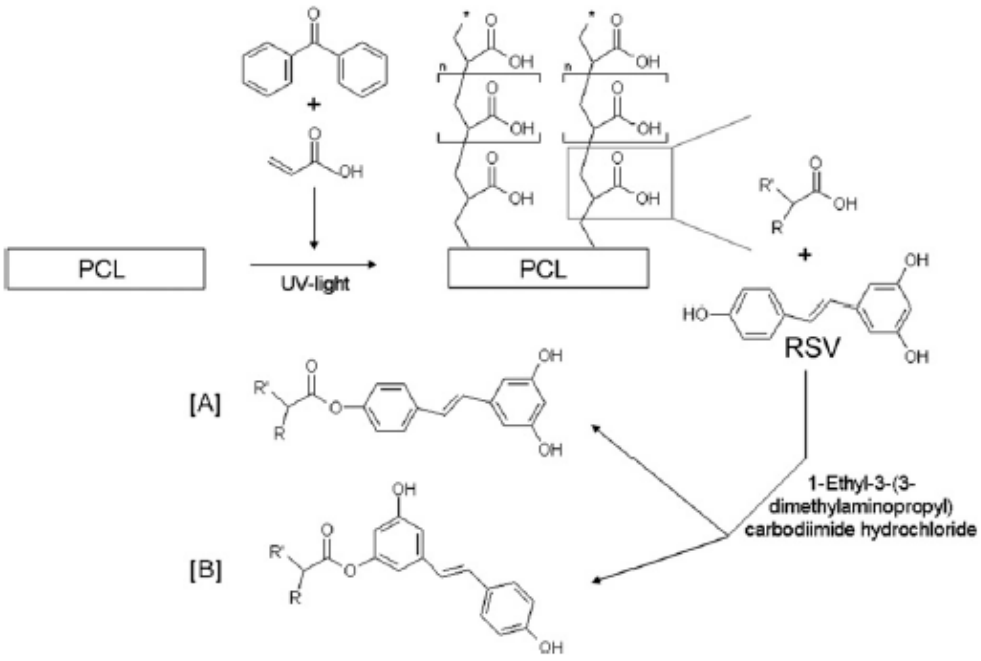


Figure 3.2 PCL functionalisation with resveratrol through covalent binding[33]

Another interesting application of covalent binding is in hydrogels field[34]. Hydrogels are innovative polymeric biomaterials characterised by hydrophilic properties, but unable to be melted in water thanks to its network structure which is due to numerous cross-links previously formed between the chains constituting the polymer itself through covalent bonds. When a hydrogel is immersed in an aqueous environment, because of its high hydrophilicity, it absorbs a large amount of water, but, because of the presence of the numerous cross-links, are not melted nor degradres, but significantly swells absorbing water molecules. So, cross-links are fundamental to obtain the desired properties that characterise this biomaterial. It is possible to induce cross-links in various ways, such as radicals formation in two different chains, which react together to obtain a covalent bond stably linking the two chains[35]. Another more innovative strategy is to exploit polyphenols as linkers to obtain a hydrogel from a liquid polymer. In a recent study, a hydrogel formation at physiological condition was optimised, using a polyethylene glycol (PEG)-based polymer modified with boronic acids (BAs), able to self-assembly *in vivo*. Cross-links are due to the presence of polyphenols, which react to form covalent bonds with the surrounding polymer molecular structures. Different polyphenols have been used, both flavonoids and non-flavonoid compounds, such as ellagic acid (EA), epigallocatechin gallate (EGCG), rosmarinic acid (RA) and tannic acid (TA). It was noted that EGCG, TA and EA give stable hydrogels, while only through EGCG and TA mechanically stiffer hydrogels are obtained. Because of the liquid state in which the polymer is at the initial

conditions and its ability to become a hydrogel under specific conditions, this biomaterial can be applied as an injectable system for tissue engineering. After implantation, in fact, forming cross-links, the liquid precursor changes its viscosity becoming a hydrogel, thanks to the presence of polyphenols and the physiological pH and temperature conditions. In Figure 3.3 the reaction through which cross-links are formed and, consequently, a hydrogel, is illustrated[36].

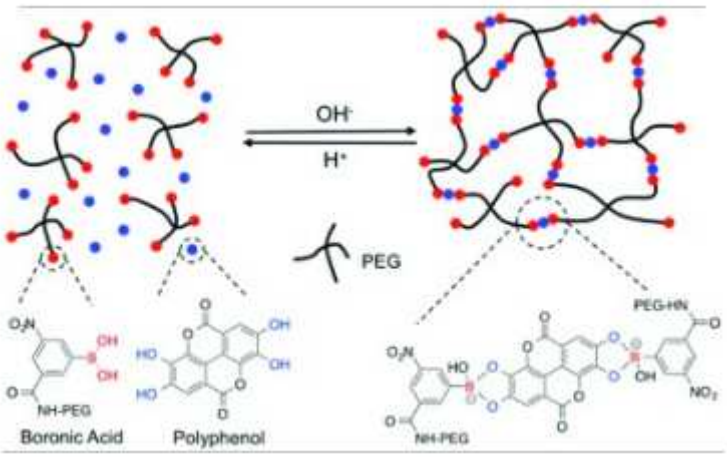


Figure 3.3 PEG-based hydrogel formation with boronic acid and polyphenols[37]

3.2.4 Multifunctional surface modification through dip-coating

Because of the limitation of the precedent techniques, such as the difficulty of multi-step procedures and pre-treatments, new ones have been studied to eliminate the problems given by material-dependence. A new functionalisation method is plant flavonoid-mediated multifunctional surface modification through dip-coating (Figure 3.4)[38].

Figure 3.4 Plant flavonoid-mediated multifunctional surface modification[39]

Dip coating is a simple technique through which it is possible to deposit a layer of any material on the surface of the target substrate. To do that, the substrate is one or more time immersed in a reservoir of liquid, generally a solution, containing the material to deposit[40]. This technique is applicable through five steps: immersion of the substrate in the solution, extraction, deposition, drainage and evaporation[41]. Dip coating permits to obtain layers of different thicknesses, varying the initial parameters: initial reactivity of the surface, immersion time, immersion speed, number of

immersion cycles, viscosity, concentration and temperature of solution and environment humidity. It is a versatile technique, through which the obtained coating is uniform and stable, and it is possible to functionalise various types of materials[42].

So, dip coating is possible to be applied to polyphenol-mediated treatment to easily obtain a high quality layer. Catechol groups which are present in polyphenols structure are exploited to bind the biomolecules to the surface: oxidation of the catechol moiety can induce self-polymerisation and π-π stacking of the polyphenols, generating a final physical assembling of catechins mediated by cation-π interactions. The physical assembling of catechins to form a dimer is reported in Figure 3.5, where it is possible to see how the sodium ion (Na^+), present in the saline solution in which polymerisation is induced, is involved in the bond between the two catechins constituting the dimer. Na^+ presence in the saline solution is fundamental to activate this process; also other cations, such as calcium (Ca^{2+}) and iron (Fe^{3+}), could participate, but with a lower efficiency. The result is a thin layer whose thickness can vary from 2 nm to 15 nm. Furthermore, other bioactive molecules can be immobilised on the surface through the functionality that is improved by the presence of catechols; it can be fundamental in tissue engineering applications, to increase biocompatibility and add specific molecules to stimulate target cells. Then, also silver ions (Ag^+) - whose antibacterial effect is well-known – can be bound to catechol groups, to be successively released to increase the intrinsic antimicrobial effect[43].

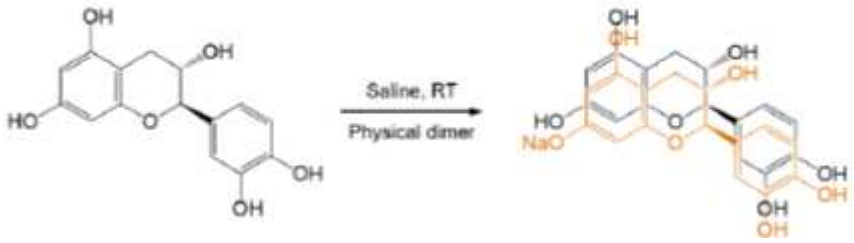


Figure 3.5 Catechin dimerisation in saline solution environment[44]

The polyphenols-coated surface obtained through this technique has interesting properties. First, the distribution of catechins is uniform and homogeneous, without any discontinuities. The roughness of the surface after the treatment does not present any significant variation. Then, the hydrophilicity of the material increases significantly: positioning a drop of water on its surface after functionalisation process, it is possible to verify that the contact angle decreases (Figure 3.6), with a visible spreading, totally absent on the not coated biomaterial[45].

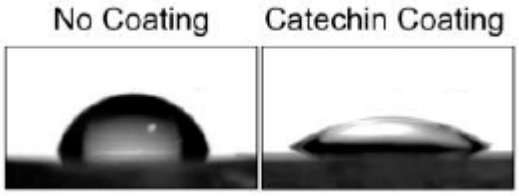


Figure 3.6 Comparison of contact angles before and after polyphenols coating[46]

Finally – but it is not less important – the intrinsic stability of the treated surface is another fundamental advantage carried by this technique. While other techniques, such as the ones based on physical adsorption or electrostatic interactions, generate weakly-bound coatings that are stable only for short times, plant flavonoid-mediated multifunctional surface modification guarantees a long-term stability, which permits a larger application field[47].

Another analogue technique is instead based on hydroxyl groups present in the molecular structure of resorcin-4-arenes. Resorcinarenes are macrocyclic polyphenols generally extracted from plants, characterised by a chain and a cyclic structure, in which hydroxyl groups are exposed. The general formula of these molecules are illustrated in Figure 3.7(A), where it is possible to see the variable chain (R-). R- can have different dimensions: CH_3- as R- gives a C-methylresorcin-4-arene (Figure 3.7(B)), while a longer chain $C_{11}H_{23}$- gives a C-undecylresorcin-4-arene (Figure 3.7(C))[48].

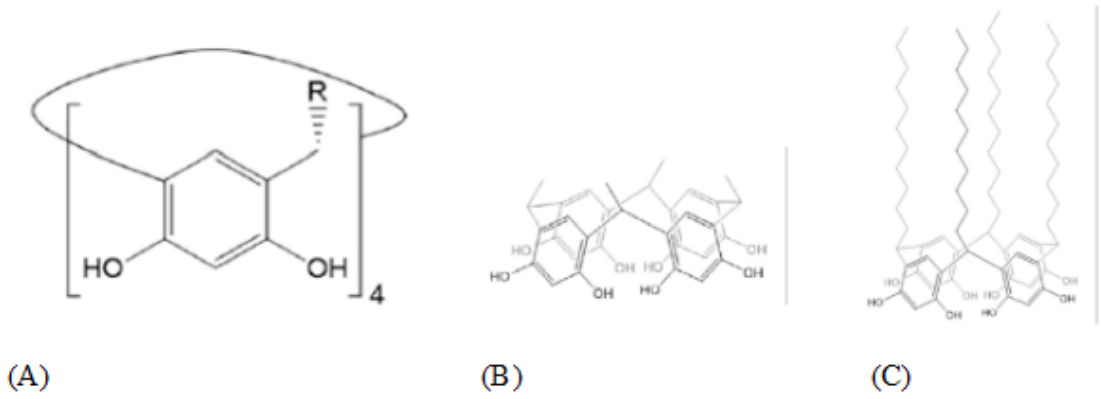


Figure 3.7 Generic resorcin-4-arene (A); C-methylresorcin-4-arene (B); C-undecylresorcin-4-arene (C)[49]

These macromolecules are obtained through an acid-catalysed condensation between resorcinol and an aldehyde, reacting in a solution of ethanol and hydrochloric acid (HCl). The obtained resorcin-4-arenes are deposited on the target surface through dip coating technique. Each molecule is composed by two parts: a head and four chains (R-). The surface-reactive head covalently binds the molecule to the target surface through its four hydroxyl groups as anchoring points; the chains R- will be exposed to the environment to permit to exploit the beneficial properties of polyphenols, that, in this way, are not loosen. The chains can be also used to let the substrate more reactive, letting possible to bind other molecules to the substrate itself exploiting the functionality of the chains R-, if the surface of the material is not compatible to bind or adsorb them: so, the deposited polyphenols layer can be utilised to permit a direct functionalisation of the material with molecules that are not compatible otherwise. In this way, the beneficial properties of polyphenols are available with the ones of the added molecule.
It is possible to apply this process to a large variety of material, equally forming a stable and homogeneous layer on each surface, thanks to covalent bonds, hydrophobic interactions, hydrogen bonds and π-π stacking. Metals, such as stainless steel, aluminium and zinc, polymers, such as polyethylene (PE), polymethyl metacrilate (PMMA) and polydimethylsiloxan (PDMS), and different types of glass, such as amine-functionalised glasses, are compatible with this versatile, single-step and material-independent technique[50].

3.2.5 Polyphenols-mediated functionalisation with bioactive molecules

Proteins play fundamental roles in human metabolism, permitting specific biological processes and catalysing particular chemical reactions which are basilar to maintain homeostasis. Anyway, they are stable only in mild conditions, similar to the ones characterising human body; manipulating them to functionalise biomaterials or for analogue applications, it is so easy denature them, with a consequent loose of functionality, which could inactivate the involved proteins[51].

A new mild technique has been studied to reduce inactivation risk and permit an efficient biomaterial functionalisation. It is possible exploit polyphenols to covalently immobilise proteins to a surface, using an aqueous-processed cross-linked polyphenols coating, capable to interact both with the material and with the protein. Through this method, it is possible to bind different types of proteins, such as immunoglobulin, and to maintain physiological bioactivity of both single and multi-domain enzymes, immobilising them through a stable bond, significantly stronger and more efficient than a non-specific intermolecular one. Multifunctional cross-linkings permit a higher stability and the ability to better interact with proteins without denaturing them. Aqueous-processed cross-linked polyphenols coating, easy to obtain on every surface thanks to the material-independence of this technique, is deposited on nanoporous surfaces, necessary to have a higher area-to-volume ratio to increase the efficiency of the treatment. The most used polyphenols for this application are tannic acid (TA) and catechins, rich of catechol and galloyl groups, fundamental to cross-link the precursors monomer and to have sufficient covalent and non-covalent interactions with the biomaterial surface. The trivalent iron ion Fe^{3+} can play an important role in these interactions[52]. The long-term stability of the obtained polyphenols layer is guaranteed also by the intrinsic stability of the structure in a wide range of pH. Polyphenols are generally stable at acidic and neutral pH, but certain molecules, such as (-)-catechin, (-)-epigallocatechin, ferulic acid and trans-cinnamic acid, can better resist to higher pH. Anyway, human body pH is acidic during inflammation – the conditions in which generally polyphenols are applied – or mildly alkaline – about 7.4 in physiological conditions – so, polyphenols could maintain their stability in medical applications[53].

As cited in the precedent paragraph, also resorcin-4-arenes are utilised to mediate a protein-functionalisation. The simplicity to coat a substrate with polyphenols molecules could be exploited to activate a mildly reactive material, which could not be functionalised with proteins or whose coating would be unstable after biomaterial implantation. The R- chains of resorcin-4-arenes, exposed to the environment as described before, permit to give the necessary functionalities to the material, while the hydrophilic heads constituting the macromolecule are stably bound to the substrate. In this way, a stable complex material-polyphenol-protein is obtained, with a stable interface[54].

3.3 Surfaces functionalisation and clinical applications

Polyphenols application in clinics is a new frontier: antioxidant, anti-radical, antimicrobial, anti-inflammatory properties and other more specific effects, such as anti-atherogenic, anti-thrombotic, anti-hypertensive, cardioprotective and anti-carcinogenic ones, are only the main properties characterising polyphenols. Protection against neurodegenerative disorders is another possible field in which polyphenols are beneficial to human health[55].

One of the possible applications is osteointegration. Polyphenols coatings on titanium alloys (Ti6Al4V) could determine a better and more specific answer from the human organism, when it is necessary to induce integration of a device in bone tissue. In a study conducted in Spain in 2015 it

was demonstrated that a titanium surface has osteogenic, anti-inflammatory and antifibrotic properties *in vitro*, especially when treated with a polyphenols coating. Precisely, quercitrin nanocoating was optimised, able to accelerate stem cells adhesion and their differentiation in mature osteoblasts, with a consequent mineralisation of the cell culture, due to the extra-cellular matrix (ECM) formation and hydroxyapatite precipitation. This phenomenon was explained by the presence of numerous catechol groups, which interact with stem cells to induce their differentiation and their consequent physiological activity. The inhibition of cells differentiation into fibroblasts prevents the formation of the dangerous fibrotic capsule, that would create a weak tissue-implant interface, mediated by fibrous tissue instead of bone tissue. Faster cells adhesion, target differentiation and ECM mineralisation conduce to a better stability of biomaterial-bone tissue interface and tissue regeneration, which is fundamental to guarantee a stable bone implant itself. Another beneficial effect of the quercitrin coating is the ability to inhibit bacteria adhesion and proliferation, reducing the risk of a septic implant failure[56].

The behaviour of polyphenols-functionalised titanium alloy can be optimised with a pre-treatment of the metallic surface. Before the functionalisation, titanium can be chemically treated on its surface to induce the formation of hydroxyl groups. These groups are able, first, to let the surface more efficiently functionalised, and, then, to better interact with inorganic body fluids to obtain an osteoinductive effect, inducing hydroxyapatite to spontaneously precipitate on the surface of the implant for an optimised osteointegration. The successive polyphenols functionalisation, using catechins extracted from *Camellia Sinensis*, is fundamental for their anti-inflammatory, antioxidant and antibacterial effects. In this way, inorganic and biological reaction are combined in a synergic activity to increase the successfulness of the implant[57].

The application of these two strategies – nanocoated titanium alloy and chemically treated bioactive titanium alloy – are applicable to dental sector and orthopaedics, to product osteointegrable dental implants and prosthesis components, able to better transmit loads from biomaterial to bone[58,59].

To promote antibacterial effect, each biomaterial surface could be coated or functionalised with polyphenols. Remaining in orthopaedic field, antibacterial and anti-biofouling properties were observed on tannic acid- treated hydroxyapatite. Hydroxyapatite is often used as a coating for bulk implants or as a bulk material itself, generally porous, to conduct bone regeneration. Because of the inflammation of the tissue around the implant, inevitable after surgery, and of the eventual third body reaction that involves the immune system, bacteria adhesion is a high risk. Tannic acid (TA, Figure 3.8) has been studied for its antimicrobial properties and anti-biofouling effects: it induces cell membrane destabilisation, increasing its permeability and altering protein-to-lipid ratios[60].

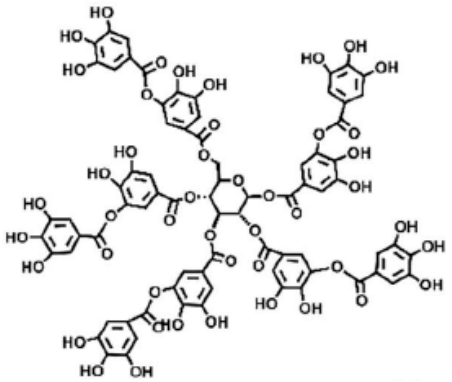


Figure 3.8 TA molecular structure[61]

Furthermore, the well-known hydrophilicity characterising polyphenols can influence the process, inhibiting bacteria adhesion, as water layer theory explains. In fact, the strong interaction between water and a super-hydrophilic surface weaken the interactions between the surface and the hydrophobic proteins, limiting non-specific protein adhesion and, consequently, bacteria activity and fibrotic capsule formation [62]. Anyway, hydroxyapatite functionalisation with TA is not sufficiently efficient, so a strategy was realised to obtain a protein-mediated functionalisation. Salivary acquired pellicle (SAP), a natural layer extracted from teeth surface containing proteins and glycoproteins, is utilised as a mediator to be adsorbed on hydroxyapatite surface and, on the other side, to constitute a strong interface with TA. The so obtained molecular system is called SAP3-TA. The efficacy of SAP to be adsorbed on hydroxyapatite is due to the presence of specific salivary proteins, especially statherin, that, through its acidic N-terminus and its α-helical structure, can easily strongly interact with hydroxyapatite. To better bind TA to SAP, TA can be modified, including three DDDEEKC peptide sequences, though which cysteine reacts with the phenolic hydroxyl of TA itself. In this way, a stable interface is created and antibacterial properties are maintained (Figure 3.9)[63].

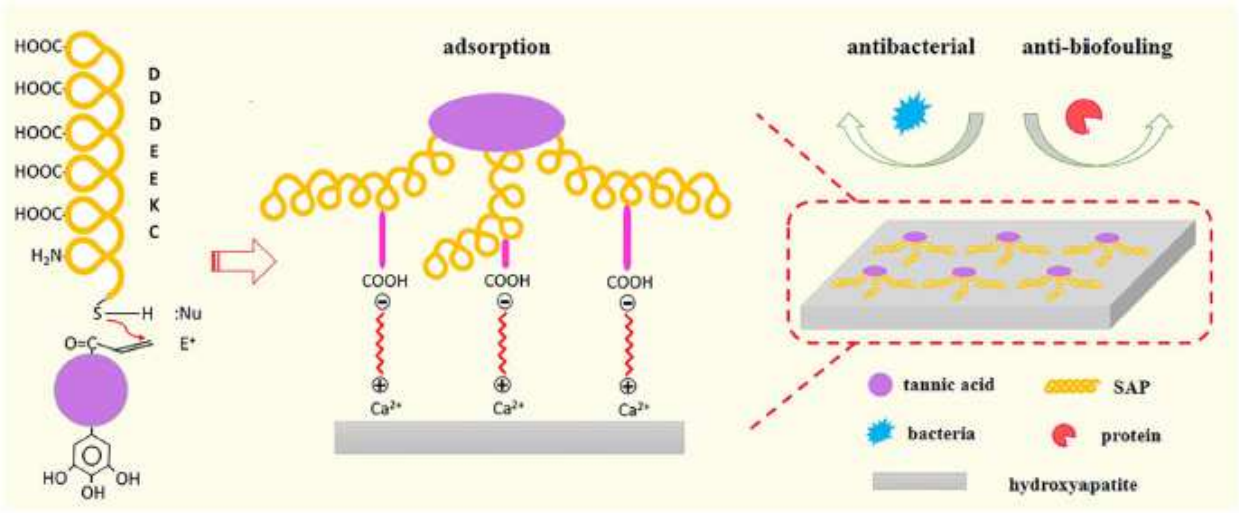


Figure 3.9 SAP3-TA antibacterial and anti-biofouling mechanism[64]

Antifouling properties are fundamental to certain application, both to prevent bacteria adhesion and to inhibit human cells adhesion, if it is necessary to not have any integration with the surrounding tissues. So a technique was studied to deposit an antifouling layer through an efficient material-independent treatment. It was demonstrated that the antifouling properties of zwitterionic materials could be optimised by the presence of polyphenols, especially TA[65]. Zwitterionic polymers, such as polyethylenimine (PEI), in fact have significant antifouling properties thanks to their hydration ability induced by electrostatic interactions and hydrogen bonds, that permit the formation of a thin water layer inhibiting cells adhesion[66,67]. Conforming this new approach, a polyethersulfone (PES) membrane having multivalent anchoring sites is used as a substrate to covalently bind TA, obtaining a functionalised polymer membrane. The homogeneity of the coating is guaranteed by the electrostatic interactions between iron trivalent ions Fe^{3+} and TA molecules, which permit a better adhesion to PES. After that, a reactive zwitterionic polymer, synthesised via the quaternisation of

PEI (Figure 3.10), is post-cross-linked on the layer though dip coating technique at room temperature[68].

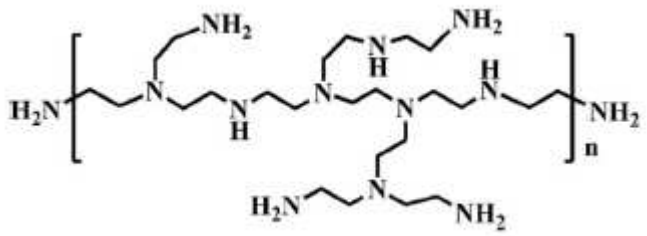


Figure 3.10 PEI molecular structure[69]

TA, rich of catechol and pyrogallol groups, is particularly versatile and applicable to various substrates, reacting with H_2N- and HS-terminated molecules, forming covalent bonds and maintaining its original antifouling properties. So, TA forms a very stable interface with the quaternised PEI, exploiting respectively oxidised catechol and amino groups that are exposed to the environment during the reaction. In this way, an optimised antifouling coating was developed to inhibit non-specific protein adsorption and bacteria adhesion, but also able to carry an anti-thrombotic effect, limiting blood platelets adhesion and, so, permitting the application of this new technology potentially also in cardiovascular field[70].

Concerning cardiovascular applications, another significant one is in anti-thrombotic surfaces generating nitric oxide (NO) as a therapeutic gas[71]. In human body, NO is produced by endothelial cells through oxidation of L-arginine, catalysed by NO synthase, principally to maintain haemostasis and to regulate vasodilatation. Anyway, NO is involved in prevention of thrombosis, leukocyte activation and tissue hypertrophy[72]. Various techniques were tested to produce a NO-generating and NO-releasing coating which was possibly material-independent. Catalytic plant-inspired gallolamine surface chemistry, through which gallic acid could be assembled to proteins exploiting a one-step phenol-amine molecular assembling process, permits a long-term stability of the coating and the ability to control the range of NO release rates. The coating has to incorporate NO donors, such as S-nitrosothiols (RSNO) – generally anchored to the matrix through covalent bonds – and a glutathione peroxidase (GPx)-like compound, such as cystamine (CySA) or selenocystamine (SeCA), having the target to catalysed the decomposition of RSNO in presence of thiols in blood[73,74]. To obtain a material-independent coating having these characteristics, it is possible to exploit polyphenol-based surface chemistry, involving different molecules, especially gallic acid. The versatility of polyphenols easily permits the formation of long-term and stable bonds between gallic acid, RSNO and CySA (or SeCA), through the cited gallolamine surface chemistry, obtaining a homogeneous coating and maintaining the desired NO-generating and releasing properties of the involved molecules. The anti-thrombotic properties are demonstrated comparing a bare metal surface (316L stainless steel) with a treated one with gallic acid and SeCA: analysing the two surfaces with SEM, it is possible to see a significant reduction of platelets adhesion on the coated surface. This result is confirmed by weighting the formed thrombi on the analysed surfaces, demonstrating a relevant difference between the masses adhered to each surface: while the bare surface presents more than 120 mg of thrombus, from the treated one less than 30 mg were extracted, which is about four times minor than the other (Figure 3.11)[75].

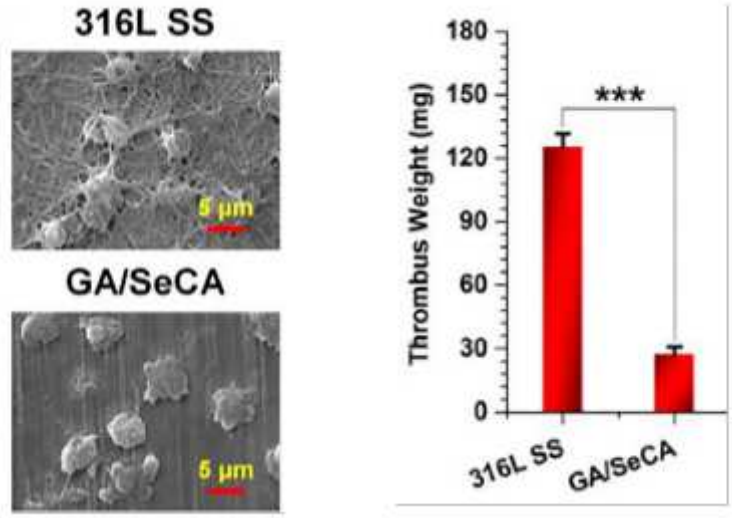


Figure 3.11 Comparing a bare stainless steel surface with a treated one to evaluate platelets adhesion respectively through SEM and weighting the generated thrombi[76]

3.4 Polyphenols application in tissue engineering

Tissue engineering is a largely developing field, thanks to the numerous new biocompatible and biodegradable materials – especially polymers – that have been synthesised. They are generally applied in tissue regeneration, to assist the formation of healthy tissue because of a precedent surgical removal of a damaged one after a wound, trauma or cancer. The material, constituting an implanted scaffold, behaves as the extracellular matrix (ECM) of the type of tissue to treat while it regenerates and, with a dynamic that is compatible with the time of the tissue regeneration, it degrades, releasing biocompatible molecules that the organism will metabolise. During the degradation of the scaffold, it is possible to induce the release also of other molecules, previously bound to the material in different ways – as described in the precedent paragraphs – which diffuse in the tissues surrounding the implant to have beneficial effects[77].

3.4.1 Scaffolds to improve anti-inflammatory effect

An important effect to associate to a device is anti-inflammatory one. After the implantation of a medical device, such as a scaffold, it is inevitable that tissues inflame as the first answer to the contact with an implanted material. This phenomenon is fundamental to the successive device integration, but it has to be controlled, to not induce dangerous chronic inflammation[78]. When a device enters in contact with a generic soft tissue, it often interacts also with adipose cells: adipose tissue inflammation can conduct to metabolic disease, because of immune cell accumulation and excessive inflammatory cytokine production. A new scaffold has been studied to prevent this process, utilising poly(lactide-co-glycolide) (PLG) to produce a porous device, loaded with resveratrol through nanoparticles, released after the scaffold implantation to promote an anti-inflammatory environment. PLG scaffold was fabricated through salt porogen leaching and, successively, resveratrol was loaded in nanoparticles of the same material using a single emulsion and solvent evaporation technique[79]. This technique consists in solubilise both polymer and resveratrol in a common organic solvent and, drop by drop, pouring it in an aqueous environment.

Inducing water phase evaporation, PLG nanoparticle are obtained, in which resveratrol is contained[80]. In Figure 3.12, the PLG scaffold treated with resveratrol is illustrated[81].

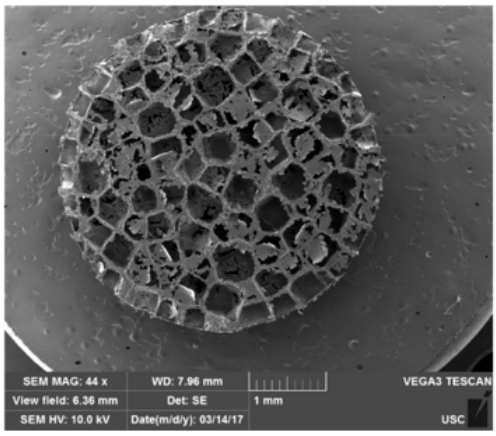


Figure 3.12 SEM image of PLG scaffold loaded with resveratrol[82]

Loading resveratrol on a scaffold is significantly more efficient than on the only particles: its encapsulation is about three times higher. Its release has a biphasic behaviour, as illustrated in Figure 3.13; it is possible to see a burst release over 3 days after the implant, followed by a plateau. More than 60% of resveratrol is released during the first 14 days following the implantation, which are the most critical[83].

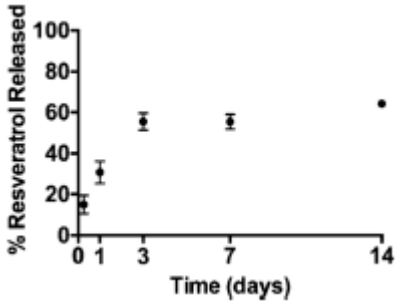


Figure 3.13 Resveratrol release over the first 14 days following the scaffold implantation[84]

After the implantation of this device in soft tissues, it is possible to appreciate a significant decreasing of monocyte and lymphocyte number and the promotion of two cytokines expression, interleukin-10 (IL-10) and interleukin-13 (IL-13), able to promote adipose tissue. Also other factors demonstrate a not negligible reduction, such as the expression of genes that are activated during the local and systemic inflammation characterizing obesity[85].

3.4.2 Scaffolds to modify enzymes catalytic activity

Polyphenols can also be loaded on scaffolds to interact with enzymes, favouring or inhibiting their activity. An example of enzyme inhibition is in cancer therapy, in which a family of enzymes, aurora kinases, are overexpressed. Aurora kinases are involved in mitosis and cell division, characterized by the same catalytic domain, but, on the basis of its function and position, they can be divided in three groups: aurora A (aurA), B (aurB) and C (aurC). After different studies have demonstrated their overexpression in colon, breast, gastric, ovarian and pancreatic tumours, it was thought to inhibit their catalytic activity with a local treatment after tumours removal, incorporating

polyphenols in a scaffold. Polyphenols have in fact the ability to interact with this family of enzymes, inactivating them and, consequently, decreasing the probability of formation of a new tumour. Three polyphenols were demonstrated to be the most efficient for this application: flavones, benzochalcones bearing pyrazoline moiety and chromenylchalcones (Figure 3.14)[86].

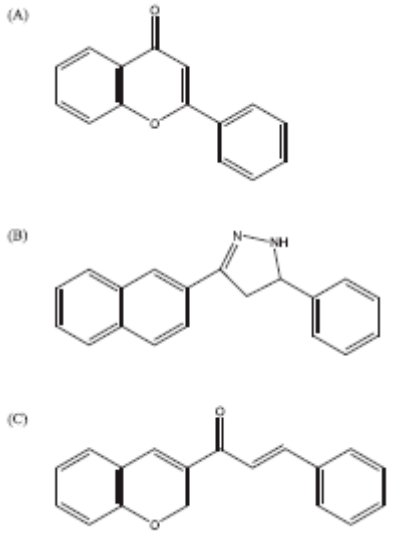


Figure 3.14 Polyphenols inhibiting aurora kinases. (A) Flavone; (B) Benzochalcone bearing pyrazoline moiety; (C) Chromenylchalcone[87]

Starting from these polyphenols, it is possible to add a specific moiety, called N'-methyleneformohydrazide, able to better interact with aurora kinases to inhibit their activity. In Figure 3.15, a modified polyphenol containing N'-methyleneformohydrazide is reported, where R_1 and R_2 are generic aromatic groups[88].

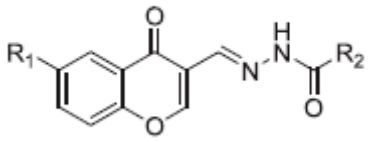


Figure 3.15 A modified polyphenol containing N'-methyleneformohydrazide molecular structure[89]

Loading these compounds on a scaffold, it is possible to implant the so-obtained device in a tissue from where a tumour was removed, to lead the regeneration of healthy tissue in an optimised environment[90].

3.4.3 Scaffolds for dermal applications

Regarding soft tissue, an interesting application of polyphenols-loaded scaffolds is in wound healing. Wound can be caused by an accident or be chronic; anyway, it is possible to surgically treat the damaged tissue and induce its regeneration through the implantation of a scaffold releasing

compounds able to limit tissue inflammation and induce the formation of healthy tissue. There are different strategies to project a device having these requisites. One of them is to obtain a nanoporous scaffold mimicking the dermal ECM through electrospinning technique, using different materials[91,92]. Resveratrol has been chosen for its ability to induce angiogenesis. In fact, from 31% to 53.4% of skin grafts fail for ischemic conditions of the wound, which induce thrombosis and necrosis, because of the imbalance between the metabolic and nutritional demand and the prevailing blood supply. Free radicals due to the peroxidation of cellular membrane lipids, the delay in neovascularisation and the accumulation of degradation products of ECM are the main causes of these failures. Resveratrol can contrast this process through its antioxidant activity; then, the regulation of inflammatory marker proteins, such as interleukin-1α (IL-1α) and interleukin-6 (IL-6), and the influence on gene expression could have important effects. Resveratrol can also control the production of chemokines and cytokines, whose accumulation could induce the failure of the implant. This polyphenol can be carried by a PCL scaffold, in addition to chitosan – a polysaccharide loaded with resveratrol on the same scaffold to improve its biocompatibility – to be released *in situ* and have a local effect; it is necessary because of the speed of resveratrol to be metabolized and its relative low bioavailability[93].

Another strategy utilises ferulic acid in synergy with resveratrol. In fact, while resveratrol limits the oxidant activity of free radicals and promotes angiogenesis, ferulic acid inhibits bacterial activity and promotes endothelial cell proliferation, modulating the productions of specific proteins, particularly vascular endothelial growth factor (VEGF) and nitric oxide synthase. To optimise their release, a core-shell nanofiber was thought; the core is constituted by chitosan, while the shell by PCL. Ferulic acid and resveratrol are both solubilised with chitosan and PCL, to respectively obtain the two solutions for the two components of the nanofiber, and successively extruded through coaxial electrospinning technique[94].

Also the morphology of the scaffold plays an important role in the success of these grafts, thanks to the nanofibrous structure and its consequent interconnected porosity, which mimicks the structure of the dermal ECM: implanting *in vivo* a so characterised device, discontinuities at the interface skin-graft are minimised and cells easily colonize the implanted scaffold[95].

3.4.4 Scaffolds in orthopaedic applications

Resveratrol is a particularly versatile polyphenol, utilised also to treat damaged cartilage and guide its regeneration. Different studies have been conducted to optimise a nano-scaffold releasing resveratrol to assist chondrocytes proliferation in a damaged site. In fact, cartilage degeneration or loss is quite common and difficult to treat because of several conditions: first, the impossibility of chondrocytes to migrate across the defect and fill the gap; then, the difficulty of blood to reach the damaged site; the presence of a single cartilage cell type; finally, the absence of a support on which cells could adhere to proliferate and induce a self-repair process[96,97].

So, to guide the regeneration of the damaged tissue, a biodegradable nano-scaffold has been studied. It was obtained through electrospinning technique[98]. The solution to extrude was composed by poly lactic acid (PLA), natural gelatin and resveratrol. PLA and gelatine were chosen to optimise cellular reaction. In fact, synthetic polymers are characterised by a relatively low hydrophilicity and low affinity to cells, but they could guarantee the necessary mechanical properties that natural polymers could not. So, PLA is mixed to gelatin, a natural polymer easy to extract from ECM of connective tissue. The presence of gelatin in the structure of the fibre could improve the hydrophilicity of the fibre itself, increasing the compatibility with the surrounding tissues and, consequently, the performance of the implant[99]. The solution to electrospin was obtained through freeze drying

technique, followed by uniform dispersion technique. The so obtained solution was electrospun to have a nanoporous structure formed by nanometre-sized fibres randomly organised. It was noted that the system is characterised by good strength and resistance, able to guarantee also the necessary biocompatibility and to biodegrade in times that are compatible with new cartilage tissue formation. After implantation of the scaffold in a cartilage defect, the efficiency of resveratrol as antioxidant, anti-ageing and anti-inflammatory factor was demonstrated, but it behaved especially as a selective activator of the SIRT1 gene, whose down-regulation conducts to different protein-mediated signalling pathways which aggravate the cartilage degeneration in osteoarthritis. So, resveratrol could prevent the tissue degeneration after the therapy, inhibiting the reaching of the conditions which caused the first degradation[100].

Resveratrol could be exploited also to improve hard tissue regeneration[101]. Craniofacial application is a developing field: defects in craniofacial tissues could be due to traumas, cancer resections or congenital abnormalities. When these tissues are injured, inflammatory signal pathways are activated, producing different factors, such as cytokines, chemokines and growth factors, and activating inflammatory cells, releasing hydrogen peroxide and generating reactive oxygen species. Oxidative stress could then ulteriorly damage intracellular components, such as mitochondrial DNA and proteins, and conduct cell to apoptosis with a consequent tissue degradation[102,103]. It is possible to include resveratrol in a scaffold to be released *in situ* to maintain the concentration of intracellular antioxidants and to behave as antioxidant itself; furthermore, regulating SIRT1 gene expression, also in this case it has beneficial effects, protecting bone from degeneration. Resveratrol can be mixed to collagen to obtain an efficient biomaterial to product a scaffold to implant in bone tissue to guide craniofacial tissue regeneration and to physiologically fill the defects, increasing the biocompatibility of the device, inducing the desired bone differentiation of the surrounding adipose-derived stem cells and adding the previously cited properties to the system[104].

Resveratrol could be substituted by epigallocatechin gallate (EGCG) in an analogue strategy. The scaffold is realised in gelatin, whose molecules are cross-linked first through EGCG molecules themselves and successively through a vacuum-heating procedure. Similarly to the just described collagen-resveratrol scaffold, also in this case adipose-derived stem cells are cultured to obtain mature bone cells (Figure 3.16)[105].

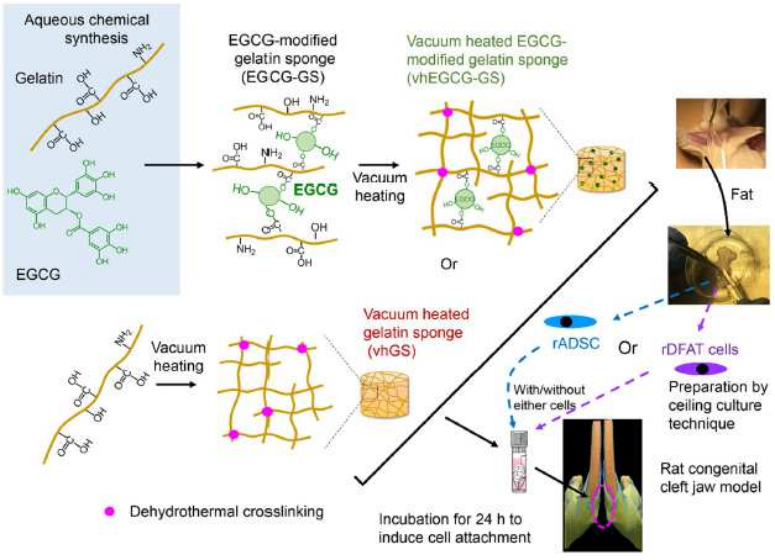


Figure 3.16 Preparation procedure to obtain vhEGCG-GS and cells culture[106]

Through this process, a scaffold mimicking the spongeous bone tissue is obtained. In Figure 3.17 the macroscopic images of these scaffold are reported, with a zoom on the nanostructure visible through SEM[107].

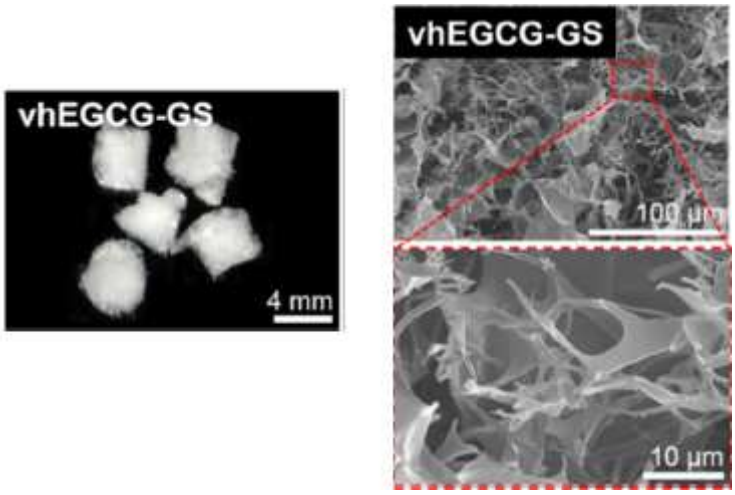


Figure 3.17 Vacuum-heated gelatin sponges modified with EGCG[108]

Though EGCG-modification, this device could increase its hydrophilicity and be enriched by other properties: EGCG induces calcium phosphate precipitation, fundamental to have an osteoinductive effect, and, through zeta potential, it is possible to note a negative surface charge, necessary to improve bone cells adhesion[109,110].
Both the described scaffold for craniofacial application, thanks to the action of collagen and gelatin – respectively – and, especially, of polyphenols, were demonstrated to implement the necessary physiological pathways to guide the tissue regeneration and to let stem cells differentiate into the target cell type[111,112].

3.5 Polyphenols application in bionanotechnology

Bionanotechnology is a recent field, developing in the last twenty years, which includes different applications: construction of nanometre-sized structures, such as carbon nanotubes, drug release through high area-to-volume ratio nanoparticles and functionalisation of nanosheets are only the main ones[113]. Polyphenols could be involved in these application, to be incorporated, immobilised in these nano-devices or released from them in the implantation zone to let therapies be more efficient[114]. Nanoparticles can be classified in two groups: nanoparticles produced with natural biomolecules, such as lipids or carbohydrates, and with synthetic materials, such as glass or metal[115,116,117,118].

3.5.1 Biomolecules-based nanoparticles

One of the most studied application of nanoparticles is for anti-cancer therapies. It is possible to incorporate polyphenols in nanoparticles constituted by different materials to be released *in situ* after their implantation for a mired therapy, thanks to their anti-cancer properties. Solid lipid nanoparticles (SLN) have been one of the first studied systems. Some molecules appertaining to polyphenol class, such as resveratrol and curcumin, are not sufficiently hydrophilic to have the necessary bioavailability when administrated to humans, so it is possible to incorporate them in lipid structures to be better metabolised and be more efficient to treat target cells – in this case,

cancer cells. To make SLN loaded with polyphenols, a possible strategy exploits hydroxypropyl β-cyclodextrin (HPβCD), a hydrophobic polymer, and gelucire 50/13[119]. Gelucire 50/13 is a water-dispersible surfactant able to solubilise and increase the bioavailability of mildly hydrophilic compounds, as resveratrol and curcumin are[120]. The nanoparticles are obtained through probe sonication, followed by freeze-drying; they are in amorphous state and their diameter appertains to a range going from 100 to 150 nm (Figure 3.18)[121].

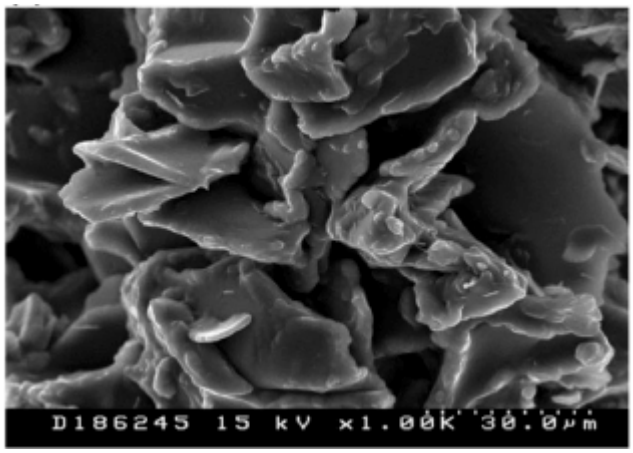


Figure 3.18 SEM image of a curcumin - gelucire 50/13 - HPβCD SLN[122]

A particular advantage of these SLNs is the reduction of the undesired phenomenon of drug burst release: with a more gradual and homogeneous release of polyphenols, their bioavailability significantly increases and their effects are prolonged. In Figure 3.19, the release of pure curcumin and resveratrol is compared with their release exploiting SLN – different measures are reported on the same graph, having all analogue trends. It is possible to appreciate the higher efficacy of the second system, characterised by a constant and more uniform polyphenols release. Furthermore, using SLN all the loaded polyphenols are released, while with other systems of release only a low percentage is available in the surrounding environment[123].

