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New theoretical highlighting on the molecular interactions of natural polyphenols: penetration in lipid membranes and oxidative dimerization

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Contents

Introduction

Polyphenols represent a large and diverse group of natural compounds found in almost all plants. As secondary metabolites, they have many important functions in plant tissues, *e.g.*, they are important in plant growth, they protect tissues against UV radiation and are partly responsible for colours of flowers and fruit. Due to their presence in plants, they are also important components of human diet. We may find them in fruit (citruses, apples, peaches, grapes *etc.*), vegetables (onions, broccoli, *etc.*), aromatic plants (salvia, chamomile *etc.*), spices (nutmeg, curcuma, *etc.*) and beverages (wine, tea, cacao, *etc.*).

Plant extracts rich in polyphenols have been widely used in traditional medicine, *e.g.,* silymarin (extracted from Milk Thistle) is used to treat liver problems. Due to their beneficial effects on human health, they have been extensively studied. It has been proved that diets rich in polyphenols protect the organism against cardiovascular diseases, various types of cancer, Alzheimer's disease and also aging. As natural products with many beneficial effects and as powerful antioxidants, they are used in pharmacology, food industry and cosmetics. This general knowledge on polyphenols is treated in Chapter 1.

They are also known as pro-oxidant agents. One of the possible explanation of this deleterious behaviour comes from the high capacity of these compounds to lose H-atoms. This process originates free radical scavenging but also the formation of phenoxyl radicals stabilized by π-electron delocalization over the aromatic rings. Even if these radicals are relatively stable they may react by following different pathways:

→ A second H-atom abstraction (HAT) to form stable quinones or semiquinons

→ Regeneration by HAT from another antioxidant or solvent, so the polyphenol may continue to act as an antioxidant

→ Degradation into metabolites

→ Adduct formation

→ Dimerization with another phenoxyl radical

If two identical phenoxyl radicals meet together, a dimer may be formed. The dimerization process exists in plants. It is often only an initial step to form oligomers or polymers of polyphenols, *e.g.,* tannins which give characteristic colours and tastes to red wine or tea. Since the dimers play on important role in the nature, one part of this work aims at elucidating the dimerization process. The compounds chosen as model compounds are *(i)* silybin and dehydrosilybin (flavonolignans extracted from Milk Thistle) and *(ii)* quercetin, which is one of the most described polyphenolic compounds. (Chapter 3)

The second part of our study aims at rationalizing the interaction of polyphenols with lipid membranes. Generally, membranes play an important role in life of cells. They are composed of different types of lipids, proteins and other molecules which give them characteristic structure and features. The lipid chains are sensitive to changes which are caused by reactive species (*e.g.,* free radicals). The radicals may initiate chain reactions by HAT from lipid chain. This process is known as lipid peroxidation and it may cause serious changes in the membrane properties. As antioxidants, polyphenols may stop this process. The capacity to inhibit lipid peroxidation depends on *(i)* the capacity to scavenge free radicals, *(ii)* the solubility in apolar solution and *(iii)* the position and orientation inside the membrane. (Chapter 4)

To study dimerization process and interaction of polyphenols with lipid membrane, the methods of computational chemistry were used. These methods are powerful tools for science. Since the dimerization process involves the reactivity of compounds, quantum chemistry was used. This allows to describe the electronic structure of molecules which is necessary to

describe chemical reactions. On another hand, membranes are too large molecular systems for which molecular dynamics was used. In Chapter 2, the basic concepts concerning the theory are proposed.

Chapter 1

Oxidative stress, antioxidants and polyphenols

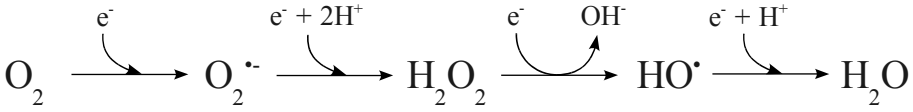
1. Reactive oxygen species and oxidative stress

During aerobic metabolism, the reactive oxygen species (ROS) are formed. Because of possible negative impact of ROS on organisms, the cells have set up strategies for their elimination. An imbalance between the production of ROS and their elimination may cause tissue damage. This imbalance is called “oxidative stress”.[1]