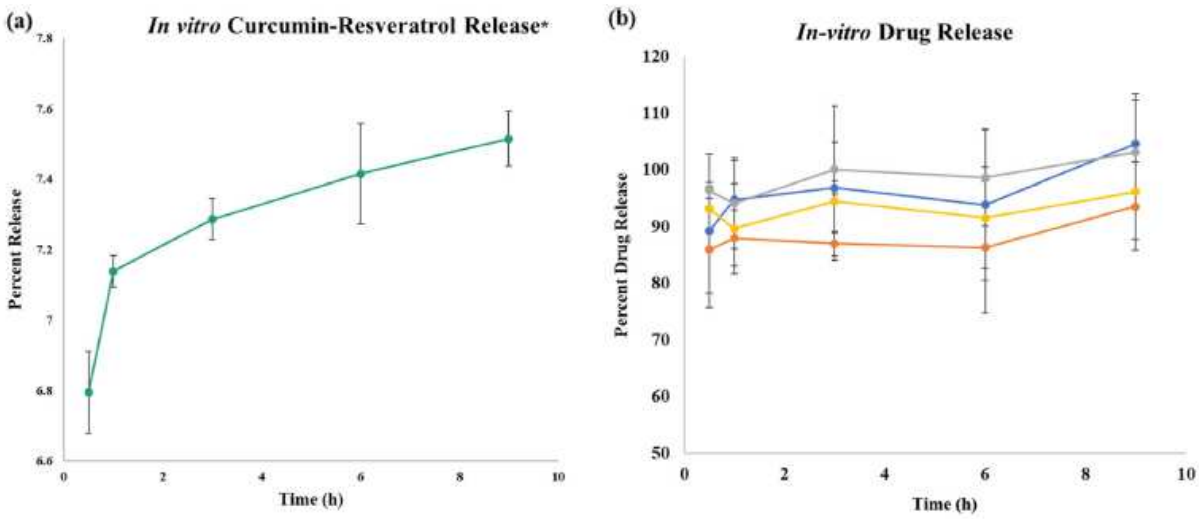


Figure 3.19 (a) Pure polyphenols release; (b) Polyphenols release through SNL[124]

Another interesting application of this device is for cataract treatment[125]. It is one of the main causes of blindness and, with the increasing of life expectation and the ageing of the population, it

is become more and more common. Cataract is due to the opacification of crystalline because of tissue degeneration and protein precipitation[126]. Polyphenols, thanks to their antioxidant and anti-inflammatory properties, could be exploited to treat this pathology, caused principally by oxidant processes. For this application, nanoparticles with a diameter going from 163 to 331 nm were tested, composed by cyclodextrin-based polymer mixed with a specific polyphenols, generally resveratrol, curcumin or dibenzoylmethane, characterised by a low water-solubility. Polyphenols-loaded SLN have demonstrated a significant enhanced penetration and mucho-adhesiveness, which improve the performance of the drug-delivering system. The efficacy of this therapy is demonstrated by a higher antioxidant activity, both *in vitro* and *in vivo*, than using pure polyphenols and by a significant increasing of the level of superoxide dismutase and glutathione *in vivo*[127].

It is possible to exploit lipids also in ethosomes. Ethosomes are phospholipid-based elastic nanovescicles, organised in a double layer containing from 20 to 45% of ethanol, able to interact with the polar head of lipid molecules, increasing the fluidity of lipids in the surrounding environment and the permeability of cells membranes, also diffusing themselves through the membrane pores. They are generally exploited for transdermal treatments, both cosmetic and clinic, because of their ability to interact with lipids and the similarity of their structure with the cell membrane[128]. In 2016 an optimised system to trans-dermally treat malignant melanoma of the skin. It is the deadliest form of cancer, invasive and rapid to metastasise, which has increased its incidence over the last decades. (-)-Epigallocatechin-3-o-gallate (EGCG) was thought to be applied to treat this form of cancer because its strong antioxidant activity, about 25-100 time higher then vitamins C and E. EGCG was encapsulated in nanoethosomes obtained by ultrasonication of a solution containing soybean phosphatidylcholine, ethanol, sugar esters, Tween-80 and, obviously, EGCG itself (Figure 3.20)[129].

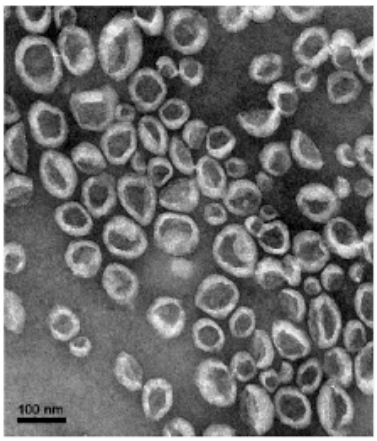


Figure 3.20 SEM image of EGCG-nanoethosomes[130]

Through the so obtained ethosomes, it is possible to have a localised therapy, efficient thanks to the high bioavailability of EGCG given by the encapsulation in a lipid system and to the efficacy of ethosomes in skin penetration. These nanosystems could be exploited also for drug delivery: docetaxel, an antitumor drug, could be added to the initial solution and encapsulated itself with EGCG and successively released with the polyphenol for a stronger therapy[131].

An innovative application of polyphenols in lipid nano-devices is for lyotropic liquid crystal nanoparticles. Dihydromyricetin (DMY) is a hydrophobic polyphenol appertaining to flavonoid class, noted for its anti-inflammatory, antioxidant, anti-microbial and anti-cancer activity. It can be

encapsulated in these crystals to be injected in human bodies to treat or prevent various pathologies. Lyotropic liquid crystals are formed by amphiphilic molecules, self-assembled in a solvent to obtain a multi-phase structure. Thanks to the presence of three different crystallographic phases, lamellar, hexagonal and cubic phases, they are characterised by high viscoelastic properties. These nanoparticles could change their viscosity thanks to phase transition from lamellar to hexagonal phase, transiting from solid-like properties to viscous liquid ones. DMY can interact with them both through nanometre-sized water channel inside the particle itself and in apolar regions of the surfactants. For these causes, this nano-device has a great potential, given by its biological properties and its versatility[132].

Another application of SLN is in infections. In 2019 a study was conducted to find a localised deliver system to contrast vaginal infections and inflammations. A hydrogel was projected to contain liposomes to release polyphenols *in situ,* basing on analogue nanotechnologies already used for cancer therapies[133].

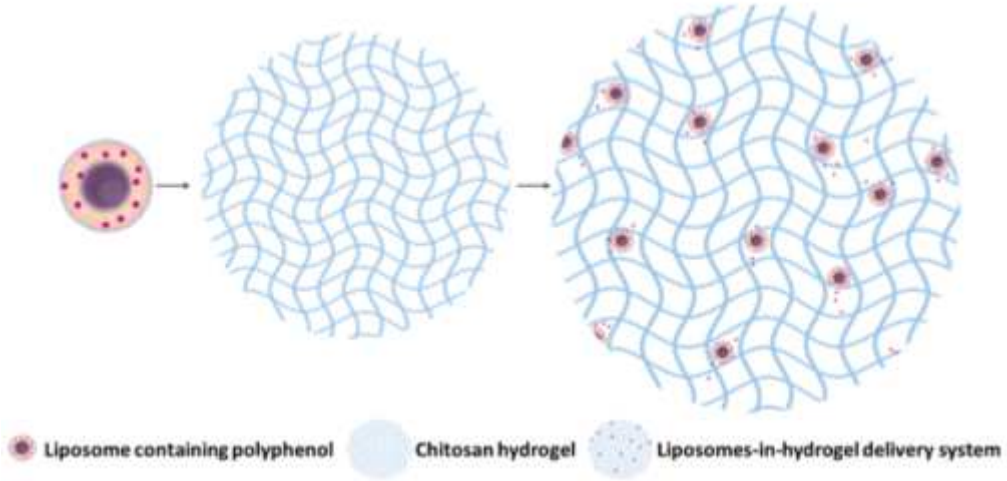


Figure 3.21 Liposomes-in-hydrogel delivery system to release polyphenols *in situ*[134]

In Figure 3.21, the delivery system is represented in its three principal components.

Concerning polyphenols, resveratrol and epicatechin were selected for their important anti-inflammatory effect and a chitosan-based hydrogel was utilised, having itself anti-inflammatory properties. The synergic activity of polyphenols and chitosan increased the beneficial effect of the treatment. These polyphenols, characterised by a low hydrophobicity, were incorporated in the hydrogel through liposomes, used as nanocarriers to increase the efficacy of incorporation. It was demonstrated that, in this way, polyphenols show a higher antioxidant activity than vitamin C and E, scavenging a major quantity of free radicals at lower concentrations. Polyphenols are gradually released in different hours, interacting with diseased cells and so contrasting inflammation[135].

Instead of lipid biomolecules, it is also possible to produce bioactive particles through carbohydrates. Chitosan is a polysaccharide obtainable treating the chitin extracted from crustacean exoskeletons[136,137]. An interesting application of polyphenols-loaded chitosan microspheres is for a parallel treatment to anti-cancer therapies. Tumours are often bombarded with γ radiation, which could damage also healthy tissues. So, the protective, mucho-adhesive and antimicrobial properties

of chitosan could be combined with the antioxidant activity of polyphenols to prevent injuries given by radiation therapies. For this application, polyphenols are extracted from pine cones of *Pinus koraiensis.* The nanoparticles, with a diameter around 100 nm, are obtained through emulsion cross-linking technique: chitosan and polyphenols are mixed with a hydrophobic phase, to obtain a single emulsion (water in oil), and stabilised through the addiction of a surfactant; cross-linking is implemented by glutaraldehyde, which induces the formation of transversal bonds. The beneficial effects given by the injection of polyphenols-loaded chitosan nanoparticles has been demonstrated in an experiment *in vivo* on mice previously exposed to γ radiations: the number of micronuclei in bone marrow cells was significantly lower in nanoparticles-treated mice than in non-treated ones. In Figure 3.22 it is possible to see a bone marrow cell having only two micronuclei: it means that the oxidant activity of radiation on healthy tissues was decreased by the released polyphenols from the chitosan nanoparticles[138].

Figure 3.22 Micronuclei decreasing in bone marrow cells after polyphenols-loaded chitosan nanoparticles treatment[139]

3.5.2 Synthesised nanoparticles

Nanoparticles could be produced by synthetic materials, such as glasses, ceramics or metals, and successively functionalised or treated to include polyphenols. Synthetic materials guarantee a higher durability of the implanted systems, because of their higher stability, both chemical and mechanical, and their lower susceptibility to be degraded through hydrolysis or enzyme-activated processes, as natural polymers are[140,141,142].

Silica is one of the most utilised glasses to produce nanoparticles, thanks to its inertly, versatility and mechanical stability, both *in vitro* and *in vivo*[143]. Furthermore, it is produced through proved and well-known aqueous based synthesis protocols, easy to implement, and it is possible to induce silanes exposure through a simply immersion in an aqueous environment to eventually chemically treat its surface[144]. The nanoparticles could be worked to obtain a mesoporous structure, to increase their area-to-volume ratio and so optimise the interactions with functionalising molecules and with the environment surrounding the implant. A usual application of silica nanoparticles is for drug delivering. Polyphenols could be used to functionalise the nanoparticle, both to protect the encapsulated drug and to be released themselves. Functionalisation is possible through different strategies: physical adsorption, condensation or covalent grafting. Covalent grafting is easily obtainable through the reaction between the carboxyl groups of polyphenols and the amino groups whose presence is induced on the nanoparticle surface. It has been noted that polyphenols iron chelating activity is maintained after silica functionalisation, but their antioxidant capacity mildly decreases. Anyway, thanks to the surface grafting, the interactions between polyphenols and the

proteins that are present in the implantation environment significantly decrease. These interactions would limit the free radicals scavenging activity of polyphenols; inhibiting their formation, it is so possible to have overall the initial antioxidant properties of the molecules grafted on the nanoparticles to obtain an efficient system[145].

Silica could be also be associated to polyethylene glycol (PEG) to better graft polyphenols. PEG has been chosen because of its high biocompatibility and their ability to form complexes, especially hydrogen bonds, with polyphenols, through which their physical adsorption on the nanoparticle is more efficient. PEG is covalent grafted to silica surface through a silanisation process, thanks to the presence of silanes, exposed on the surface of the nanoparticles when immersed in an aqueous environment. In fact, the ethoxysilane moieties of the silanes appertaining to PEG molecules are able to react with the silanes of the nanoparticle, forming Si-O-Si bonds between silica and PEG, which constitute cross-linking structures. After that, through the use of an aqueous ethanol solution in which polyphenols are dissolved, it is possible to let polyphenols adsorb on the prepared nanoparticles with an about ten times more efficient functionalisation. Furthermore, varying the molecular weight of PEG, it is possible to obtain different levels of adsorption capacity. Comparing two samples of nanoparticles, respectively treated with two types of PEG, characterised by different molecular weights (respectively, 2000 and 5000 g/mol), it is shown that, increasing the molecular weight, also the adsorption capacity increases (Figure 3.23). Finally, the antioxidant activity of polyphenols has been evaluated: through DPPH test, it was demonstrated that free radicals scavenging capacity of the adsorbed molecules is maintained after functionalisation[146].

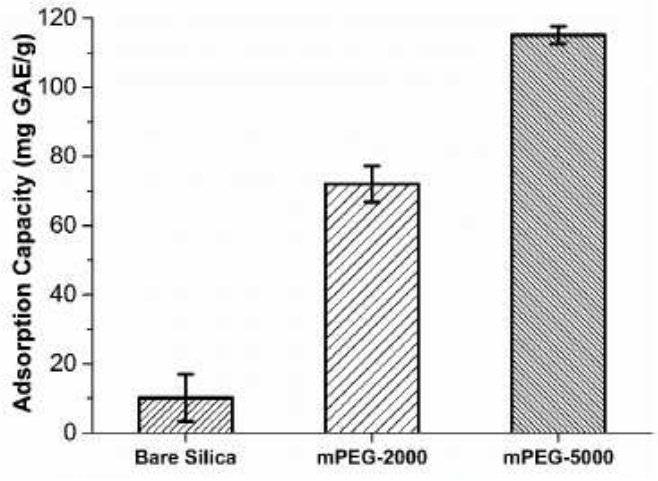


Figure 3.23 Polyphenols physical adsorption of bare silica and PEG-grafted nanoparticles[147]

Ceramics nanoparticles, especially hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$) ones, can be synthesised to be used for bone treatments in orthopaedic applications. HAp as a dispersed phase in a composite material having polymers as the main matrix is one of the most recently studied application. HAp has been chosen for its biocompatibility, bioactivity and osteoconductive properties, but its ability to form homogeneous dispersions in nanocomposite materials with an advantageous energy balance at the interface is also a significant characteristic. It is possible to treat HAp nanoparticles with polyphenols – precisely, grape seed polyphenols (GSP) – to improve bacteriostasis, antiviral and anticancer properties. Functionalisation with GSP, appertaining to the family of condensed tannins, could also improve the ability of nanoparticles themselves to increase

colloid stability in aqueous phase. Functionalisation is possible through a process based on chemical precipitation nanoparticles in an aqueous alkaline environment containing GSP and other compounds able to release calcium and phosphate ions. The chemical reaction through which HAp is formed is the following:

$$CaCl_2 + Na_2HPO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2 + NaCl$$

The producing process is illustrated in Figure 3.24[148].

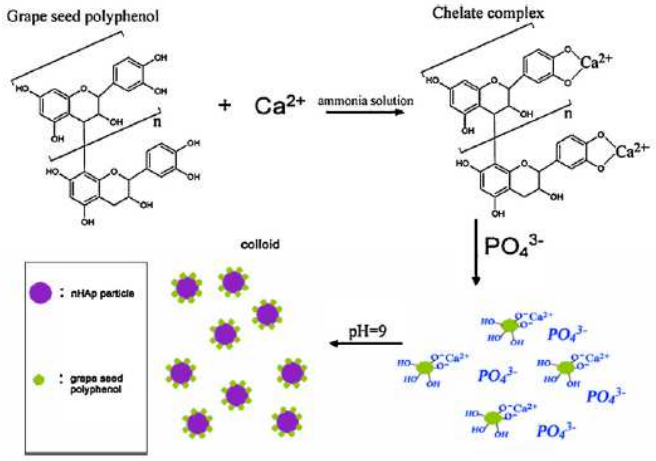


Figure 3.24 GSP-functionalised HAp nanoparticles production[149]

The obtained particles have a diameter around 20-50 nm (Figure 3.25). The ability of GSP to strongly be bound to HAp is due to phenolic hydroxyl groups; -OH groups that are not involved in covalent grafting are available to ulteriorly induce other eventual functionalisations[150].

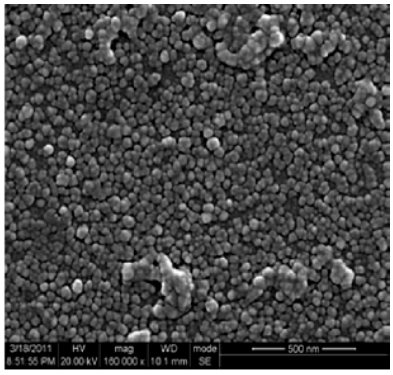


Figure 3.25 SEM image of GSP-functionalised HAp nanoparticles[151]

Another studied synthetic nanoparticle is gold nanoparticle. Gold is one of the first materials to be studied for nanoparticles production since the last decade of XX century, because of its inert behaviour in different environments and its low propensity to oxide – gold is, in fact, a noble metal[152]. Furthermore, gold is particularly stable after implantation, also when carried in systemic circulation. Radiopacity of gold is another fundamental characteristic, through which it is possible to detect the position of injected nanoparticles in human body utilising imaging techniques. Finally, gold nanoparticles could be easily functionalised to implement target therapies against various types

of tumours, both to assist and to potentially substitute chemotherapy. The neologism "nanochemoprevention", born in 2010, refers in fact to the ability of the developing bionanotechnology to improve targeted release of drugs or phytochemicals to treat cancers[153].
Nanoparticles for cancer therapy have been chosen because of their high surface reactivity consequent from the high area-to-volume ratio, through which functionalisation becomes more efficient and lets it possible to graft different types of molecules[154].
Tea polyphenols have been used to functionalise gold nanoparticles to obtain an anti-cancer system to treat liver tumour. Green tea catechins (GT) and (-)-epigallocatechin-3-gallate (EGCG) confer to the system a significant stability, higher cell affinity and a particular antioxidant capacity, which polyphenols do not have if not associated to nanoparticles because of their relatively low bioavailability. In fact, absolute bioavailability of GT and EGCG orally administrated reaches a maximum of 30% of the initial quantity. So, through nanoparticles, the anti-cancer effect of polyphenols is optimised. Different tests have demonstrated the selectivity of polyphenols towards different types of cells, confirming the targeted effects: GT and EGCG have a hepatoprotective activity towards healthy cells, while they are toxic to liver cancer cells, appertaining to the same organ. It is due to the influence they have on various physiological pathways: down-regulation of anti-apoptotic proteins, which are overexpressed in presence of a tumour, is one of the main polyphenol-induced effects[155].

Gastric cancer is another lethal pathology, one of the most lethal cancers all over the world, especially in China, where it is the third tumour causing death. For this application, EGCG grafting was optimised through the addition of fucose-carboxymethyl chitosan to gold to obtain nanocomposite nanoparticles (Figure 3.26)[156].

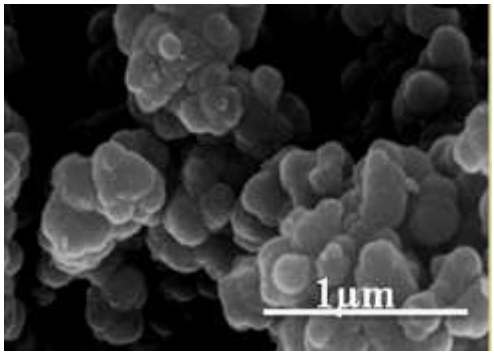


Figure 3.26 SEM image of fucose-carboxymethyl chitosan gold nanoparticles functionalised with EGCG[157]

In this way, functionalisation with EGCG becomes more efficient and mechanical and biocompatibility properties of the nanoparticle itself are increased. Also in this case, the utilisation of nanoparticles consents to peritumorally target the polyphenol therapy and amplify the effects of the pure molecules administration. EGCG induces a higher number of apoptosis in cancer cells, while it protects the surrounding healthy cells which constitute the internal tissues of stomach[158].

Metal nanoparticles could be also utilised to have magnetic effects to be applied as magnetic resonance imaging (MRI) contrasting and in hyperthermia field to deliver drugs or treat cancer. Iron nanoparticles have been studied for decades because of their magnetic properties, but they could be

optimised with a polyphenols surface functionalisation to increase their biocompatibility, conferring to the system antioxidant properties through which iron is chelated. The functionalisation is obtainable in mild conditions, at physiological pH, without using toxic compounds; at the end of the procedure, stable core-shell nanoparticles are obtained, in which the core is constituted by iron and the shell by gallic acid and polygallate. The stability of the interface core-shell is guaranteed by the modalities of functionalisation. First, gallic acid is induced to be adsorbed on the surface of the nanoparticles, immersing them in a gallic acid solution at physiological pH; gallic acid easily interact with the nanoparticle thanks to the presence of catechol groups: the two hydroxyl groups in ortho-position react with iron ion Fe^{3+}, chelating it and forming a stable bond. Then, polymerisation, spontaneous in biological conditions, has place directly on the nanoparticle surface, producing polygallate molecules (Figure 3.27), through the formation of ester and ether bonds between the monomers, permitting the desired stability both at the interface core-shell and intrinsically inside the shell itself[159].

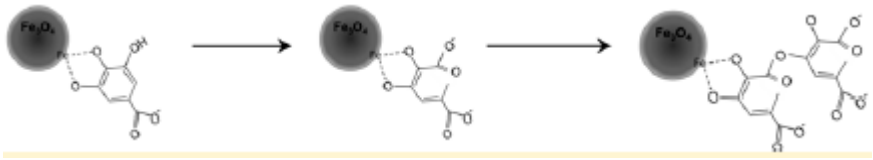


Figure 3.27 Gallic acid polymerisation on iron nanoparticle surface[160]

3.5.3 Nanofibres and nanosheets

Nanofibres are easily obtainable through electrospinning technique, extruding them from a syringe containing a previously prepared solution. They are characterised by a high area-to-volume ratio and are generally exploited to produce membranes or films because of the high porosity obtainable just assembling them. Layer by layer (LbL) is a common technique utilised to assembly thin films to have a multilayer system, whose thickness and composition are easy to control. Polyethylene glycol (PEG) could be electrospun to obtain nanofibres to make successively multilayer films to release polyphenols. Tannic acid – precisely – is included in the nano-device during the LbL step, to create a multilayer structure able to release it during the disassembling of the membrane; during the procedure, tannic acid and PEG are assembled alternating a layer of tannic acid and a layer of PEG, as illustrated in Figure 3.27. The stability of the assembled layers is due to hydrogen bonds, which also permit the successive disassembling after the implantation of the device, which causes the release of tannic acid for therapeutic effects. In Figure 3.28 the section of a PEG nanofibre is illustrated, zooming the PEG/tannic acid coating obtained through LbL technique[161].

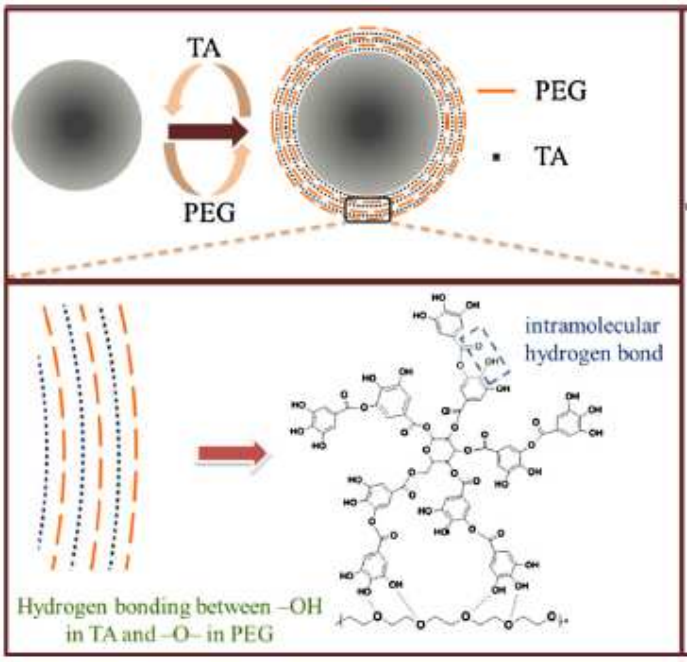


Figure 3.28 Section of a PEG nanofibre coated with PEG and tannic acid assembled layers[162]

Thanks to the reversible nature of hydrogen bonds and the alternated structure of the layers themselves, a gradual release of the assembled layers is guaranteed, which consequently induces a gradual release of tannic acid and a gradual degradation of PEG, which, in this way, could be easily metabolised. This permits to maintain the antioxidant activity of the released polyphenol and to increase its bioavailability for a higher efficiency, without compromising the bulk properties of the structure[163].

An innovative application of polyphenols is for nanosheets production. Nanosheets are nanometres-sized structure able to improve drug releasing, thanks a synergic effect of chemotherapy, photothermal therapy and antibacterial effect in association with near infrared (NIR) radiations. Transition metal dichalcogenide (TMD) is utilised to produce nanosheets for anticancer applications. Anyway, to optimise the production process, facilitating the exfoliation and the drug loading, polyphenols are exploited. TMD is treated with a top-down approach, through which the bulk material is exfoliated in an aqueous environment; the solution is sonicated to facilitate this process, obtaining TMD monolayers or few-layers aggregations. Adding polyphenols to the solution, it is possible to limit nanosheets aggregations after their formation, letting also possible to increase the final concentration of nanosheets themselves, maintaining anyway the same properties: polyphenols in fact behave as stabilisers, decreasing electrostatic interaction between the sheets of the exfoliated material. The production process is illustrated in Figure 3.29[164].

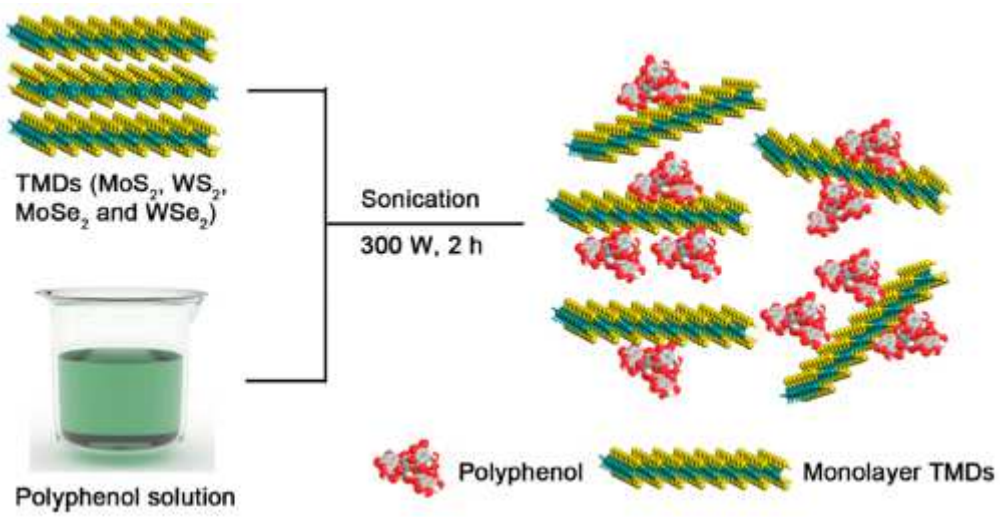


Figure 3.29 TMD nanosheets production process[165]

Different polyphenols have been tested for this application, such as epicatechin gallate, epigallocatechin gallate, epigallocatechin, tannic acid and tea catechins, to demonstrate the versatility of the technique. As TMD, molybdenum disulfide (MoS_2) has been used. These nano-devices could be utilised as drug nanocarriers for their high area-to-volume ratio – drugs loading could be so more efficient – but also to regulate antibiotic release exploiting their photothermal effect, increasing the penetration of the drug in biofilms for a more efficient antimicrobial treatment, with the synergic action of polyphenols antibacterial activity[166].

3.5.4 Nanometre-sized biosensors to detect polyphenols

Glassy carbon electrodes (GCE) are generally utilised to quantify the presence of polyphenols in a solution. Anyway, to increase the sensitivity of this detection method, a new electrochemical biosensor was developed, importing a modification through a dispersion of carbon nanotubes (CNTs). Single-walled CNTs have been functionalised with polytyrosine to obtain a homogeneous dispersion to apply to GCE. The functionalisation is possible immersing CNTs in dimethylformamide to be activated and successively treated with polytyrosine: the protein reacts with the activated functional groups of the nanotube to form a covalent bond between the two compounds. The polytyrosine-functionalised CNT molecular structure is reported in Figure 3.30[167].

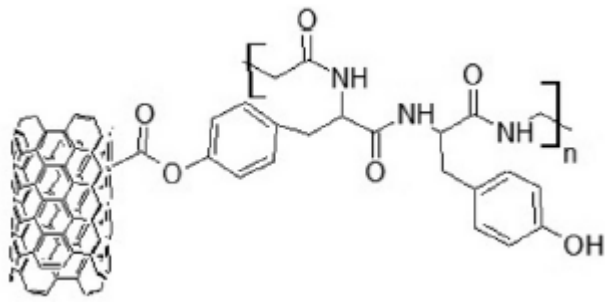


Figure 3.30 CNT covalently functionalised with polytyrosine[168]

Polytyrosine has been chosen because of its amphiphilic properties. Pure CNTs, in fact, without any other treatments, agglomerate each-others through Van der Waals interactions or π-π stacking,

decreasing their availability and their potential efficacy for application in biosensors field. So, polytyrosine is exploited as a stabiliser and dispersing agent: through its amphiphilicity, it could interact with CNTs limiting the formation of agglomeration inducing an efficient dispersion of the nanostructures. These conditions permit a better interaction between this system and the polyphenols to detect, whose measured quantity is expressed in gallic acid equivalent (GAE)[169,170].

3.6 Other applications

In the last years, because of their antioxidant properties, polyphenols have been studied to be applied in orthopaedics, not only to promote osteointegration or limiting bacterial activity in the tissues surrounding the implant, as described in the precedent paragraphs, but also as stabilisers of ultrahigh molecular weight polyethylene (UHMWPE). It is a polymeric material characterised by a high density – around $5*10^6$ g/mol – utilised for total joint replacement applications. The two most frequent applications in this field are for the tibial insert of the total knee replacement, to be positioned between the tibial and the femoral components, and for the total hip replacement, to make the polymer cup to be positioned between the metal back and the head of the femoral component (Figure 3.31)[171,172].

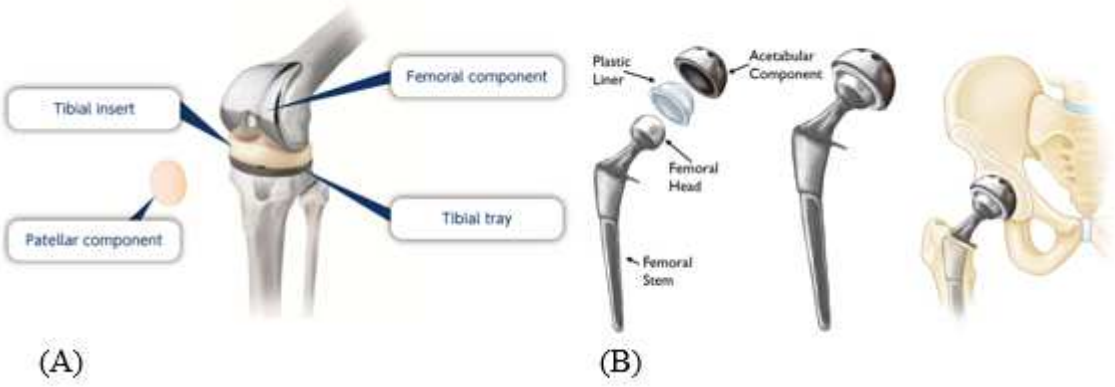


Figure 3.31 Components of (A) total knee replacement[173] and (B) total hip replacement[174]

UHMWPE is a stiff polymer, but it could be damaged during sterilization process, generally obtained through γ irradiation: the aggressiveness of these rays and its oxidant power induce radical formation on the polyethylene chains, with a consequent decreasing of the necessary molecular weight to be applied in arthroprosthesis. Polyethylene characterised by lower molecular weight is in fact not sufficiently stiff to be loaded with the tensions normally reached in a lower limb joint[175]. The implantation of a not sufficient stiff material certainly causes friction and usury, with consequent debris formation, which conducts to ulterior usury: the final result will be the failure of the implant. To limit this undesirable phenomenon, different strategies have been planned. The first was a thermal stabilisation process after sterilisation, through which the residual free radicals are induced by the high temperatures to react together to obtain cross-links between the chains of different polyethylene molecules and, so, to have a final stronger material[176]. Successively, it was thought to treat the sterilized material with antioxidant molecules, such as vitamin E, to scavenge the residual free radicals: the results were promising, but not particularly efficient. Polyphenols were tested to be used for this application, obtaining interesting results: the radical scavenging power was significantly higher than vitamins one and the material demonstrated the sufficient

stiffness to satisfy the mechanical requisites and the intrinsic properties derived by the presence of polyphenols[177].
Different types of polyphenols were tested: epigallocatechin gallate (EGCG), lipid-soluble tea polyphenols (lsPPT) and lipid-soluble epigallocatechin gallate (lsEGCG). The particular efficiency of these biomolecules is due to hydrogen donation phenomena. The presence of multiple phenolic hydroxyl groups inside the single molecules, characterised by a very high antioxidant power, permits the donation of hydrogen ions to scavenge the residual free radicals of sterilised UHMWPE. The higher efficacy of polyphenols is explained by the number of phenolic hydroxyl groups, higher than in vitamin E, which has only one per molecule, as reported in Figure 3.32[178].

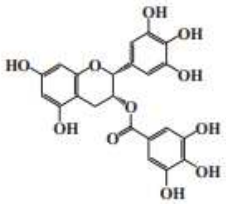
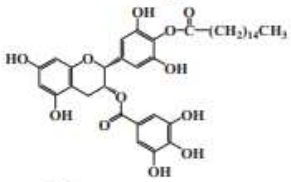
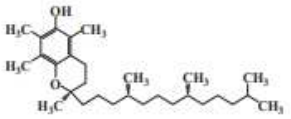
Abbreviation	Molecular formula	Chemical structure	Numbers of phenolic hydroxyl groups
EGCG	$C_{22}H_{18}O_{11}$		8
*ls*EGCG	$C_{38}H_{48}O_{12}$		7
VE	$C_{29}H_{50}O_2$		1

Figure 3.32 Comparison of the molecular structure of polyphenols and vitamin E and evaluation of the respective content of phenolic hydroxyl groups[179]

So, polyphenols have demonstrated to have different potential applications, in various fields, from diseases prevention to tissue engineering, from cosmetics to cancer therapies. Various researches are carried at the moment to study and deepen the numerous beneficial properties and the way to exploit them in medicine and biomedical field, to limit the use of invasive drug and treatments which have potentially dangerous collateral effects and ideally substitute them with natural biomolecules. Now, clinical applications are limited, but the optimal results of the actual studies are particularly promising and in the next decades the application of polyphenols in clinical practices will be ordinary[180,181].

1

Jung Seung Lee, Jong Seung Lee, Min Suk Lee, Soohwan An, Kisuk Yang, Kyueui Lee, Hee Seok Yang, Haeshin Lee, Seung-Woo Cho, “Plant flavonoids-mediated multifunctional surface modification chemistry: catechin coating for enhanced osteogenesis of human stem cells”, Chemistry of Materials, 2017

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CHAPTER 4
MATERIALS AND METHODS

4.1 Introduction

This current research is contextualised in an International project: NAT4MORE (NATural molecules on the surface of bioactive materials FOR MOdulating the host REsponse to implants), involving European and South American universities, finalised to better biomaterials behaviour after their implantation in human body. Titanium, hydroxyapatite and two different types of bioglasses are superficially treated with natural molecules to add peculiar surface properties maintaining the specific characteristics of the starting materials. Italy, Iceland and Brazil are cooperating to study the behaviour of these substrates, analysing the efficacy of the applied treatments, the biocompatibility and the cellular response. The natural molecules used to functionalise the substrates are a chitosan-based polymer and polyphenols: at the interface between the implant and the human tissue, they are capable to beneficially interact with human cells and decrease the probability of implant failure.

The target of the work carried on in the Italian Athenaeum is to functionalise different biomaterials with polyphenols, characterise them, compare their behaviour to the one of the pure materials and study their properties. This, because, as diffusely illustrated in the precedent chapters, polyphenols are discovered to have interesting and beneficial properties for human health. Their antioxidant, antibacterial and anti-inflammatory power can be exploited in implantology applications: the mechanical and chemical properties of bulk materials are paired with the chemical and biological properties of the superficial film of polyphenols to obtain a better biomaterial to implant in human body, that could reduce inflammatory response and bacteria adhesion. In this way, the life-time of implants could be increased and the percentage of failed ones will be reduced.

Three different substrates are investigated:

- Hydroxyapatite (HAp), industrially sintered or in laboratory;
- Chemically treated (CT) titanium alloy (Ti6Al4V): before its functionalisation, it is double etched, as illustrated in the next paragraphs, to obtain a porous and biocompatible superficial oxide;
- Bioglasses: two types of bioglasses are studied, SCNA and SCNbis, characterised by different oxide composition and surface reactivity, as will be successively described.

4.2 Polyphenols

4.2.1 Extraction

The polyphenols (PPHE) which are used for this application are extracted from red grape pomaces (Barbera type). This process is implemented by CREA, an Italian research center in Asti, Piedmont, Italy, involved in the project, from which they are acquired. By the protocol, peel, seeds, stalk and pulp have to be separated; seeds, stalk and peel are successively treated in a heater at 60°C for 36 hours to be dried[1]. Then, to obtain a higher surface-to-volume ratio, they are ground in a ceramic mortar: in this way, a higher surface will be exposed to solvents to increase the efficacy of the treatments which will follow. Polyphenols were extracted by conventional solvent extraction in a water:ethanol (50:50) mixture. After ethanol evaporation, the extracted biomolecules, to guarantee a better conservation, are then lyophilized (Figure 4.1).

Figure 4.1 Lyophilised polyphenols

So, not a single, specific type, but a mix of polyphenols is obtained. It could present residues of carbohydrates: in fact, to not use aggressive processes which could damage biomolecules, it is impossible to remove completely all carbohydrates during the process of purification of polyphenols: this situation is preferred to a high percentage of denatured molecules at the end of extraction caused by the aggressiveness of the treatment.
The extraction process is here reported.

1. 10 g of grape peel are weighted;
2. 100 ml of 50% v/v ethanol water solution are prepared: 50 ml of ethanol and 50 ml of water;
3. The dried and ground peels are mixed with the solution and introduced in containers with screw cap with a solid-to-liquid ratio of 1:20;
4. The containers are put and agitated in a thermostatic bath at 60 °C for an hour;
5. The solutions are filtered and put on a Petri dish in an incubator at 37°C to let ethanol completely evaporate to obtain the polyphenols extract;
6. The obtained and extract polyphenols are suspended in water, freezed and put in a lyophilizer for 48 hours: in this way the final polyphenols mix is obtained, that will be used for biomaterials functionalization[2].

4.2.2 Preparation of polyphenol solutions

To functionalise the materials, they are immersed in polyphenol solutions. Preparing them, it is necessary to keep attention to some aspects.
First, polyphenols are very sensitive to pH. They conserve their properties to acid pH, ergo lower then 7.4: it is so necessary to work much below this threshold and, if it will be not possible – like it will be explained -, these biomolecules are solved in solutions characterized by pH around this value or, possibly, slightly lower. In fact, over pH=7.4, polyphenols start a time-depending degradation process, whose speed increases with the increase of pH values. Working with HAp and bioglasses, a critical situation could be reached: because of high surface reactivity of these materials, significant ion release can happen upon contact with solutions with consequent pH increase. This point should be taken into account and controlled tailoring solution composition as well as exposure time.
Another significant characteristic of polyphenols is their propensity to oxidise if exposed to visible light and to UV radiations. It is necessary to keep these molecules in dark containers to protect them

from radiations and, while working with solutions containing polyphenols out of their containers, their exposure to light has to be as shortest as possible.

The functionalising solutions are the following:

- ultra-pure water with polyphenols (5 mg/ml) – H_2O+PPHE;
- ultra-pure water and ethanol 50% v/v solution with polyphenols (5mg/ml) – H_2O+EtOH+PPHE;
- TRIS/HCl buffer with $CaCl_2$ (292 mg/l, as in simulated body fluid solution) solution with polyphenols (5mg/ml) – TRIS/HCl+$CaCl_2$+PPHE.

The first two solutions are applied to functionalize HAp and bioglasses, while the third is applied to CT.

The first two different solutions (H_2O+PPHE and H_2O+EtOH+PPHE) are prepared to treat the same material for a comparison to optimise the functionalising protocol. Not all polyphenols are, in fact, soluble in water: so, the extracted mixture is not completely solved in the first solution and particles of polyphenols are visible. To obviate to this problem, a second solution containing ethanol is tested, because some of polyphenols are soluble in this alcohol. The behaviour of polyphenols in this two solutions will be compared to find the more efficient functionalising method.
It is necessary to work under a chemical hood to reduce the dispersion of vapour and volatile substances, especially when manipulating ethanol.
The water and polyphenols solution is prepared in this way:

1. The desired volume of ultra-pure water is spilled in a beaker;
2. The necessary quantity of lyophilised polyphenols (5 mg/ml) is weighted and added to the spilled water;
3. To make the solution more homogeneous, it is put under magnetic stirring for an hour;
4. The pH of this solution is acidic (3.45±0.1).

The water, ethanol and polyphenols solution has instead a more articulated preparation:

1. The desired volume of ultra-pure water is spilled in a beaker;
2. The same volume of ethanol is added;
3. The necessary quantity of lyophilised polyphenols (5 mg/ml) is weighted and added to the just obtained solution;
4. To make the solution more homogeneous, it is put under magnetic stirring for an hour;
5. pH is increased to 7.5, buffering the functionalising solution with a NaOH solution 0.05 M, adding it drop by drop, until the desired pH value is reached.

The last step is fundamental to optimise the following functionalisation process and is done to activate the hydroxyl groups of ethanol: increasing pH value, they deprotonate, becoming more reactive. In this way, the probability of interaction between polyphenols and the substrate is significantly increased.

The third solution – TRIS/HCl+$CaCl_2$+PPHEsolution – is instead applied to functionalise titanium. It has been chosen in order to exploit the ability of Calcium ions to mediate the grafting of polyphenols to the titanium surface through the formation of complexes [3, 4]. Tris(hydroxymethyl)aminomethane (TRIS) is an organic compound added to the functionalising

solution because of its buffering action: after its addition, pH, having an extremely acid value (2.0±0.1), reaches the desired value, 7.4±0.1, that is the physiological pH.
The necessary steps to produce 1000 ml of TRIS/HCl+$CaCl_2$ solution are the following (adapted from the protocol for the preparation of SBF[5]):

1. Weight 0.292 g of $CaCl_2$;
2. Weight 6.118 g of TRIS;
3. Spill 700 ml of ultra-pure water in a plastic beaker;
4. Left it some minutes in the incubator at 37°C to reach temperature T=(36.5±1.5)°C;
5. Working on a magnetic stirrer, pour in the water first the weighted quantity of $CaCl_2$;
6. Spill 39 ml of 1M HCl;
7. Ascertain that the volume of the solution is 900 ml and, if it is not, add the necessary quantity of water to reach this value;
8. With a pH-meter, ascertain that pH is 2.00±1.00;
9. Pour TRIS in the solution till pH=7.45±0.01 and stop when the condition is reached;
10. Add, drop by drop, a 1 M HCl solution till pH reaches 7.42±0.01;
11. Repeat Step 7 and Step 8 till all the weighted TRIS has been utilised;
12. Add, drop by drop, the 1 M HCl solution till pH is 7.40±0.01;
13. Remove the pH-meter and pour the water utilised to wash it in the just prepared solution;
14. Let the obtained solution reach a temperature of (20.0±0.1)°C;
15. At this temperature, add ultra-pure water to have a total volume of 1000 ml.

This solution has to be conserved in a refrigerator at 5-10°C and has to be utilised within 30 days from the moment it has been prepared.
To obtain its relative functionalising solution, TRIS/HCl+$CaCl_2$+PPHE, with a concentration of 5 mg/ml of polyphenols, the necessary quantity of lyophilised biomolecules is weighted and added to TRIS/HCl+$CaCl_2$ solution. It has to be left 1 hour on a magnetic stirrer to let polyphenols be dissolved in the solution. The pH of this solution is 7.4.
It is fundamental to manipulate polyphenols in dark containers to limit UV-visible radiations aggression: these molecules are quite sensitive to these radiations and can be damaged or degraded, losing their properties. If dark jars are not available, it is necessary to cover transparent ones with aluminium film to protect the contained solution.

4.2.3 Variations to functionalising solutions

To optimise the functionalising solutions, some variations were ported, both to volume percentage of ethanol and to polyphenols concentration.
The first regards the relative quantity of ethanol and water. Six different types of polyphenols solution were prepared and compared:

- 0% EtOH – 100% H_2O (pure water)
- 20% EtOH – 80% H_2O
- 40% EtOH – 60% H_2O
- 60% EtOH – 40% H_2O
- 80% EtOH – 20% H_2O
- 100% EtOH – 0% H_2O (pure ethanol)

The necessary steps to obtain each of these solutions are the following:

1. The desired volume of ultra-pure water is spilled in a beaker;

2. The proportional quantity of ethanol is added;
3. The necessary quantity of lyophilised polyphenols (5 mg/ml) is weighted and added to the just obtained solution;
4. To make the solution more homogeneous, it is put on a magnetic stirrer for an hour;
5. pH is increased to 7.5, buffering the functionalising solution with a NaOH solution 0.05 M, adding it drop by drop, until the desired pH value is reached.

Analysing and characterising these solutions, as it will be discussed in Chapter 5, it is possible to note eventual differences in their behaviour and the eventual different solubility of polyphenols in them.
The other ported variation in functionalising solutions is the concentration of polyphenols. To characterise the biomaterials – if not otherwise specified – the 5 mg/ml solution is used, but also 1 mg/ml one was tested. The process through which the 1 mg/ml solution is prepared is the same of the previous one for each of the cited solvents, with the only difference consisting in polyphenols concentration. A lower concentration has thought to be tested to verify the necessity to use a higher quantity of molecules to functionalise biomaterials and to comprehend if the concentration itself could influence the efficiency of the biomaterial to interact with polyphenols.

4.2.3 Uptake solutions

The described solutions are applied to functionalise the four biomaterials which are analysed in this study. Functionalising protocol is in detail described for each material in the following paragraphs. Anyway, at the end of the treatment, the solution that was utilised to functionalise a certain sample is extracted. It is called uptake solution. Similar to the functionalising one, it is characterised by eventual modifications in the composition due to its interactions with the biomaterial during the chemical process. In fact, during the treatment, diffusion phenomena, ionic exchanges and other interactions have place between polyphenols and their solvent and the biomaterial surface. To evaluate these potential chemical variations, the uptake solutions are considered different solutions from the ones before functionalisation and, so, newly analysed.

4.3 Hydroxyapatite

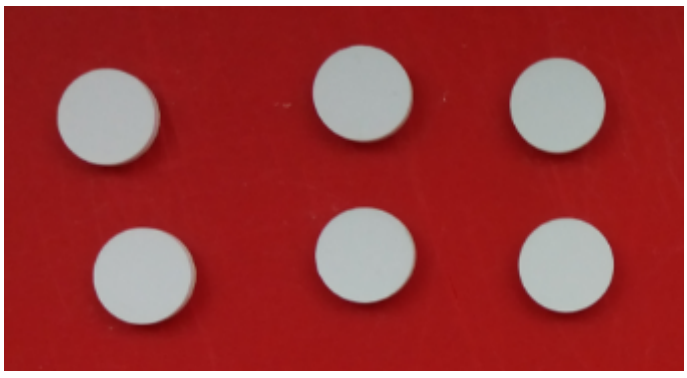

Figure 4.2 Hydroxyapatite samples

Hydroxyapatite (HAp) is the main inorganic component of the bone extracellular matrix. It is a double salt appertaining to the apatite family, having a crystalline reticule characterised by a hexagonal elementary cell. The bone can synthesize it to form the hard phase of its extracellular

matrix, fundamental to let the tissue have the necessary mechanical properties to sustain the load graving on skeletal structure.
Its chemical formula is the following:

$$[Ca_{10}(PO_4)_6](OH)_2$$

HAp is often utilised in biomimetic devices, especially in orthopaedic and dental applications. In fact, after the implant of a device covered with HAp, the surrounding bone has a contact first with this phase, that the bone recognize as part of the tissue itself: this situation decreases the inflammatory response and stimulates the bone to self-remodel.
In the current work two type of HAp will be analysed:

- HAp sintered in laboratory;
- HAp industrially sintered.

Both the types of HAp are provided in thin, 10 mm diameter disks of sintered powder (Figure 4.2): these disks are the samples which will be used in this research. The particles composing the lab made HAp, because of the lower applied pressures and temperatures, are not perfectly sintered, so the sample is highly porous, friable, easier to abrade and it is not perfectly reproducible because of the intrinsic variability in the process parameters. Industrially prepared HAp, instead, is more stiff, resistant and difficult to abrade, because the particles are more compact thanks to the more aggressive sintering conditions; the variability of parameters in industrially produced HAp is lower thanks to the higher control, which lets to obtain more reproducible samples.

4.3.1 Functionalisation

To functionalise HAp, H_2O+PPHE solution is chosen. HAp samples are put in a container – a single container for each sample – in which 5 ml of the functionalising solution were previously poured. Possibly, opaque and dark containers have to be used to protect the polyphenols present in the solution from UV radiations; if it is not possible, it is necessary to cover them with an aluminium film to protect the biomolecules. To distinguish the upper side of the disk, a "X" is scratched on the down side of the sample through a diamond tip; the side marked with the scratch has to be put in contact with the bottom of the container to let the upper side be better functionalised.
So, the functionalising procedure is the following:

1. Prepare the functionalising solution as described in Paragraph 4.2.2;
2. Pour 5 ml of the solution in each of the containers – one for each HAp sample;
3. Scratch the mark on the lower side of the sample;
4. Immerse the samples inside each container paying attention to put the upper side in the right way to be functionalised;
5. Keep all the containers in an incubator at 37°C for 3 hours;
6. Extract the containers from the incubator and the samples from the functionalising solution;
7. Wash each sample with ultra-pure water, immersing each of them through a pair of tweezers in a beaker for a few second;
8. Put the functionalised samples to dry under a chemical hood.

The functionalisation process needs to have place for three hours in an incubator at 37°C to be more efficient. This temperature has been chosen for two reasons. The first is that 37°C is the physiological temperature: it is necessary to evaluate the behaviour of polyphenols in conditions that are similar to the implant ones. The second reason is that these molecules are sensitive to

temperature: to not denature polyphenols, it is necessary to work at temperatures lower than 60°C and 37°C is the right compromise between the acceleration of chemical kinematics and the conservation of the properties of the molecules.
The final sample washing is necessary to remove the eventual polyphenols present on the surface of the sample that not have adhered or covalently bound to HAp: without this last step, it would be possible to overestimate the quantity of biomolecules which have stably interacted with the sample itself.

4.4 Bioglasses

Bioglasses constitute a particularly important class of biomaterials, composed by different oxides and amorphous phases. When put in contact with body fluids, they could be more or less bioactive, on the basis of the nature different oxides they are constituted with: varying the concentrations of the constituents, it is possible to obtain bioglasses characterised by largely different properties.
Bioactivity is the property for which hydroxyapatite precipitates on the surface of a material when it is put in contact with body fluids. The formation of this new phase at the interface between the implanted material and bone tissue implements a better and stronger interaction between the two elements. Bioactivity could be modulated varying the composition of the bioglass: adding silica, bioactivity is inhibited, while sodium and calcium oxides increase the bioactive behaviour of the bioglass[6,7].
During this research, two different types of bioglasses have been tested: SCNA and SCNbis. Both the materials are available in thin disks, characterised by a 10 mm diameter and a thickness of 2 mm, obtained in laboratory through a series of procedures which will be illustrated in this paragraph. In Figure 4.3, bioglass samples are reported.

Figure 4.3 SCNbis samples

4.4.1 SCNA

The first type of analysed bioglass is SCNA, composed by quartz (SiO_2), sodium oxide (Na_2O), calcium oxide (CaO) and aluminium oxide (Al_2O_3) in the percentage illustrated in Table 4.1:

	SiO_2	**Na_2O**	**CaO**	**Al_2O_3**
Molar weigth (g/mol)	60	62	56	102
Weight (g)	3420	372	1904	306
%mol	57	6	34	3
%wt	56.98	6.20	31.72	5.10

Table 4.1 SCNA components.

In nature it is possible to find only quartz and aluminium oxide, but not sodium and calcium oxides. So, to synthesise SCNA it is necessary to use their respective precursors: sodium carbonate (Na_2CO_3) and calcium carbonate ($CaCO_3$). To calculate the oxide weight percentages that is necessary to respect the correct proportions, this equation has to be solved:

$$\%wt=(weight/W_{tot})*100$$

where:

- weight=(molar weight)*%mol; it is the weight of the oxide;
- $W_{tot}=\Sigma_i(weight)_i$; it is the total weight, where the index i=1,2,3,4 referring to the four precursors.

In the following table (Table 4.2), the correct mass of precursors to weight to obtain the desired bioglass and the mass of the other two oxides are reported.

Oxide	Precursor	Molar weight (g/mol)	Weight (g)
SiO_2	**SiO_2**	60	56.98
Na_2O	**Na_2CO_3**	106	10.60
CaO	**$CaCO_3$**	100	56.65
Al_2O_3	**Al_2O_3**	102	5.10

Table 4.2 SCNA precursors molar weight and weight.

To obtain the correct precursors weights, this equation is applied:

$$weight=(w\_glass/100)\ *\%wt*(molar\_weight\_carbonate/molar\_weight\_oxide)$$

where:

- w_glass is the total weight of the bioglass desired to be obtained (for the current application, w_glass=100 g);
- %wt is the weight percentage of the oxide;
- molar_weight_carbonate is the molar weight of the carbonate that is the precursor of the respective oxide;
- molar_weight_oxide is the molar weight of the considered oxide itself.

To prepare 100 g of SCNA, it is necessary to weight the four precursors and mix them. The precursors are industrially available as a thin powder. Each precursor is weighted through a weight scale; to add the dust to weight each compound, a spatula is utilised. Then, the four components of SCNA are put in a jar, paying attention to not lose material during the transfer of the powder from the container in which they have been weighted to the jar itself, and successively they are shaken to obtain a homogeneous mixture, without any kind of agglomerate.

The obtained mix of powder is melt in a specific oven in a platinum crucible, as it will be described in Paragraph 4.4.3.

4.4.2 SCNbis

The second type of bioglass is different from the precedent one because it does not contain aluminium oxide and has different molar and weight percentages of the other substances, as appreciable in Table 4.3.

	SiO_2	**Na_2O**	**CaO**
Molar weigth (g/mol)	60	62	56
Weight (g)	3336	1407.4	1215.2
%mol	55.6	22.7	21.7
%wt	55.99	23.62	20.39

Table 4.3 Percentages of SCNbis components.

Analogously as for SCNA production, also for SCNbis it is necessary to use sodium and calcium carbonate as the respective precursors of sodium and calcium oxides.
In the following table (Table 4.4), the correct mass of precursors to weight to obtain the desired bioglass is reported.

Oxide	**Precursor**	**Molar weight (g/mol)**	**Weight (g)**
SiO_2	**SiO_2**	60	55.99
Na_2O	**Na_2CO_3**	106	40.38
CaO	**$CaCO_3$**	100	36.41

Table 4.4 SCNbis precursors molar weight and weight.

These values are obtained with the same equations used for the precedent case.
To prepare 100 g of SCNbis, it is necessary to weight the three precursors and mix them. Analogously to SCNA ones, SCNbis precursors are industrially available as a thin powder. Each precursor is weighted through a weight scale; to add the dust to weight each compound, a spatula is utilised. Then, the three components of SCNbis are put in a jar, paying attention to not lose material during the transfer of the powder from the container in which they have been weighted to the jar itself, and successively they are shaken to obtain a homogeneous mixture, without any kind of agglomerate.
The obtained mix of powder is melt in a specific oven in a platinum crucible, as it will be described in Paragraph 4.5.3.

4.4.3 Samples production

At the end of the previously illustrated procedures, two jars are obtained respectively containing the two powder mixtures constituting the necessary precursors of SCNA and SCNbis. To melt the

powder of the two precursors, the protocol wants they are put in an oven and left for two hours at 1450 °C. It is necessary melt the two types of glass in two respective different times.
For each bioglass precursor, its respective powder is poured in a platinum melting pot. Platinum is chosen because of its high stability at high temperatures: it does not corrode or deteriorate and does not release substances that could contaminate bioglasses.
Before to take the powder in the melting pot, a series of precautions and controls are necessary.
First of all, it is necessary to control that the powder is sufficiently homogeneous and, if not, it has to be shaken a second time. Successively, it is poured, step by step, in small quantities and it is made more compact with a pestle, excluding eventual residual air which could have been insinuated between the precursors particles. The eventual presence of air could induce the formation of bubbles inside the matrix of the final product: this situation conduces to have a not homogeneous material and deteriorates its mechanical properties, through the concentration of internal stresses locally around the bubble. Then, it is necessary that the melting pot is not totally filled till its upper limit because in the oven bioglass become more voluminous and, once reached critical temperatures, boils and splashes: if the melting pot is excessively filled, the melt powder could go out and contaminate the oven, carrying a high probable damage. This critical phenomenon is not only due to the high temperatures reached during the melting process, but also to the exothermic reaction for which carbonates are chemically transformed in the respective oxides of which they are precursors. Another aspect to pay attention at to limit the material going out and contaminate the oven is to put a cover on the melting pot until the oven reaches 1000 °C. It is possible to add to the precursors powders also some pieces of already formed glass, obtained by precedent melts, which do not satisfy the desired characteristics: for example, the presence of bubbles could induce the experimenter to reject a certain sample of bioglass. Through this expedient, not only previous glass can be recycled, but it is possible to obtain a higher volume of final bioglass at the end of this procedure than if we used only precursors powder. It is explained by the fact for which in the remelted glass carbonates had already been transformed during the precedent melting and, so, all the volume is effectively formed by the final oxides: it limits the net volume reduction that normally has place after the melting process.
After having prepared the materials to be melt and have controlled the necessary aspects, the oven is switched on, imposing the final temperature to reach (1550 °C) and the melting time (2 hours). The platinum melting pot, containing the material to be melt, is put inside the oven and covered; when the temperature reaches the value of 1000 °C, after about 1 hour and 40 minutes, the cover is removed.
After the 2 hours of melting, other two steps are necessary before to obtain the final material: casting and annealing. So, when the bioglass is melt, it is casted in a cylindrical mould whose diameter is 10 mm to give it the desired shape and put it in another oven for 10 hours to anneal. Annealing is an important procedure to potentially remove from the material all the residual internal stresses which could arise otherwise; it consists in maintaining a temperature that is minor than the melting one, but higher than the environment one. For these bioglasses, it was optimised to be 600 °C. At the end of the precedent procedures, a cylindrical piece of glass is obtained, characterised by a 10 mm diameter, and it is left to be cooled till it reaches room temperature.
From the room temperature 10 mm diameter glass cylinder, it is possible to obtain the desired samples, cutting it transversely with a 2 mm thickness for each cut. A cutting machine is utilised: the cylinder is put horizontally in the machine and bound to the gripping, so that the blade cuts it transversally. The speed of the blade has to be imposed: it was optimised to 0.015 mm/s. Before to start the cuts, it is necessary to verify the lubricant flow: it is fundamental to not corrode or damage

the blade during the procedure. After that, it is possible to proceed with the glass cutting, translating the cylinder 2 mm by 2 mm for each cut, regulating the movements of the glass cylinder through a knob which is present in the external part of the cutting machine.
The disks extracted from the cutting machine are however quite irregular. In fact, in spite of annealing, the intrinsic fragility of glass is a persistent characteristic, so it is impossible to obtain perfectly smooth surfaces from the cutting process. To optimise polyphenols functionalisation, it is so necessary to abrade the two surfaces of the samples – upper and lower – polishing them. A polishing machine is utilised, on which sandpaper sheets are put with smaller and smaller grains step by step, from the roughest to the finest: each granulometry is applied in series to each bioglass disk, to refine surfaces irregularities. Precisely, the following granulometry is utilised: P120, P320, P600, P800, P1000, P2500, P4000. For each step, the sandpaper characterised with a precise granulometry is fixed inside the polishing machine on a rotating platform; water, flowing on the platform from a small sink present in the device, is used because of its lubricant effect and for the removal of abraded particles. The sample to be refined is so put on the sandpaper. Then, the sample is located on the platform and the polishing machine is switched on, so the platform starts to rotate with an angular velocity which can be selected by the user, usually in the order of a few hundreds of rpm (rotation per minute). Because of the absence of adequate gripping – the disks have small dimensions and eventual gripping could damage it – it is necessary to impose manually a low pressure on the sample to let it remain in the desired position. The pressure to apply to the sample is quite critical: it has to be as more homogeneous as possible to not abrade the surfaces asymmetrically and to let its two sides be parallel. Each side of the disk must be treated for 30 s: 15 s in the initial position and 15 s after a 90° rotation around the axis that is normal to the surface, to limit eventual inhomogeneities only on one side.
After the first granulometry treatment, to distinguish the two sides of the samples, the most irregular one id chosen and a “X” is scratched on it with a diamond tip. Then, the samples are treated with the finer granulometries.
Finally, to purify all the bioglass samples from the cutting and polishing residues and from eventually adsorbed contaminations, a last washing treatment is applied. For bioactive glasses the washing step is also used as surface activation step in order to expose hydroxyl groups, useful for molecular grafting. This is obtained with four washing steps: the first with acetone, the others with ultra-pure water[8,9]. For each of these steps, it is necessary to put all the glasses in a beaker – the polished surfaces have to be put upward, pour on them a thin film of fluid just to cover them completely and treat them for five minutes with ultrasounds.
Through this treatment 10 mm diameter polished disks are obtained, that will be the samples to use for this research.

4.4.4 Functionalisation

To functionalise SCNA and SCNbis, H_2O+PPHE solution is utilised. Analogously to HAp samples, each bioglass disk has to be put in a dark and opaque jar containing 5 ml of functionalising solution. So, 5 ml of each solution are spilled in each jar with a pipette and, through a pair of tweezers, the samples are immersed in it to be completely covered by the solution, paying attention to put the side to functionalise upward and the lower side, recognisable by the previously scratched “X”, in contact with the bottom of the jar. Then, the jars are put in the incubator and left in it for 3 hours at 37°C to let the surfaces interact with the polyphenols solutions.

So, the functionalising procedure is the following:

1. Prepare the functionalising solution as described in Paragraph 4.2.2;
2. Pour 5 ml of the solution in each of the containers – one for each bioglass sample;
3. Immerse the samples inside each container paying attention to put the upper side in the right way to be functionalised;
4. Keep all the containers in an incubator at 37°C for 3 hours;
5. Extract the containers from the incubator and the samples from the functionalising solution;
6. Wash each sample with ultra-pure water, immersing each of them through a pair of tweezers in a beaker for a few second;
7. Put the functionalised samples to dry under a chemical hood.

4.5 Chemically Treated Titanium Alloy

The second material to work with is titanium aluminium-vanadium alloy (Ti6Al4V). It is industrially provided in 10 mm diameter disks, obtained from a cylindric bar successively cut with a cutting machine imposing a 2 mm of thickness. It is preferred to buy industrially-produced disks to work with more reproducible samples – as industrial products are – and decrease the variability that could characterise laboratory-made samples. Before to implement the functionalisation with polyphenols, titanium has to be processed to obtain a Chemically Treated titanium alloy (CT). This chemical process is applied to make the metal more biomimetic. In fact, the target of this chemical treatment is to obtain micro- and nanostructuration of its superficial oxide, while, on the other side, lets the material expose -OH groups to increase interactions with polyphenols, to induce osteoblasts adhesion. The chemical treatment consists in a double etching attack, as it will be described in this paragraph. The greater advantage of CT on the classical titanium alloy is the micro- and nanoporously structured surface which will constitute the more osteophilic interface between the implant and the bone tissue: it has the necessary morphology and chemical characteristics for which osteoblasts better adhere, proliferate and grow on and in the material. An ingrowth effect is in this way observable. It could be explained by the only presence of titanium, a highly biocompatible metal, without the addition of other biomaterials at the interface, which could induce a probable mechanical instability. Then, the morphology, similar to the one characterising the extracellular matrix of bone tissue, permits a better integration with the implant, significantly reducing discontinuities, implementing cellular migration and, so, conducing to the desired ingrowth effect. Ingrowth effect can guarantee a higher stability of the device thanks of the induction of the bone to grow inside the material itself, limiting the formation of gaps or other discontinuities at the interface between the metal and the human tissue.

Before to apply the chemical treatment to obtain CT, it is necessary to polish the titanium samples to eliminate turning lines. To choose the side of the disk to polish, it is necessary to inspect it and select the side on which turning lines are less visible: the polishing treatment will be more efficient. On the other side the number of each sample is scratched, impressing the respective number on it with an electric pen. Then, through a polishing machine, each disk is polished – only on the upper side that will be functionalised – with different granulometries. Three different granulometries sequences are tested and then compared to understand the more efficient one to treat CT with:

- P320 and P400: this treatment leaves a rougher surface;
- P320, P600, P800, P1000: a finer granulometry makes the metal surface smoother;

- P320, P600, P800, P1000 and P2500: refining the surface with a further fine granulometry, the surface becomes significantly smooth and homogeneous (if not otherwise specified, the utilised samples have been treated with this granulometry).

Each granulometry has to be applied to each side of the disk for 30 s: 15 s in the initial position and 15 s after a 90° rotation around the axis that is normal to the surface, to limit eventual inhomogeneities. At the end of the treatment, one side polished disks are obtained, on which turning lines are disappeared and the surface to be functionalised is sufficiently homogeneous.

To remove the impurities due to the polishing process and the eventual contaminations from the atmosphere, three washes are necessary: the first of them with acetone, the others with ultra-pure water. For each wash, all the disks are put in a beaker, disposed with the polished surface upward, and covered, respectively, with a thin film of acetone for the first wash and of water for the last two. To increase the washing efficacy, the beaker is sonicated: the acetone wash has to have place for five minutes, while each of ultra-pure water ones is ten minutes long. Finally, the disks are taken under the chemical hood to dry.

After to have obtained the polished and washed samples, the last step to prepare CT – and so the reactive surfaces ready to be treated with polyphenols – is the chemical treatment, to obtain the nanotextured oxide layer rich in OH groups. The treatment applied to the samples is the following patented chemical treatment[10,11]: an acid etching (HF), for the removal of the native oxide and a controlled oxidation in hydrogen peroxide (H_2O_2), to form a new nano-porous layer of titanium oxide (TiO_2). After the treatment, CT samples (Figure 4.4) are so dried under the chemical hood.

Figure 4.4 CT samples

4.5.1 Functionalisation

Because the superficial oxide of titanium has a significant passivating effect, before proceeding with the functionalisation, it is necessary to make the surface more reactive, a process finalised to optimise the functionalisation itself. The samples of CT are put under UV radiations for an hour to eliminate the carbon contaminations that the surface of the metal eventually adsorbed from the atmosphere and to activate hydroxyl groups, to optimise their grafting ability.

The functionalising process wants CT be immersed in 5 ml of TRIS/HCl+$CaCl_2$+PPHE solution (5 mg/ml): 5 ml of the functionalising solution is spilled in each container, one for each CT sample, in which, through a pair of tweezers, the CT disks are put with their polished side upward. The containers are taken 3 hours in the incubator at 37°C to let polyphenols react with the biomaterial surface. So, the necessary steps to functionalise CT samples are the following:

1. Treat the samples with UV radiation for an hour to activate them;
2. Prepare the functionalising solution as described in Paragraph 4.2.2;
3. Pour 5 ml of the solution in each of the containers – one for each CT sample;
4. Immerse the samples inside each container paying attention to put the upper side in the right way to be functionalised;
5. Keep all the containers in an incubator at 37°C for 3 hours;

6. Extract the containers from the incubator and the samples from the functionalising solution;
7. Wash each sample with ultra-pure water, immersing each of them through a pair of tweezers in a beaker for a few second;
8. Put the functionalised samples to dry under a chemical hood.

4.6 pH

The evaluation of the pH value characterising a solution is an important step while analysing the properties of a sample. In fact, pH could influence or be influenced by the presence of certain ions or molecules in the system and be the cause of conservation or degradation of the sample itself.
The measurement of pH is possible through a digital pH-meter, a sensing electrode connected to a tablet on whose screen the pH value and the temperature of the sample are displayed. pH is in fact temperature-dependent: it is fundamental to refer a pH value to the temperature at which it had been measured, because low temperature variations could induce significant alteration of the pH of the solution.
pH-meter is a sensitive instrument: it has to be calibrated once every seven days. To calibrate it, three different solutions with three different pH values have to be used:

- acid: pH=4.01;
- neutral: pH=7;
- alkaline: pH=9.21.

Furthermore, when not used, the electrode of the device has to be stored in a specific solution to maintain its performances. Finally, before to effectuate a measure, between the analysis of two different solutions and after to have used it, it is also necessary to wash the sensor, pouring ultra-pure water on the electrode through a pipette, to limit eventual contaminations.
pH measurements were performed on functionalising solutions and uptakes, to evaluate eventual variations on polyphenol solutions before and after functionalisation process: the lower quantity of polyphenols in the solvent after the treatment and the presence of the biomaterial ions, caused by diffusion.

4.7 UV spectroscopy

Ultra-violet (UV) spectroscopy is a characterisation technique through which a solid or liquid sample is analysed by UV-Vis irradiation. The behaviour of the sample is evaluated quantifying its absorbance or transmittance, reported in function of the wavelength with which the sample is irradiated[12]. The instrument (Figure 4.5) is composed by a UV source, a UV detector and a PC, through which the acquired signals could be conditioned and displayed.

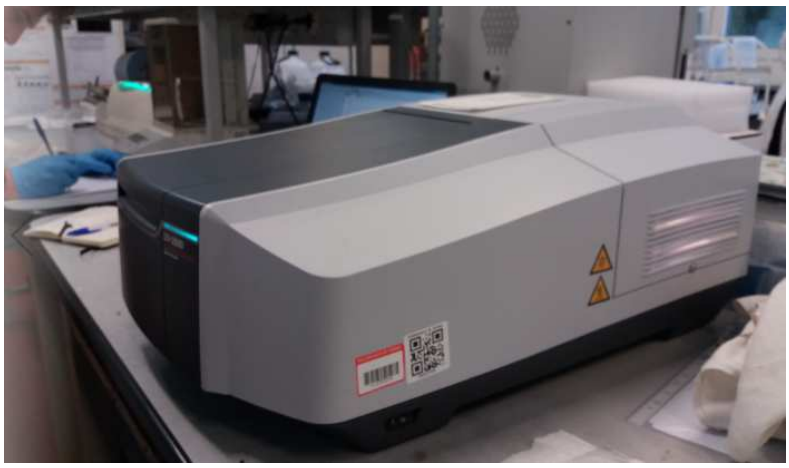

Figure 4.5 UV spectroscopy

Liquid samples are analysed by pouring them in a cuvette; to analyse solid ones, it is possible to process them in analogue way if the sample is transparent, while it is necessary to utilise an integrating sphere if it is opaque. In both cases, the sample has to be put between the source and the detector. The source emits a radiation appertaining to UV-Vis spectre; a fraction of it is absorbed by the sample, a fraction is transmitted, a fraction is reflected and the remaining part is scattered. This phenomenon is due to energy accumulation after irradiation, which is successively emitted and sensed by the detector. The detector can work in absorbance, transmittance or reflectance modality and, respectively for each case, it is able to evaluate the fraction of light of interest. The UV-Vis spectre characterising the sample is given in output at the end of the measurement[13]. So, through spectroscopy, it is possible to characterise a substance analysing its peculiar peaks due to tendency of the atoms, functional groups and complexes constituting the compound itself to absorb, transmit or reflect UV radiation at precise wavelength[14].

UV spectroscopy is utilised in the context of the current research in two possible modalities: spectrum and photometric modality. The first one evaluates the response of the sample in function of the variations of the wavelength with which it is irradiated and gives in output a UV-Vis spectrum, while through the second one the sample is irradiated with a single wavelength and a single value is given in output. Spectrum modality is utilised to study the characterising peaks of the analysed compound; photometric modality is instead exploited to implement other characterisation techniques, such as Folin and Ciocalteu test and DPPH test (respectively treated in paragraph 4.8 and paragraph 4.9).

To study the characterising peaks of polyphenols, both solved in solutions and bound to a biomaterial surface, it is necessary to evaluate the full UV-Vis spectre, whose wavelength is included between 190 and 1000 nm.

Before to proceed with the measurements, a baseline has to be acquired by the instrument. To do that, two different procedures have to be implemented, on the basis if the samples are liquid or solid. For liquid samples, it is necessary to acquire a spectre of the solvent of the analysing solution, using the solvent itself as a reference. For solid samples, a reference white sample, constituted by barium solphate, provided by the supplier industry, has to be evaluated and the acquired value has to be used as a reference.

A criticality which could appear analysing liquid samples is their concentration. Regardless of the selected modality (absorbance, transmittance or reflectance), it is necessary to verify that the

instrument do not saturate, phenomenon which often conduces to the alteration of the results; if it is, the solutions have to be adequately diluted before to be inserted in the instrument to be analysed.

4.8 Folin and Ciocalteu test

As illustrated in Chapter 1, Folin and Ciocalteu (F&C) test, based on the evaluation of the absorbance through UV-Vis spectroscopy, is finalised to quantify total phenols as Gallic Acid Equivalents (GAE), that are eventually present in a solution or on a solid sample. Through this test, it is possible to evaluate the presence of polyphenols on a surface after a functionalisation process, to study the eventual variation of concentration of polyphenols in a solution before and after have used it to functionalise a solid sample or, finally, to verify the polyphenols release in water. Gallic acid (GA), in fact, is a phenolic acid which, thanks to its simple structure and representative properties, is considered as a good model molecule for polyphenols and exploited in this way[15].

The test is based on a redox reaction between gallic acid (GA) or polyphenols (in the sample to be analysed) and phosphotungstic/phophomolybdic acids (in the Folin&Ciocalteu reagent used for the test). GA/polyphenols reduce phosphotungstic/phophomolybdic acids (which are yellow) to phosphotungstic/phophomolybdic oxides (which are blue). The extent of blue colouration can be measured by means of UV spectroscopy and quantified through a proper calibration curve, as described in the following. Since the Folin&Ciocalteu test measures the result of a redox reaction it does not only quantify the amount of phenols but also their redox activity[16].

To quantify the presence of polyphenols, GAE concentration has been chosen as a valid method. It is imposed through a calibration curve, which has to be obtained before to take the measures of interest. To calibrate the instrument, it is necessary to impose 7 calibration points, preparing 7 solutions of water and gallic acid, respectively characterised by 7 different concentrations. Each of the 7 solutions has to be triplicate: three solutions for each value of concentration have to be prepared. In this way, the 760 nm-absorbance is measured three times for each imposed concentration: averaging the three values of absorbance, a single value is obtained. It constitutes the one that is associated to the relative concentration to have a point of the calibration curve. So, also the error of the instrument can be quantified through statistics.

The 7 values of concentration to impose to the instrument are the following:

	Concentration (mg/ml)
Solution 1	0.0010
Solution 2	0.0025
Solution 3	0.0050
Solution 4	0.0100
Solution 5	0.0200
Solution 6	0.0300
Solution 7	0.0400

Table 4.5 Concentrations of calibration solutions

So, 7 couples of values (concentration, absorbance) are obtained from this process, which constitute the 7 points from which the calibration curve can be extracted through the linear regression method. It imposes a linear relation between the two parameters: Abs=a*C+b, where:

- Abs is the absorbance;
- C is the concentration (mg/ml);
- a is the angular coefficient;
- b is the offset.

This curve is based on gallic acid concentrations, but it is utilised to quantify polyphenols appertaining to every class. For this reason, "GAE concentration" has to be specified: it means that the analysed solution has the same behaviour to light irradiation of a hypothetical equivalent solution of gallic acid.

Once obtained the calibration curve, it is possible to proceed with the measurements. Following the protocol of the procedure, each sample to be analysed has to chemically react for 2 hours with two compounds, Folin and Ciocalteu reagent and sodium carbonate water solution (Na_2CO_3 20% w/v), before to be analysed. During the 2 hours, these substances react with the polyphenols eventually present in the sample to produce a blue solution, whose intensity is proportional to the quantity of polyphenols. The blue solution is, so, studied through a spectrophotometer in photometric modality to evaluate its absorbance of blue wavelength (λ=760 nm). Higher the quantity of polyphenols in the sample, higher the absorbance value: the polyphenols content is quantified through a calibration curve, able to impose a linear dependence between GAE concentration and the measured absorbance[17].

First of all, Na_2CO_3 20% w/v solution has to be prepared. ultra-pure water is spilled in a beaker and then extracted with a pipette to pour the necessary volume in the container where the solution is produced. Successively, using a weight scale, the 20% w/v of Na_2CO_3 powder is taken and poured in the jar containing the just spilled water. To let Na_2CO_3 be solved and to make this solution more homogeneous, it is left on a magnetic stirrer for 1 hour to be better shaken.

Folin and Ciocalteu reagent is instead industrially available.

The next step is the preparation of the samples to analyse. If the samples are solid, they do not need a previous treatment, while, if the samples are liquid, constituted by polyphenols solutions, they have to be diluted in a proportion of 1:60 to not saturate the machine. In fact, the solutions utilised during this research – the functionalising solutions and the uptake ones – are very concentrated: 1 mg/ml and 5 mg/ml concentrations are able to saturate the device, which, in this case, could give in output altered results with not characterised errors.

To let F&C test have place, it is necessary to let the reagents and the samples react. As cited before, through this test it is possible to analyse both liquid and solid samples: there are respectively two different techniques to do that.

4.8.1 Preparation of liquid samples

Liquid samples are functionalising solutions or uptakes and, as cited before, they need to be diluted in 1:60 ratio. So, before to carry on the test, all the solutions have to be diluted.

For each sample to analyse through F&C test, it is necessary to extract and spill in a container the following compounds in this precise order:

- 6 ml of ultra-pure water
- 2 ml of the sample to analyse (previously prepared)
- 0.5 ml of F&C reagent

- 1.5 ml of the Na_2CO_3 20% w/v solution (previously prepared)

Each container is closed and left under the hood. It is necessary to wait for 2 hours, before analysing them, to let the reactions have place.
As highlighted previously, it is preferred to utilise dark containers to limit the aggression of UV-visible radiations: polyphenols and F&C reagent are in fact photosensitive and an eventual damage could alter the test. Then, to manipulate Na_2CO_3 solution and F&C reagent, it is necessary to work under a chemical hood because of the toxicity of the alkaline solution and the aggressive reagent.

4.8.2 Preparation of solid samples

Solid samples are the samples of the four analysed biomaterials: HAp, CT, SCNA and SCNbis. They can be pure or functionalised with polyphenols. To prepare each of them for the test, it is necessary to put in a container in the following order:

- the dried sample to analyse (previously prepared)
- 8 ml of ultra-pure water
- 0.5 ml of F&C reagent
- 1.5 ml of the Na_2CO_3 20% w/v solution (previously prepared)

Analogously to the precedent case, a dark container is preferred to limit the aggression of UV-visible radiations. Each sample has then to be put in each of these jars: as for liquid samples, it is necessary to work under the chemical hood; furthermore, the solid sample has to be put in the container using a pair of clean tweezers and, if functionalised, paying attention to put the treated side upward – eventually also looking at the lower side marked with the symbol "X".
Finally, the jars are closed and left under the hood for two hours to let the reactions have place.

4.8.3 Evaluation of absorbance

At the end of the 2 hours that are necessary to the reaction to have place, it is possible to measure the absorbance of the obtained solution. If the test is done on solid samples, before to proceed, it is necessary to extract them from the containers and put them under the hood to dry; this samples are not utilisable other times for eventual successive analysis, because they have been contaminated by F&C reagent and Na_2CO_3 solution.
As previously cited, to measure the absorbance of the solutions, a UV spectrophotometer in photometric modality is exploited. The absorbance of the sample is evaluated at a single wavelength, precisely the blue wavelength (λ=760 nm): basing on the previously obtained calibration curve, it is possible to relate the absorbance of blue light of a sample with its concentration of polyphenols.
The obtained calibration curve applied during the current study is the following:

$$Abs=22.523*C+0.0201$$

where:

- Abs is the absorbance;
- C is the GAE concentration (mg/ml);
- a=22.523 is the angular coefficient;
- b=0.0201 is the offset.

The calibration curve has to be imposed to the instrument each time before to effectuate a set of measures. The last step before to evaluate the absorbance of the samples to analyse is to give a reference to the instrument. Two cuvettes are in fact to be inserted inside the instrument: in the first one the solution to analyse is spilled, while in the second one the utilised solvent – in which polyphenols have been previously solved – is poured. Before to proceed with the analysis of interest, a first 760 nm-absorbance acquisition has to be done, in which there is the same solvent in both the cuvettes: it constitutes the reference value for the next measures.
Obtained the entire set of absorbance value, through the calibration curve is possible to calculate the GAE of the polyphenols that are present in the solution. If diluted liquid samples are analysed, the calculated values of concentration are referred to the diluted solution: to know the real concentrations of interest of the starting solution, it is necessary to consider the proportion with which it has been diluted and apply it to the obtained concentration values. Otherwise, if the solution has not been diluted or if a solid sample has been evaluated, the obtained value of concentration has not to be further conditioned.

4.9 DPPH

α-diphenyl-β-picrylhydrazyl (DPPH) is a free radical, highly reactive and toxic molecule to human body, capable to exert a significant pro-oxidant activity in the environment it is. DPPH could be exploited to evaluate the antioxidant power of molecules, quantifying the capability of the molecule to deactivate the radical. In the context of the current research, the antioxidant activity of polyphenols is studied, both when they are in solution and bound to a biomaterial surface after functionalisation.
DPPH test consists in making the sample to analyse in contact with a DPPH solution in order to evaluate its ability to quench the radical. Ethanol is used as the solvent to prepare DPPH solution because of the high solubility of the radical in this solvent; when dissolved in it, a dark purple solution is obtained. When polyphenols are added to this solution and can react for a sufficient time, they exert their antioxidant power binding to DPPH and deactivating it: a lighter purple solution is obtained after the process. The intensity of purple characterising the solution is proportional to the quantity of active radical: lighter the intensity of the colour after the treatment, higher the antioxidant power of the analysed molecules. The concentration of the active radical of the mother solution of DPPH is evaluated before the test and, successively, after it has been in contact with the antioxidant sample a sufficient time to let it exercise its properties. To make a quantitative measure, UV spectroscopy in photometric modality is newly used, evaluating the absorbance of the analysed solutions before and after the polyphenols action; the utilised wavelength is 515 nm, *ergo* the wavelength corresponding to purple.
DPPH technique is used both on solid and liquid samples, applying respectively two different protocols.

The protocol to analyse solid samples is the following[18,19,20]:

1. weight 9.858 mg of DPPH (according to the resolution of the weight scale); anyway, it is necessary to write down the precise weighted mass of the radical which the instrument gives in output, to calculate the exact molarity of the solution;
2. with a pipette, extract 25 ml of ethanol and pour it in a beaker with the just weighted DPPH;
3. leave the beaker for 20 minutes in ultrasounds to have a homogeneous solution, eliminate all the eventual particulate and completely dissolve DPPH in ethanol;

4. filter the solution through a 0.2 μm PTFE filter to eliminate eventual inhomogeneities;
5. dilute the solution in a 1:10 proportion, that is 1 ml of solution and 9 ml of pure ethanol;
6. measure 515 nm absorbance of the diluted solution, verifying the instrument is not saturating (after have done the auto-zero with the two cuvettes both containing pure ethanol);
7. prepare a set of containers and put the samples in, one for each container;
8. pour 3 ml of the diluted solution for each container to completely cover the sample;
9. close the containers and leave all the samples under the chemical hood, in a dark environment, for 4 hours;
10. measure 515 nm absorbance of the samples after the 4 four hours reaction;
11. repeat the measure after 24 hours.

The protocol to analyse liquid samples is the following[21,22]:

1. weight 8 mg of DPPH;
2. with a pipette, extract 20 ml of ethanol and pour it in a beaker with the just weighted DPPH, to obtain a 1 mM concentrated solution;
3. leave the beaker for 10 minutes in ultrasounds to have a homogeneous solution, eliminate all the eventual particulate and completely dissolve DPPH in ethanol;
4. filter the solution through a 0.2 μm PTFE filter to eliminate eventual inhomogeneities;
5. dilute the solution in a 1:10 proportion, that is 1 ml of solution and 9 ml of pure ethanol – a 0.1 M solution is obtained;
6. measure 515 nm absorbance of the diluted solution, verifying the instrument is not saturating (after have done the auto-zero with the two cuvettes both containing pure ethanol);
7. prepare a set of containers and, through a pipette, pour 3 ml of solution: 2950 μl of the diluted DPPH solution and 50 μl of the liquid sample to analyse;
8. close the containers and leave all the samples under the chemical hood, in a dark environment, for 4 hours;
9. measure 515 nm absorbance of the samples after the 4 four hours reaction;
10. repeat the measure after 24 hours.

Regardless the samples to analyse are solid or liquid, an additional liquid sample – the control sample – has to be added to the experimental set to evaluate its absorbance at the two experimental times (4 hours and 24 hours). For solid samples set, it is constituted by 3 ml of the diluted DPPH solution; for liquid samples set, instead, it is constituted by a 3 ml solutions composed in this way: 2950 μl of the diluted DPPH solution and 50 μl of pure ethanol.
While carrying on the pre-processing of the samples – both solid and liquid ones – it is necessary to pay attention to limit the exposition of DPPH to light. Because of its photosensitivity, a partial degradation is possible, which could alter the results of the test. So, the containers in which the test has place have to be dark and opaque; if such containers are not available, it is preferred to cover them with a film of aluminium to limit light aggression.
Another criticality is constituted by the volatility of ethanol: the samples have not to be exposed to air for long time, maintaining them into covered containers; a further foresight could be covering the cuvettes during 515-absorbance measurement, finalised to limit ethanol evaporation while the measure is having place.

So, at the end of the test, two values are given in output for each of the two experimental times: the values of absorbance of the control (A_0) and of each of the N samples (A_i, where i=1,2,3,…,N). Through evaluating these absorbances, it is possible to quantify the variation of the pro-oxidant activity of DPPH by comparing the initial absorbance value to the ones obtained at the end of each experimental step. Radical Scavenging Activity (RSA) parameter constitutes the quantification of the antioxidant activity of the analysed samples:

$$RSA\%=[(A_0-A_i)/A_0]*100$$

RSA is a semi-quantitative parameter that illustrates the behaviour of polyphenols and their level of antioxidant activity when solved in a solution or bound to a biomaterial surface. Higher the difference between the two measured absorbances, higher RSA value, higher the ability of the sample to scavenge free radicals. Starting from the same control conditions (A_0), the antioxidant activity is more intense for the samples characterised by a lower A_i value. It means that a major deactivation of DPPH, causing a lighter intensity of purple of the solution in which the sample had been immersed or solved, induces a significant decreasing of the 515-absorbance: the difference of the oxidant behaviour between the initial and the final condition is so more appreciable.

4.10 Zeta potential

As described in Chapter 1, zeta potential is measured to electrically characterise sample surfaces and solutions, analysing how the electrical charges vary as a function of pH variations. The instrument gives in output zeta potential value of the analysed samples in function of pH and their isoelectric point. Isoelectric point is defined as the value of pH for which there is not a net charge on the analysed surface, so that zeta potential is null; for pH values that are lower than isoelectric point, the net charge is positive, otherwise the surface is negatively charged.

During the current study, both solid and liquid samples are analysed.

Solid samples are:

- Bare HAp;
- Polyphenol-functionalised HAp (HAp+PPHE);
- Bare CT;
- Polyphenol-functionalised CT (CT+PPHE);
- Bare bioglasses (SCNA, SCNbis);
- Polyphenol-functionalised bioglasses (SCNA+PPHE, SCNbis+PPHE).

The liquid sample are 5 mg/ml polyphenols solutions, having respectively water and HCl/TRIS+$CaCl_2$ as the solvent of polyphenols.

For both solid and liquid sample, KCl 0.001 M solution is used as the electrolyte. In the case of liquid solution, the electrolyte has been used instead of water in the solution preparation.

Two different instrument have been utilised to analyse respectively solid and liquid samples. They both have two sensors: pH-meter and conductivity probe. The first one has been installed to measure the pH values that have to be imposed buffering the analysed sample during the measurement process; the second one is utilised to measure the conductivity of the solution. To evaluate zeta potential, a software is exploited, intrinsic to the instrument, through which the potential difference – measured at the two extremes of the cell – is converted in the value of interest.

The applied equation is the following[23]:

$$\zeta = \frac{dI_{str}}{d\Delta p} \times \frac{\eta}{\varepsilon \times \varepsilon_0} \times \frac{L}{A}$$

where:

$dI_{str}/d\Delta p$ is the streaming current coefficient;
η is the viscosity of the solution;
$\varepsilon^*\varepsilon_0$ is dielectric coefficient of the electrolyte solution;
L/A is the cell constant;
L is the length of cell;
A is the cross-section of the cell.

The previously cited sensors – pH and conductivity electrodes – are particularly sensitive, so they have to be calibrated regularly: pH-meter ones a week and conductivity probe ones a month.
To calibrate the pH-meter, three different solutions with three different pH values have to be used:

- acid: pH=4.01;
- neutral: pH=7;
- alkaline: pH=9.21.

Instead, to calibrate the conductivity probe, a KCl 0.1 M solution (745.5 mg of KCl in 100 ml of ultra-pure water) is utilised.
Once imposed the requested solutions to the instrument, calibration process has place automatically.

4.10.1 Solid samples analysis

The instrument evaluates zeta potential through the measurement of an electrokinetic effect, due to the relative movement of the two involved phases – solid sample and electrolyte. It is defined as a coupling of a mechanical and electrical force; this force, which could either be mechanical or electrical, is able to implement the relative movement[24]. The main electrokinetic effects are illustrated in the next figure (Figure 4.6).

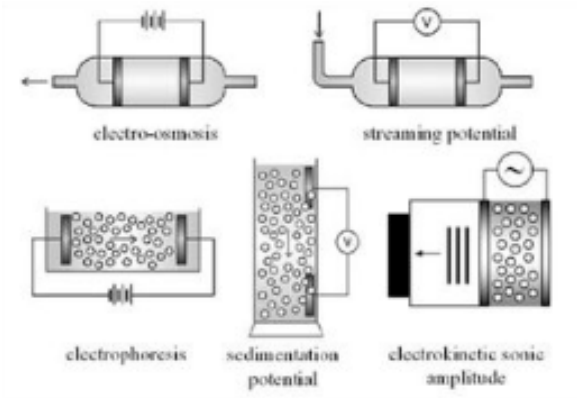

Figure 4.6 Electrokinetic effects[25]

The relative movement of the electrolyte and the solid samples generates a difference of potential, called streaming potential[26]. It is the main effect of interest to evaluate while measuring zeta

potential on solid samples. To implement streaming potential measurements, an electrokinetic analyser is exploited (Figure 4.7).

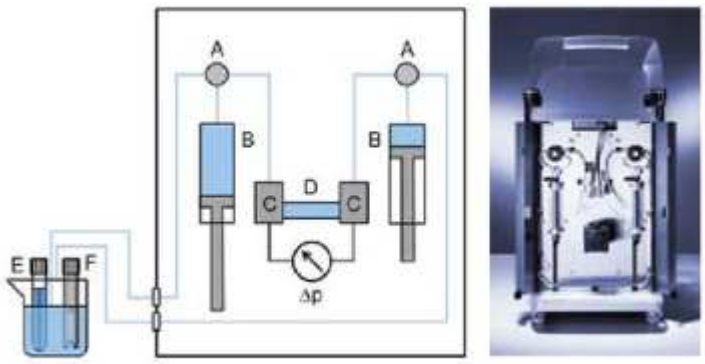

Figure 4.7 Components of the electrokinetic analyser[27]

It is composed by:

A) 3-way valves;
B) Syringes to let the electrolyte flow inside the cell;
C) Pressure transducers and electrodes;
D) Cell;
E) pH electrode;
F) Conductivity probe.

The flow of the electrolyte, contained inside the syringes, is regulated through the valves; the pressure transducer monitors the pressure inside the cell, whose value depends on the gap between the two solid samples and on the flow of the electrolyte itself.
The pH electrode and the conductivity probe are instead externally positioned to monitor respectively the pH and conductivity values of the flowing solution.
To optimize the measurements, because of the sensitivity of the device, it is necessary to pre-process the samples to analyse, assuring the absence of impurities eventually present on the surface. To effectuate the measures on a certain material, it is necessary to install inside the cell of the instrument two samples of the material to analyse. The solid sample that are utilised in this study are constituted, as described in the precedent paragraphs, by 10 mm disks: with double-sided adhesive tape, the two samples are attached in the cell of the device, paying attention to align them to the support – in fact, they must not stick out of the support, especially during the analysis – and to collocate them with their surface disposed parallelly: a constant distance and the consequent homogeneous interface between the samples are necessary to not alter the measure. Moreover, the distance between the two samples has to be close to 100 μm: it is the optimised value for which the electrolyte flows between the two surfaces with a laminar regime during the measurement, permitting to obtain the most precise results. This is possible thanks to a particular cell – the adjustable gap cell – through which the distance between the two solid samples could be controlled and regulated by the user itself (Figure 4.8)[28].

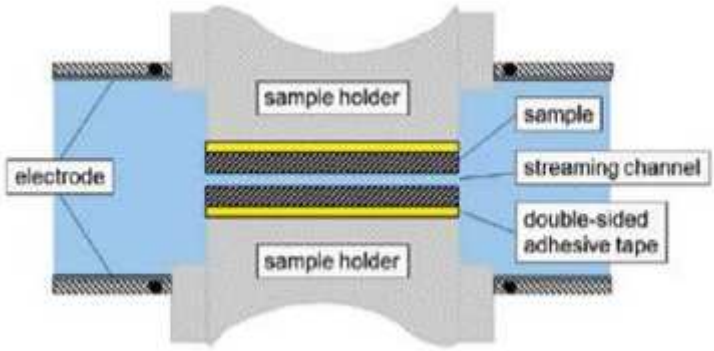

Figure 4.8 Adjustable gap cell for zeta potential analysis of solid samples with planar surface[29]

The solution flowing between the solid samples is, as previously cited, KCl 0.001 M solution: 45 mg of KCl are added to 600 ml of ultra-pure water in a beaker and it is inserted inside the instrument.

To evaluate zeta potential as a function of pH, it is necessary to buffer the flowing electrolyte. So, after to have measured the initial value of pH characterising the surface to analyse, the solution is buffered to induce pH to decrease and, successively, to increase with two different buffering solutions. They respectively are:

- as an acid buffer, HCl solution 0.05 M: 410 ml of HCl (37% v) are spilled in 99.59 ml of ultra-pure water;
- as an alkaline buffer, NaOH solution 0.05 M: 0.2 g of KCl are dissolved in 100 ml of ultra-pure water.