Damage could affect all types of biological molecules, *i.e.,* DNA,[2][3] lipids,[4][5] proteins[6] and carbohydrates. Numerous studies concerning the role of the oxidative stress in human diseases has been published. The contribution of ROS seems to be important in cardiovascular diseases, cancer, Alzheimer's disease, aging *etc.*[7][8][9][10][11][12]

1.1. Reactive oxygen species

Oxygen exists in the air as a diatomic molecule (O_2). Living organisms use it to oxidise hydrocarbons to obtain energy necessary for their life. During this process, molecular oxygen is reduced into non toxic water. The reaction goes through three intermediate steps in which superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) are formed.[13]



In atoms and molecules, electrons occupy orbitals. Each orbital can maximally bear two electrons with antiparallel spins. Free radicals are defined as chemical species (atoms or molecules) which contain unpaired electrons. Such species are more reactive.

Some of free radicals are generated in the respiratory chain ($O_2^{\bullet -}$, $^{\bullet}OH$, $HO_2^{\bullet}$), other radical species derived from fatty acids can be generated in the organism, namely peroxyl

radicals ($ROO^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$). All the reactive species containing O-atoms are denoted reactive oxygen species (ROS).

Together with ROS, there exist several radicals with the unpaired electron on carbon ($R^{\bullet}$), sulphur ($RS^{\bullet}$) or nitrogen ($RN^{\bullet}$).

ROS are also non-radical compounds, *i.e.,* hydrogen peroxide (H_2O_2), singlet dioxygen ($^1O_2$) or hypochlorous acid (HOCl). These compounds are relatively stable but they can be precursors for the generation of more reactive species. Most of the ROS may cause serious damages to macromolecules.

Free radicals could be stabilized in several ways:

→ If two radicals meet, they can combine their unpaired electrons and create a covalent bond.

→ If the free radical reacts with a non-radical molecule two cases may arise:

i) The free radical may release its unpaired electron (reducing radical).

ii) The free radical may accept the electron from the other molecule (oxidizing radical).

When the radical gives or accepts the electron from a non-radical molecule, the latter becomes a radical. Thus, the whole process has a character of chain, which is terminated when two radicals meet.

1.1.1. Representatives of reactive species

1.1.1.1. Superoxide anion radical ($O_2^{\bullet -}$)

Superoxide is the one-electron reduction product of dioxygen. It is synthesized by phagocytic cells to inactivate pathogens or it could be involved in inter-cellular signalization processes and grown regulation.[14] It does not belong to highly toxic substances for cells. It is

a weak oxidizing agent but a stronger reducing agent. It can oxidise ascorbic acids or thiols and it can reduce iron complexes such as cytochrome-c or ferric-EDTA. It is also the main precursor for H_2O_2 which is decomposed into $^{\bullet}OH$.[13]

In the organism, there exist several sources for formation of superoxide.

→ The major process is the mitochondrial respiratory chain. In this process, oxygen is reduced to water via several steps. One of the intermediates is $O_2^{\bullet-}$. The whole process is catalysed by the cytochrome oxido-reductase. Dioxygen is bonded inside the HEME-type active site of cytochrome.[15]

$$O_2 + e^- \xrightarrow{\text{Cytochrome oxido-reductase}} O_2^{\bullet-}$$

→ Superoxide can be also directly formed when metal ion is oxidized by molecular oxygen (M^{n+} can be Fe^{2+}, Cu^+ *etc.*).[15]

$$M^{n+} + O_2 \rightarrow M^{(n+1)+} + O_2^{\bullet-}$$

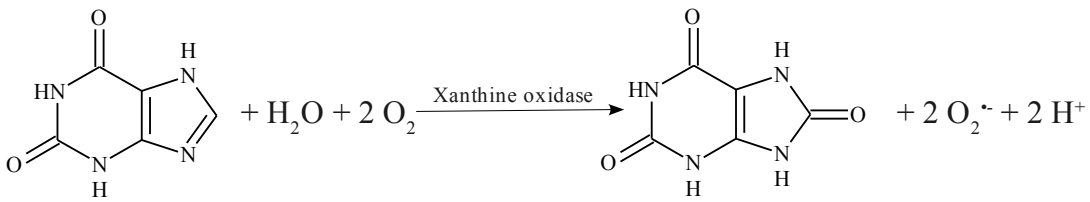
→ Another possible source of this substance is the autoxidation of ubisemiquinone inside the mitochondria membrane.[16]

$$UQ^{\bullet-} + O_2 \rightarrow UQ + O_2^{\bullet-}$$

→ Re-oxidizing process of flavin by molecular oxygen is the forth possibility for $O_2^{\bullet-}$ formation. The process is catalysed by enzymes, *i.e.,* xanthine oxidase.

$$\text{Enz-Flavin-H}_2 + O_2 \xrightarrow{\text{Xanthine oxidase}} \text{Enz-Flavin-H} + O_2^{\bullet-} + H^+$$

→ Xanthine oxidase can also produce $O_2^{\bullet-}$ via the transformation xanthine into uric acid: [17]



1.1.1.2. Hydroxyl radical (•OH)

In our organism, hydroxyl radical is the most aggressive free radical which can attack almost all biological molecules at diffusion controlled rates. This means that it reacts as soon as it meets another molecule. In the organism, there are several sources of •OH:[13]

→ It is a major product arose during radiolysis of water:

$$H_2O \rightarrow {}^{\bullet}OH + H^{\bullet}$$

→ This radical can also be formed during biological processes, for example, it is produced by the decomposition of H_2O_2 via two possible approaches:[18][19]

i) *Fenton reaction:* $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$ (iron may be also replaced by copper)

$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

ii) *Haber – Weiss reaction:* $H_2O_2 + O_2^{\bullet-} \rightarrow {}^{\bullet}OH + HO^- + O_2$

1.1.1.3. Perhydroxyl radical ($HO_2^{\bullet}$)

Perhydroxyl radical is a protonated form of $O_2^{\bullet-}$ and *in vitro* it is more reactive than $O_2^{\bullet-}$. Since the physiological pH is around 7.4, not a lot of $HO_2^{\bullet}$ radicals appear in the organism. Superoxide itself does not enable to initiate lipid peroxidation but $HO_2^{\bullet}$ has this capacity *in vitro*. However, there is no evidence that $HO_2^{\bullet}$ really has this effect in living organisms.[4]

1.1.1.4. Hydrogen peroxide (H_2O_2)

The main source of hydrogen peroxide is dismutation of $O_2^{\bullet-}$ which is catalysed by superoxide dismutase (SOD).

$$O_2^{\bullet-} + 2\,H^+ \xrightarrow{SOD} H_2O_2$$

There are other oxidase enzymes in the organism (*e.g.*, urate oxidase, glucose oxidase, D-aminoacide oxidase) which can produce H_2O_2 directly via transfer of two electrons to dioxygen.

Generally, hydrogen peroxide is a weak oxidant and a weak reducing agent. Its uncharged covalent structure makes it relatively stable. It may oxidise a SH group on glyceraldehyde-3-phosphate dehydrogenase and thus block glycolysis. It is also capable to form highly reactive hydroxyl radicals via metal-catalysed Fenton reaction or Haber-Weiss reaction.[18][19]

i) *Fenton reaction:* $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$ (iron ion can be replaced with copper)

$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

ii) *Haber – Weiss reaction:* $H_2O_2 + O_2^{\bullet-} \rightarrow {}^{\bullet}OH + HO^- + O_2$

Its structure is similar to H_2O. Therefore, it can be easily transported both intracellularly and extracellularly. This allows H_2O_2 to react far from the place where it is formed. Hydrogen peroxide is eliminated from the organism, mainly by the action of catalase or gluthation peroxidase, but other types of peroxidases may be also involved in this process.[13]

1.1.1.5. Singlet dioxygen ($^1O_2$)

Singlet dioxygen is a more reactive form of molecular oxygen. It is characterized by the rearrangement of electrons in the electronic structure of oxygen. Instead of two unpaired electrons which are common for molecular oxygen, the singlet state does not have any unpaired electron. Excitation of O_2 in the organism to singlet state can occur when biological pigments, *e.g.*, retinal, flavin or porphyrins, are illuminated in the presence of O_2.[13]