It is preferred to evaluate first zeta potential in the acid range and, successively, in the alkaline one. This order is chosen on the basis of the degrading mechanism of the analysed biomaterial: polyphenols are in fact more sensitive to high pHs, which easily induce damages on the biomolecules that are present on the samples, degrading them and, in this way, altering the measure. Anyway, between the evaluation of the two different ranges of pH, it is necessary to wash the cell containing the samples with ultra-pure water.

If the number of available samples is sufficient, the optimal strategy to decrease the variability due to external factors and the probability to have intrinsic alterations is to apply two different pairs of samples to the cell while evaluating zeta potential respectively in the two different ranges of pH.

The last step before to proceed with the measurements is the check of the instrument. It is necessary to verify that pH is 5.5, conductivity is 15 mS/m and the distance between the two interfaced surfaces is 100 μm – in the admissible range of error, it is preferred to have a slightly higher value to permit the electrolyte to flow better in the gap and not to be obstructed. The flow of KCl solution has then to be forced to be 100 ml/minute and its behaviour has to be evaluated while the pressure is varying between 0 and 300 mbar. The ideal condition is obtained when 100 ml/minute flows with an imposed 100 mbar pressure, but some variations could have place due to the properties of the single sample: if the sample is particularly thin or porous, a higher pressure is necessary to obtain the desired flow. The instrument, during this last step, verifies also if the outward flow and the return flow are characterised by the same flow-pressure curve. If hysteresis is not negligible, it is necessary to reset the cell, repositioning the samples, and repeat the check to verify that all conditions are satisfied.

During the measurement, a curve is visible on the display, reporting the zeta potential as a function of pH, and it is real-time updated. The device starts analysing the initial solution (KCl), which has a pH value around 5.5, as it has been checked during pre-processing. Then, it proceeds adding HCl solution to decrease pH value of the flowing electrolyte: for each pH value, the measurement is repeated four times and the results averaged, so that they are visible on the display. This is repeated 15 times to obtain 15 points-curves, adding respectively, for each step, first 0.1 ml of HCl and then a quantity of buffering solution that goes from 0.2 ml to 2.6 ml, 0.2 ml by 0.2 ml.
An analogue procedure is done for the second part of the measurement, in which the electrical properties of the samples are evaluated in the alkaline range. The 15 points-curve is obtained starting from the initial KCl solution, successively adding, for each step, 0.01 ml of NaOH solution the first time and then a quantity of buffering solution that goes from 0.02 ml to 0.26 ml, 0.02 ml by 0.02 ml. Also in this case, for each drop of added buffer, the measure is effectuated four times and plotted in the same way as for the acid range evaluation.
At the end of the total measurement, two paired arrays of pH and zeta potential values (one for pH<5.5 and one for pH>5.5) are given in output by the instrument, which have to be elaborated, combining them to obtain the final curve that describes the behaviour of the analysed sample over the full range of pH. The zeta potential instrument also gives in output the isoelectric point of the material.

4.10.2 Liquid samples analysis

Zeta potential evaluation is implemented through an electrophoretic measurement. Electrophoresis is a phenomenon for which the particulate dispersed in a fluid fluxes under the effect of an imposed electric field (through the application of a difference of potential); it often has place at the same time of electrosmosis, for which, after the application of an electric field through a difference of potential, the fluid itself, charged, fluxes from the positive to the negative pole[30].
In the current study, the particulate dispersed in the electrolyte is constituted by the polyphenols particles which, under the effect of the applied electric field, become electrically charged. Because of the flux – the imposed movement with which these particles respond to this stimulation – the distribution of the charges surrounding the single moving particle varies as illustrated in Figure 4.9; it is due to the fact that the double layer of ions surrounding the particle moves close to the particle itself[31].

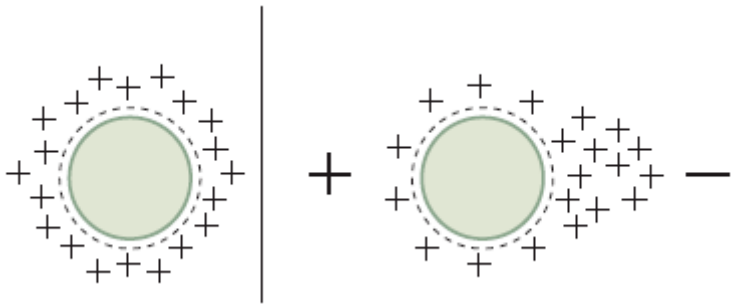

Figure 4. 9 Variation of charges distribution due to the movement of a spheric charged particle in a fluid[32]

So, the movement of the charged particle can be characterised by a parameter, called electrophoretic velocity (u_e), defined as the maximal velocity imposed by the electric field, which, for a stationary and laminar flux, is present at the centre of the tube in which the electrolyte flows[33,34]. Zeta potential is so evaluable as:

$$\zeta = \frac{4\pi\mu u_e}{\varepsilon X}$$

where:

- μ is the viscosity of the electrolyte;
- u_e is the electrophoretic velocity;
- ε is the dielectric constant of the electrolyte;
- X is the intensity of the electric field.

The instrument utilised to implement zeta potential measurements on liquid samples, through a light scattering system, is able to evaluate the average of the electrophoretic velocities of the flowing particles inside the electrolyte; zeta potential is calculated through a software and given in output at the end of measurement process[35].
To proceed with the evaluation of zeta potential of liquid samples, it is necessary to prepare a KCl 0.001 M solution using, as the solvent, the solution to analyse. To have results that are comparable between liquid and solid samples, the same electrolyte has been used. In this way, the evaluation of the electrical properties of the solutions and the surfaces of biomaterials are rigorously comparable with no interferences due to different electrolytes.
Two liquid samples have been studied, *ergo* the two main functionalising solutions:

- H_2O+PPHE (5 mg/ml);
- H_2O+$CaCl_2$+PPHE (5 mg/ml).

The second one does not contain TRIS/HCl (typically introduced in the functionalising solutions) because of its aggressive buffering action which could bias the pH value of the solution to analyse around 7.4, highly limiting the effect of the buffer – necessary to vary pH values of the sample during zeta potential evaluation. In any case the aim of this measurement is to evaluate the effect of calcium ions on the solution zeta potential.
The pre-processing before to utilise the instrument is analogue to the one that is applied to solid samples analysis: the check of the flow and of he other parameters is the same. Anyway, this instrument does not automatically buffer an initial solution provided by the user, but every single solution characterised by different pHs has to be buffered manually and imposed to the device to be measured. The buffering solutions are the same which are exploited for solid samples:

- as an acid buffer, HCl solution 0.05 M: 410 ml of HCl (37% v) are spilled in 99.59 ml of ultra-pure water;
- as an alkaline buffer, NaOH solution 0.05 M: 0.2 g of KCl are dissolved in 100 ml of ultra-pure water.

Manually adding drop by drop respectively the acid and the alkaline buffer, nine samples for each type of functionalising solution are obtained with nine pH values:

- pH=2.5;
- pH=3;
- pH=3.5;
- pH=4;
- pH=5;
- pH=6;
- pH=7;
- pH=8;
- pH=9.

So, each sample is provided to the instrument step by step; for each pH value, zeta potential is measured three times and the average is given in output.

4.11 FTIR ATR

Attenuated total reflectance Fourier-transform infrared spectroscopy (FTIR ATR) is a technique through which an infrared (IR) spectrum characterising the analysed material – both solid and liquid samples – is obtained.

The instrument (Figure 4.10) is composed by a IR source, a IR detector, a small platform and a conditioning system, constituted by a PC. The sample to analyse has to be positioned onto the platform and put in contact with the source. If it is solid, it has to be positioned with the surface to analyse in contact with source itself; if it is liquid, a drop of the sample has to be delicately put on the platform in correspondence to the source. Between two different measures, the platform has to be adequately washed through pure ethanol.

Figure 4.10 FTIR instrument – a zoom on ATR accessory

An IR beam is generated by a source inside the instrument and directed towards an optically dense crystal, which, thanks to its high refractive index, creates an evanescent wave; this wave is extended beyond the surface of the crystal and reaches the sample. In the areas of the sample in which the wave is absorbed, an attenuated signal is emitted and detected as an interferogram signal by a sensor in the IR spectrometer inside the instrument. The final output is a IR spectrum, displayed on the screen of PC[36].

For this study, FTIR ATR is exploited to evaluate the presence of the peaks characterising the polyphenols eventually present in solutions and on functionalised surfaces. In fact, in organic compounds atoms are bound each other through covalent bonds, precisely σ bonds; these atoms are free to vibrate in four different ways, as illustrated in Figure 4.11. Stretching vibrations (symmetric or asymmetric) are due to a back and forth movement around an average separation distance, corresponding to the bond length; instead, bending vibrations (scissoring or rocking) are due to a 2D movement of the bond axis on the plane that the bond itself defines with another bond[37,38].

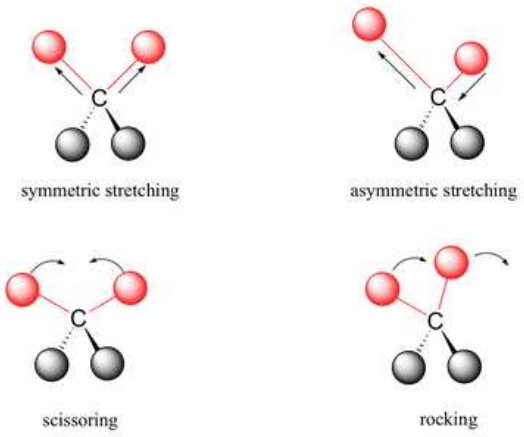

Figure 4.11 Chemical bonds vibrations[39]

The energy difference between stretching and bending vibrations – constituting two different energy levels of a molecule – appertains to the IR region of electromagnetic spectrum, whose wavelengths are included in a range going from 2.5 μm to 15 μm. So, stimulating an organic compound with IR radiation, the radiation itself induces vibration movements in the molecular structures which, vibrating, absorb the emitted radiation at precise wavelengths, depending on the analysed compound. This phenomenon is quantified measuring the transmittance of the compound in function of the wavelength (or the wavenumber, defined as $k=2\pi/\lambda$, where λ is the wavelength) at which radiation is emitted. The obtained spectrum constitutes a sort of "fingerprint" of the analysed chemical compound. In fact, the absorbance of the stimulating radiation lowers the transmittance value in correspondence of precise wavelengths (or wavenumbers), inducing the presence of the characterising peaks in the spectrum, so that each chemical compound is characterised by a specific IR spectrum, where each peak corresponds to a precise chemical bond[40].

4.12 Fluorescence

Polyphenols are naturally fluorescent compounds[41], so this property could be exploited to easily quantify their presence on a surface through fluorescence techniques. No previous treatments nor pre-processing are necessary.

Fluorescence is a phenomenon due to the emission of electromagnetic radiations by fluorescent molecules, generally in the visible spectrum, when excited by an incident radiation; the re-emitted light is at a lower energy level[42]. Excitation is due to the raising of the electrons of the stimulated molecules to a higher and unstable energy level; because of this instability, the energy in excess is re-emitted as fluorescence light to let the atom return to a stable state[43]. The response of the stimulated material has place almost instantaneously: the radiation is re-emitted after around 10 ns, so fluorescence stops as soon as the exciting radiation is removed[44].
Through fluorescence microscopy, polyphenols are stimulated with visible radiation: they re-emit red light, which could be detected by a sensor. Higher the intensity of red fluorescence, higher the quantity of the biomolecules on the surface of the functionalised sample. Fluoresce observation allows not only to verify the presence of polyphenols on surfaces, but also to investigate their distribution on the surface itself. The fluorescent microscope is an instrument where a source of visible light excites the sample, previously placed in a cell, and a sensor detects the re-emitted radiations. A PC is connected to the instrument to select the measure parameters and condition the acquired signal; through that, it is also possible zooming the image or translating the sample to analyse a different fraction of its area. A real-time image is given in output by the instrument.
In the current study, biomaterials have been analysed, both bare and functionalised, to evaluate the eventual presence of polyphenols and their distribution on the surface.

The analysed samples are the following:

- Bare HAp;
- Polyphenol-functionalised HAp (HAp+PPHE);
- Bare CT;
- Polyphenol-functionalised CT (CT+PPHE);
- Bare bioglass (SCNbis);
- Polyphenol-functionalised bioglass (SCNbis+PPHE).

The parameters at which the measurements have been carried on are the following:

- Zoom: x 20;
- Exposure time: 1 s;
- Source power: maximum;
- Filter: Rhodamine 573 nm (red).

4.13 FESEM

Field Emission Scanning Electron microscopy (FESEM) is a characterisation technique, able to provide two different informations to the user: the morphology of the sample and its semi-quantitative chemical composition. The technique is based on the emission of an electron beam by a source inside the instrument, which stimulates the sample to analyse and induces in it a response; it is read by a sensor and conditioned to convert the acquired signal in the information of interest. The instrument emits three different signals:

- secondary electrons give the information about topography: with high resolution, the morphology of the surfaces could be evaluated and the presence of eventual fractures both in porous and bulk materials are visible through a significant depth; a grey-scaled image of the sample is given in output by the instrument, with a previously selected zoom;

- backscattered electrons give the information about material composition, through the evaluation of the atomic weight of the single components of the surface: lighter the emitted colour, higher the atomic mass of the element; the instrument gives in output an image in which the greyscale corresponds to different atomic weights of the elements and allows a distinction of different morphological features with eventually different composition, but does not indicate the chemical elements which constitutes the surface;
- characteristic X-rays give a semi-quantitative compositional analysis: Energy Dispersion Spectroscopy (EDS) detector collects characteristic X-rays emitted from the sample, estimating what element corresponds to: this analysis gives in output a spectrum of the zone of interest, where each peak can be assigned to a specific element, and the elemental composition can be calculated as atomic or weight percentage.

Before to analyse a sample through FESEM, a criticality has to be considered. In fact, surfaces have to be conductive to let electrons flow through the material and adequately respond to the electronic stimulation to be analysed. If the samples are conductive, they are directly analysable, while, if they are not, it is necessary to pre-process them, applying to their surface a nanometric metal coating. For the current research, titanium samples are immediately analysable, while hydroxyapatite, SCNA and SCNbis have to be covered by a nanometric layer of chromium: electrons, in this way, do not accumulate on the material, but can flow through it so that analysis can be optimised. The choose of the appropriate metal coating for a certain material is quite critical: the added film do not have to produce interferences during the instrument elaboration: in this case, chromium has been chosen instead of the more common gold because in the Energy Dispersive X-ray Spectrometry (EDS) spectrum the peak of gold is very to the phosphorus one and this situation can compromise the correct interpretation of measures.

4.14 Polyphenols release in water

Polyphenols, previously grafted to a biomaterial surface, could be characterised through a release test, to evaluate their behaviour when immersed in water/water-base media for a programmed experimental time. Also if the polyphenols-biomaterial interface is sufficiently stable, it is possible that some ion exchanges have place and a fraction of the grafted biomolecules (or evenly the superficial atoms constituting the first layer of the biomaterial sample) are released in water because of diffusive and degradative phenomena.

The study of this phenomenon is important in order to understand if the mode of action of polyphenols can be related to a local action from the surface or to molecular release in physiological fluids.

The previously functionalised samples of biomaterials are immersed in 15 ml of ultra-pure water. Through a pipette, 15 ml of ultra-pure water are spilled in each container, one for each of the samples to analyse. Then, each sample is put inside each container through a pair of tweezers. Finally, all the set of containers is left in the incubator at 37°C for 28 days. The effects due to the release in water are evaluated in four experimental times after the start of the test:

- 1 day;
- 7 days;
- 14 days;
- 28 days.

For each of the experimental steps, each sample is extracted from the release solution and put in the incubator at 37°C immersed in other 15 ml of new ultra-pure water. Then, the 15 ml of water in which the samples were previously immersed are analysed. At every experimental step, each solution is studied through pH evaluation, F&C test and UV spectroscopy. At the final experimental step (28 days), also the solid samples are analysed through F&C test, DPPH test, FTIR ATR and fluorescence; the results are compared with the ones obtained from just functionalised samples.

4.15 Sterilisation

Two macro-groups of samples were prepared to be compared: the first one was only treated with polyphenols solution before to be analysed, while the second one was functionalised at the same time of the first one and then sterilised. In this way it had been possible to investigate the effect of sterilisation minimising the effect of storage.

Sterilisation is a fundamental step, usually the last step at the end of device production, to guarantee the absence of bacteria and other potentially dangerous sources of infection after the device implantation in human body.

Sterilisation was implemented by Gammatom (Guanzate, Como, Italy), an Italian industry specialised in sterilisation processes on different types of materials in different fields of application.

The samples were packed and treated with γ radiations at 25 kGy (standard sterilisation for medical devices); sterilisation was monitored through a dosimeter previously applied to the packaging.

To be sterilised, each sample – at which a 3D Cartesian reference system is associated – is inserted in an irradiation container, characterised by fixed dimensions to let the intensity of the dose be known in every point of the volume. Through the reference system, it is also possible to control the orientation of the sample in space and to modulate its distance from the source. The sample is inserted in the irradiation container and extracted at the end of the process by a conveyor, driven by a completely automatised mechanical system[45].

The functionalised samples are so analysed before and after sterilisation through UV spectroscopy, F&C test, FTIR-ATR and fluorescence.

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CHAPTER 5
RESULTS

5.1 Polyphenols functionalising solutions

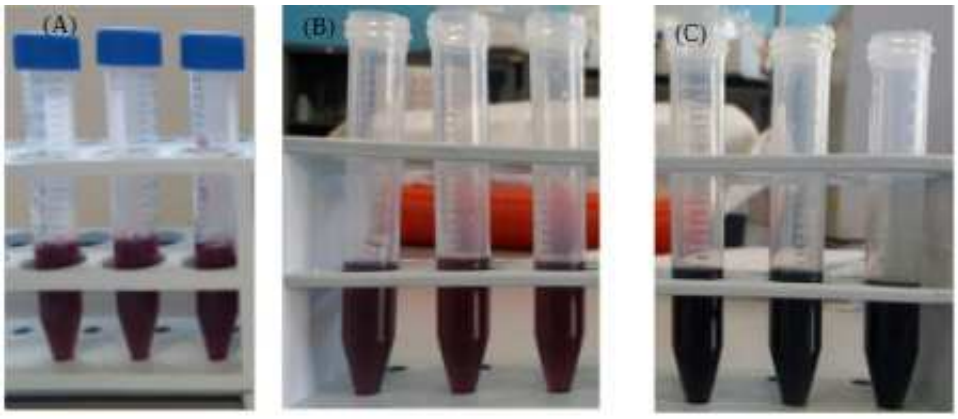

Figure 5.1 Polyphenols solutions (5 mg/ml):
(A) H_2O+PPHE; (B) TRIS/HCl+$CaCl_2$+PPHE; (C) H_2O+EtOH 50% v/v +PPHE

In Figure 5.1 the three functionalising solutions are illustrated. First, it is possible to appreciate the difference of the colour characterising each solution. While H_2O+PPHE is purple, the same colour characterising lyophilised polyphenols, TRIS/HCl+$CaCl_2$+PPHE is appreciably darker and tends to be quite black, with a blue nuance, due to the formation – as successively described – of the complex between polyphenols and calcium; H_2O+EtOH 50% v/v +PPHE is instead quite black because of the presence of EtOH, which influences the solubility of polyphenols. As successively reported in the next paragraphs, these differences are due to significant variations of pH and the formation of molecular complexes. The different colours typical of the first two solutions is compared in the following image (Figure 5.2), where the blue/black nuance of TRIS/HCl+$CaCl_2$+PPHE solution is easier visible.

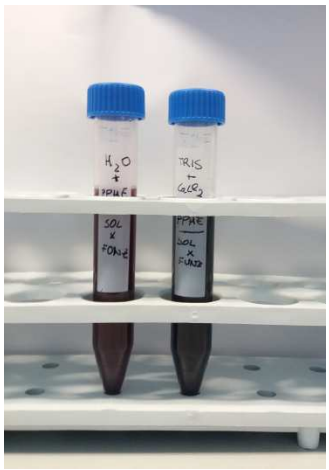

Figure 5.2 H_2O+PPHE and TRIS/HCl+$CaCl_2$+PPHE solutions comparison

5.1.1 pH

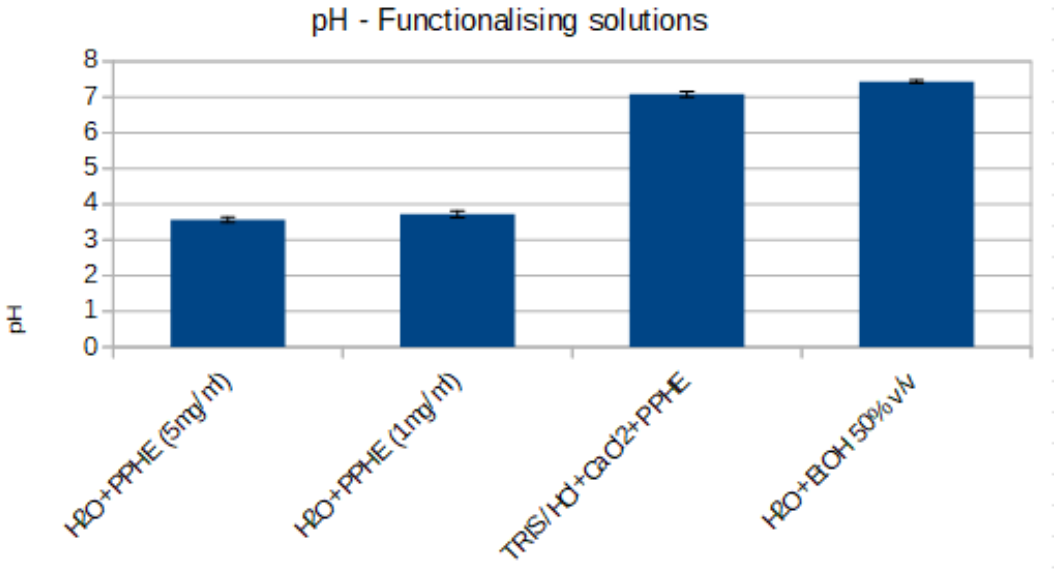

Figure 5.3 pH values characterising functionalising solutions

As appreciable in Figure 5.3, the pH values of H_2O+PPHE solutions are comparable, regardless of the concentration of polyphenols. It means that the presence of polyphenols is sufficient in both the solutions to take the pH value in an acidic range, typical of polyphenols around 3.55 or lower; the value depends on the nature of the phenolic compounds[1].
The other two functionalising solutions, TRIS/HCl+$CaCl_2$+PPHE and H_2O+EtOH 50% v/v, are characterised by a higher pH value: it is intrinsic to the preparation method, through which pH is imposed by the buffering action of TRIS and NaOH solution 0.05 M, respectively.

5.1.2 UV spectroscopy

H_2O+PPHE and TRIS/HCl+$CaCl_2$+PPHE solutions have been investigated through UV spectroscopy and compared.
As illustrated in Figure 5.4, in the UV spectrum characterising H_2O+PPHE solution three main peaks are visible:

- λ=250 nm corresponds to isoflavones;
- λ=280 nm corresponds to flavanols;
- λ=500 nm corresponds to anthocyanins.

The first two peaks characterise the most part of polyphenols: different classes of polyphenols are characterised by the presence of absorbance peaks in correspondence of 250 and 280 nm[2]. The analysed solution contains a mixture of polyphenols, appertaining to different classes. In the current study, these absorbance peaks are so frequently present in the obtained spectra when polyphenols are dissolved in a solution or grafted to a solid substrate. A study demonstrated that the peak corresponding to 250 nm characterises isoflavones, while the one at 280 nm is due to the presence of flavanols, especially epicatechins[3]. The third peak, corresponding to 500 nm, is instead proper of anthocyanins[4].

A peak in correspondence of 270 nm is less visible, but present, to confirm the presence of hydroxycinnamic acids in the analysed solution[5].

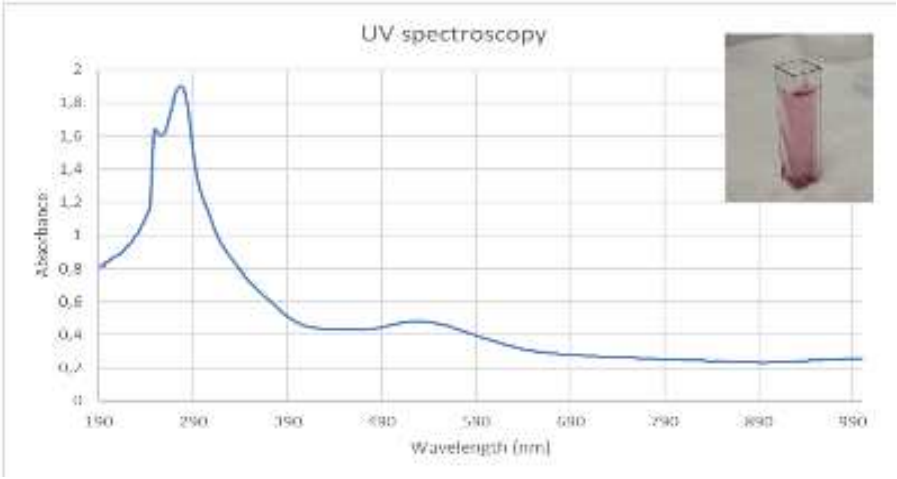

Figure 5.4 UV spectrum of H_2O+PPHE solution and the 1:60 diluted sample

TRIS/HCl+$CaCl_2$+PPHE solution is instead characterised by the spectrum reported in Figure 5.5. The absorbance peaks at 250 and 280 nm are appreciable also in this case: it can be induced that there are no alterations in the molecular structure of isoflavones and flavanols caused by the solution with a pH of 7.4 and the presence of calcium ions (TRIS/HCl+$CaCl_2$+PPHE)[6]. Also the hydroxycinnamic acids peak is maintained[7].

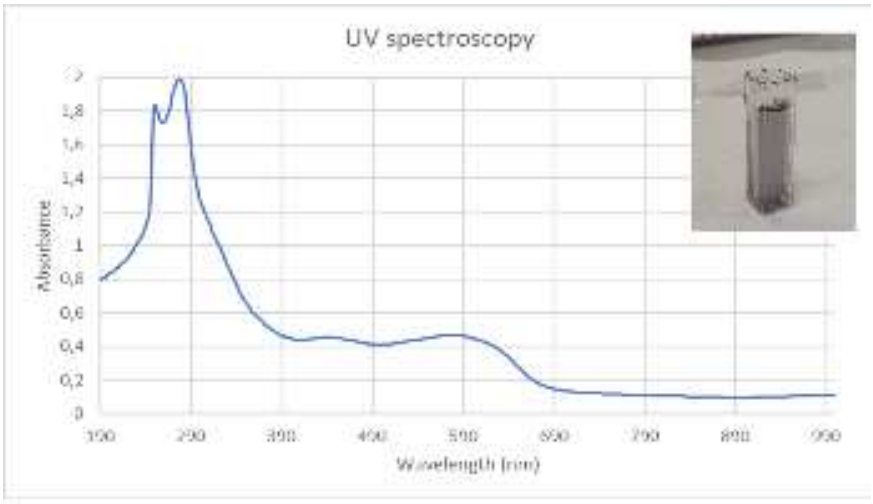

Figure 5.5 UV spectrum of TRIS/HCl+$CaCl_2$+PPHE solution and the 1:60 diluted sample

Anyway, this spectrum presents two significant differences.
First, an appreciable absorbance local maximum is present around 600 nm, which can be attributed to the molecular complex which polyphenols form with calcium ions Ca^{2+}. The formation of this complex is due to the presence of Ca^{2+} in the solvent and to the dissociated hydroxyl group appertaining to COOH group of phenolic compounds (COO^-). Ca^{2+} is obtained through the dissociation of $CaCl_2$ in an aqueous environment[8]:

$$CaCl_2 \rightarrow Ca^{2+}+2Cl^-$$

Exploiting gallic acid (GA) as a model for a generic polyphenol, the dissociation of its OH group, characterised by a pK_a value of 4.4 – lower than the pH value characterising TRIS/HCl+$CaCl_2$+PPHE solution (pH=7.4) – has place as illustrated in Figure 5.6[9]. Polyphenols, negatively charged in aqueous environment, easily interact with calcium cation. The phenomenon of dissociation and formation of the molecular complex with Ca^{2+} is analysed in paragraph 5.1.5.

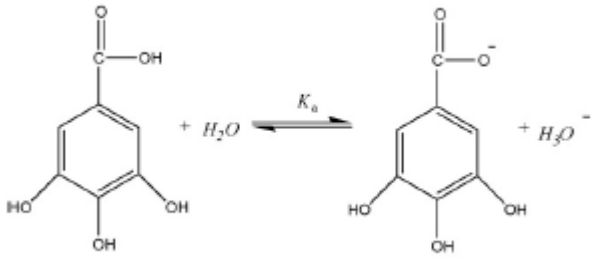

Figure 5.6 GA carboxyl group dissociation in aqueous environment[10]

The second difference is constituted by the shift of the peak corresponding to anthocyanins: in this case is not present at 500 nm as in H_2O+PPHE solution, but at 450 nm. In fact, utilising TRIS/HCl+$CaCl_2$ as solvent, and, consequently, imposing neutral pH, de-protonation occurs at pH=7, causing this shift of the absorbance peak of anthocyanins, more sensitive to this phenomenon. At pH=7.4 – the imposed value during solution preparation – de-protonation is almost complete and this effect is visible through the cited peak shift[11]. De-protonation causes also variations in the colour of the solution, as cited in the previous paragraph: the number of hydroxyl groups in B ring induces a displacement in chromaticity, while pH increases[12]. So, the purple solution becomes darker and assumes a blue-black nuance. Gallic acid, because of de-protonation, assumes a green colour, a phenomenon that is covered by the dark colouring induced by the formation of the complex between polyphenols and calcium – typical of the iron-gallate inks, utilised still Antique Greece[13].

The comparison of the UV spectra characterising the functionalising solution is reported in the following plot (Figure 5.7).

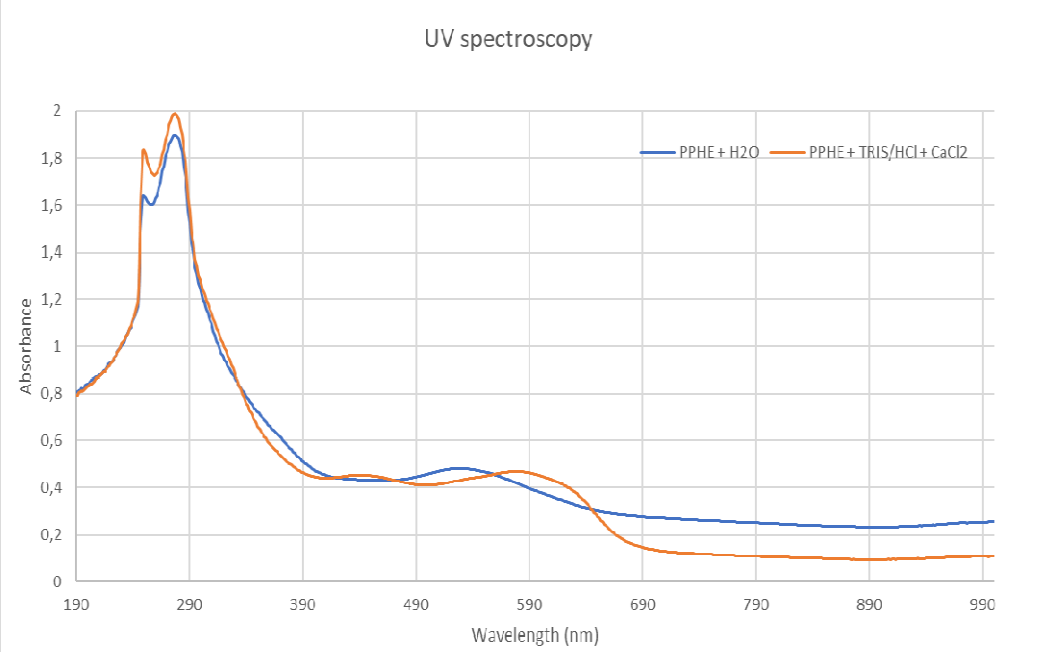

Figure 5.7 UV spectra of H_2O+PPHE and TRIS/HCl+$CaCl_2$+PPHE solutions

The higher intensity of the peak at 280 nm characterising TRIS/HCl+$CaCl_2$+PPHE solution spectrum is caused by the signal due to π-system of aromatic rings at pH>7, which is added to the before cited signal induced by the presence of flavanols[14].

5.1.3 FTIR-ATR

FTIR-ATR analysis has been carried on to evaluate the transmittance peaks characterising polyphenols and the functionalising solutions.

The spectrum characterising the lyophilised polyphenols – utilised to prepare the functionalising solutions – is reported in Figure 5.8.

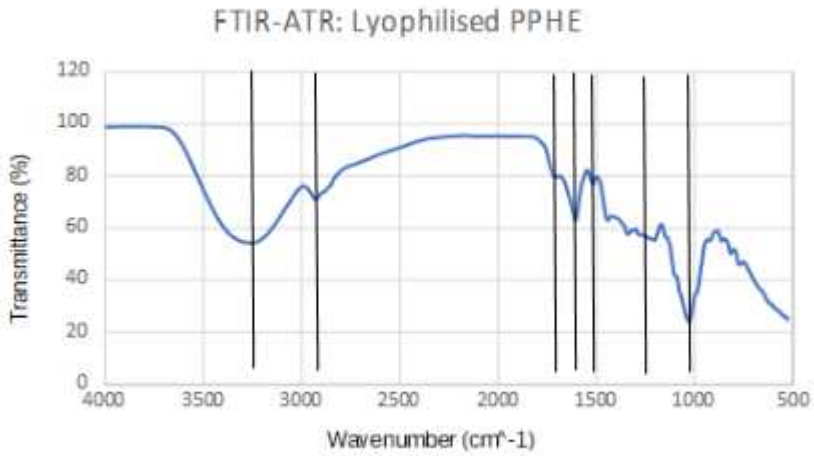

Figure 5.8 FTIR-ATR spectra of lyophilised polyphenols

Analysing the spectrum, it is possible to distinguish the characterising transmittance peaks in correspondence of the following wavenumbers (k)[15,16,17]:

- k=3600-2900 cm^{-1}: OH bonds;
- k=3010-2700 cm^{-1}: CH bonds;
- k=1715 cm^{-1}: aromatic ring;
- k=1700 cm^{-1}: COOH group;
- k=1637 cm^{-1}: C=O stretching vibrations;
- k=1250-1203 cm^{-1}: phenolic OH groups bending vibrations;
- k=1200-750 cm^{-1}: carbohydrates fingerprint.

These peaks and bands correspond to the cited bonds, characterising phenolic compounds. The 1250-1203 cm^{-1} transmittance band is peculiar of the polyphenols and corresponds to the in-plane bending vibration of phenolic OH groups which are present at 3, 4, 5 positions of the aromatic rings[18]. Furthermore, the presence of carbohydrates in the mixture is significant: as illustrated in Chapter 4, to not degrade polyphenols, the extraction process is not aggressive, so carbohydrate traces are present in the lyophilised polyphenol mix. The carbohydrates that are naturally bound to polyphenols molecules in plants are so still present and bound also after extraction and lyophilisation: the polyphenols utilised in the current study are so in the glycosylated form.
In Figure 5.9, the IR spectra of the two functionalising solutions – H_2O+PPHE and TRIS/HCl+$CaCl_2$+PPHE solutions – are reported. It is evident that the peaks of the solvent (H_2O), more intense, cover the ones characterising the solute. So, both the solutions are characterised by the same IR spectrum, which corresponds to the water one. Four peaks are, in fact, typical of water spectrum[19,20]:

- k=3600-2900 cm^{-1}: OH bonds;
- k=2100 cm^{-1} (low intensity): overtone of OH bonds rotation;
- k=1600 cm^{-1}: HOH bending;
- k=900-500 cm^{-1}: molecular vibration.

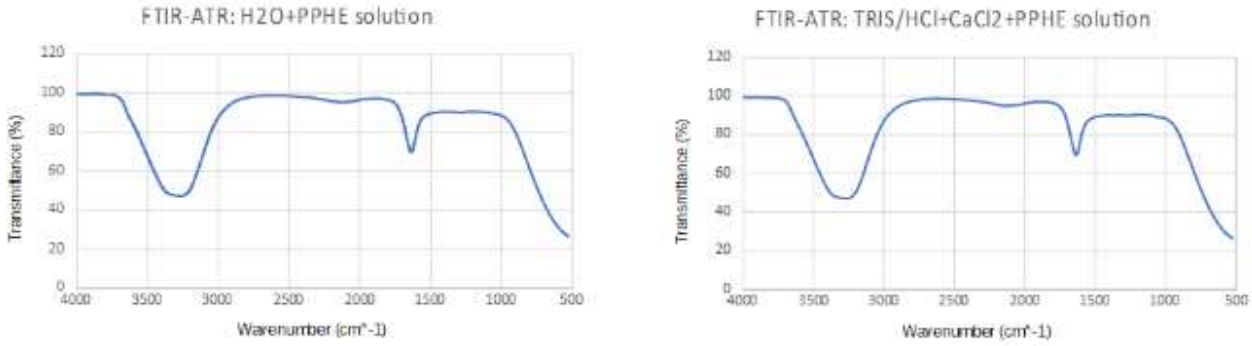

Figure 5.9 FTIR-ATR spectra of functionalising solutions

Moreover, the broadening of the signal in the 3000-3600 cm^{-1} region together with the disappearance of the characteristic fingerprint has been attributed to the partial polymerization of polyphenols in solution[21].
The comparison between the spectra of the lyophilised polyphenols and the two analysed solutions is appreciable in Figure 5.10, where also the perfect match of the spectra of the functionalising solutions is well visible.

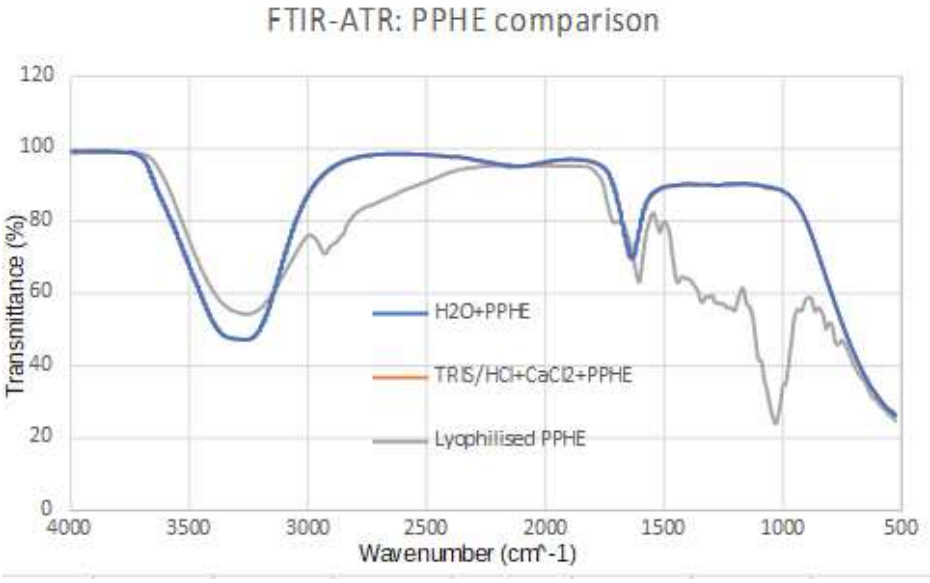

Figure 5.10 Comparison of FTIR-ATR spectra

5.1.4 F&C test

the total concentration of polyphenols (as Gallic Acid Equivalents – GAE) characterising the functionalising solutions has been evaluated through F&C test.

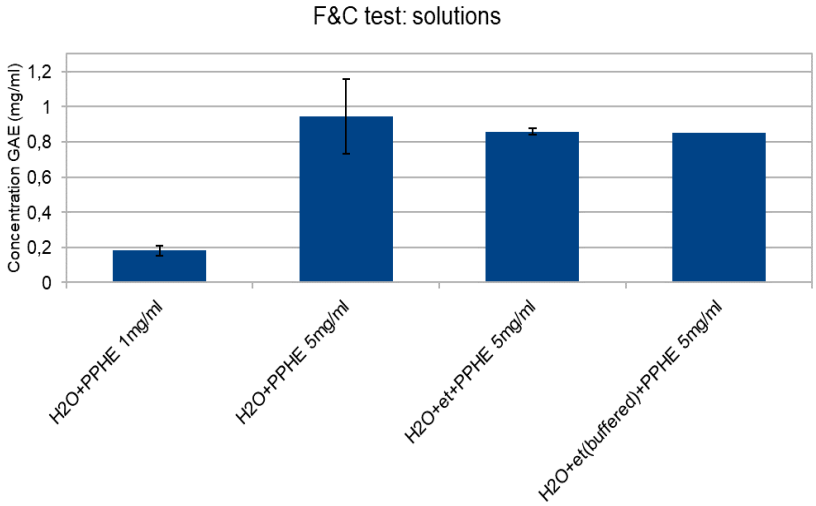

Figure 5.11 F&C test: polyphenols GAE concentrations of functionalising solutions

As appreciable in Figure 5.11, the most polyphenols-enriched solution is H_2O+PPHE (5 mg/ml), characterised by an average concentration of 0.911 mg/ml GAE. The other aqueous solution, H_2O+PPHE (1 mg/ml), instead, is not adequate to functionalise biomaterials because of the low polyphenols concentration, which does not even reach 0.200 mg/ml GAE: for this reason, 5 mg/ml has been chosen to treat the biomaterial surfaces.
H_2O+EtOH 50% v/v solution has been evaluated before and after being buffered: there are not significant variations of the polyphenols content caused by the imposed pH variation.

5.1.5 Zeta potential

Zeta potential of H_2O+PPHE and TRIS/HCl+$CaCl_2$+PPHE solutions has been measured in function of pH. Different solutions, buffered with HCl or NaOH, have been prepared for the different pH values to be explored, as described in the materials and methods section. In Figure 5.12 the buffered solutions are reported, where it is possible to appreciate the colour differences: the solutions becomes darker when pH increases, as cited in the previous paragraphs, going from grape-purple to indigo, quite black for pH=8-9.

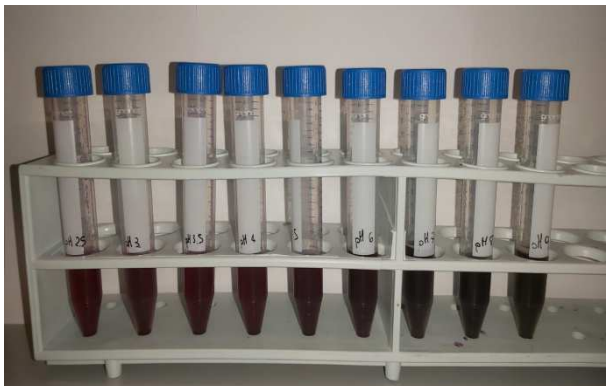

Figure 5.12 Buffered functionalising solutions (TRIS/HCl+$CaCl_2$+PPHE) to be characterised through zeta potential

Zeta potential characterising H_2O+PPHE and TRIS/HCl+$CaCl_2$+PPHE solutions is reported in Figure 5.13. Isoelectric point has been calculated through interpolation: it corresponds to pH=2.00 and pH=2.21, respectively.
The first significant aspect to highlight is the fact that polyphenols, when dissolved in an aqueous environment, are characterised by a negative zeta potential for the evaluated pH range. It is due to the extremely low isoelectric point value and means that polyphenols are negatively charged when dissolved in an aqueous solution.

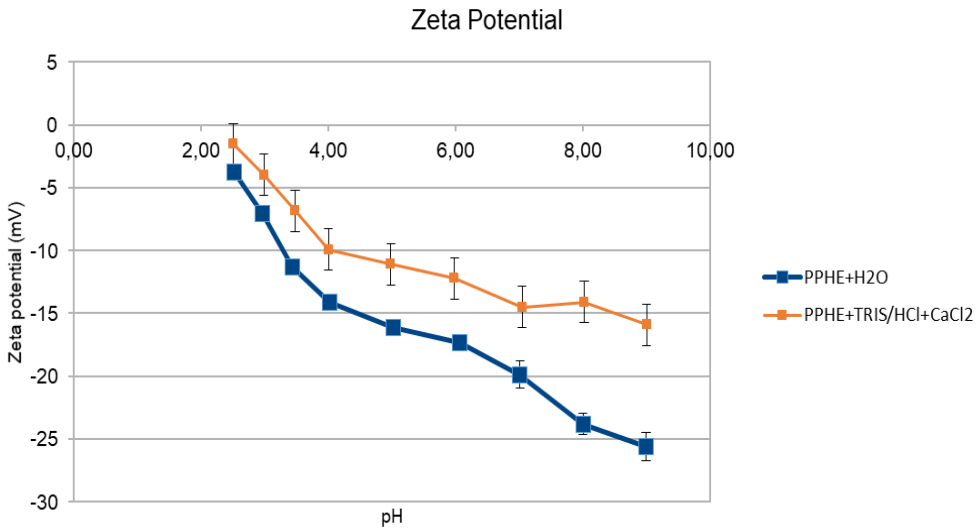

Figure 5.13 Zeta potential of H_2O+PPHE and TRIS/HCl+$CaCl_2$+PPHE solutions

It is possible to see how the absolute value of the charge increases when pH increases. The trend of the curves is non-linear: in fact, an important aspect characterising both solution is the variation of the slope around pH=4. This fact could be explained through the dissociation of OH groups appertaining to the carboxyl groups that are present in the molecular structure of polyphenols. Gallic acid (GA), often exploited as a model of a generic polyphenol, is characterised by pK_a=4.4: as illustrated in Figure 5.14, COOH group is not dissociated under this value, while, over this threshold, it is. Thanks to this phenomenon, grafting processes can have place, so that surfaces could be easier functionalised[22].

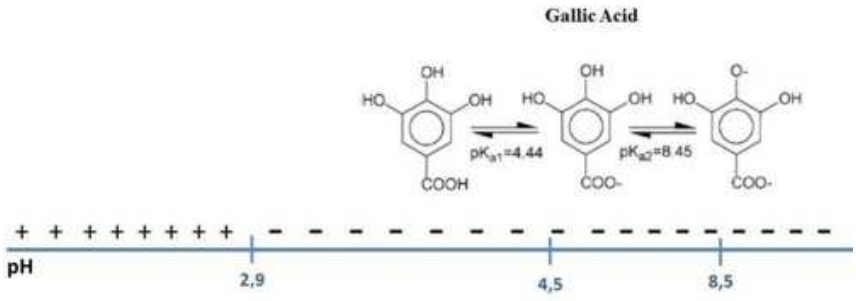

Figure 5.14 pH-dependent dissociation of OH group in GA[23]

A peculiarity characterising only TRIS/HCl+$CaCl_2$+PPHE solution is the presence of a second variation of slope around pH=7. It could be explained through another dissociation process, which, in this case, involves phenolic OH groups, whose dissociation has place around pH=8. In fact, reporting GA as a model (Figure 5.14), phenolic OH dissociation has place at pK_a=8.5[24]. This variation of slope causes the reaching of a plateau around ζ= -15 mV, which characterises

exclusively TRIS/HCl+$CaCl_2$+PPHE solution, probably due also to the non-negligible presence of the positive calcium ion; H_2O+PPHE zeta potential has instead a decreasing trend.
The difference between the two curves at alkaline pHs could be explained by the fact that, because of the presence of calcium ion Ca^{2+} – obtained by the dissociation of $CaCl_2$ – in TRIS/HCl+$CaCl_2$+PPHE solution, phenolic OH groups dissociate simultaneously, once reached a certain pH value; without Ca^{2+}, it is supposed that the dissociation of these hydroxyl groups has place progressively when pH increases[25]. This consideration could conduce to the hypothesis for which the polyphenols-calcium complex is involved in the chemical reactivity variation of polyphenols, inducing the same acid force in phenolic hydroxyl groups after the formation of the complex itself.

5.2 Ethanol solutions

The six H_2O+EtOH solutions, respectively characterised by the six different v/v concentration ratios illustrated in Chapter 4, are reported in Figure 5.15.

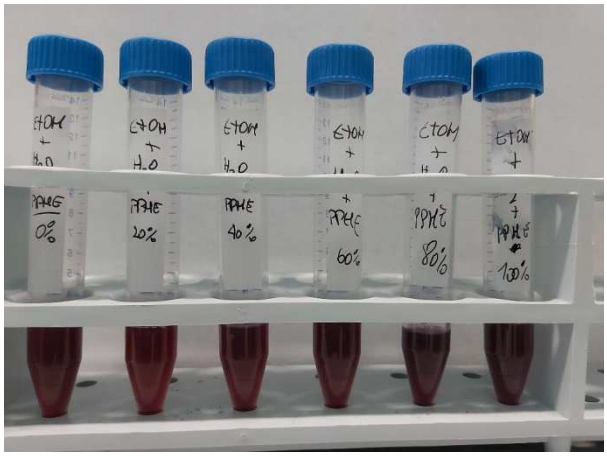

Figure 5.15 H_2O+EtOH solutions:
0% EtOH, 20% EtOH, 40% EtOH, 60% EtOH, 80% EtOH, 100% EtOH

First, it is possible to appreciate the differences of the colour characterising each solution: increasing EtOH percentage, the solution becomes darker. It is due to the significant pH alterations induced by the higher EtOH content – as successively illustrated – which probably cause a partial degradation of the dissolved polyphenols[26].
In Figure 5.16, the pH values characterising EtOH solutions are reported. The trend of pH of the two functionalising solutions is opposite: while pH of EtOH+H_2O+PPHE increases according to the increase of EtOH content, EtOH+TRIS/HCl+$CaCl_2$+PPHE is characterised by a continuous decreasing trend. In fact, increasing EtOH content, the TRIS percentage becomes lower in the analysed solution, so that its buffering action becomes less efficient. Furthermore, the pHmeter is particularly sensitive to EtOH: the error associated to the instrument increases when this substance is present in the solution to analyse.

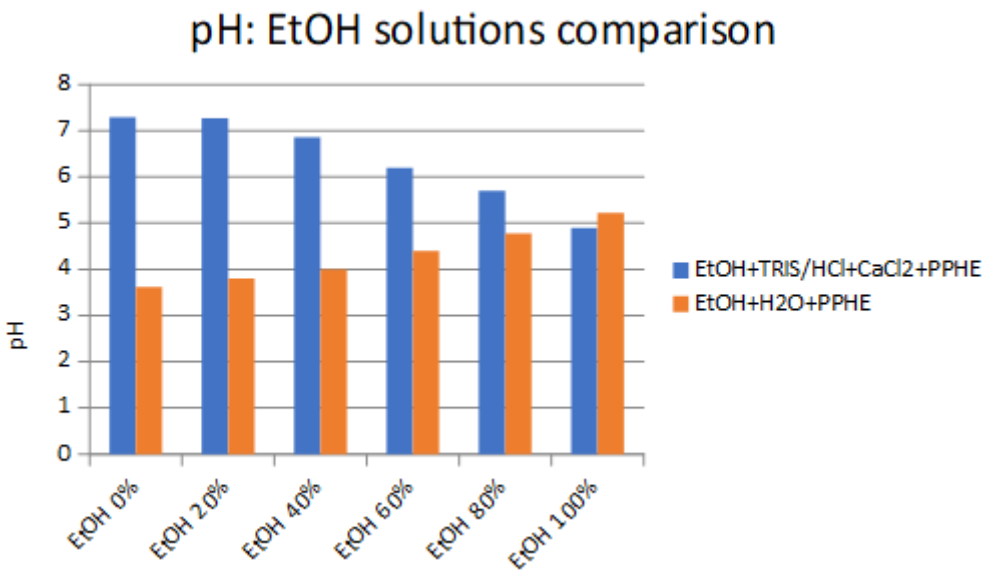

Figure 5.16 pH values of ethanol solutions

The concentration of polyphenols has been evaluated through F&C test, to determine the GAE concentrations characterising each solution. Two solutions have been diluted and compared. The first one, EtOH+TRIS/HCl+$CaCl_2$+PPHE (5 mg/ml), has been diluted for each EtOH percentage with TRIS/HCl+$CaCl_2$+PPHE solution, with a ratio of 1:12.5; finally, it has been filtered (200 nm). The second one, EtOH+H_2O+PPHE (5 mg/ml), has been diluted for each EtOH percentage with H_2O+PPHE solution, with a ratio of 1:30. These two dilutions have been necessary to not saturate the instrument during the measurements. The histogram reported in Figure 5.17 illustrates how the polyphenols content is significantly higher in EtOH+TRIS/HCl+$CaCl_2$+PPHE solution, despite the filtering action, at the same EtOH concentration.

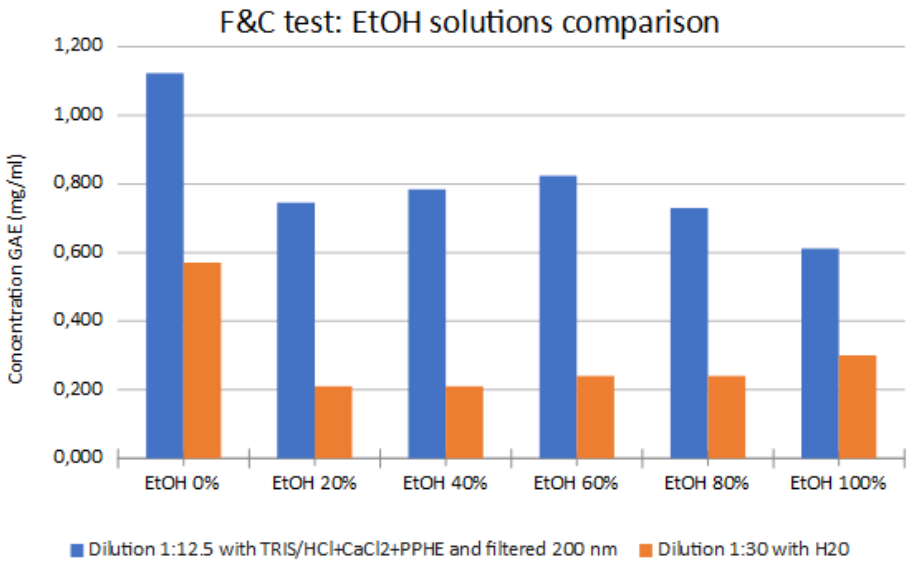

Figure 5.17 Concentration GAE of ethanol solutions

The decreasing of GAE concentration in the solutions containing EtOH could be explained by the fact that the increasing of the ionic strength of the medium – from 10^{-3} to 10^{-1} M –, in response to the increasing of EtOH percentage, induces aggregation and polymerisation processes, due to Van der Waals interactions and hydrogen bonds. In fact, both electrostatic and polar interactions are possible thanks to the amphiphilic nature of polyphenols, thanks to the presence of the hydrophobic aromatic rings and the hydrophilic OH groups. So, high ionic strengths induce the decreasing of solubility of polyphenols in the medium because of the aggregation of the biomolecules: these aggregates and – generally – molecules characterised by a higher degree of polymerisation are less soluble[27].

5.3 Hydroxyapatite

After the functionalisation process, both solid samples – HAp samples – and the functionalising solutions – called uptakes after the functionalisation process – are analysed.

In Figure 5.18 HAp lab functionalised samples are observable.

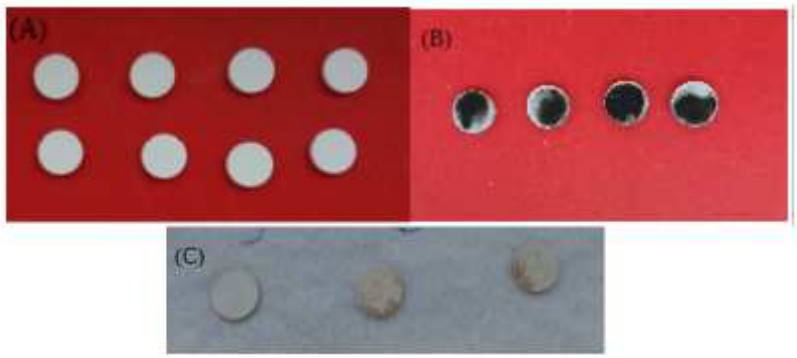

Figure 5.18 HAp lab: (A) before functionalisation; (B) after functionalisation (5 mg/ml); (C) after functionalisation (1 mg/ml).

Because of the relative bland sintering conditions, HAp lab samples are characterised by a significant porosity and a high surface-to-volume ratio, which increases the efficacy of the functionalisation process. In Figure 5.18(B), it is possible to see a quite inhomogeneous distribution of polyphenols on the biomaterial surface: because of the high surface-to-volume ratio and the reactivity of HAp, polyphenols are concentrated only in certain areas, while others are approximately bare; the covered areas present a high polyphenols content, as visible by the concentrated colour characterising these areas. In fact, as appreciable in Figure 5.18(C), for lower polyphenol concentrations (1 mg/ml), the grafting process is not efficient: the surface is only partially covered by the biomolecules and the low intensity of the colour indicates a low polyphenols content.

Industrially sintered HAp – reported in Figure 5.19 – is instead characterised by lower porosity and surface-to-volume ratio because of the more aggressive sintering conditions. So, the distribution of polyphenols on the surface of the samples is quite inhomogeneous: the grafted biomolecules occupy only a fraction of the available surface and are distributed as spots. Also in this case the difference between the effects of the two functionalising solutions (respectively 1 mg/ml and 5 mg/ml) are visible at the end of the functionalisation process. The less concentrated solution causes a relative

more homogeneous distribution with a low polyphenols content, as appreciable through the low intensity of the colour on the surface; 5 mg/ml solution induces a spotted distribution, characterised by a higher local polyphenol content.

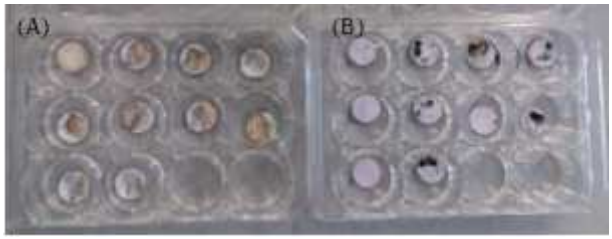

Figure 5.19 HAp ind: (A) after functionalisation (1 mg/ml);
(B) after functionalisation (5 mg/ml).

The uptakes are reported in Figure 5.20: H_2O+PPHE (1 mg/ml) solution is significantly lighter than the H_2O+PPHE (5 mg/ml) solution, which instead is characterised by an intense purple, index of the higher polyphenols concentration.
These observations are quantified through successive analysis, as illustrated in the next paragraphs.

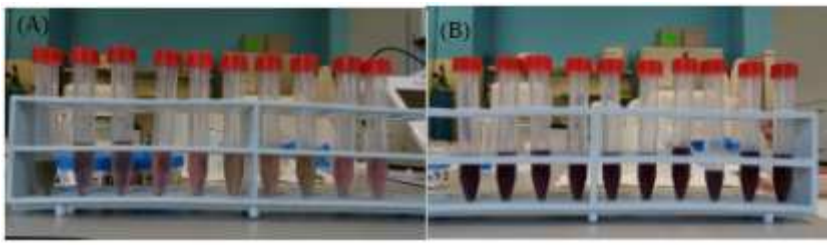

Figure 5.20 HAp uptakes: (A) 1 mg/ml; (B) 5 mg/ml.

5.3.1 pH

The uptake solutions are characterised by the following pH values (Figure 5.21); the functionalising solution utilised to treat HAp samples, H_2O+PPHE (5 mg/ml), has been reported as control.

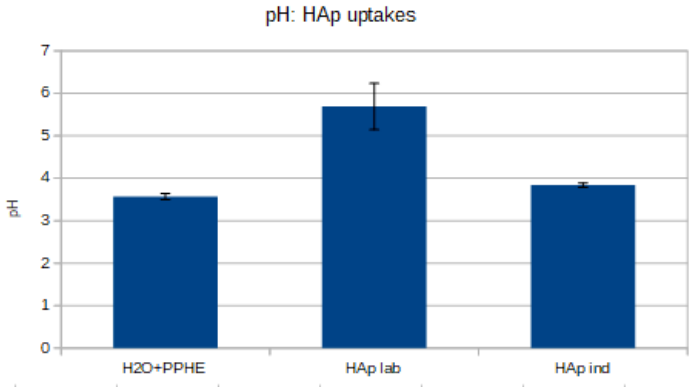

Figure 5.21 pH values of HAp uptakes

The qualitative considerations, illustrated in the previous paragraph, are confirmed. HAp lab is in fact more reactive: not only a higher quantity of polyphenols is grafted to HAp lab samples, but ionic and molecular exchanges have place in both directions during functionalisation process: it induces a significant pH increase and a higher variability of the measurements (quantified by the standard deviation). This is due to the exchange of hydrogen ions, calcium ions and phosphates in the functionalising solution: the presence of H^+ and Ca^{2+} in fact induces a significant variation of pH toward alkaline values[28]. The high surface-to-volume ratio amplifies this phenomenon.
HAp ind is instead quite stable when immersed in the functionalising solution, so that pH increasing is minor than for HAp lab uptake; the pH value of the HAp ind uptake is in fact comparable to the one characterising the functionalising solution itself. It is due to the lower porosity of the material and the lower surface-to-volume ratio: the exposed surface to the functionalising solution is lower, so that ionic exchanges are minor than for HAp lab.

5.3.2 UV spectroscopy

The UV spectra characterising respectively bare and functionalised HAp are reported in Figure 5.22. The reflectance of the samples has been analysed.

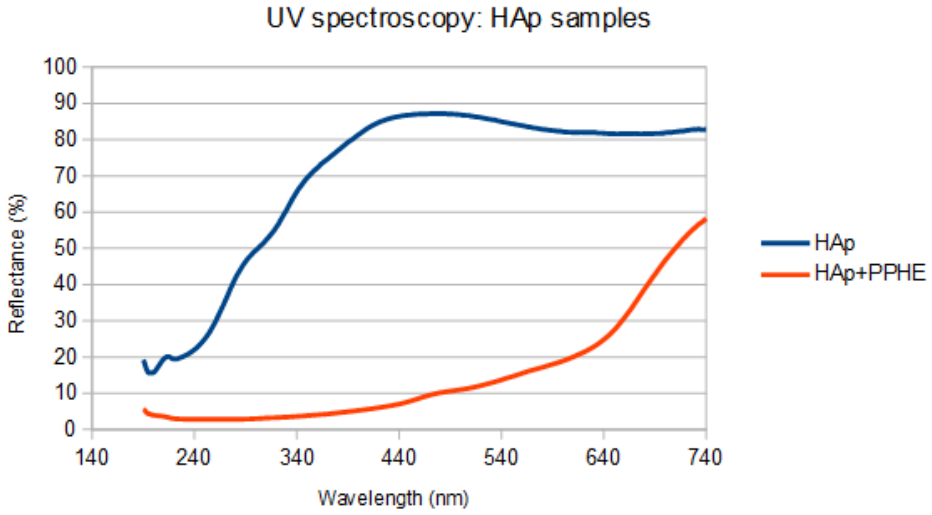

Figure 5.22 UV spectra of bare and functionalised HAp lab samples

It is possible to see a more intense signal associated to bare HAp, whose reflectance band is quite large and involves almost the entire evaluated wavelength range; a local peak is present around 450 nm. The functionalised sample is instead characterised by a low signal – almost a plateau – for low wavelengths and an increasing trend till 740 nm.
The difference between the two spectra could be explained through the schielding action exercised by polyphenols in the UV and visible wavelength spectrum on the sample surface. HAp lab samples are in fact homogeneously covered by the grafted biomolecules, which interact with the stimulating radiation, so that the reflectance signal becomes lower than the one detected from the bare sample.

5.3.3 FTIR-ATR

FTIR-ATR spectra characterising bare and functionalised HAp lab samples (Figure 5.23) have been acquired.

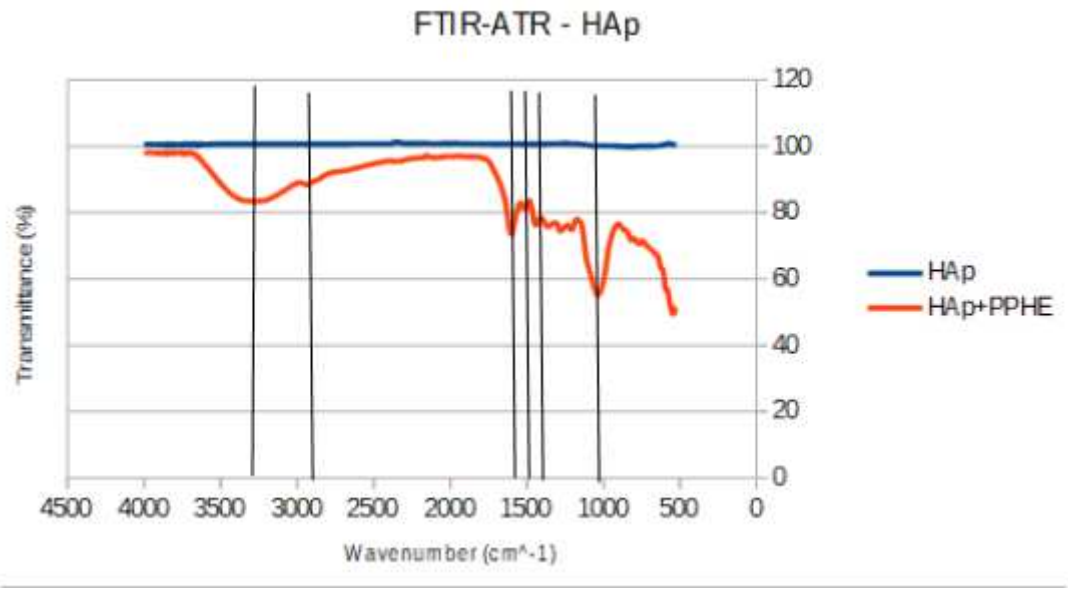

Figure 5.23 FTIR-ATR spectra of bare and functionalised HAp lab samples

Bare HAp is characterised by a quasi-constant spectrum, whose value is around 100%. The OH peak, typical of hydroxyapatite, is not visible in this measure because of the low contact between the analysed sample and the sensor: this loading error is frequent while working in ATR modality.
On the spectrum of functionalised samples, instead, the typical peaks of polyphenols are present in correspondence of the following wavenumbers and are well defined[29,30,31]:

- k=3600-2900 cm^{-1}: OH bonds;
- k=3010-2700 cm^{-1}: CH bonds;
- k=1715 cm^{-1}: aromatic ring;
- k=1700 cm^{-1}: COOH group;
- k=1637 cm^{-1}: C=O stretching vibrations;
- k=1250-1203 cm^{-1}: phenolic OH groups bending vibrations.

The first and the last cited transmittance bands are particularly intense to confirm the high quantity of grafted polyphenols on the analysed surface.
The spectrum characterising the functionalised sample is better defined: it is probably due to the fact that the polyphenols layer, also if nanometric, significantly contribute to increase the contact between the sample and the detector of the instrument; a higher signal-to-noise ratio is so obtained.

5.3.4 F&C test and F&C test modified for solid samples

In Figure 5.24, the experimental outcome of the F&C test carried on HAp solid samples is illustrated. In order to quantify the molecules with redox activity grafted on the solid samples, the F&C protocol (suitable for a solution) was modified in order to be applied to a solid sample as described in Chapter 4. According to previous results, this modified protocol is effective, but it is not completely clear at this stage if it quantifies the adsorbed molecules which are released into the F&C reagent solution during the test or the total amount of the grafted (unreleased) and adsorbed (released) molecules.

Concerning HAp lab samples, it is possible to appreciate the significant difference between the samples treated with H_2O+PPHE solutions characterised by the two different concentrations, respectively 1 mg/ml and 5 mg/ml. First, the high reactivity of the hydroxyapatite sintered in laboratory is appreciable: for 5 mg/ml solution, the high GAE concentrations of the grafted polyphenols on the surface (averaged, 0.5 mg/ml GAE) and the intrinsic variability of HAp functionalisation, which causes a high value of standard deviation – as visible through the error bars – are graphically appreciable. The samples treated with 1 mg/ml solution are instead covered by a significantly lower polyphenol quantity, 0.2 mg/ml GAE; it means that, despite the high reactivity of the material, the low concentration of this functionalising solution is not sufficient to obtain an efficiently functionalised sample.

Concerning HAp ind samples, the material responds analogously to the different treatments – respectively with H_2O+PPHE (5 mg/ml) and H_2O+EtOH 50% v/v +PPHE (5 mg/ml) solution. In fact, as visible in the histogram (Figure 5.24), the GAE concentration of the grafted polyphenols is comparable. Nonetheless, HAp ind samples functionalisation through H_2O+PPHE (5 mg/ml) solution is characterised by a higher variability of the process, as appreciable through the relative error bar, while the treatment with H_2O+EtOH 50% v/v +PPHE (5 mg/ml) solution is less variable. It could be explained by the phenomena correlated to the presence of ethanol in the functionalising solution: the aggregation of small molecules does not consent an efficient functionalisation process, increasing the interaction between polyphenols and, consequently, reducing the affinity with the surface[32]. Ionic release is reduced by the presence of EtOH: the release of calcium ions is so limited; this could induce the formation of a reaction layer able to adsorb polyphenols. Generally, it is possible to affirm that the presence of ethanol in the functionalising solutions decreases the efficacy of the treatment. In fact, the GAE concentration of grafted polyphenols characterising the HAp ind samples treated with H_2O+EtOH 50% v/v +PPHE (5 mg/ml) solution is lower than the one obtained with 1 mg/ml solution for HAp lab samples: it does not reach 0.2 mg/ml GAE.

Two main hypothesis could be developed:

1. a higher degree of polymerisation characterising the dissolved polyphenols in functionalising solution implies a higher dimension of the biomolecules themselves: it would induce a major surface covering and a higher number of exposed OH groups;
2. the polymerised polyphenols are more stable in the ethanol solution, so that they tend to not graft to the surface of the biomaterial, but to rest dissolved in the solution itself.

The apparent signal detected while analysing bare HAp is due partially to the off-set error of the instrument and to the reactivity of the material[33].

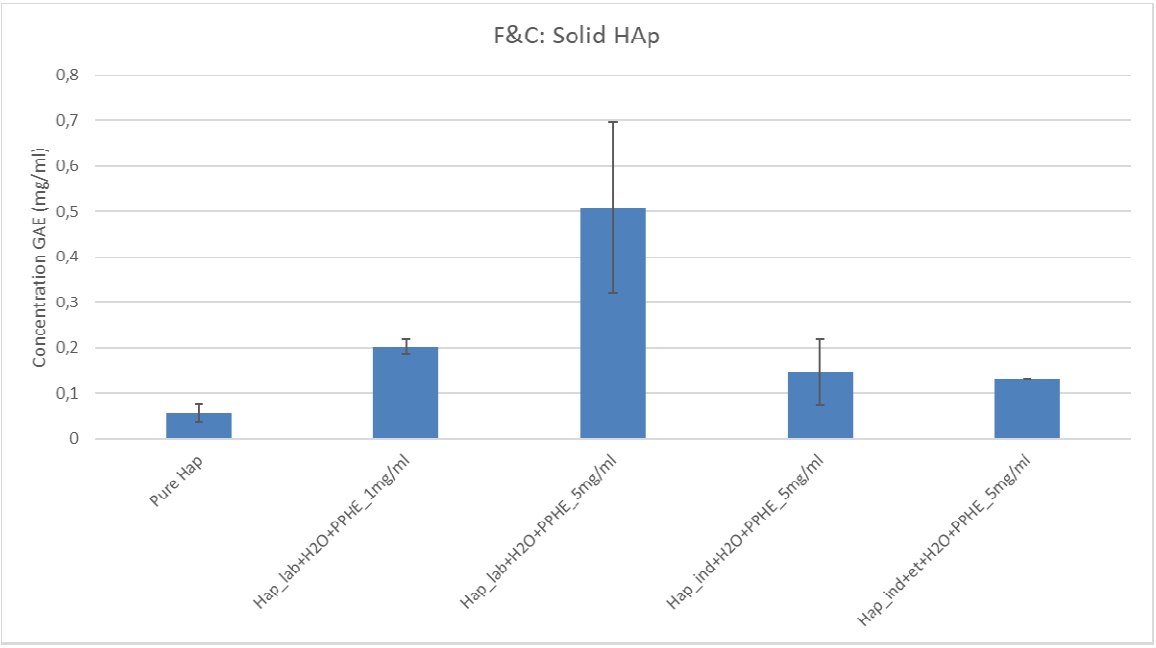

Figure 5.24 F&C test: polyphenols GAE concentrations on HAp samples

Uptakes have also been analysed through F&C test, whose results are illustrated in Figure 5.25. The histogram reports the GAE concentrations of the HAp uptakes – H_2O+PPHE (5 mg/ml) and H_2O+EtOH 50% v/v +PPHE (5 mg/ml) – and the relative functionalising solutions. There are not significant differences between the uptakes and their relative functionalising solutions, both characterised by comparable GAE concentrations. This phenomenon can be associated with the limited amount of molecules that can be grafted to the sample surface during functionalisation, which cannot change significantly the global concentration of the solution.

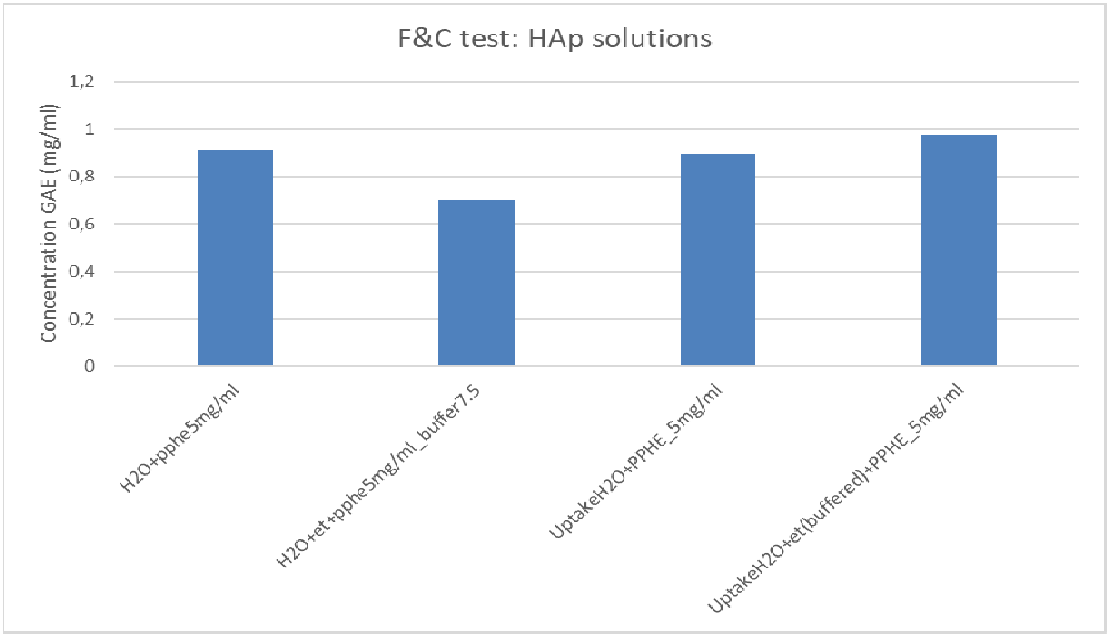

Figure 5.25 F&C test: polyphenols GAE concentrations of HAp uptakes

5.3.5 Zeta potential

Bare and functionalised HAp lab samples have been analysed through the evaluation of zeta potential.

In the following plot (Figure 5.26) zeta potential characterising the bare biomaterial is reported.

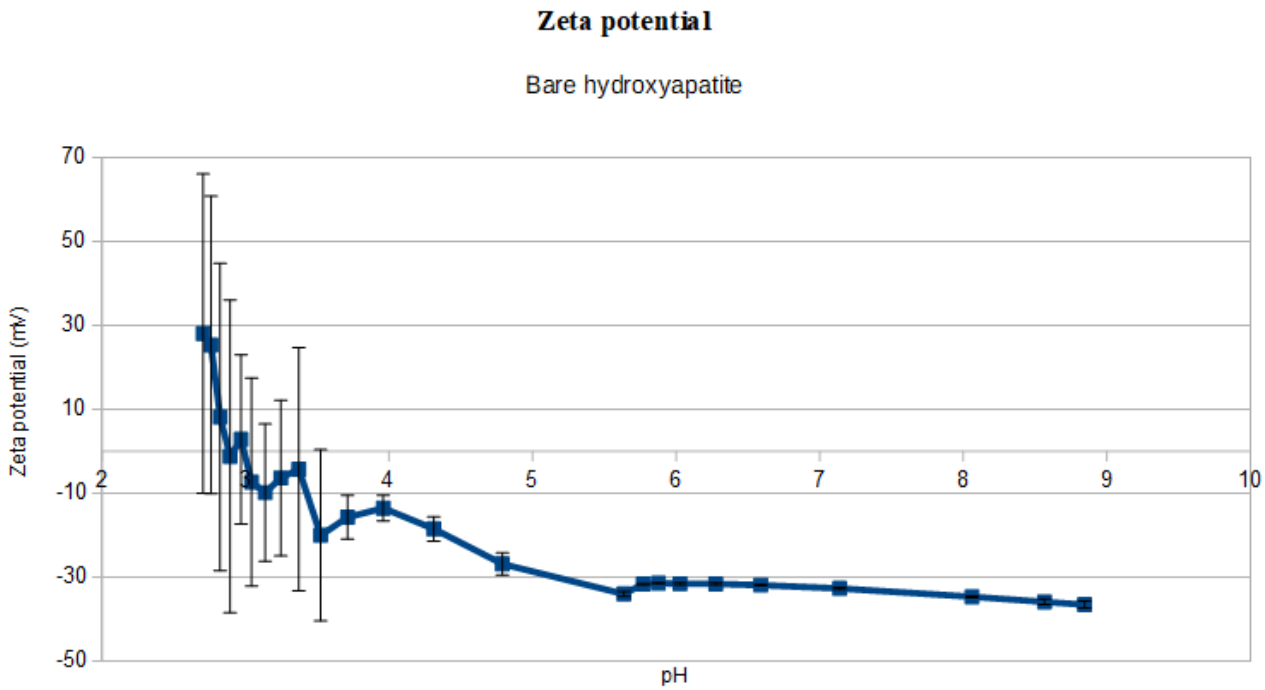

Figure 5.26 Zeta potential of bare HAp lab samples

The first significant aspect to highlight is the strong reactivity of HAp for low pH values (pH<3.5): the high standard deviation values characterising the measures in this pH range is due to the chemical instability of the material at strongly acid pHs. This is of relevance considering that during inflammatory phenomena (as it occurs just after implantation), pH can go down to values around 4, causing chemical instability of implanted HAp.

The hydroxyl groups appertaining to the lattice structure of HAp are completely dissociated for pH>5.5: in correspondence of this point, the slope of the curve decreases to reach a plateau, which tends to -35 mV. The material becomes particularly stable, so that the standard deviation of the curve significantly decreases for high pHs. The complete dissociation of OH groups for pH>5.5 confers an acid behaviour to the biomaterial[34]. In fact, despite the variance due to the reactivity, the isoelectric point, measured by the instrument and corresponding to pH=2.97, appertains to the acidic range. The acidic range of a material – whose surface is characterised by an acid-alkaline behaviour and the absence of functional groups – is so defined for pH<4, which is the reference value under whose threshold the isoelectric point is defined acid[35].

HAp is so positively charged and highly reactive at very low pHs (below 3), while it acquires a negative charge when pH increases. This fact explains the high reactivity of HAp during the functionalisation process, when it is exposed to an acid environment, as the functionalising solution is.

In Figure 5.27, the zeta potential of bare and functionalised HAp is reported. The functionalised samples are characterised by similar behaviour of the bare ones: the positive charge at acidic pHs, the tendency to reach the same plateau (-35 mV) for alkaline pH values. Isoelectric point, appertaining to acidic range, corresponds to pH=3.46 for the functionalised biomaterial (slightly higher than the one of bare hydroxyapatite, even if the high standard deviations of the bare material can affect the exact measurement of the isoelectric point). Nonetheless, comparing the standard deviation of the curves characterising the bare and functionalised samples, it is possible to see a decreasing of the biomaterial reactivity when polyphenols are grafted: the curve corresponding to HAp+PPHE samples is in fact characterised by a homogeneous standard deviation, which remains quite constant along the curve. This evidences the presence of a continuous layer of grafted biomolecules on the surface of the functionalised samples and the shielding ability of the grafted polyphenols with respect to surface reactivity of implanted HAp during inflammatory events and low pH physiological conditions.

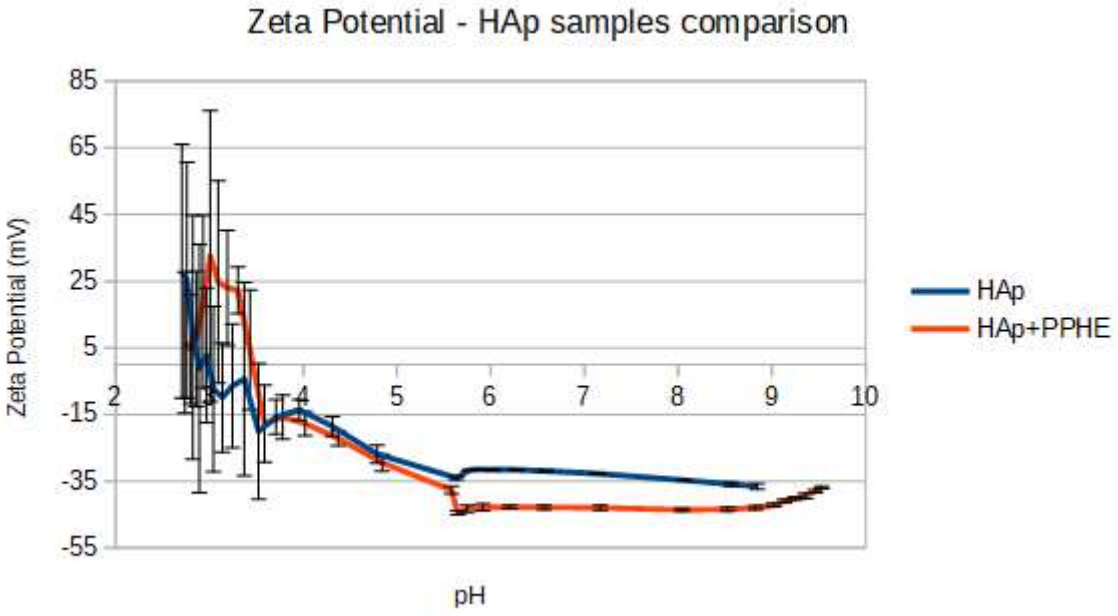

Figure 5.27 Zeta potential of bare and functionalised HAp lab samples

5.3.6 DPPH test

The antioxidant activity of the functionalised sample has been evaluated through DPPH test (Figure 5.28). Both HAp lab and HAp ind samples have been analysed, comparing the effects of H_2O+PPHE solutions characterised by the two polyphenols concentrations – respectively 1 mg/ml and 5 mg/ml. The control is constituted by the DPPH-EtOH solution without any solid sample immersed in it.

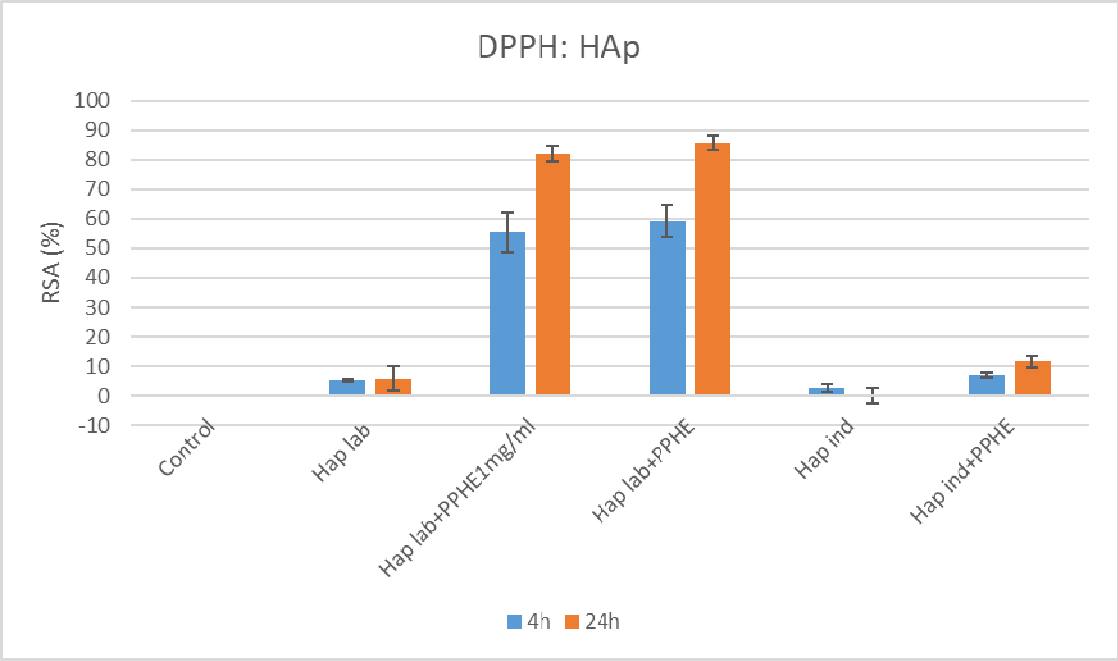

Figure 5.28 DPPH test on HAp solid samples (5 mg/ml)

Through this test, the antioxidant activity of the grafted polyphenols is quantified, which not necessarily corresponds to the quantification of the grafted biomolecules, because it is supposed that DPPH is sensitive to the outermost layer and not to the overall amount of grafted molecules. The antioxidant power of the samples functionalised with 1 mg/ml solution is comparable to the activity characterising the ones previously treated with 5 mg/ml solution: it means that in both cases there is a uniform layer of adsorbed molecules and the outermost layer is comparable, despite the difference in the total amount of adsorbed molecules. Moreover, they have a higher scavenging power when grafted to HAp lab samples than to HAp ind ones. As discussed in the previous paragraphs, the elevate surface-to-volume ratio permits a more efficient functionalisation. Then, the intrinsic porosity of the material consents to the biomolecules not only to be grafted to the surface, but also to enter in the porosities and be efficaciously physically adsorbed[36]. Physical adsorption does not saturate hydroxyl groups, which are still available to scavenge DPPH radicals. So, RSA values characterising functionalised HAp lab samples reach elevate percentages, almost around 85%.
The phenolic compounds on industrially sintered HAp, instead, not only are present in a lower quantity, but also their radical scavenging activity is significantly lower (RSA~10%), comparable to the one characterising bare samples. The chemical stability of this material, in fact, does not consent an efficient functionalisation; furthermore, quite all the polyphenols that are present on the samples are grafted, because physical adsorption phenomena are limited by the low porosity of the surface[37].
Finally, considering RSA in function of time, it is also possible to appreciate how the antioxidant power of the functionalised samples increases with time. The ability of polyphenols to scavenge DPPH radicals is higher when evaluated after 24 hours than after 4 hours. This phenomenon is particularly appreciable for HAp lab samples because of the cited higher activity of polyphenols when grafted to this material. It is due to the different reaction times: caftaric and caffeic acid are the most rapid phenolic acids to react to the aggressive environment, while (+)-catechin and (-)-epicatechin need more time. The chemical response of the polyphenolic mixture utilised in this

study does not degrade and guarantees an efficient antioxidant protection over time. This is an important characteristic which could be useful for future clinical application: the antioxidant activity of polyphenols is not altered by time, nor by the contact with a pro-oxidant environment[38].

5.3.7 Fluorescence microscopy

Bare and functionalised HAp lab samples have been analysed through fluorescence microscopy (Figure 5.29).

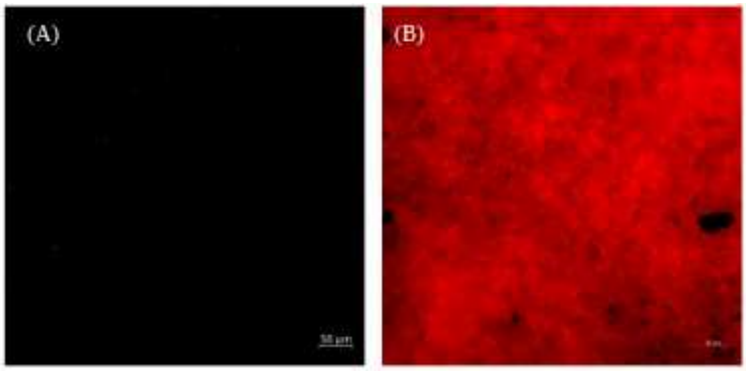

Figure 5.29 Fluorescence microscopy
HAp lab samples (A) before and (B) after functionalisation

The homogeneity of the distribution of polyphenols on the analysed surface is appreciable in Figure 5.29(B). Because of sintering process, the surface of the sample is not planar; the morphology of HAp is defined and its intrinsic porosity is visible under the grafted polyphenols layer. Thanks to this morphology, characterised by a high surface-to-volume ratio, polyphenols are both chemically grafted and physically adsorbed to the sample[39]. The biomolecules well adhere to the surface, whose reaction layer is particularly thick. It is due to the efficient chemical binding, thanks to the presence of numerous hydroxyl and carboxyl groups, adjacently distributed, though which grafting process easily has place[40].

The control, constituted by the bare biomaterial, does not respond to the light stimulation because of the total absence of fluorescent compounds nor polyphenols on its surface.

5.4 Chemically Treated titanium samples (CT)

After the functionalisation process, both solid samples – CT samples – and the functionalising solutions – called uptakes after functionalisation process – are analysed.

In Figure 5.30 bare and functionalised CT samples are reported to be compared.

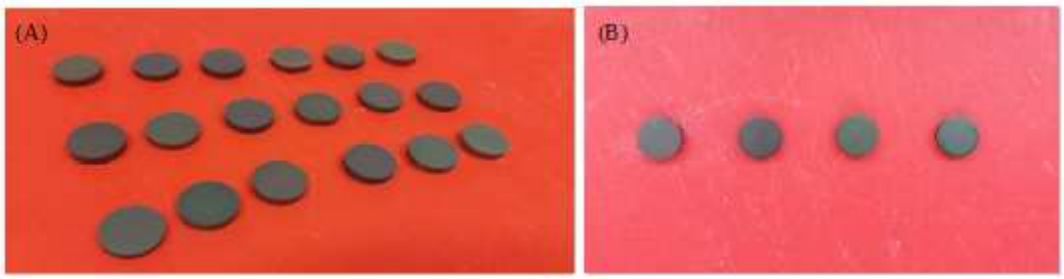

Figure 5.30 CT (A) before and (B) after functionalisation

The difference between bare and functionalised samples is not quite appreciable by the naked eye because of the similarity of the colours characterising respectively the porous titania chemically induced on the surface of the samples and the grafted polyphenols. Anyway, it is possible to note a different opacity of the surfaces of the samples: bare CT is more reflective, while the samples become slightly opaque after the treatment with polyphenols.

Uptake solutions have been analysed (Figure 5.31): the colour of the uptake – TRIS/HCl+$CaCl_2$+PPHE (5 mg/ml) – is very dark purple, quite indigo. It is possible to appreciate the trend of this solution to be darker than the functionalising solution before the functionalising process.

Figure 5.31 CT uptake (5 mg/ml)

This dark colour is due to the dissociation of the hydroxyl groups of the phenolic compounds which easily interact with the calcium ions that are present in the solvent, forming a molecular complex. Because of the high pH value (pH>7), the dissociation of OH groups bound to B ring is complete: as cited in paragraph 5.1.2, this phenomenon induces a displacement in chromaticity, so that the purple solution becomes darker[41]. This phenomenon is amplified by the functionalisation process: three hours permanence in the incubator at 37°C accelerates de-protonation. Furthermore, because of the presence of calcium ions, for pH>7 phenolic OH groups dissociation has place simultaneously[42]: this aspect ulteriorly contributes to let this phenomenon be visually appreciable. At last, a non-negligible aspect to consider is the high affinity between calcium ions and titania[43].

These aspects are deepened in the next paragraphs.

5.4.1 pH

The following histogram (Figure 5.32) reports the pH value of CT uptake, compared to the functionalising solution.

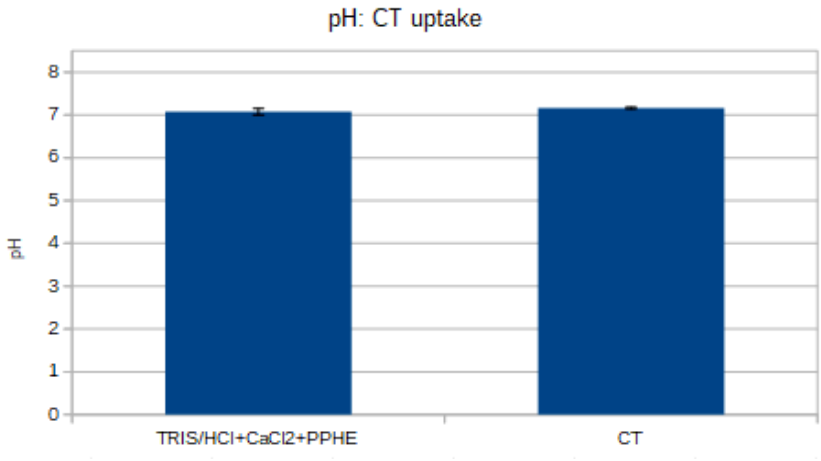

Figure 5.32 pH value of CT uptake

As graphically appreciable, pH values are comparable: there are not significant pH variation during functionalisation. It means that the buffering action of TRIS is maintained during the treatment. Moreover, as expected, no ion release can be supposed from the material surface in the case of CT samples. Nonetheless, it is possible to see that the solution tends to stabilise during the functionalisation process, as visible through the decreasing of the already low standard deviation.