1.1.1.6. Peroxyl radicals (ROO•), alkoxyl radicals (RO•) and radicals located at carbon (R•)

All these radicals are formed during oxidation of unsaturated fatty acids. This process can be initiated by the attack of a chemical species that has sufficient reactivity to abstract hydrogen from fatty acids (the mechanism is explained later). The greater the number of double bonds on fatty acid chain, the easier the H-atom abstraction, so the formation of radicals.[4]

Metal ions can be also involved in production of peroxyl and alkoxyl radicals:

$$M^{n+} + ROOH \rightarrow ROO^{\bullet} + H^{+} + M^{(n-1)+}$$

$$M^{(n-1)+} + ROOH \rightarrow RO^{\bullet} + HO^{-} + M^{n+}$$

1.1.1.7. Hypochlorous acid (HOCl)

Hypochlorous acid is a strong oxidative agent. It plays a major role in immune defence by neutrophils. Neutrophils contain myeloperoxidase (a HEME-containing enzyme) which catalyses the reaction of H_2O_2 with chloride ions.[5]

$$H_2O_2 + Cl^{-} + H^{+} \xrightarrow{\text{myeloperoxidase}} HOCl + H_2O$$

Hypochlorus acid reacts on a wide range of biomolecules including DNA, proteins or fatty acids. It gives rise the most powerful •OH radical by an iron-independent reaction:

$$HOCl + O_2^{\bullet-} \rightarrow {}^{\bullet}OH + Cl^{-} + O_2$$

In the presence of iron ion, HOCl may be decomposed into •OH and chloride:

$$HOCl + Fe^{2+} \rightarrow {}^{\bullet}OH + Cl^{-} + Fe^{3+}$$

1.1.1.8. Oxide of nitrogen (NO•)

Oxide of nitrogen (NO•) is a relatively stable radical. It is synthesized by the family of nitric oxide synthases (NOS) from amino acid L-arginine.

$$\text{L-arginine} + O_2 \xrightarrow{\text{NOS}} NO^{\bullet} + \text{L-citrulin}$$

It plays an important role in living organisms, *i.e.,* vasodilator agent, neurotransmitter or it can be involved in defence against pathogens. Its excess is toxic for tissues. It fast reacts together with superoxide to form peroxynitrite ($ONOO^-$).

$$NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^-$$

Peroxynitrite is a powerful oxidant which can oxidise thiols, initiate lipid peroxidation or cleave DNA.[20]

1.1.1.9. Thiyl radical ($RS^{\bullet}$)

Thiyl radical is the general name for a group of radicals with one unpaired electron located on sulphur. They are formed when thiols, which are abundant in living organisms, are oxidized by enzyme or other ROS.[21]

$$R_1^{\bullet} + R_2SH \rightarrow R_1H + R_2S^{\bullet}$$

The reductive ability of thiols (mainly gluthation) and their presence in cell make them effective antioxidants *in vivo* or *in vitro*. They can also combine with O_2 to generate oxo-sulphur radicals ($RSO_2^{\bullet}$ or $RSO^{\bullet}$) which may damage biological tissues.[21][22]

1.2. Toxic effects of ROS on biomolecules

1.2.1. Membrane and lipid peroxidation

Membranes are highly viscous liquids which play important role in cell life. They form the boundaries of cells. Many processes occur through membranes, *e.g.*, signal transfers between cells and exchanges of ions and other molecules from (to) the extra- to (from) the intracellular domains.

Mammalian membranes consist mainly of lipids (phospholipids, cholesterols, glycolipids), then proteins and other smaller hydrocarbon compounds.[23][24][25] Phospholipids constitute the major part of the mass of membrane. In membranes, there are four major phospholipid types:

→ phosphatidylcholine

→ phosphatidylethanolamine

→ phosphatidylserine

→ sphingomyelin

Other types of phospholipids are also present in small quantities. Even if their concentration is not so high, they influence the membrane properties and they play an important role in its function.[26]

A phospholipid unit consists of one polar head group and two hydrophobic hydrocarbon tails (fatty acids) (Fig. 1.1). This structure gives them amphipathic character. Due to amphipathic character, the individual phospholipids are organized into bilayers (Fig. 1.1), the hydrophobic chains are oriented "head to head" to form the inside part of membrane. The hydrophilic moieties are located in outside part of the bilayer making internal and external surfaces. Thus, lipid bilayers are thermodynamically stabilized by hydrophobic effect.