5.4.2 UV spectroscopy

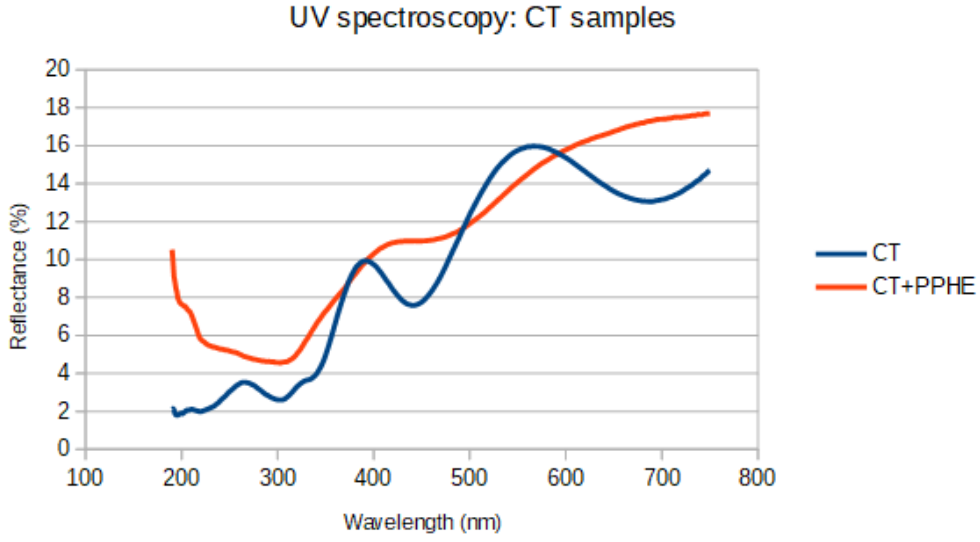

Figure 5.33 UV spectra of bare and functionalised CT samples

As appreciable in Figure 5.33, bare CT spectrum is characterised by multiple reflections: this phenomenon is due to the transparency of titania layer at visible light and to the reflection of radiations at the interface with the bulk metal underneath it[44,45]. After functionalisation, this phenomenon disappears because of the shielding effect exercised by the grafted polyphenols. In fact, it is possible to see a similar trend characterising the two spectra in the plot, where the reflectance peaks are significantly attenuated by the presence of the polyphenols on the surface.

5.4.3 FTIR-ATR

FTIR-ATR spectra characterising bare and functionalised CT samples are reported in Figure 5.34. Bare samples present a plateau, a quasi-constant spectrum almost for the entire wavelength evaluated range. The OH peak, frequently appreciable in CT spectrum, is not visible in this case because of the low contact between the analysed sample and the sensor: this loading error is frequent while working in ATR modality.

The presence of grafted polyphenols on the treated samples is confirmed by the transmittance peaks which are appreciable on their relative spectrum. The spectrum characterising the functionalised sample is better defined: it is probably due to the fact that the polyphenols layer, also if nanometric, significantly contribute to increase the contact between the sample and the detector of the instrument; a higher signal-to-noise ratio is so obtained.

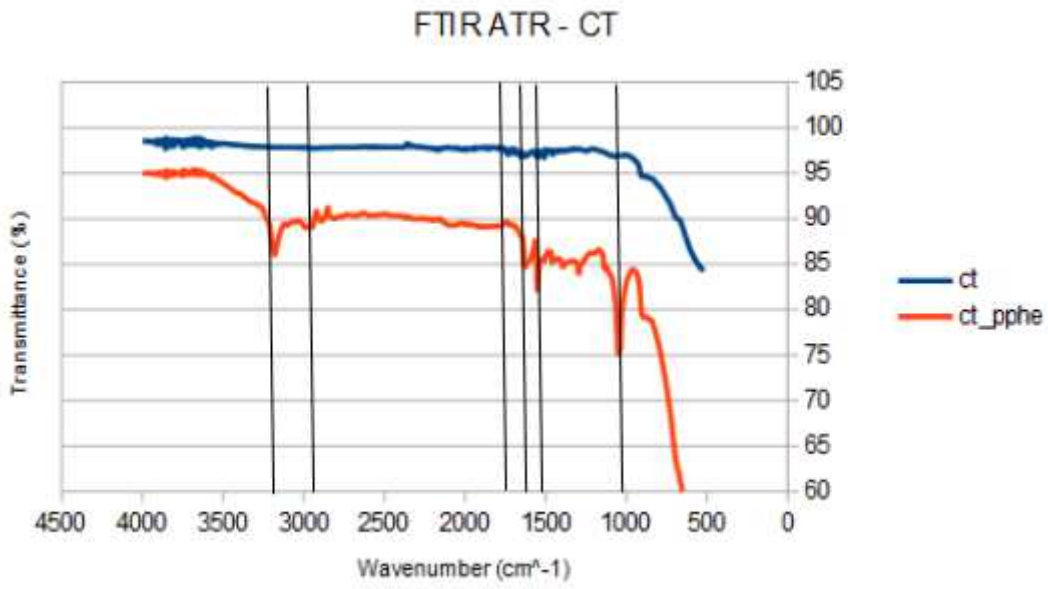

Figure 5.34 FTIR-ATR spectra of bare and functionalised CT samples

Analysing the spectrum of the functionalised samples, it is possible to distinguish the transmittance peaks characterising polyphenols in correspondence of the following wavenumbers[46,47,48]:

- k=3600-2900 cm^{-1}: OH bonds;
- k=3010-2700 cm^{-1}: CH bonds;
- k=1715 cm^{-1}: aromatic ring;

- k=1700 cm^{-1}: COOH group;
- k=1637 cm^{-1}: C=O stretching vibrations;
- k=1250-1203 cm^{-1}: phenolic OH groups bending vibrations.

The peaks are well defined: it means that the quantity of polyphenols on the analysed surface is significant.

5.4.4 F&C test and F&C test modified for solid samples

F&C test has been implemented to quantify the presence of polyphenols on the functionalised samples. In order to quantify the molecules with redox activity grafted on the solid samples, the F&C protocol (suitable for a solution) was modified in order to be applied to a solid sample as described in Chapter 4. According to previous results, this modified protocol is effective, but it is not completely clear at this stage if it quantifies the adsorbed molecules which are released into the F&C reagent solution during the test or the total amount of the grafted (unreleased) and adsorbed (released) molecules.

The results concerning the solid samples are reported in the following histogram (Figure 5.35).

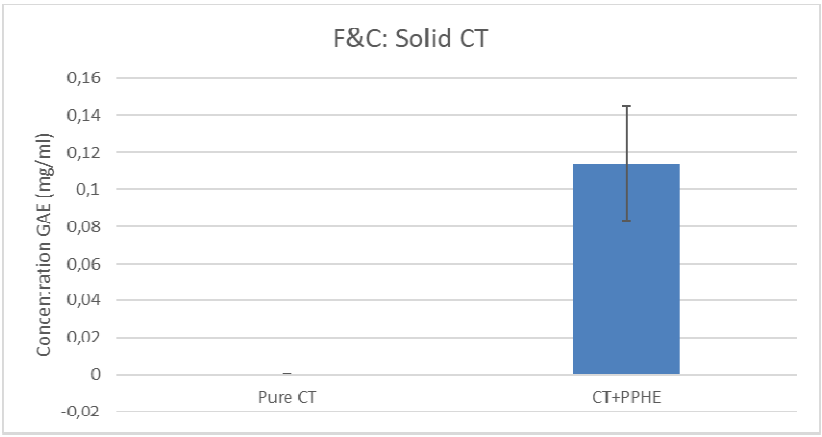

Figure 5.35 F&C test: polyphenols GAE concentrations on CT samples

Through these measurements, it is possible to see the significant difference between bare and functionalised samples. While the test does not detect any functional groups or compound with redox ability on bare CT, the GAE concentration on the functionalised samples is appreciable. The averaged GAE concentration value is particularly high: it quite reaches 0.12 mg/ml. The interconnected porosity of the titania layer permits a significant physical adsorption of the biomolecules, which are able to penetrate inside the material. Furthermore, thanks to the slightly alkaline functionalising environment, the dissociation of the phenolic hydroxyl groups permits a more efficient interaction between the polyphenols and the biomaterial through Ca^{2+} ions mediation. They are able to interact to the OH groups that are present on the surface of CT, whose exposition have been induced by the washing treatment with acetone and water and the successive 1 hour exposure to UV[49].

The uptake, extracted at the end of the functionalising process, has also been analysed through F&C test (Figure 5.36) and compared with the functionalising solution. There are not relevant differences concerning the polyphenols content before and after the treatment. As said before, this phenomenon

can be associated to the limited amount of grafted polyphenols, compared to the initial high concentration of the functionalisation solution.

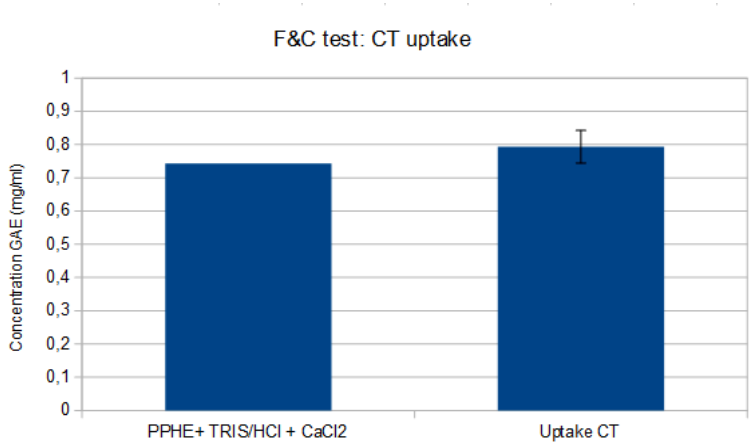

Figure 5.36 F&C test: polyphenols GAE concentrations of CT uptake

As discussed in the following paragraph, CT is stable in mildly alkaline environments: while polyphenols easily interact with the exposed surface, the material does not release any ion or molecule so that the characteristics of the solutions are not altered during functionalisation process.

5.4.5 Zeta potential

Zeta potential titration curves have been measured both on the bare and functionalised CT samples surfaces. In the following plot (Figure 5.37), it is possible to see zeta potential titration curve of CT in function of pH. The isoelectric point, calculated by interpolation, corresponds to pH=2.80.

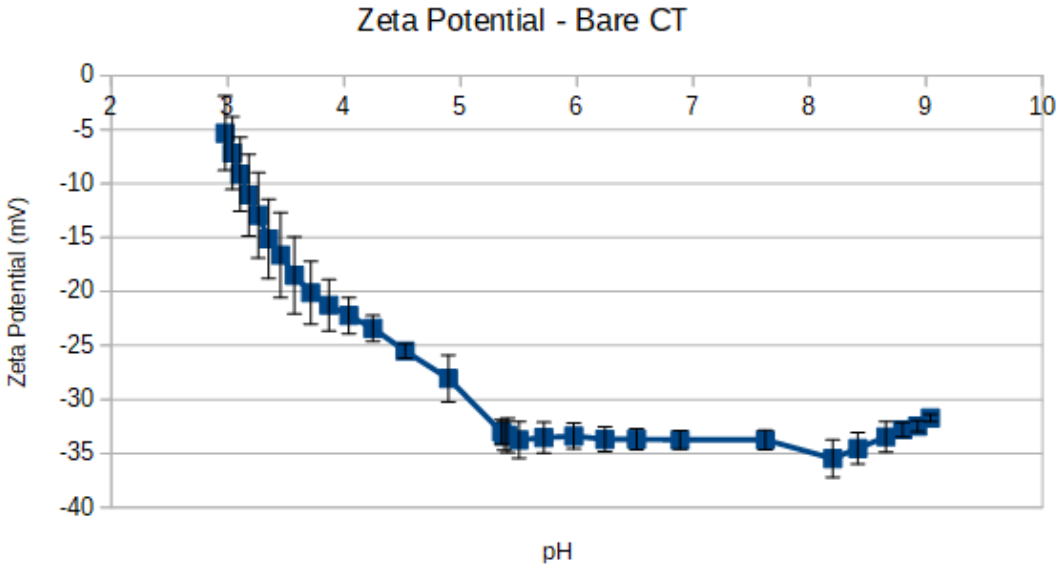

Figure 5.37 Zeta potential of bare CT samples

As previously cited, the intrinsic stability of the material, especially for alkaline pHs, is confirmed by the low standard deviation characterising each measurement. The curve presents a decreasing

trend along the acid pH range and around pH=5 reaches a plateau, tending to -35 mV. This value corresponds to the complete dissociation of the OH groups that are present on the surface of the samples. For higher pH values, in fact, there are not significant variations of the surface zeta potential of bare CT[50].

The isoelectric point (pH=2.80) appertains to acidic field: for the entire evaluated pH range, the surface is negatively charged, because of the described de-protonation phenomenon[51].

In Figure 5.38, the zeta potential titration curve of functionalised samples is reported. The isoeletric point characterising this material, given in output by the instrument, corresponds to pH=3.10.

First, it is possible to see a significant step around pH=5, both moving toward the basic and the acidic range. The plateau in the basic range could be due to the dissociation of phenolic OH groups, which, because of the presence of calcium ions, have place abruptly. Furthermore, this phenomenon is associated to the formation of the molecular complex with calcium itself[52]. For pH>5, thanks to OH groups dissociation, the complex is formed; for lower pH values, instead, COOH groups are not dissociated: decreasing pH, the simultaneous protonation of carboxyl groups induces the removal of calcium ions and, consequently, of the molecular complex[53]. This conduces to have an elevate slope of the curve and a significant reactivity of the analysed surface.

Finally, the high affinity between calcium ions and titania amplifies these effects[54]: when polyphenols interact with the surface to be grafted, also calcium cation is attracted by the negative charge that is present on the surface. The molecular complex involving phenolic compounds and calcium ions is so forced, close to the functionalised surface. Another explanation of the titration curve could be the following: around pH~4-5, the complex compound involving Ca^{2+} ions becomes unstable and this involves the detachment of the grafted biomolecules, as it can be induced from the step registered in this range of pH both moving to the acidic and basic range and from the high standard deviation registered at pH=4.5. After the detachment of the biomolecules, the titration curve becomes close to that of the substrate. Complementary characterisation is needed in order to confirm these hypotheses. If this second hypothesis would be confirmed, it would be of great interest because polyphenols are released in a smart way from the material when pH goes down to 4-5 that means when an inflammatory even occurs.

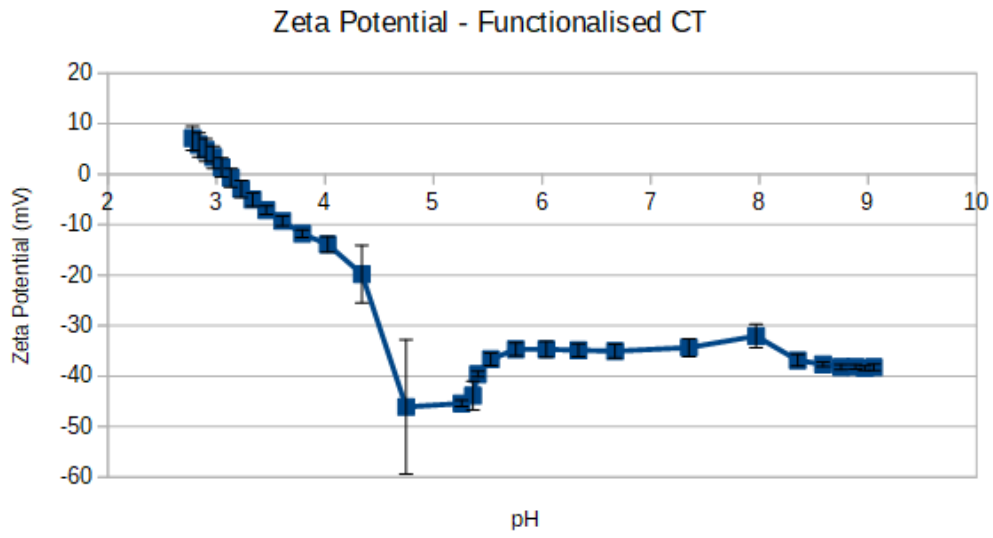

Figure 5.38 Zeta potential of functionalised CT samples

The comparison between bare and functionalised CT samples is illustrated in Figure 5.39, where the difference of reactivity is appreciable for pH~5. Both the materials reach a plateau for alkaline pHs, where zeta potential tends to -35 mV.

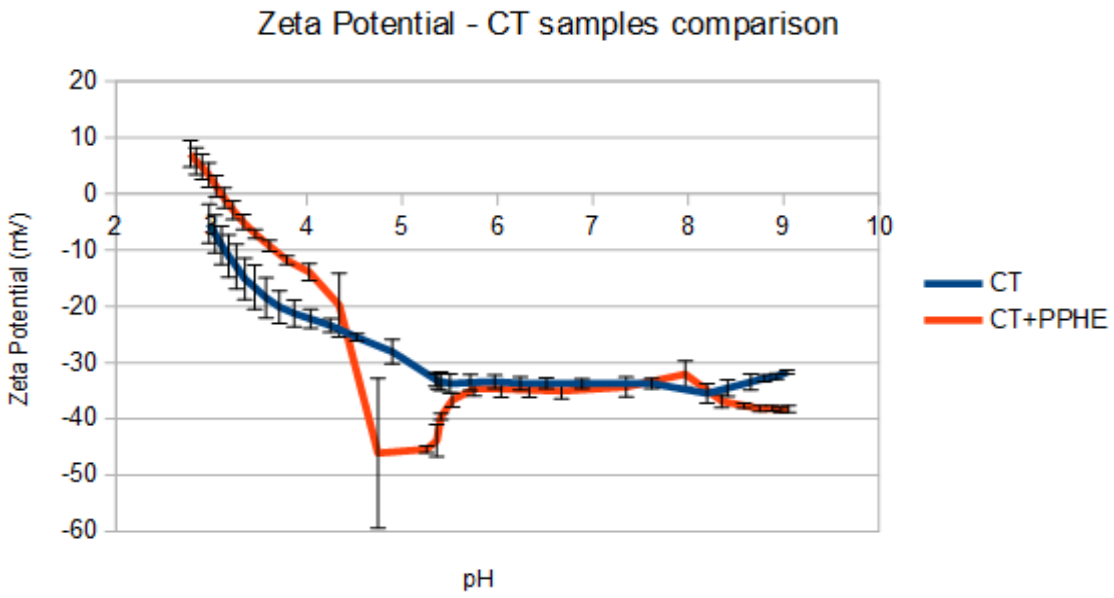

Figure 5.39 Zeta potential of bare and functionalised CT samples

5.4.6 DPPH test

The radical scavenging activity of the functionalised samples has been evaluated and compared with the one characterising the bare material (Figure 5.40).

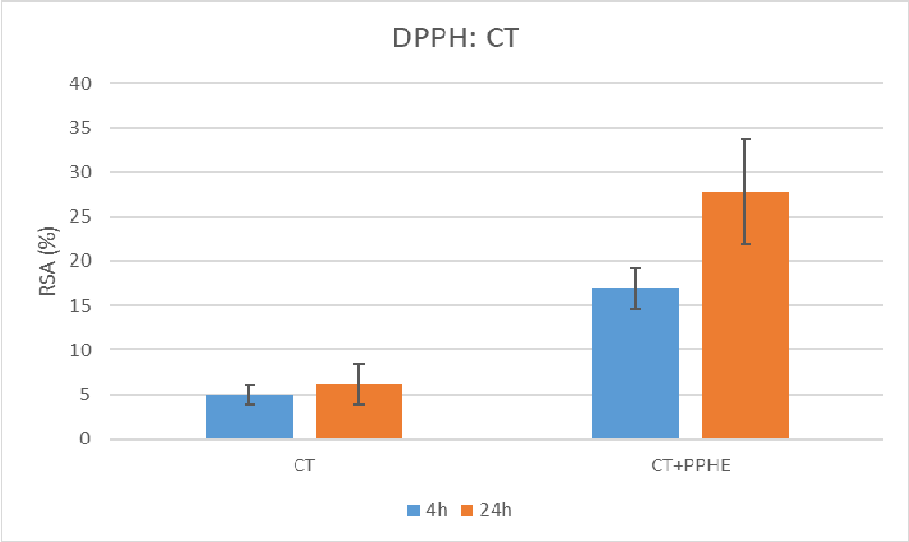

Figure 5.40 DPPH test on CT solid samples

As visible by the histogram, the difference of the antioxidant activities characterising respectively the bare and functionalised samples is significant. Anyway, RSA does not reach particular high values, which, after 24 hours, is around 20-30%.

5.4.7 Fluorescence microscopy

The following image (Figure 5.41) reports the functionalised surface of CT samples, compared with the bare biomaterial.

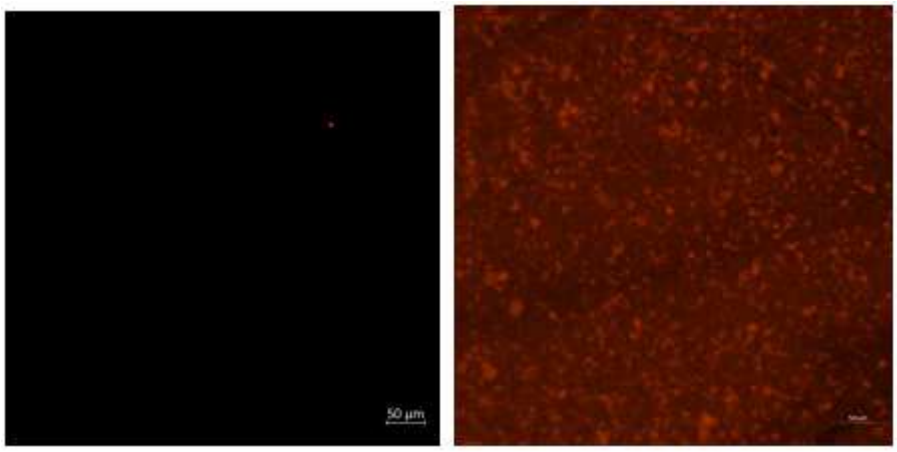

Figure 5.41 Fluorescence microscopy
CT samples (A) before and (B) after functionalisation

Polyphenols are characterised by a uniform distribution on the surface of the material: a thin background layer is clearly visible. Numerous small agglomerates are well distributed on the material, whose dimensions are comparable with superficial porosity obtained by the chemical treatment to the native oxide. It could be due to the fact that the biomolecules enter in the porosity of the material and form the small agglomerates in these areas. Probably calcium ions are involved in this phenomenon, as cited in the previous paragraph: the high affinity both between calcium ions and titania and between these ions and polyphenols induces an elevate concentration of the ions and the biomolecules close to the porous titania surface[55,56].

5.5 Bioactive glass samples SCNA

After the functionalisation process, both the solid samples – SCNA samples – and the functionalising solutions – called uptakes after functionalisation process – are analysed.

In Figure 5.42 the SCNA uptake solution can be observed.

Figure 5.42 SCNA uptake solution

The colour of the uptake is the same of the functionalising solution – H_2O+PPHE (5 mg/ml). It induces to suppose a low reactivity of the material causing a low interaction between the surface of the sample and the solution at which it is exposed during the functionalisation process. This fact is confirmed by the following experimental results.

5.5.1 pH

The pH value characterising SCNA uptake is reported in the next histogram (Figure 5.43), compared to the functionalising solution, as control sample.

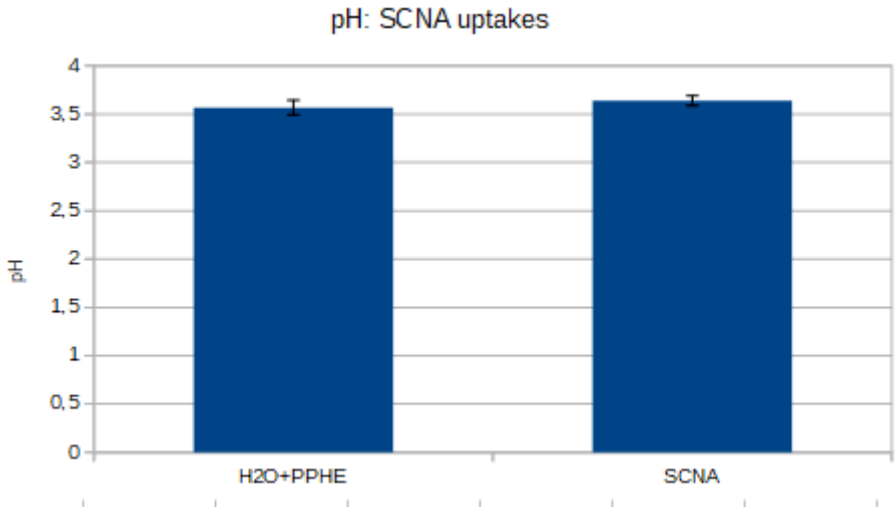

Figure 5.43 pH value of SCNA uptake

The pH value of the analysed uptake is slightly higher than the one characterising the functionalising solution, but quite comparable. The low difference between these two values is explained, as cited in the previous paragraph, by the low reactivity of the biomaterial. SCNA, in fact, contains alumina (Al_2O_3), a particularly stable compound, which increases the stability of the bioglass overall[57].

5.5.2 F&C test and F&C modified for solid samples

F&C test has carried on to quantify the GAE concentration of the grafted polyphenols to SCNA samples surface. In order to quantify the molecules with redox activity grafted on the solid samples, the F&C protocol (suitable for a solution) was modified in order to be applied to a solid sample as described in Chapter 4. According to previous results, this modified protocol is effective, but it is not completely clear at this stage if it quantifies the adsorbed molecules which are released into the F&C reagent solution during the test or the total amount of the grafted (unreleased) and adsorbed (released) molecules.

Through this analysis, polyphenols have not been detected. This fact constitutes an ulterior confirmation of the negligible interactions between the biomaterial and the polyphenols dissolved in the functionalising solution: so, the functionalisation process is not possible because of the excessive stability of SCNA in this environment[58,59].

5.5.3 DPPH test

The radical scavenging activity of SCNA has been evaluated through DPPH test. As appreciable by the histogram (Figure 5.44), there are not significant differences between bare and functionalised samples. RSA values are very low, whose average does not even reach 10%. As induced also by the previously illustrated measurements, the low reactivity of the material and the consequent negligible presence of grafted polyphenols cause the low antioxidant activity of functionalised samples, the same characterising the bare ones.

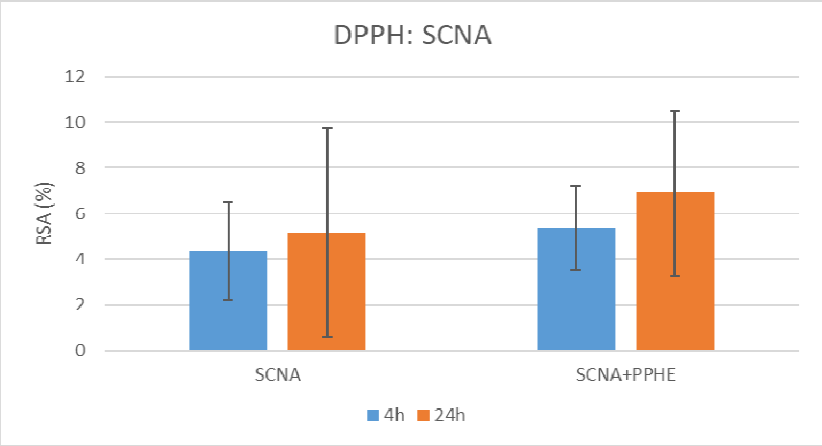

Figure 5.44 DPPH test on SCNA solid samples

5.6 Bioactive glass samples SCNbis

After the functionalisation process, both the solid samples – SCNbis samples – and the functionalising solutions – called uptakes after functionalisation process – are analysed.

In the following image (Figure 5.45), bare and functionalised SCNbis samples are compared. It is possible to appreciate the presence of a dark film on the samples treated with polyphenols: it is due to the presence of grafted molecules and the consequent efficacy of the functionalisation process.

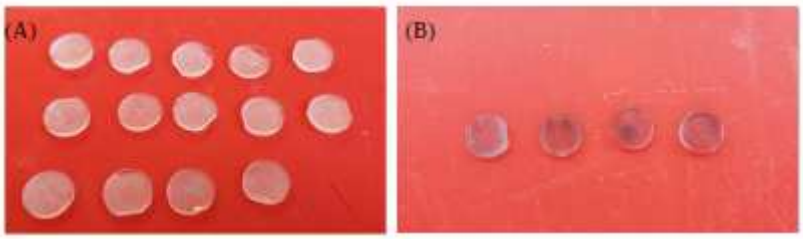

Figure 5.45 SCNbis (A) before and (B) after functionalisation

In Figure 5.46, the SCNbis uptake solution is observable. The colour of the solution is still purple, but becomes slightly darker after the functionalisation process. It is due, as successively explained, to the higher reactivity of this bioactive glass.

Figure 5.46 SCNbis uptake solution

5.6.1 pH

The pH values characterising SCNbis uptakes have been evaluated (Figure 5.47). As appreciable through the histogram, pH increases during functionalisation and becomes less acid, reaching 4.5. Furthermore, it is possible to see the increasing of standard deviation characterising the measure of the uptake. Both the phenomena are caused by the high reactivity of SCNbis: the absence of the chemically stable alumina permits to the other oxides composing the material to react with the acid environment[60].

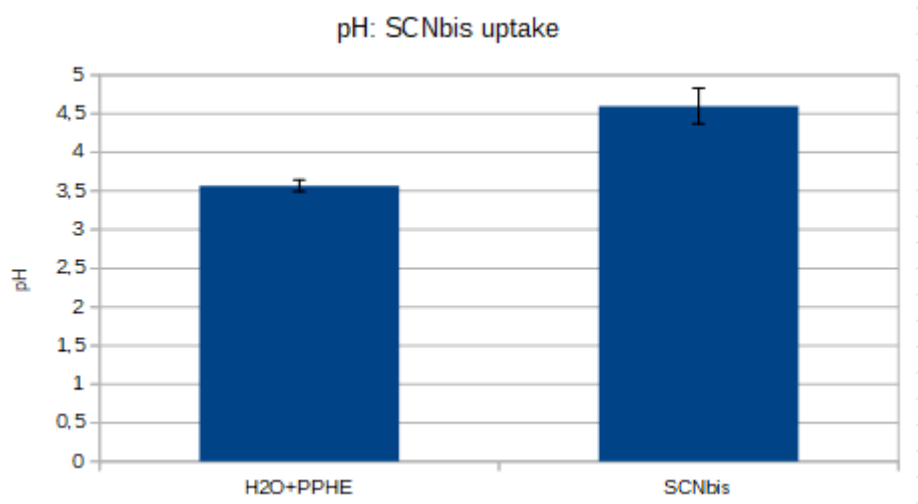

Figure 5.47 pH value of SCNbis uptake

5.6.2 UV spectroscopy

The solid SCNbis samples have been investigated through UV spectroscopy, evaluating their transmittance (Figure 5.48).

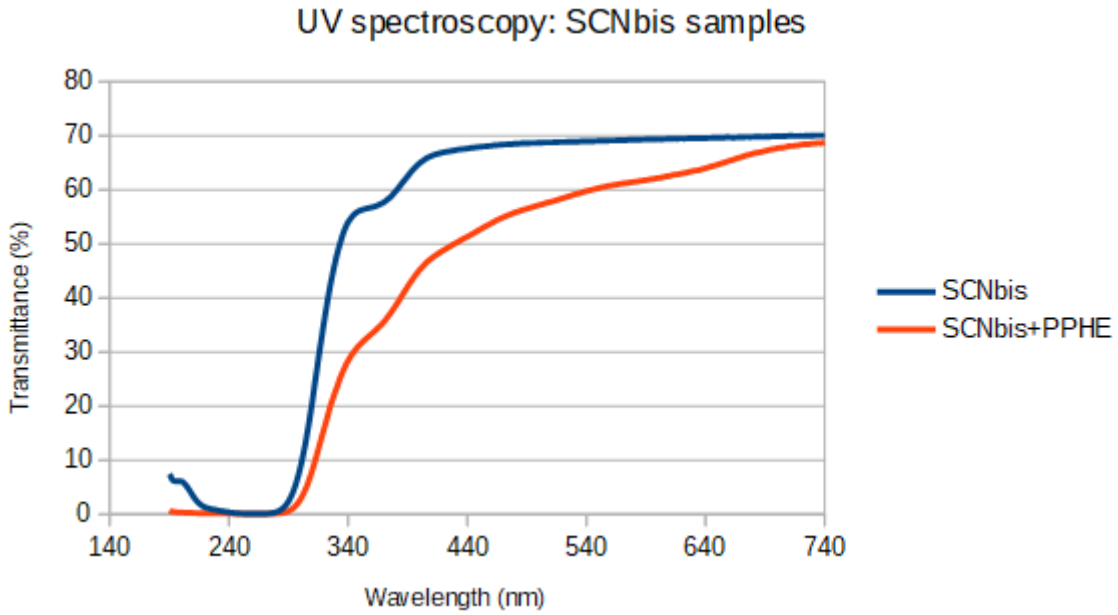

Figure 5.48 UV spectra of bare and functionalised SCNbis samples

Quite for all the evaluated wavelength range, the transmittance of the bare samples is particularly high and reaches a plateau (T~70%) in correspondence of wavelengths around 400 nm: it is due to the transparency of the bioactive glass and the absence of colour characterising its matrix. The spectra of bare and functionalised samples are characterised by analogue trends, but, after the functionalisation treatment, the transmittance of SCNbis decreases. It is due to the presence of a

layer of grafted polyphenols on the surface of the material, which decreases the transmitted radiation through the sample[61].

5.6.3 FTIR-ATR

FTIR-ATR spectra characterising bare and functionalised SCNbis samples have been compared (Figure 5.49).

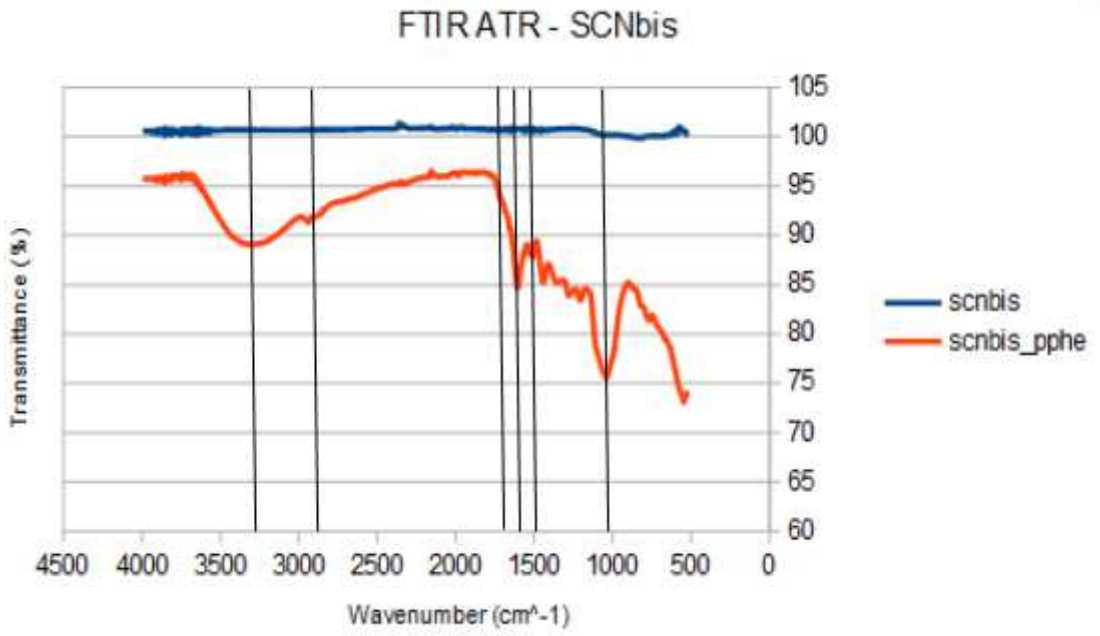

Figure 5.49 FTIR-ATR spectra of bare and functionalised SCNbis samples

It is possible to see that the spectrum of bare SCNbis is almost constant: it is constituted by a plateau around T=100%. It means that an elevate fraction of the stimulating radiation is transmitted through the sample; furthermore, similarly to HAp and CT samples, the low contact between the analysed sample and the sensor contribute to obtain a low signal, which, in these case, is not detectable by the instrument. As previously cited, this loading error is frequent while working in ATR modality. The spectrum characterising the functionalised sample, instead, is better defined: it is probably due to the fact that the polyphenols layer, also if nanometric, significantly contribute to increase the contact between the sample and the detector of the instrument; a higher signal-to-noise ratio is so obtained. So, functionalised SCNbis spectrum presents well-defined peaks, that are typical of polyphenols: analysing it, it is possible to distinguish the characterising transmittance peaks in correspondence of the following wavenumbers[62,63,64]:

- k=3600-2900 cm^{-1}: OH bonds;
- k=3010-2700 cm^{-1}: CH bonds;
- k=1715 cm^{-1}: aromatic ring;
- k=1700 cm^{-1}: COOH group;
- k=1637 cm^{-1}: C=O stretching vibrations;

- k=1250-1203 cm^{-1}: phenolic OH groups bending vibrations.

A non-negligible peak is also present in correspondence of k=900-1000 cm^{-1}. It could correspond to the fingerprint of the carbohydrates that are present in the mixture of lyophilised polyphenols[65]. In this band, also the peak typical of silica (Si-O) is present: it is originated by the matrix of SCNbis, but it was not appreciable on the bare material, as previously explained, because of the low contact between the sample and the detector. Another hypothesis attributes this peak to stretching vibrations of M-OH complexes (where M represents a generic metal) appertaining to metal hydroxides, that are present on the surface of the biomaterial[66]. In fact, the metal oxides composing the matrix of reactive bioglasses, when in contact with an aqueous environment as the functionalising solution is, interact with water and are hydrated[67]. SCNbis, as confirmed by pH values and UV spectroscopy, is quite reactive and a hydrated layer is formed on its surface when immersed in functionalising solution; this phenomenon implements a more efficient polyphenol grafting.

5.6.4 F&C test and F&C test modified for solid samples

The GAE concentration of the grafted polyphenols has been evaluated analysing SCNbis solid samples through F&C test (Figure 5.50). In order to quantify the molecules with redox activity grafted on the solid samples, the F&C protocol (suitable for a solution) was modified in order to be applied to a solid sample as described in Chapter 4. According to previous results, this modified protocol is effective, but it is not completely clear at this stage if it quantifies the adsorbed molecules which are released into the F&C reagent solution during the test or the total amount of the grafted (unreleased) and adsorbed (released) molecules.

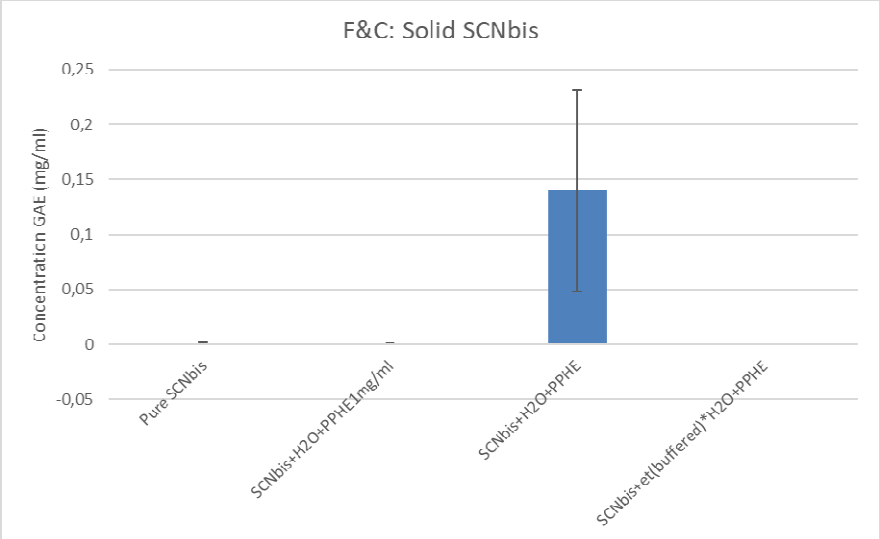

Figure 5.50 F&C test: polyphenols GAE concentrations on SCNbis samples

The histogram reports the measurements effectuated on SCNbis, respectively bare and previously treated with three different functionalising solutions. As graphically appreciable, the presence of polyphenols on the analysed surfaces has been detected only on the sample functionalised through H_2O+PPHE 5 mg/ml solution. The 1 mg/ml solution and the other one, containing 50% v/v of ethanol, are not adequate to efficaciously graft polyphenols to SCNbis: through this test, no

polyphenol has been detected on the surfaces treated with this two solutions, as on the bare sample, so that only H_2O+PPHE 5 mg/ml solution has been utilised to treat SCNbis.
The efficaciously functionalised samples present a moderate concentration of grafted biomolecules on their surface. The high error bar is, then, an ulterior confirm of the intrinsic reactivity of SCNbis.

In the following histogram (Figure 5.51) the measured GAE concentrations of SCNbis uptakes are reported.

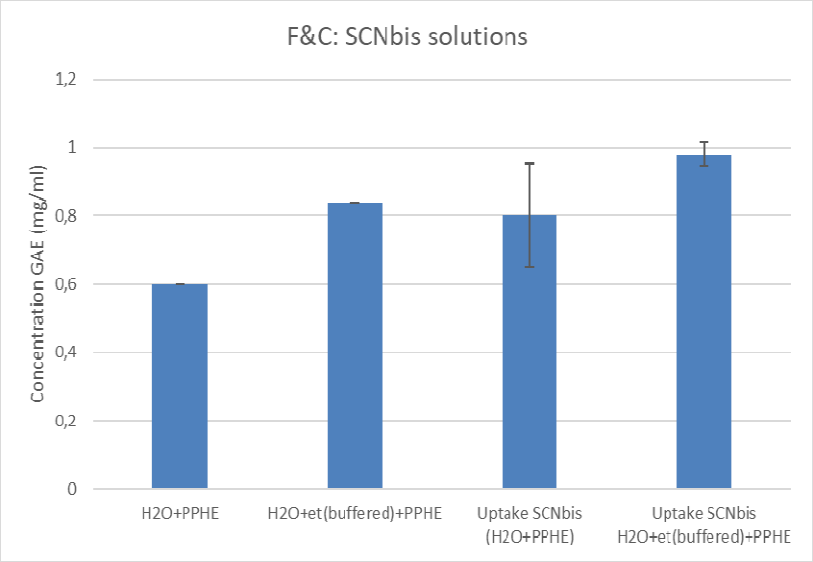

Figure 5.51 F&C test: polyphenols GAE concentrations of SCNbis uptakes

The polyphenols content of these uptakes is comparable with the one characterising the respective functionalising solutions. No significant variations have been detected. Anyway, a print of the effectuated functionalising process is revealed by the increasing of the standard deviation characterising the uptakes, higher than the one characterising the functionalising solutions. The ionic exchanges due to the reactivity of SCNbis surface induces this phenomenon.

5.6.5 DPPH test

The antioxidant activity of SCNbis solid samples has been evaluated through DPPH test. As appreciable by the following histogram (Figure 5.52), RSA is null for bare SCNbis: the bare biomaterial does not exercises any antioxidant action. The functionalised samples present a significant scavenging activity: the grafted polyphenols to SCNbis surface do not lose their antioxidant power during the functionalisation process.

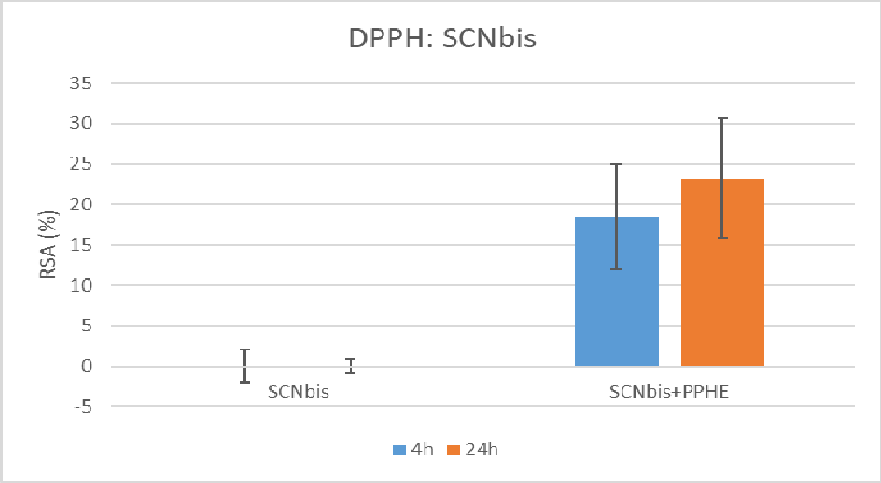

Figure 5.52 DPPH test on SCNbis solid samples

5.6.6 Fluorescence microscopy

The following image (Figure 5.53) illustrates the bare and functionalised SCNbis samples surfaces; the bare SCNbis is reported without any carbonates in its matrix. As all bioactive glasses, in fact, also SCNbis is characterised by the presence in its matrix of calcium and sodium carbonates, which, nevertheless, are fluorescent: it would not permit to appreciate the functionalisation effects[68]. So, to better detect the presence of the grafted polyphenols on the analysed surface, the absence of carbonates in the matrix of the bioglass is preferred.

Carbonates have been removed by SCNbis surface immersing the control sample in water for 3 hours at 37°C, in the incubator. This treatment simulates the functionalisation process: nonetheless, polyphenols are not dissolved in the solution, which is only finalised to carbonates removal[69].

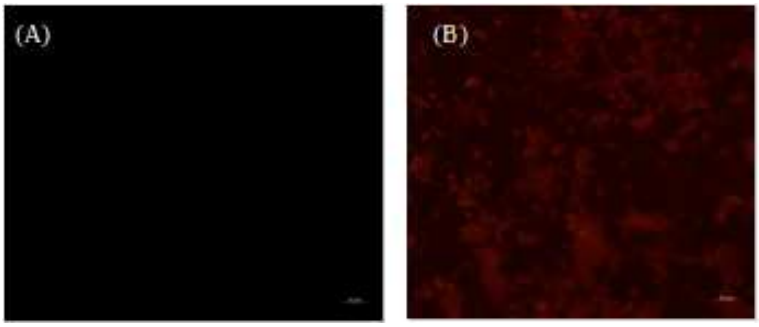

Figure 5.53 Fluorescence microscopy on SCNbis samples:
(A) bare – without carbonates, (B) functionalised

The quantity of polyphenols is elevate; they are organised on the functionalised surface in a thin background layer and numerous and large agglomerates. The distribution is less homogeneous then on the other substrates and the characteristic dimension of each agglomerate is included in a wide range[70]. It could be due to the particular reactivity of the material.

The agglomerates are probably formed because of the presence of calcium on the surface of SCNbis, coming from the matrix constituting the sample. Due to the washing in acetone and water and the following functionalisation process – through which the bioglass enters in contact with aqueous

environments – the surface, as cited in the previous paragraphs, tends to hydrate[71]. This thin reaction layer permits a more efficient functionalisation: calcium and sodium ions, positively charged, easily interact with the dissolved polyphenols, inducing them to precipitate on the surface in agglomerates.

5.7 Biomaterials comparison

After the evaluation of the properties of every biomaterial, to better appreciate their different chemical behaviour and their different response to the functionalisation process through polyphenols, the analysis of the studied biomaterials is reported as a function of the analysing technique.

5.7.1 pH

First, pH values characterising the analysed solutions have been evaluated: both the functionalising and uptake solutions are compared in the following histogram (Figure 5.54).

Concerning CT, HAp ind and SCNA, it is possible to see that the functionalising solutions and their corresponding uptakes present similar and comparable pH values, which do not significantly vary during the functionalisation process: it is due to the low reactivity of the samples themselves, for which a low ion and molecular exchange is observable between the samples and the solution in which they are immersed. The more reactive samples – SCNbis and HAp lab – cause, instead, an appreciable increasing of pH: the uptakes relative to these two biomaterials are characterised by a higher pH than the functionalising solution. It is due to the ionic exchanges at the interface between the surface of the samples and the polyphenols solution: the exchange of H^+ ions while functionalising SCNbis and HAp lab samples – respectively due to the hydrated layer and the high surface-to-volume ratio – induces an alteration of pH in the environment[72].

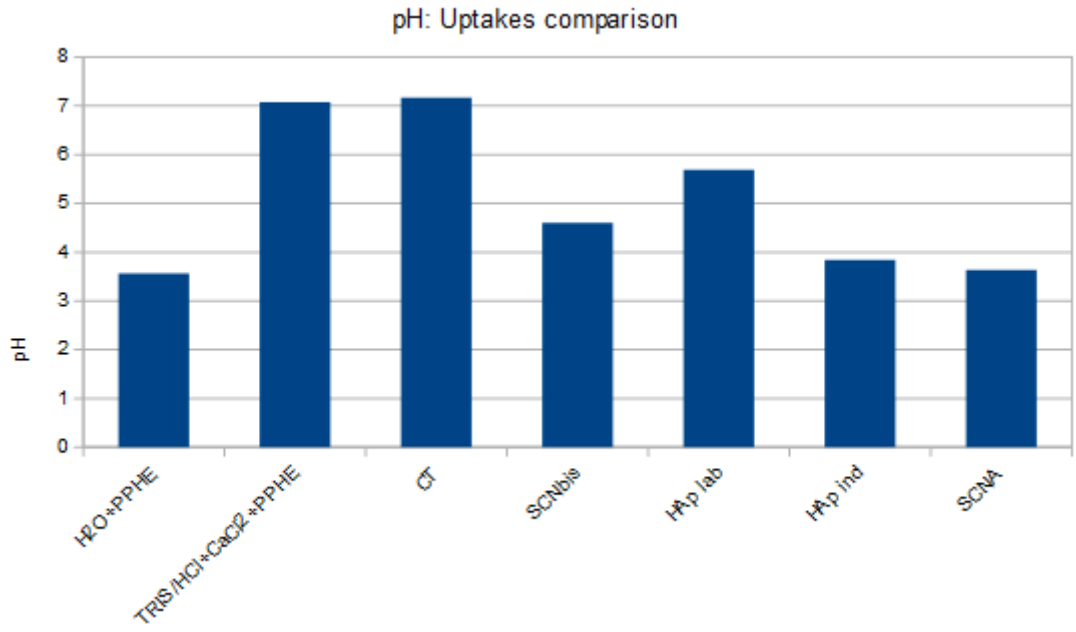

Figure 5.54 pH values of functionalising solutions and uptakes: comparison

5.7.2 FTIR-ATR

In the following plot (Figure 5.55) the FTIR-ATR spectra characterising the analysed material are reported to be compared.

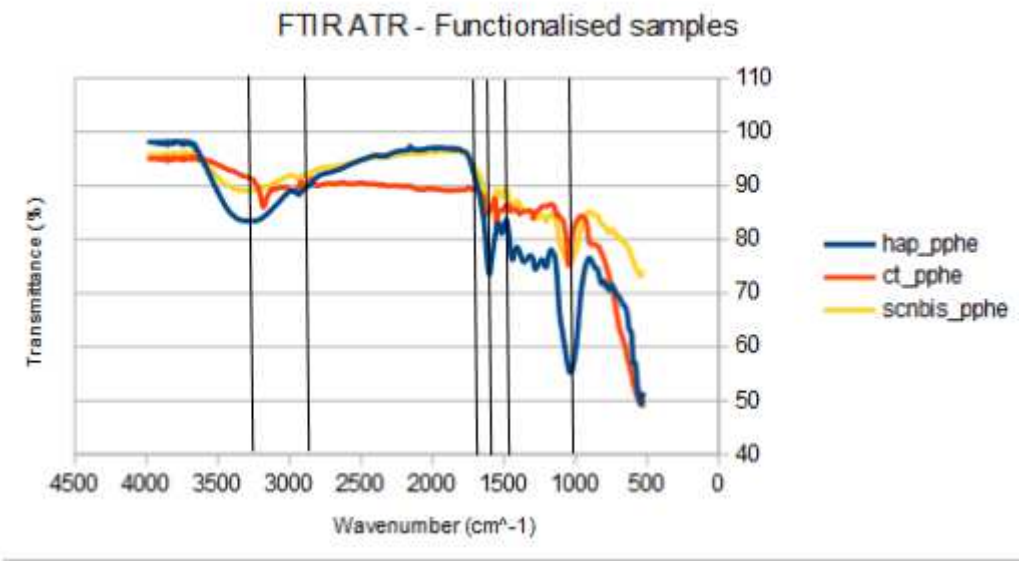

Figure 5.55 FTIR-ATR spectra of functionalised samples: comparison

The first aspect to consider is the fact that functionalised HAp lab presents a well-defined spectrum, where all the peaks characterising the polyphenolic mixture are particularly visible and defined. Especially the 3600-2900 cm^{-1} band, corresponding to the hydroxyl groups, presents a particularly high transmittance peak; anyway, also the peaks in correspondence of 1700 cm^{-1} (COOH groups), 1715 cm^{-1} (aromatic rings) and 1250-1203 cm^{-1} (phenolic OH groups) are well-defined[73,74,75]. It is due to the elevate quantity of grafted and adsorbed polyphenols, which are present on the surface and inside the porosities of this biomaterial. This phenomenon, as cited before, is possible thanks to the high reactivity of HAp lab and its high surface-to-volume ratio, that permits a better interaction between the surface of the sample and polyphenols during the functionalising process[76].

The other analysed biomaterials – CT and SCNbis – have a FTIR-ATR spectrum where the transmittance peaks, also if they are present and appreciable, are not as intense as the ones characterising HAp. It is caused by the lower surface-to-volume ratio characterising their surface and the minor reactivity.

5.7.3 F&C test and F&C test modified for solid samples

Through F&C test, the GAE concentration of the grafted polyphenols on each tested sample has been evaluated (Figure 5.56). As previously explained, in order to quantify the molecules with redox activity grafted on the solid samples, the F&C protocol (suitable for a solution) was modified in order to be applied to a solid sample as described in Chapter 4. According to previous results, this modified protocol is effective, but it is not completely clear at this stage if it quantifies the adsorbed molecules which are released into the F&C reagent solution during the test or the total amount of the grafted (unreleased) and adsorbed (released) molecules.

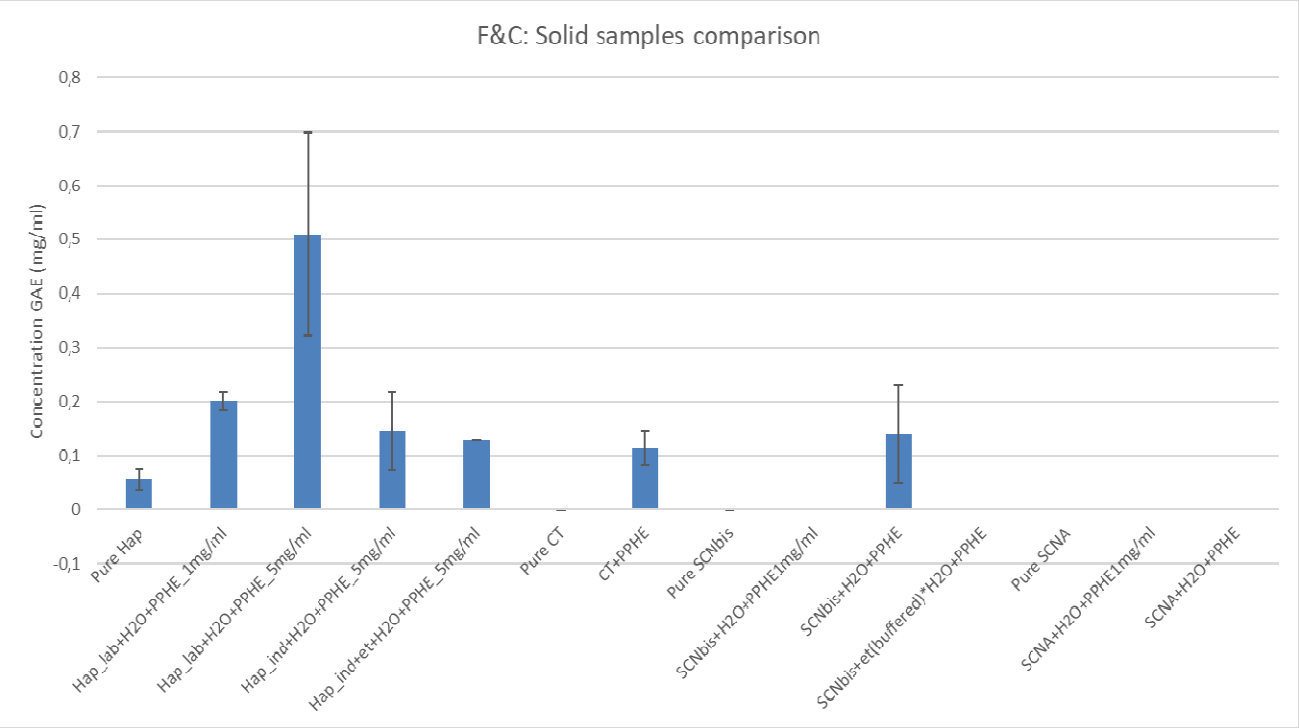

Figure 5.56 F&C test: polyphenols GAE concentrations on solid samples

It is possible to appreciate the higher polyphenols content on the HAp lab samples, despite the utilised functionalising solution; as previously demonstrated, the most efficient is H_2O+PPHE 5 mg/ml, which permits a significantly higher number of grafted biomolecules. HAp lab samples functionalised with H_2O+PPHE 5 mg/ml are the best functionalisable samples: the averaged concentration characterising them reaches 0.5 mg/ml GAE, while the other functionalised samples hardly reach 0.2 mg/ml GAE.
On other samples, such as SCNbis (when treated with 1 mg/ml solution) and SCNA (despite the utilised functionalising solution), the presence of polyphenols is not detected by the instrument: GAE concentration is null as it is for bare biomaterials.

Also GAE concentration of solutions – both functionalising solutions and uptakes – has been investigated (Figure 5.57).

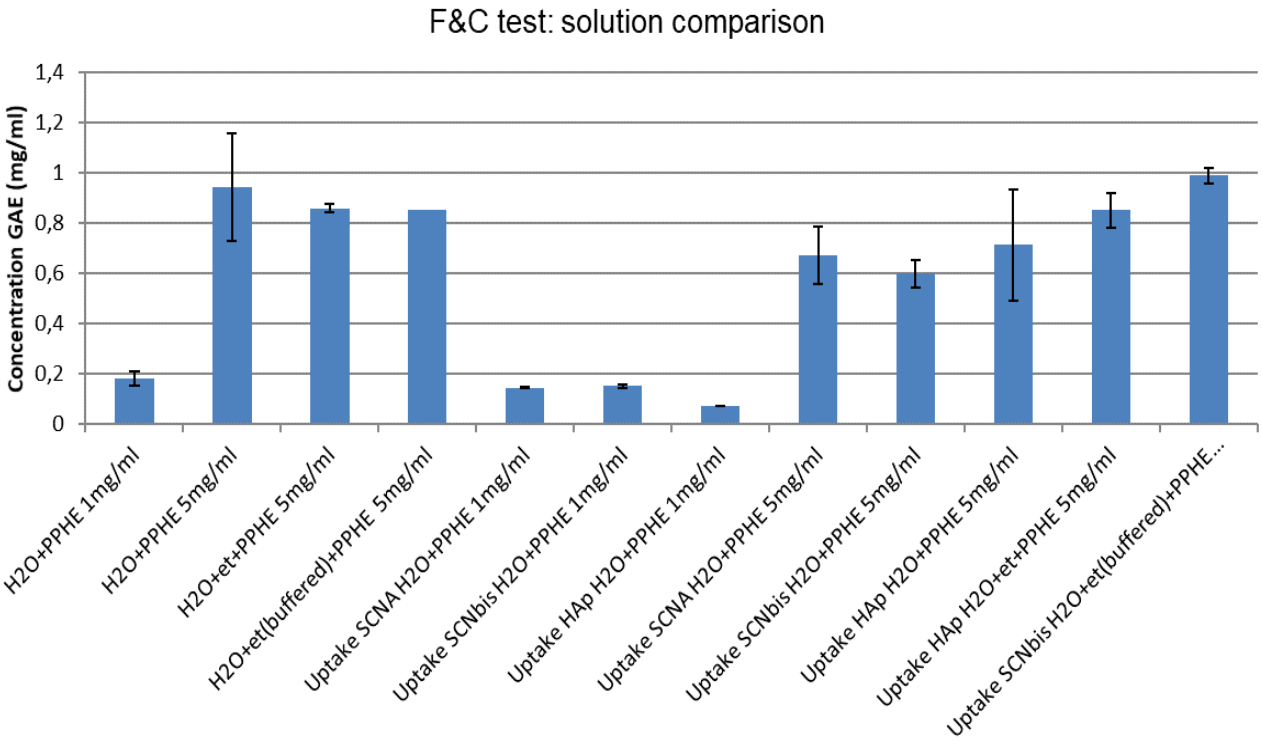

Figure 5.57 F&C test: polyphenols GAE concentrations of liquid samples

By the histogram, it is possible to appreciate that the concentrations characterising the uptakes and their respective functionalising solutions are comparable, despite the utilised solvent – H_2O, EtOH+H_2O 50% v/v or TRIS/HCl+$CaCl_2$ – and the imposed polyphenols concentration – 1 mg/ml or 5 mg/ml. It is due to the low fraction of the grafted polyphenols with respect to the total amount dissolved in the functionalising solution before the functionalisation process, which does not alter the detectable GAE concentration of the analysed liquid samples.

5.7.4 DPPH test

The radical scavenging activity of the analysed solid samples is compared in Figure 5.58.

DPPH test is able to evaluate the activity of the most external layer of the analysed surface. In fact, the samples characterised by a higher surface-to-volume ratio present a higher antioxidant activity, because of the higher quantity of the grafted polyphenols that are exposed to the environment. This is confirmed by the fact that HAp lab, also if functionalised through 1 mg/ml solution, exercises a significantly higher scavenging power than other samples treated with more concentrated solutions.

Also functionalised CT has a non-trascurable antioxidant power, thanks to the induced porosity of the superficial oxide, comparable to the one exercised by SCNbis.

Functionalised SCNA and HAp ind are characterised, instead, by particularly low RSA values, almost comparable with the one typical of bare samples: polyphenols are not active when grafted on their surface, so that their antioxidant power is not significant.

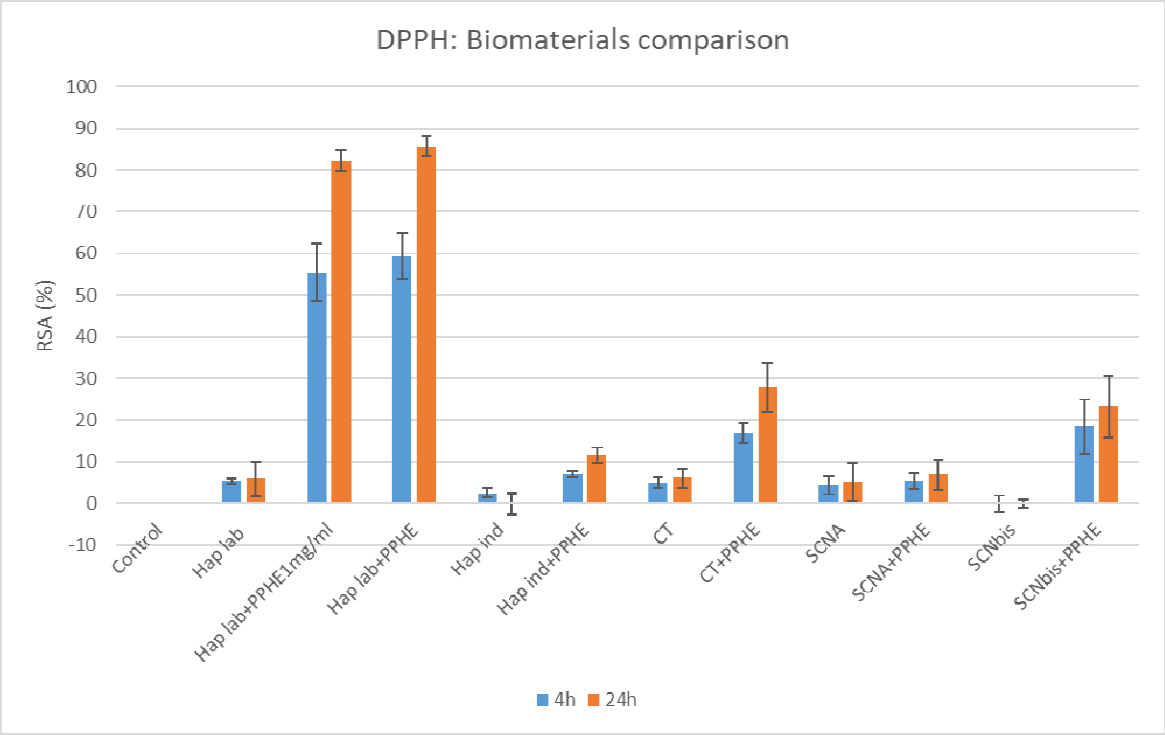

Figure 5.58 DPPH test on solid samples: comparison

5.7.5 Fluorescence microscopy

The functionalised surfaces of HAp lab, CT and SCNbis, analysed through fluorescence microscopy, are compared in the following image (Figure 5.59).

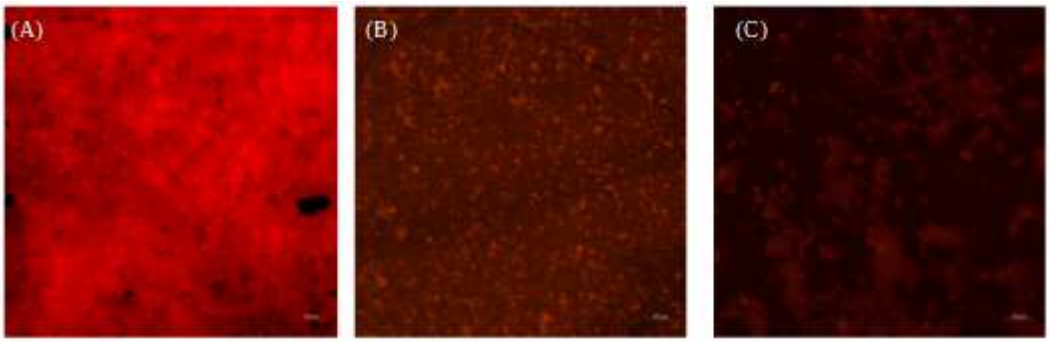

Figure 5.59 Fluorescent microscopy
(A) HAp+PPHE; (B) CT+PPHE; (C) SCNbis+PPHE

The first characteristic to highlight is the appreciably different distribution of polyphenols on the three analysed samples.

The higher quantity of grafted biomolecules on HAp lab sample and the elevate thickness of its reactive layer, with the consequent elevate thickness of polyphenols film, is well-visible.

CT and SCNbis are characterised by similar polyphenols distributions: the background layer with the numerous agglomerates. Anyway, on CT the grafted biomolecules are more homogeneous: the background layer is thicker and the agglomerates are small, numerous, and homogeneously

distributed. On SCNbis sample, instead, the background layer is thinner and polyphenols are almost present as large agglomerates, whose morphology is various; these agglomerates are inhomogeneously distributed on the surface: some areas are completely covered by the biomolecules complexes, while other ones present only the thin background layer.
The difference between the distributions could be attributed to the calcium ions. In fact, because of the high calcium content in HAp samples and their high reactivity, calcium ions are significantly present at the sample-solution interface during the functionalisation process; analogously, calcium ions are widely dissolved in the functionalising solution (TRIS/HCl+$CaCl_2$). So, polyphenols – negatively charged for pH>2.21 – easily interact with Ca^{2+} cations: they are attracted by the surface of the biomaterials where the concentration of calcium is higher: HAp, which contains calcium in its matrix, is so completely and deeply covered by polyphenols; CT presents a homogeneous distribution thanks to calcium complex. SCNbis, instead, despite its non-negligible reactivity and the presence of calcium carbonate in its matrix, does not release a sufficient calcium ions to have a homogeneous distribution of polyphenols. Calcium is present quite only on the hydrated external layer and its concentration in the uptake is not sufficient: so, polyphenols tend to form agglomerates close to this layer, but the calcium content is not sufficient to form calcium-polyphenols complex[77,78].

5.8 Release in water

In the following images, the functionalised samples before the release test are reported and compared with the same samples extracted from water and evaluated at every experimental time.
HAp lab samples (Figure 5.60) conserve the same physical aspect till the fourteenth day, then the colour of the film of grafted polyphenols becomes lighter at the end of the test (28 days). As successively confirmed, it means that the release of polyphenols has taken place after a long time: the polyphenols-substrate interface remains stable for the first 14 days, than polyphenols are released in a huge quantity at the same time, after 14 days of immersion in water.

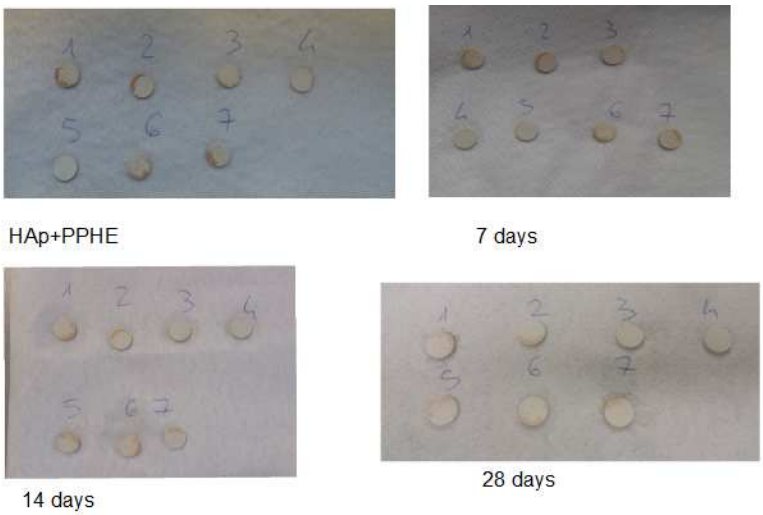

Figure 5.60 Release in water: HAp lab samples

The aspect of CT and SCNbis samples is observable in Figures 5.61-62. For these two biomaterials, it is possible to appreciate the main difference between the functionalised sample and the ones evaluated after seven days of immersion in water: in fact, the film of grafted polyphenols is visible

by the naked eye – especially on SCNbis samples, more hardly on CT ones because of the similarity of the colours characterising the nano-porous oxide and the polyphenols layer – only on the functionalised samples, but not more after seven days; evaluating the samples at the other experimental times, there are not any significant differences. It means – as confirmed by successive analysis – that a significative release of polyphenols has taken place quite immediately, during the first hours and not later than seven days.

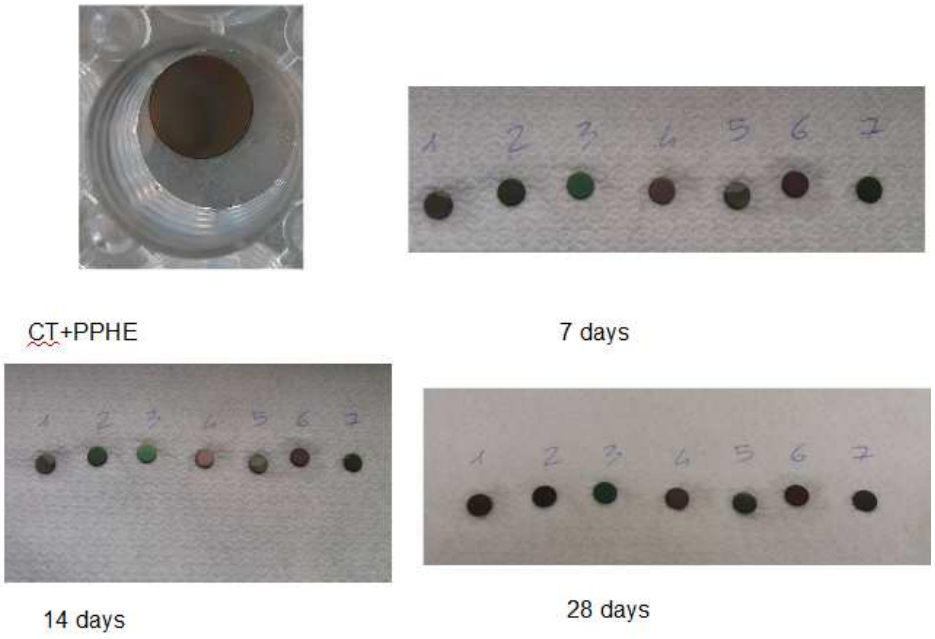

Figure 5.62 Release in water: CT samples

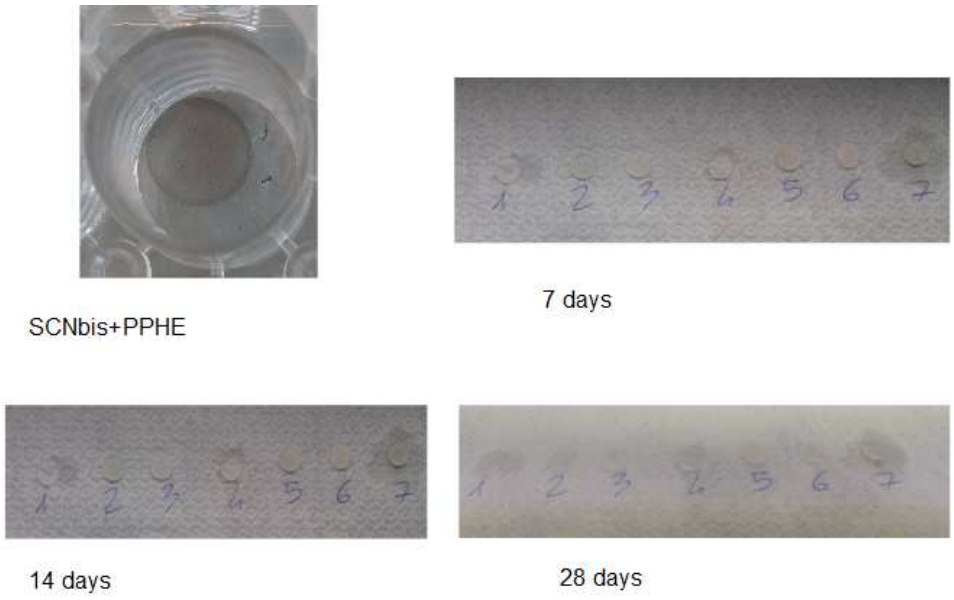

Figure 5.62 Release in water: SCNbis samples

Figure 5.63 illustrates the samples of the three analysed biomaterials at the initial experimental time and at the end of the test.

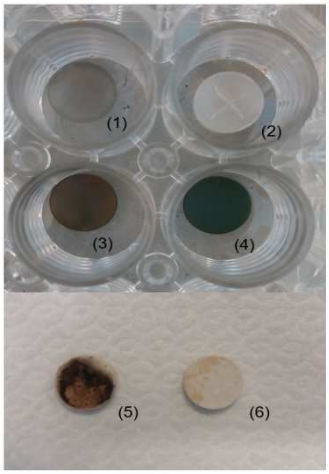

Figure 5.63 Release in water: solid samples comparison
(1) SCNbis+PPHE; (2) SCNbis+PPHE 28d; (3) CT+PPHE; (4) CT+PPHE 28d;
(5) HAp+PPHE; (6) HAp+PPHE 28d

Through this image, the difference between the samples are better appreciable: the lower polyphenols content on the surface of the samples – when extracted from water at the end of the test – is evident.
Also the release solutions have been visually analysed: by naked eye, the presence of polyphenols has not been detected and no differences between these solutions and ultra-pure water are appreciable.

5.8.1 pH
At every experimental time, the release solutions have been tested and characterised through pH measurement (Figure 5.64); ultra-pure water was utilised as control.

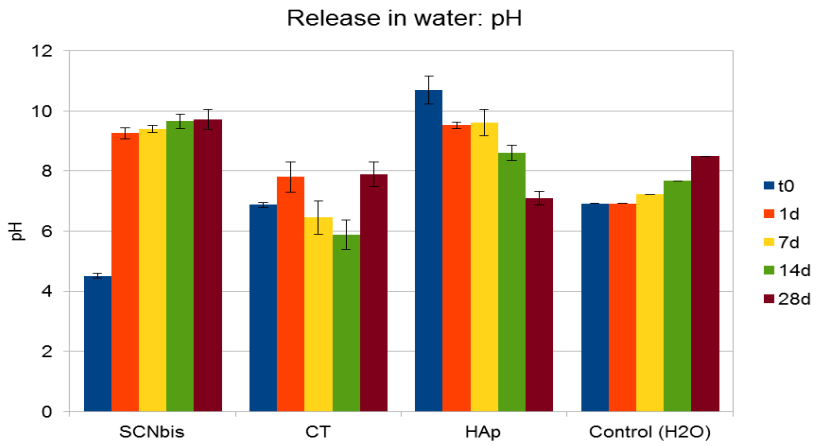

Figure 5.64 pH values of release solutions

Through the histogram, it is possible to appreciate two different behaviours. The solutions where CT+PPHE samples are immersed present low pH variations: the stability of the substrate is particularly high and, 28 days-immersion in water does not significantly alter the system[79,80].
Instead, SCNbis and HAp lab samples induce a pH alteration, increasing the initial value: the release solutions tend to become alkaline, reaching also high pH values. This phenomenon, immediate for HAp and slower for SCNbis, is due to the reactivity of these biomaterials.
HAp release solutions reach pH~11 after only one day of immersion; then, the released polyphenols tend to decrease pH values. SCNbis release solutions are instead initially acid and becomes strongly alkaline (pH~10) after few days; successively, pH remains quasi-constant when evaluated at the following experimental times.

5.8.2 UV spectroscopy
The UV spectra characterising the analysed solutions at the four evaluated experimental times are reported.
The release solutions after one day are characterised by the following spectra (Figure 5.65)

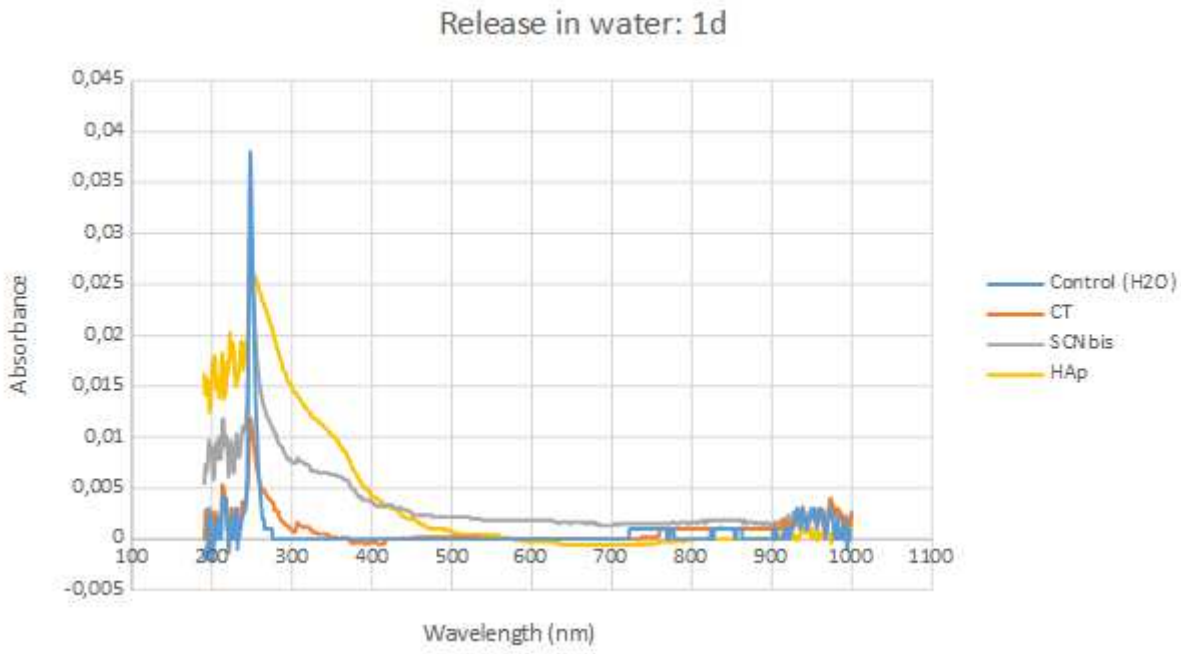

Figure 5.65 UV spectra of release solutions after 1 day

The signal is low, that means a low polyphenol concentration in the analysed solutions. Anyway, a peak is appreciable in correspondence of λ=250 nm, attributed to the presence of isoflavones[81]. For CT and HAp solutions, the peak is larger: it is due to partial polymerisation of the released polyphenols and the eventual amount of hydroxycinnammic acids[82,83].
After 7 days, the spectra characterising the release solutions are the following (Figure 5.66).

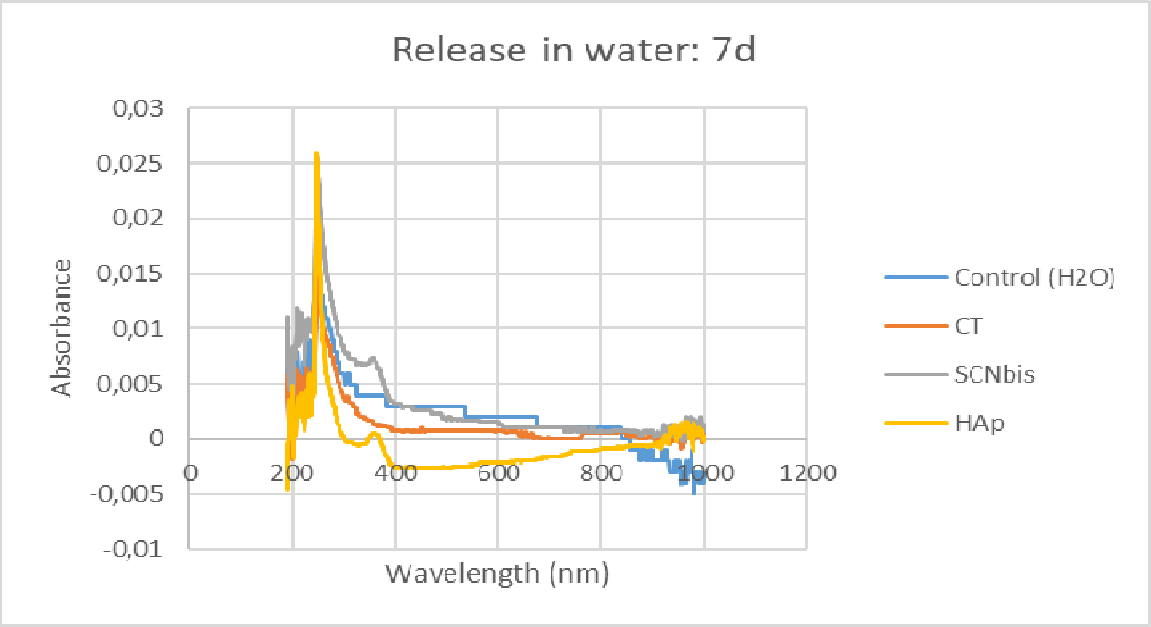

Figure 5.66 UV spectra of release solutions after 7 days

The signal is ulteriorly lower: the polyphenols concentration is lower at the second experimental time. Anyway, the peaks characterising polyphenols are better defined. In correspondence of λ=250 nm, confirming the presence of isoflavones, a peak is clearly visible. Furthermore, around 380 nm, a low, but well-defined peak is appreciable, which could correspond to aromatic rings[84].
The spectra evaluated after fourteen days (Figure 5.67) are quite null and do not present significant peaks: the release of polyphenols has place during the first seven days after the start of the test. Only SCNbis release solution presents two peaks in correspondence of 250 nm (isoflavones) and 380 nm (aromatic rings), but their intensity is comparable to the background noise[85].

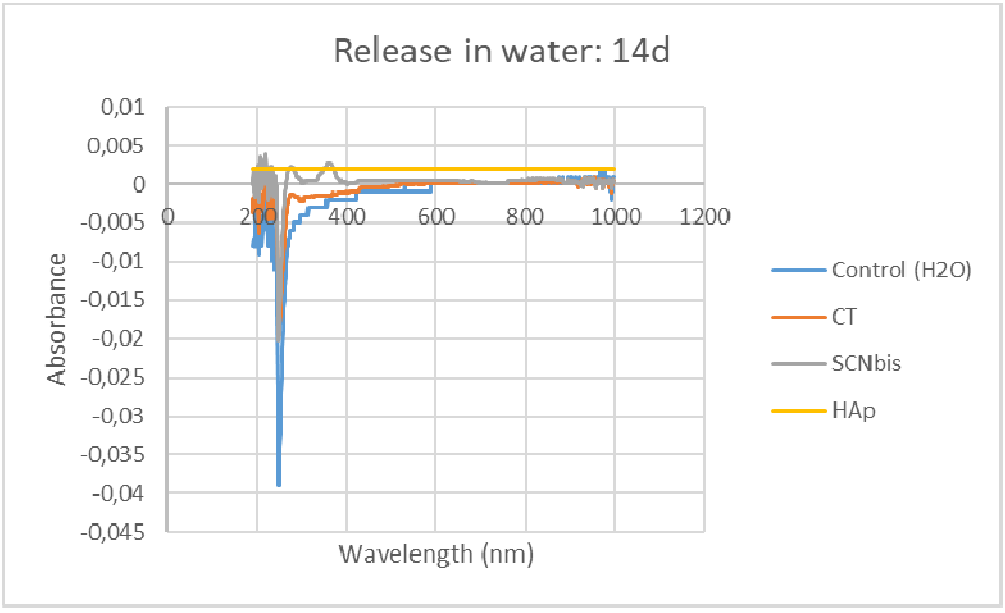

Figure 5.67 UV spectra of release solutions after 14 days

Finally, the release solutions have been analysed after 28 days. The characterising UV spectra are reported in Figure 5.68.

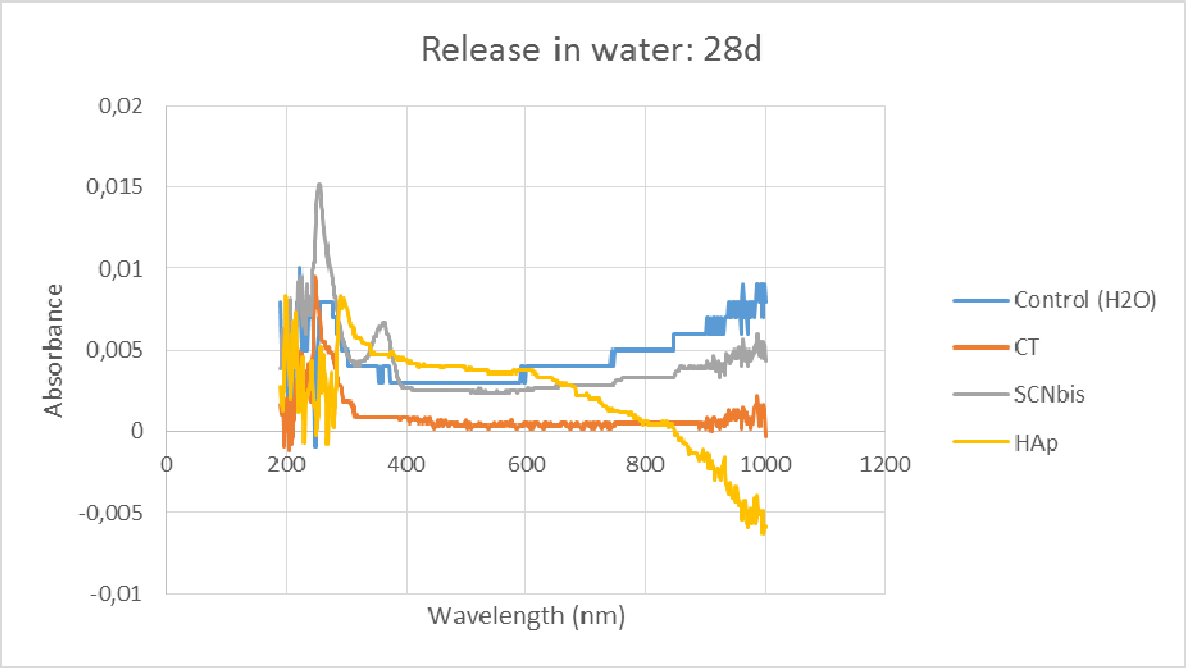

Figure 5.68 UV spectra of release solutions after 28 days

In this last case, the detected signal is higher and its intensity is comparable with the ones characterising 1 day and 7 days spectra. The 28 days spectra newly present the peaks characterising polyphenols: all the biomaterials present the same peak at 250 nm, corresponding to isoflavones; for HAp, it is not well-defined because of electronic noise, but the flavanols peak (280 nm) is appreciable[86]. SCNbis presents a particularly intense isoflavones peak and is the only sample characterised by a second peak – 380 nm peak, corresponding to aromatic rings[87].
So, it is possible to affirm that polyphenols are continuously released during the first seven days and then, abruptly, after 28 days. It is supposed that during the first seven days the released biomolecules are the ones that are more weakly grafted to the substrates: their release is continuous, so that their presence is detected through UV spectroscopy both after 1 day and after 7 days. Successively, after a month, also the strongly bound polyphenols are partially released in the solution.

5.8.3 FTIR-ATR

The functionalised samples have been analysed and evaluated through FTIR-ATR spectra, at the end of the release test. In Figure 5.69, these spectra are reported and compared with the ones characterising the bare and the just functionalised samples.
It is possible to appreciate the tendency of the spectra characterising the samples after the release test to match with the ones that are proper of the bare biomaterials. This phenomenon, particularly visible on HAp lab and CT surfaces, is due to the low polyphenols content on their surface, which is hardly detectable by the instrument.
Also the spectrum of SCNbis+PPHE after the release test tends to match with the one characterising the bare bioglass, but has a peculiarity. Both these spectra present a peak in correspondence of 900-

500 cm^{-1}, but it is better defined and more intense on SCNbis+PPHE sample after the release test. This peak, typical of water, could be attributed to the hydrated reaction layer[88,89]. Because of the 28 days immersion of the sample in an aqueous environment, water molecules interacted with its surface all the test long; this phenomenon has been amplified after the release of the most part of the grafted polyphenols, which protected the biomaterial from oxidation, exercising a shielding action. The reaction layer has become particularly hydrated, so that the 900-500 cm^{-1} peak is particularly visible, intense and well-defined after the release test.

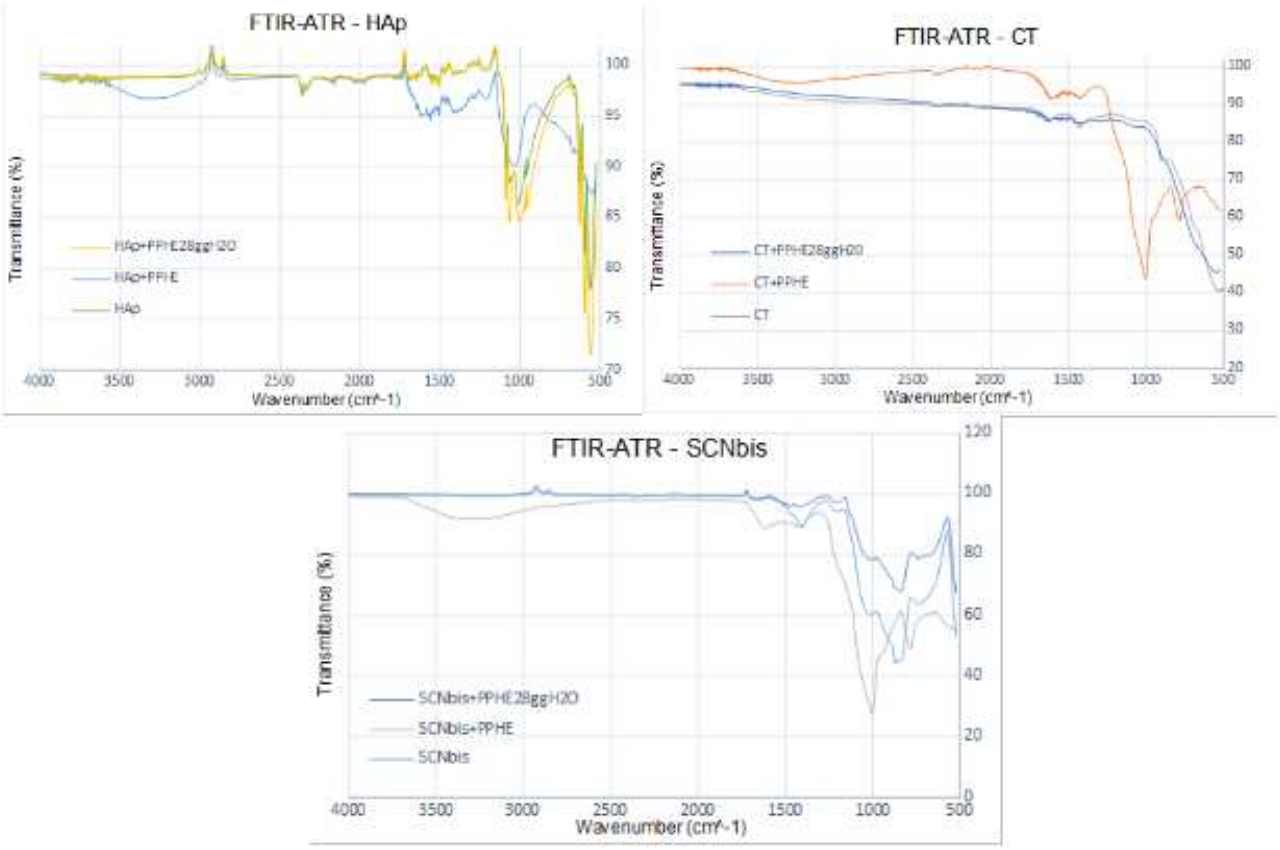

Figure 5.69 FTIR-ATR spectra of bare and functionalised samples, before and after the release test

5.8.4 F&C test and F&C modified for solid samples

The polyphenols content has been quantified also by F&C test, evaluating – at every experimental time – GAE concentrations of the release solutions and of the solid samples themselves at the end of the release test.

As previously explained, in order to quantify the molecules with redox activity grafted on the solid samples, the F&C protocol (suitable for a solution) was modified in order to be applied to a solid sample as described in Chapter 4. According to previous results, this modified protocol is effective, but it is not completely clear at this stage if it quantifies the adsorbed molecules which are released into the F&C reagent solution during the test or the total amount of the grafted (unreleased) and adsorbed (released) molecules.

The outcomes of F&C test carried on the release solutions are reported in Figure 5.70. As visible by the histogram, concerning SCNbis and CT, polyphenols release has taken place almost at the first experimental time, confirming the previous considerations, while, regarding HAp samples, the most part of polyphenols are released after 28 days.
Specifically, SCNbis releases polyphenols quite homogeneously among the experimental times, but the higher quantities are detected in the solutions after 1 day and after 28 days; CT presents instead a bulk release after 1 day, while the concentration of polyphenols at the following experimental times is negligible.
A significantly higher quantity of biomolecules are complexly released by HAp, probably due to the high surface-to-volume ratio and porosity, in which polyphenols are retained to be released only at the end of the test. In fact, the GAE concentrations of polyphenols at the first experimental time is particularly low, while, after 28 days a bulk release has place.