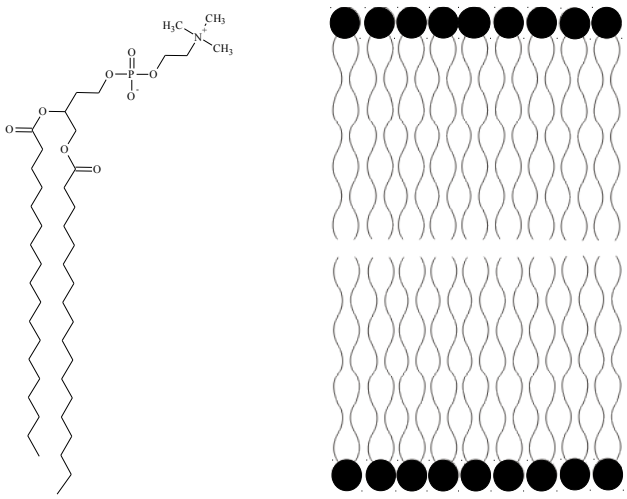


Figure 1.1: *Example of phospholipid (phosphatidylcholine) and the structure of membrane*

Membranes may differ from each other by their composition (*e.g.*, type and concentration of the individual proteins which they contain, asymmetry in phospholipid composition, presence of cholesterol). Composition, temperature, length and degree of unsaturation of fatty acids influence the fluidity of membranes. Unsaturated lipid chains disfavour membrane packing, which indirectly induces a higher fluidity.[27]

Changes in membranes can cause perturbations in their structure, which can affect intracellular processes. Some of such changes are attributed to reactions of reactive species with lipid chains. This reaction is known as lipid peroxidation.

1.2.1.1. Lipid peroxidation

Lipid peroxidation is an oxidative process which has a chain character. As all radical chain reactions, the whole process is divided into three steps – initiation, propagation and termination.[4][28]

(1) Initiation of lipid peroxidation

Lipid peroxidation is initiated by the attack of the reactive species which have the capacity to abstract H-atom from fatty acid chains. The $^{\bullet}OH$ radical is the most probable initiator in the living cells. The most sensitive positions of the polyunsaturated fatty acids for HAT are the methylene groups close to double bonds, which help the relative stabilization of the subsequent carbon-centred radical ($R^{\bullet}$) by π-electron conjugation.[4][5]

$$R\text{-}H + {}^{\bullet}OH \rightarrow R^{\bullet} + H_2O$$

(2) Propagation of lipid peroxidation

Because of the instability of carbon-centred radicals, the initiation step is followed by the fast reaction with dioxygen molecules. During this reaction, a peroxyl radical of fatty acid ($ROO^{\bullet}$) is formed.

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$

$ROO^{\bullet}$ may react by HAT with adjacent fatty acids to produce lipid peroxides and new carbon-centred radicals.

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$$

The cycle is repeated until termination. The number of cycles depends on many factors (*e.g.* concentration of oxygen, type of fatty acid).

(3) Termination of lipid peroxidation

Termination occurs when two radicals react together. Lipid peroxidation can be terminated by several ways:

→ two peroxyl radicals meet: $2\ ROO^{\bullet} \rightarrow ROOR + O_2$

→ two carbon-centred radicals meet: $2\ R^{\bullet} \rightarrow R\text{-}R$

→ peroxyl radical and carbon-centred radical meet: $ROO^{\bullet} + R^{\bullet} \rightarrow ROOR$

These three possibilities can occur if the concentration of radicals is high enough to meet. To protect against membrane damage, the membranes include different chain-breaking molecules (antioxidants (A)) which also interrupt the chain reaction by providing a H-atom.

$$ROO^{\bullet} + AH \rightarrow A^{\bullet} + ROOH$$

The radical derived from the antioxidant is not sufficiently reactive to attack new fatty acids and thus the chain reaction is terminated.

1.2.2. Proteins and their damage by ROS

Proteins are other basic structural component of all living organisms. They belong to biopolymers which are made of amino acids. Individual amino acids are connected by the peptide bond (-NH-CO-). Even if there exist several hundreds of naturally occurring aminoacids, only 20 L-α-aminoacids are structural units for proteins. They are quoted by one-letter of three-letter symbols.