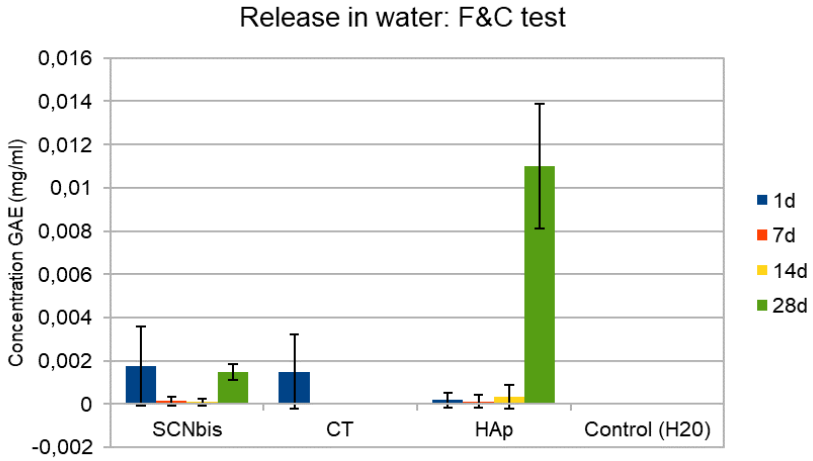

Figure 5.70 F&C test: GAE concentrations of release solutions

In the following histogram (Figure 5.71), GAE concentrations characterising the analysed samples are reported: the functionalised samples after the release test have been analysed and compared with some just functionalised samples of the same biomaterial.

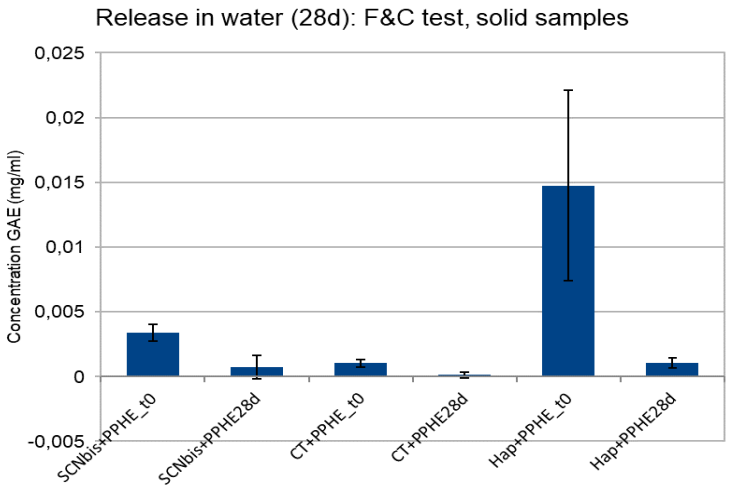

Figure 5.71 F&C test: GAE concentrations on solid samples

As appreciable, through the F&C test the detected polyphenols at the end of the release test are significantly lower than the ones initially grafted to their respective surfaces. It means that during release test, almost all polyphenols are released in water; in fact, at the last experimental time (28 days), the quantity of grafted polyphenols is negligible. These results are in accordance with the previously described analyses on the solutions. The F&C test quantifies the redox activity of polyphenols: the detected signal is so approximately null because of the negligible presence of grafted biomolecules.

5.8.5 DPPH test

At the end of the release test, the antioxidant power of the residually grafted polyphenols have been evaluated and compared with the one characterising the same samples just after their functionalisation (Figure 5.72).

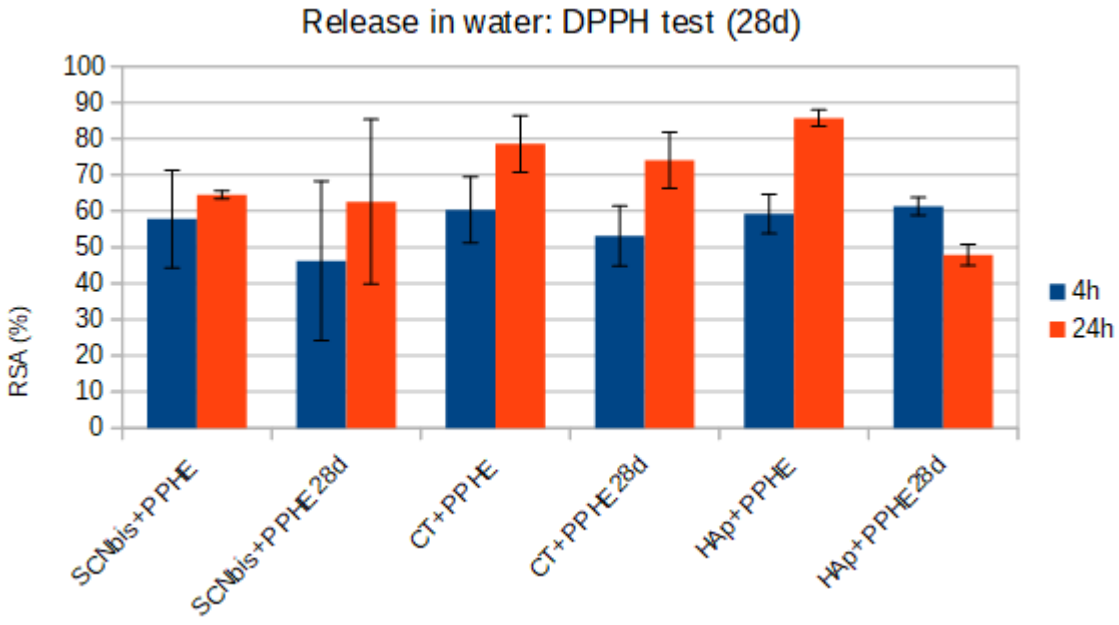

Figure 5.72 DPPH test on solid samples after 28 days of release in water

This test demonstrates the high antioxidant activity of the tested samples after 28 days: it means that there is a surface layer of residual phenolic compounds that are active and able to exercise their radical scavenging activity. In fact, as appreciable by the histogram, the RSA values characterising the samples before and after the release test are comparable.

If we compare the F&C results obtained on the solid samples after the release test (28 days) and these results, it seems that there is a fraction of strongly grafted molecules still unreleased after 28 days of soaking which are detected from the DPPH test, but not from the F&C test modified for the solid samples. This supports the hypothesis that the modified F&C test is sensitive only to the fraction of molecules released into the F&C reagent solution during the test, that means probably to the adsorbed fraction. The modified F&C and DPPH tests are complementary in order to have a complete characterisation of the functionalisation outcome.

5.8.6 Fluorescence microscopy

The three biomaterials have been analysed through fluorescence microscopy before and after the release test. All the analysed biomaterials present a significantly lower polyphenols content, but each of them differently respond to the treatment: the distribution of the residual grafted polyphenols is different for each biomaterial.

In Figure 5.73 the functionalised HAp lab samples are evaluated at the end of the release test and compared with the just functionalised samples.

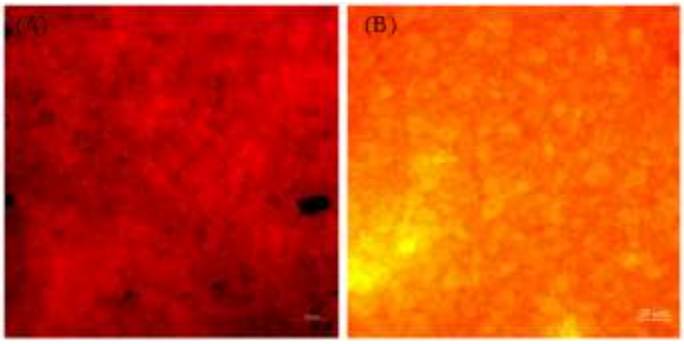

Figure 5.73 Fluorescence microscopy
(A) HAp lab + PPHE; (B) HAp lab + PPHE 28 days

After 28 days in water, a lower quantity of grafted polyphenols are present on HAp surface. It is appreciable by the lower intensity of the fluorescent signal: the intense red characterising the just functionalised HAp becomes light orange, almost yellow in certain areas of the sample. It could signify a lower polyphenol content or an alteration of their molecular structure. This aspect could be deepened through other analysis.

Anyway, the distribution of the grafted biomolecules remains the same, as homogeneous as it is before release test: the observed bulk release at the last experimental time does not exclude the presence of a surface layer of polyphenols on the substrate.

The next image (Figure 5.74) illustrates CT+PPHE samples before and after the release test.

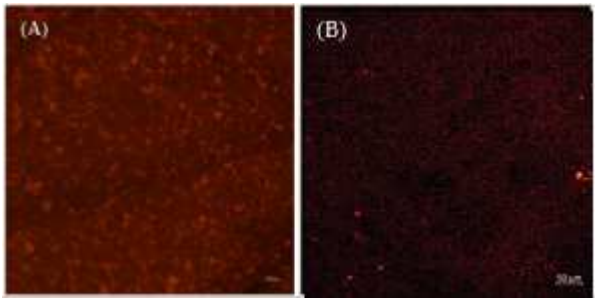

Figure 5.74 Fluorescence microscopy
(A) CT + PPHE; (B) CT + PPHE 28 days

In this case, the polyphenols content after 28 days is particularly low, as the intensity of the signal reveals. A thin film, difficultly appreciable but well-distributed, is present on the analysed sample. In fact, almost all polyphenols are released among the 28 days and only a thin layer of them is visible on the analysed surface.
Finally, SCNbis functionalised samples (before and after the release test) have been analysed through fluorescence microscopy and compared (Figure 5.75).

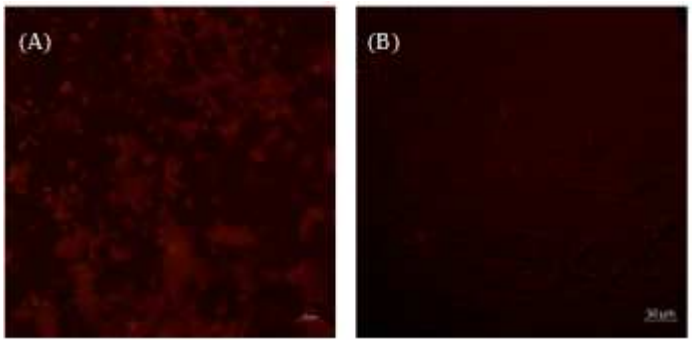

Figure 5.75 Fluorescence microscopy
(A) SCNbis + PPHE; (B) SCNbis + PPHE 28 days

At the end of release test, it is possible to see that polyphenols agglomerates have been completely released. The background layer, anyway, has been almost completely conserved during the 28 days, probably because of the stronger interactions between polyphenols and the hydrated layer[90]. In fact, the polyphenols layer well adhere to the substrate and, through it, it is possible to see the morphology of the polished sample.

5.9 Sterilisation
HAp lab, CT and SCNbis functionalised samples have been sterilised after an adequate packaging through γ irradiation – 25 kGy, as uniformed for medical devices. The packaged and sterilised samples are reported in Figure 5.76(A).

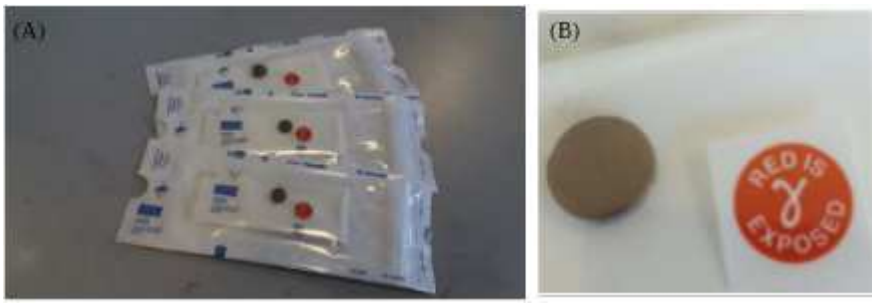

Figure 5.76 (A) Packaged samples after sterilisation
(B) Zoomed package: sterilised sample and dosimeter

A dosimeter has been applied to each package before sterilisation to verify if each sample is effectively treated with the imposed dose of γ radiation. During irradiation, the dosimeter – because its chemical and physical properties – is able to vary its colour. In this case, the red dosimeters

guarantee the efficacy of sterilisation. In fact, as visible in Figure 5.75(A), but better appreciable in Figure 5.75(B), the red colour characterising the dosimeter implies an efficient sterilisation process.

The samples have been extracted from the package and compared. In Figure 5.77, the functionalised biomaterials are illustrated and compared before and after sterilisation.

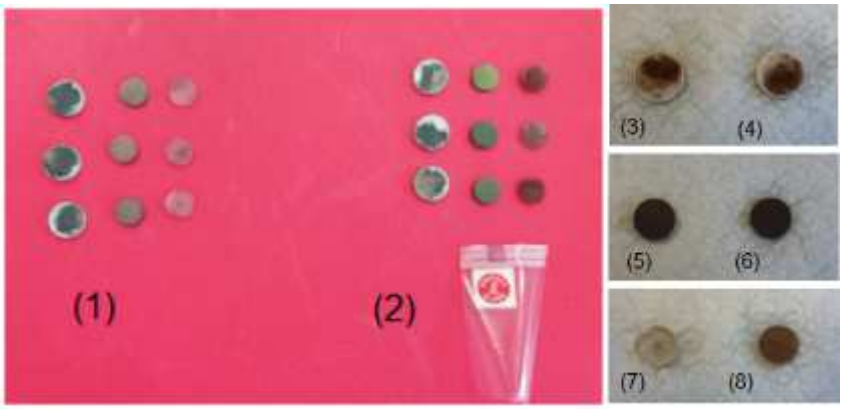

Figure 5.77 Functionalised samples (1) before and (2) after sterilisation:
(3) HAp lab + PPHE; (4) HAp lab + PPHE after sterilisation;
(5) CT + PPHE; (6) CT + PPHE after sterilisation;
(7) SCNbis + PPHE; (8) SCNbis + PPHE after sterilisation.

Visually analysing the samples, the eventual alterations of polyphenols content are not particularly appreciable by naked eye, but it is possible to see a particular variation of colour on SCNbis samples: after sterilisation through ^{60}Co γ irradiation, the colour of the biomaterial itself significantly changes from the initial transparency and absence of colour, becoming brown at the end of the process. The intensity of the colour is proportional to the radiation dose applied to the sample[91]. Irradiating at 25 kGy, the exposure of bioglass at γ radiations causes two main processes: displacement and electron rearrangement. The impact of the ionising radiation depends on the morphology of the surface, the eventual presence of superficial defects and on the modifier oxides content. The presence of structural defects and vacancies amplifies the effects of irradiation: the induced variations in electronic distribution of glass could accelerate bond breaking phenomena inside the bulk material[92]. Furthermore, the presence of certain chemical elements – such as K, Na, Mg, Zn and Fe – in the matrix of the material could contribute to alter the chemical structure of a bioglass during the sterilisation process[93]. The presence of sodium, introduced in SCNbis as sodium oxide, induces the formation of non-bridging oxygens: these structures are sensitive to γ radiations, contributing to induce the presence of defects[94]. The presence of these defects on the material and the alteration of its molecular induce a significant variation of the absorbance UV-Vis spectrum – which induce the visually appreciable alteration of the colour – and the increasing of optical density sensitivity. In fact, because of irradiation, free electrons and holes are concentrated in vacancies, interstitials and near to impurities and defects: this induces the glass lattice to differently absorb the light: the alteration of absorbance on the visible spectrum – alteration of optical transmission and refractive index – causes the macroscopic change of colour. These phenomena are amplified by the presence of the modifier oxides, which increase the presence of interstitial elements and impurities

in the glass lattice: sodium is so the main contributor in formation of colour centres in SCNbis matrix[95].

5.9.1 UV spectroscopy

The UV spectra characterising sterilised and non-sterilised functionalised samples are reported in the following plots (Figure 5.78).

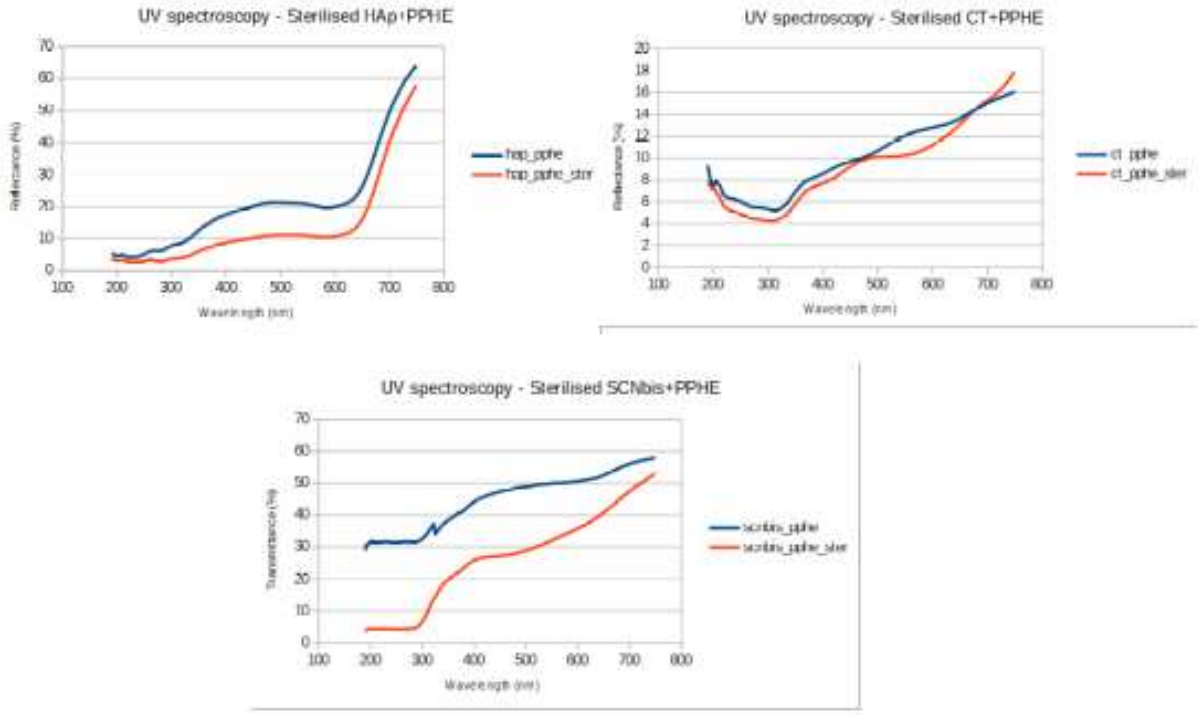

Figure 5.78 UV spectra of functionalised samples before and after sterilisation

Analysing the acquired spectra, it is possible to see an analogue response to the sterilisation process characterising all the studied materials. The reflectance and transmittance spectra – respectively evaluating HAp and CT, and SCNbis behaviour – conserve the same trend before and after sterilisation: it means that polyphenols are still present after irradiation and their presence is detectable by the instrument. The lower intensity of the signal is due to the lower concentration caused by the ionising radiation. The antioxidant power of polyphenols limits the ionising effects: the grafted biomolecules scavenge the reactive oxygen species induced by the treatment, so that only a low fraction of the functionalised layer is damaged and the desired effects of sterilisation – elimination of contamination and bacteria – are guaranteed, without any significant alterations of the sample[96,97]. The phenomenon of limited alteration of the grafted molecules after γ irradiation is amplified by the sterilisation modalities. The environment in which sterilisation had been implemented was dry, so that the only effect of radiation was due to the aggressivity of the associated energy. The eventual presence of water and a high humidity level in the sterilisation environment would induce an additional stress due to the water-derived radiolytic free radicals[98].

Evaluating specifically the single plots, the difference between the initial and the final intensity varies in function of the biomaterial. HAp and CT samples do not strongly resent of γ irradiation: it is possible to appreciate a low decrease of the intensity of the signal after the treatment, that means

a low decrease of grafted polyphenols content. This difference is minimal on CT samples: the two spectra – before and after sterilisation are almost comparable. SCNbis, instead, present a higher alteration of the intensity of the initial spectrum: it is due to the cited formation of colour centres in the matrix of the glass. It is probably due to the described variations of the structure of the glass lattice, which induce an alteration of the hydrated layer, where polyphenols are grafted: the most part of defects and impurities are in fact present on the surface[99].

5.9.2 FTIR-ATR

FTIR-ATR spectra, acquired before and after γ irradiation, are reported in Figure 5.79.

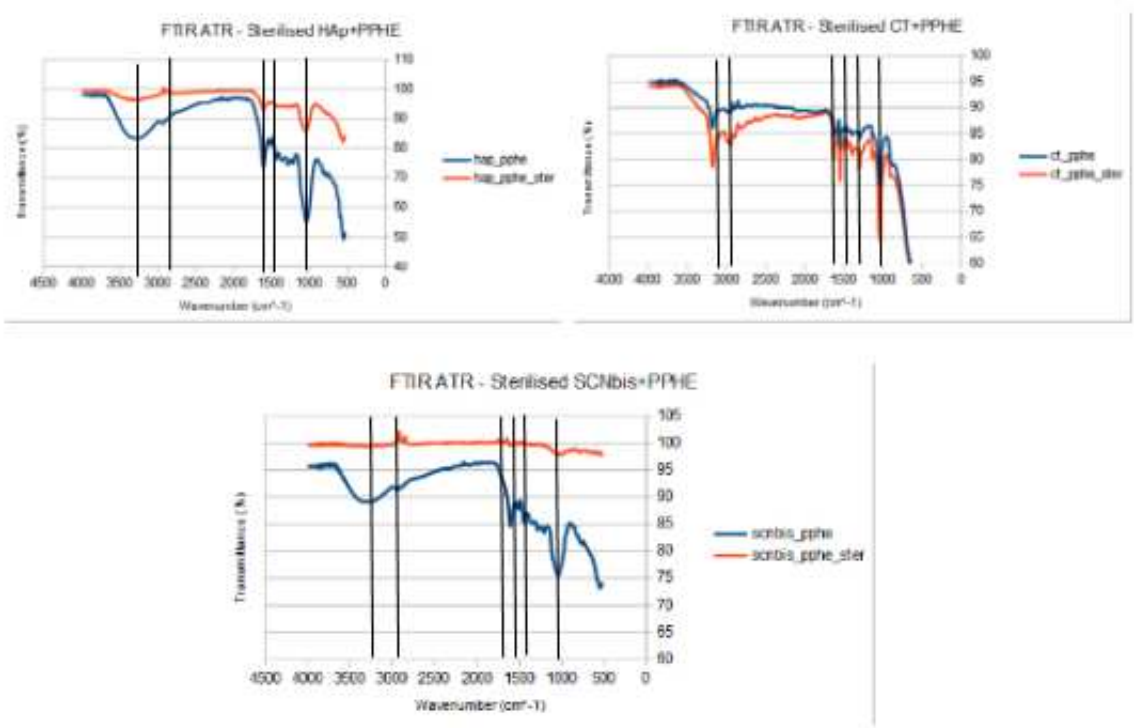

Figure 5.79 FTIR-ATR spectra of functionalised samples before and after sterilisation

As observed for UV spectroscopy, the difference of intensity between the initial and the final spectra is minimal for CT samples, followed by HAp, for which, despite the lower intensity, the peaks are well-defined. At last, SCNbis is characterised by a significant difference between the two transmittance spectra: while before sterilisation polyphenols peaks are present and well-visible, after the treatment the curve is quasi-constant, characterised by a low ripple around 3000 cm^{-1} (corresponding to C-H bonds) and around 1200 cm^{-1} (corresponding to phenolic hydroxyl groups bending vibrations)[100,101,102].

5.9.3 F&C test

To quantify the residual grafted polyphenols after γ irradiation, the sterilised samples have been analysed through F&C test.

As previously explained, in order to quantify the molecules with redox activity grafted on the solid samples, the F&C protocol (suitable for a solution) was modified in order to be applied to a solid sample as described in Chapter 4. According to previous results, this modified protocol is effective, but it is not completely clear at this stage if it quantifies the adsorbed molecules which are released into the F&C reagent solution during the test or the total amount of the grafted (unreleased) and adsorbed (released) molecules.

The GAE concentrations characterising each sample are reported in Figure 5.80 and compared with the respective just-functionalised one.

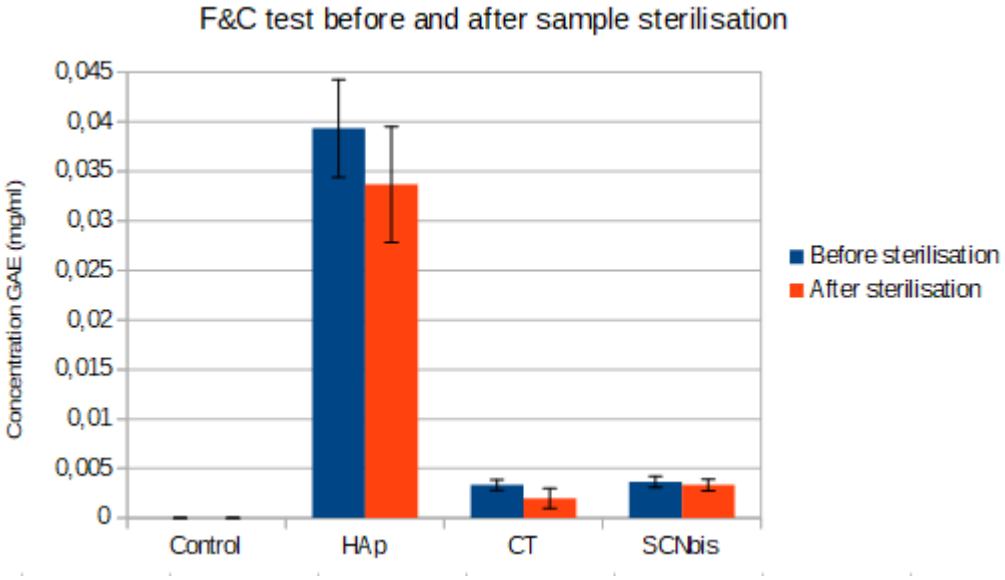

Figure 5.80 F&C test: polyphenols GAE concentrations on sterilised samples

As appreciable by the histogram, the quantity of grafted polyphenols does not present significant alterations during sterilisation: the GAE concentrations characterising the analysed surfaces is comparable before and after γ irradiation.

Analysing UV and FTIR-ATR spectra, the effective quantity of residual polyphenols has been evaluated; through this test, instead, it is possible to appreciate the different redox activity of phenolic compounds on each surface. By F&C test, in fact, GAE concentration of polyphenols on CT and SCNbis remains approximately constant during the treatment, while HAp presents a low decrease when analysed after sterilisation. As described in Chapter 1, it is reported in literature that sterilisation can induce a decrease in the total amount of polyphenols (in a solution) from one side and an increase in their redox activity on the other side. It seems to be the same on the solid samples investigated in this research.

5.9.4 Fluorescence microscopy

In Figure 5.81, functionalised HAp samples, respectively before and after sterilisation, are observable.

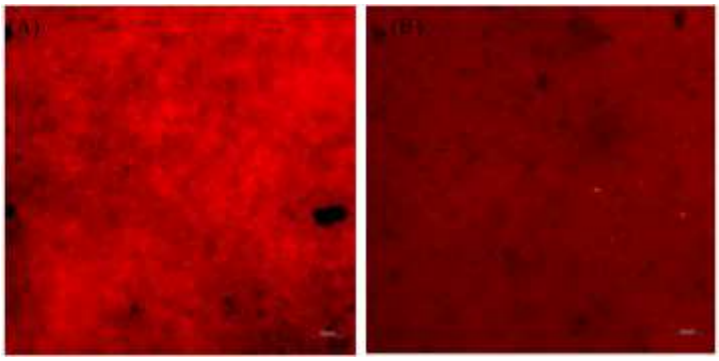

Figure 5.81 Fluorescence microscopy
(A) HAp lab +PPHE; (B) HAp lab + PPHE after sterilisation

As visible by Figure 5.81, the distribution of polyphenols does not vary significantly after sterilisation: the homogeneity of the functionalisation layer is not altered by the treatment. The decreasing of fluorescence intensity is probably associated to a low thinning of the reaction layer.
Sterilised CT sample is reported in Figure 5.82 and compared with the just functionalised one. It is possible to see that the background layer is not altered during sterilisation: it is homogeneous and polyphenols are well-distributed. A significant variation is instead appreciable concerning the dimension of agglomerates: the characteristic dimension of agglomerates averagely increases and the distribution of their diameters is less homogeneous. It is probably due to a polymerisation process implemented by γ radiation.

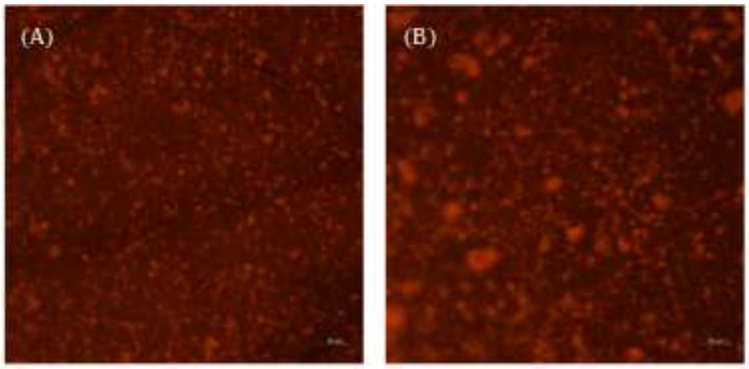

Figure 5.82 Fluorescence microscopy
(A) CT+PPHE; (B) CT + PPHE after sterilisation

The grafted polyphenols on SCNbis surface – reported in Figure 5.83 – present an opposite alteration, compared with the ones bound on CT. In fact, after sterilisation, the agglomerates do not present significant variations, while the background layer thins during the treatment, as appreciable

through the lower intensity of the fluorescent signal. This analysis shows a low decrease of polyphenols content after irradiation, apparently discordant with FTIR-ATR spectroscopy outcome. Anyway, the low transmittance signal of sterilised SCNbis sample is probably due to the variations of optical properties of the glass lattice, caused by γ radiation: it exercises a screening action, so that the residual polyphenols are not detected through FTIR-ATR technique[103].

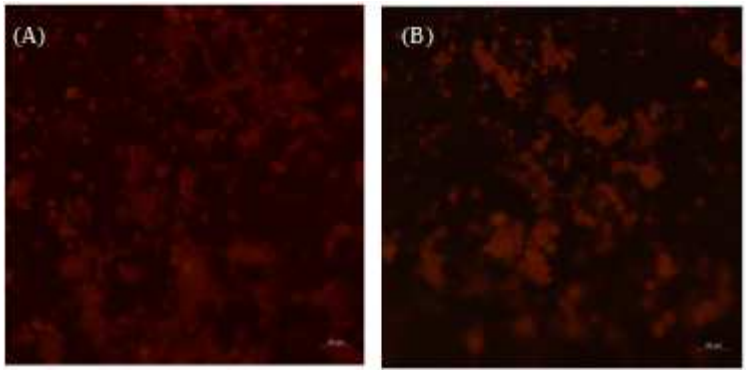

Figure 5.83 Fluorescence microscopy
(A) SCNbis+PPHE; (B) SCNbis + PPHE after sterilisation

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CONCLUSION

In the current study, the functionalisation process of biomaterials with polyphenols has been implemented and the obtained surfaces have been analysed and characterised. Hydroxyapatite – with two different porosities, respectively sintered in laboratory and industrially -, Ti6Al4V – previously chemically treated to obtain a nano-porous and bioactive surface – and two types of bioglass – SCNA and SCNbis – have been considered. The samples have been tested through various and complementary techniques, to better evaluate their chemical and physical properties and their response to functionalisation. A test of release in water was carried to study the associated diffusive phenomena. The effects of sterilisation with γ radiation have also been studied.

A mixture of lyophilised polyphenols has been extracted from red grape (type Barbera) and utilised to prepare a series of different solutions to functionalise the analysed biomaterials. Different solvents, concentrations of polyphenols and pH values have been imposed and compared. The most efficacious polyphenols concentration has been found as 5 mg/ml. Ultra-pure water has been chosen as the best solvent to treat hydroxyapatite and bioglass, while TRIS/HCl+$CaCl_2$ resulted the most efficient solvent to implement the functionalisation of titanium alloy. This is because Ca^{2+} ions in solution at pH=7 are able to form a complex compound with polyphenols and act as linkers with the titanium treated surface, which shows de-protonated (negatively charged) hydroxyl groups at pH=7. Because of this grafting mechanism, polyphenols are released from the titanium treated surface at pH=4: this material acts as smart with a specific release action in inflammatory condition. Ethanol has been excluded as a potential solvent even if the reason why is to be much more investigated.

The efficacy of functionalisation has been quantified through Folin and Ciocalteu test (redox activity of the adsorbed biomolecules)and the antioxidant activity of the grafted biomolecules through DPPH test. FTIR-ATR spectroscopy, UV spectroscopy, zeta potential and fluorescence microscopy have been exploited to characterise both biomaterial samples and solutions. Through these analysis, the efficiency of the optimised functionalisation process has been confirmed for hydroxyapatite sintered in laboratory, titanium alloy and SCNbis; SCNA and industrially sintered hydroxyapatite were excluded because of the low capacity to graft polyphenols. Polyphenols act as a protective layer on HAp, reducing its natural chemical instability at pH=4 in inflammatory conditions.

Through the release test, a high polyphenols release has been noted, which has place with different trends for each biomaterial. At the end of the test, the grafted polyphenols content on each surface was significantly reduced, but a thin and almost continuous layer of grafted biomolecules maintained a high antioxidant power.

Finally, the effects of sterilisation of functionalised samples have been evaluated: the quantity of grafted polyphenols did not significantly decrease and the antioxidant activity was not significantly altered.

The positive outcomes concerning functionalisation process, the elevate antioxidant power of the grafted biomolecules and the low effects of sterilisation on the analysed samples permit to hypothesise a future clinical application of polyphenols-functionalised biomaterials to musculoskeletal implants and eventually an industrial production.

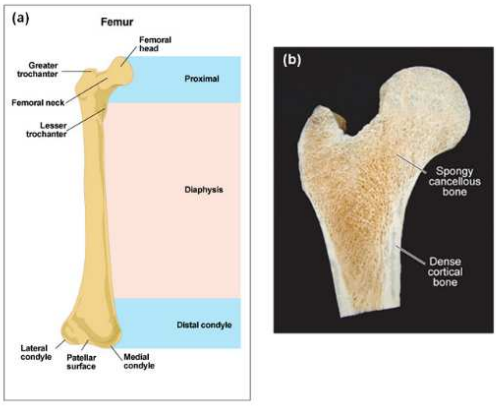
APPENDIX

BIOMATERIALS SELECTION FOR A JOINT REPLACEMENT: METALS, CERAMICS, POLYMERS

To select the best materials for a joint replacement, it is necessary to follow an appropriate methodology, considering three important aspects:

1. Function: what does the component do?
2. Constraints: what essential conditions must be met?
3. Objectives: what physical quantity is to be maximized or minimized?

First of all, after the definition of the function of the component we are interested to, we focus on constraints, defining the various criteria that must be satisfied. Then, we have to rank on objectives, ordering the selected materials that satisfy these criteria. So, once obtained the top candidate materials, we interface with specialists, from whom we can get more fundamental information. The last things to consider is the definition of local conditions: the preferred suppliers, the process capability and the location. So, we obtain the final biomaterials selection.



Now, we focus on the hip replacement, considering two of the components of the prosthesis: first, femoral stem and, then, femoral head.

Femoral stem.

Its function is sustaining compressive load from external forces resulting in shear and bending of the femoral stem itself.

Examining the constraints, the material:

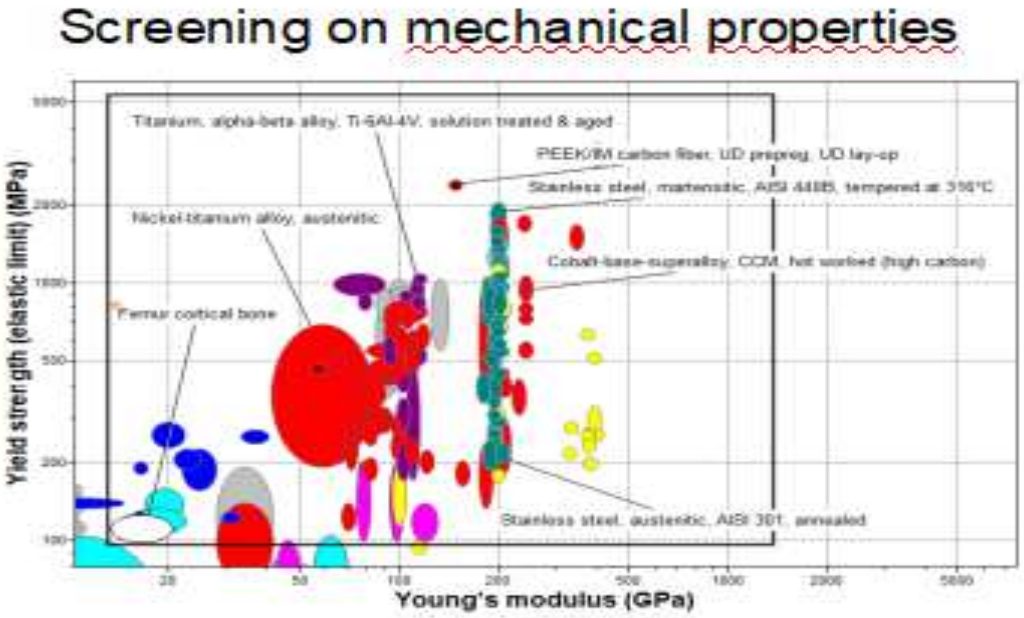
- has to be biomedical
- has to be stiff and strong not less than the cortical bone (E>10 GPa)
- has to have a sufficient fracture toughness to avoid fast fracture (K_I>11 MPa*$m^{0.5}$)
- has to be a bulk material, not filled, non-magnetic.

At last, the two principal objectives are maximizing specific strength and minimizing costs.

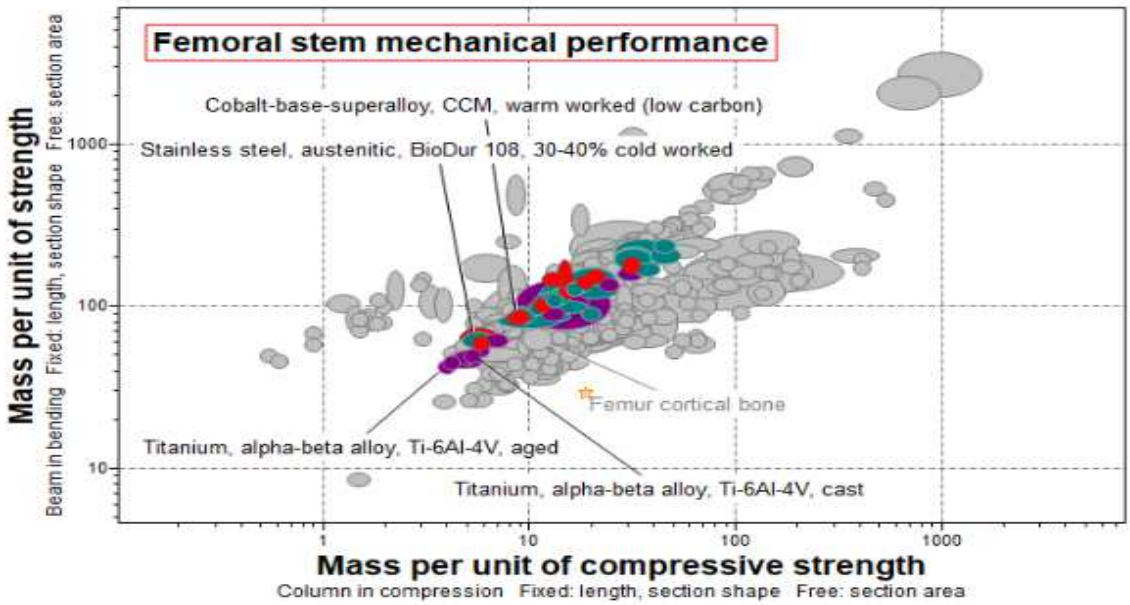
Analysing experimental data, we determine different parameters to be minimized. Selecting different types of loads, the software give us in output these parameters:

- compression → ρ/σ_c (ρ=material density, σ_c= compression stress)
- torsion → $\rho/\sigma_y^{2/3}$ (σ_y=torsion stress)
- bending → $\rho/\sigma_f^{2/3}$ (σ_f=flecture stress)

Then, we can plot the yield strength as a function of Young's modulus (E) of the biomaterials we are interested to: stainless steel, Ti6Al4V, cobalt-base-superalloy (CCM), alumina, zirconia, and, obviously, cortical bone are reported in the following plot.



In the same way, we also plot, for each of these materials, the mass per unit of strength as a function of the mass per unit of compressive strength. Visualizing the behavior of these parameters can be a great help to select the best materials.



Another important factor is the fracture toughness (K_I): considering it must be at least 11 MPa*$m^{0.5}$, we see only metals satisfying this condition, while ceramics not.
Last, but not least, we have to consider costs: platinum and palladium have a correct fracture toughness, but they are too expensive materials to be used for this application. We can so chose other metallic materials, such as titanium, cobalt or stainless steel.

Femoral head.

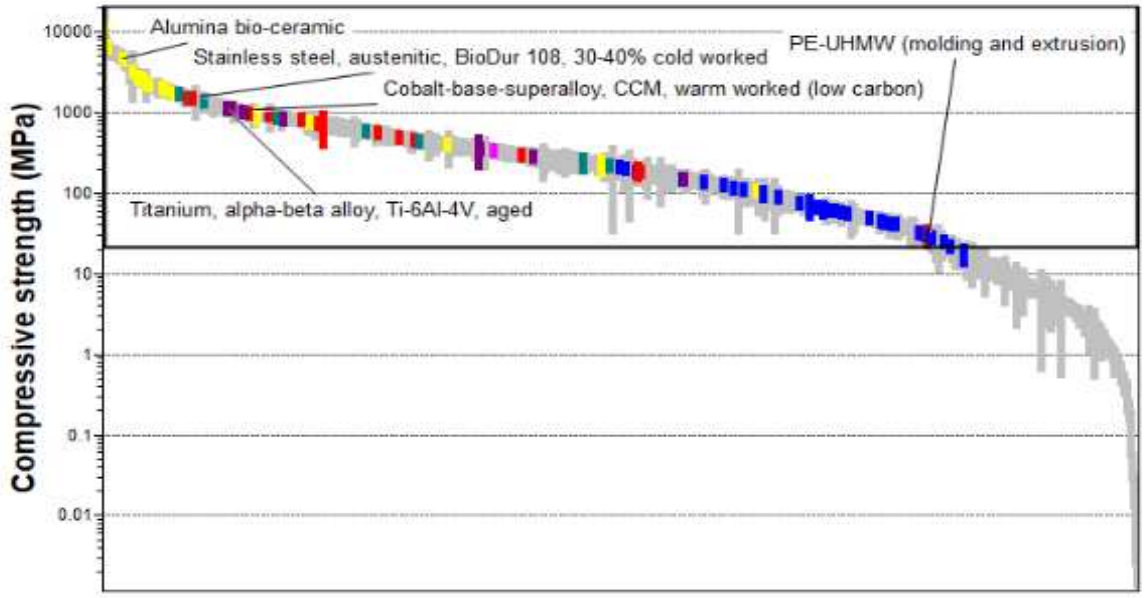
Its function is sustaining compressive load and wear resistance for both the femoral head itself and the linear/acetabular cup.

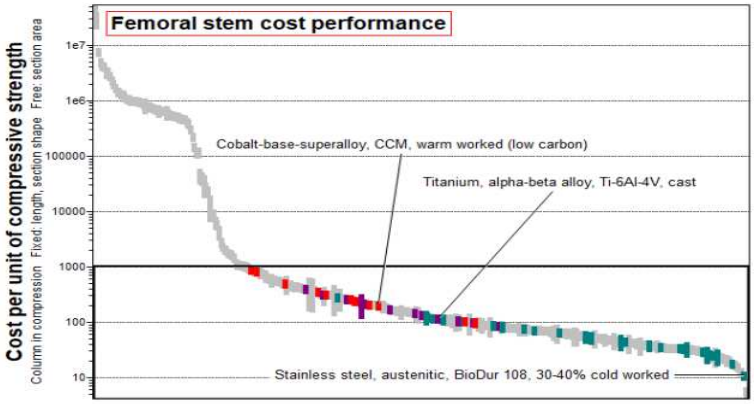
The most important constraint is that the material has to be optimal for a total joint replacement.

There are two principal objectives: maximizing compressive strength and minimizing wear and blunt abrasion.

There are different types of artificial joints: metal-metal, ceramics-ceramics and metal-ceramics adding polyethilene (UHMWPE).

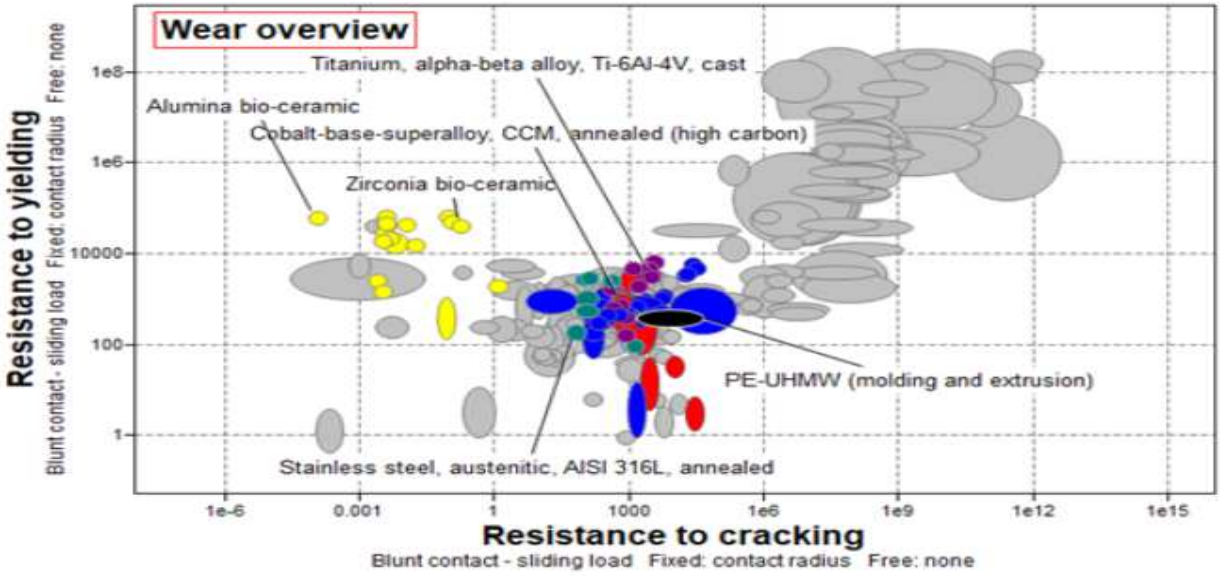
We can plot the compressive strength of the biomaterials we are considering, ranking them decreasingly: the most strong material is alumina, followed by zirconia, titanium, stainless steel, CCM and, very far in the distribution, UHMWPE. For this reason, PE is introduced to optimize compressive strength in a part of the joint that is not particularly stressed.





We can also evaluate the cost per unit of compressive strength to chose the best biomaterials to use for femoral head: gold is extremely expensive, so we exclude it because we need a significant quantity of bulk material for this application; then, we prefer other metallic materials, such as cobalt, titanium and stainless steel, which have good properties, but maintaining sustainable costs.

Another important aspect to consider is resistance to yielding in function of resistance to cracking.



We see that the best properties in this sense are given by ceramic biomaterials, while metallic ones have a good resistance to cracking with a relatively low resistance to yielding; titanium is an optimal compromise between these two properties. PE has a significant resistance to cracking, too: there are a lot of devices containing PE that were approved by FDA; this characteristic is given by the cross-linking process that makes the linear molecule of PE the UHMWPE we desire, significantly more resistant.