Except of their structural function, they also catalyse nearly all biological pathways in the organism. In case of specific changes in their structure, enzymes can lose this catalytic function. Over the past years, it has been proved that the reactive species may lead to serious changes which inhibit their function.[6]

ROS can directly attack the polypeptide backbone to induce oxidation processes (Fig. 1.2). In the presence of molecular dioxygen, peroxides or hydroxyl derivatives are formed. If dioxygen is absent, two carbon-centred radicals may react together to form protein–protein cross-linked derivatives.[6]

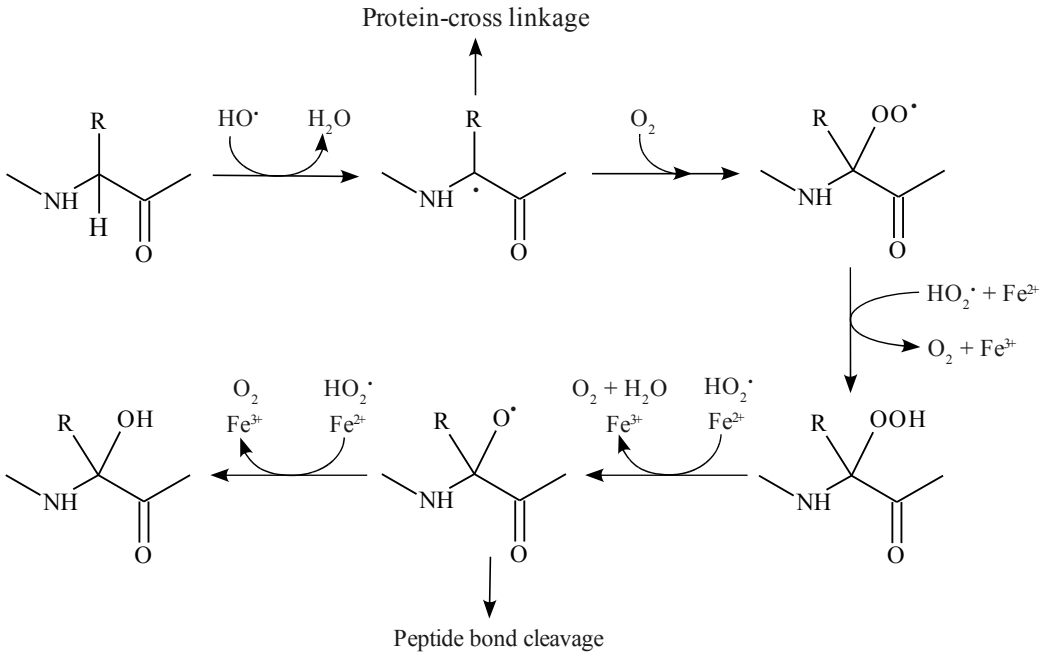


Figure 1.2: *Oxygen free radical-mediated oxidation damage of proteins*

Alkoxyl radicals may cleave peptide bonds by either the diamide or the α-amidation pathways. In the former mechanism, CO_2, NH_3 and carboxylic acid are formed. The latter pathway yields NH_3 and free α-ketocarboxylic acid. Cleavage of peptide bond can also occur by ROS attack on glutamyl, aspartyl and prolyl side chains.

All amino acids are sensitive to the direct oxidation by ROS. Probably the most sensitive are residues containing sulphur as cysteine and methionine. Cysteines are converted to disulfides and methionines are converted to methionine sulfoxide. These changes can be easily repaired because cyclic oxidation-reduction of sulphur residues is one of antioxidant mechanism. The other amino acids, *e.g.,* with aromatic rings (Phe and Tyr) are also sensitive to ROS attacks. The possible oxidation products are summarized in Table 1.1.

Table 1.1: Possible oxidation changes in structure of amino acids.

Amino acid	Products of oxidation
Trp	Hydroxytryptophans, nitrotryptophan, kynurenins ...
Phe	Hydroxyphenylalanines ...
Tyr	Dihydroxyphenylalanine, tyr-tyr cross-linkage, tyr-O-tyr cross-linkage ...
His	Oxohistidine, asparagine, aspartic acid
Arg	Glutamic semialdehyde
Lys	Aminoadipic semialdehde
Pro	Pyrrolidone, hydroxyproline, glutamic semialdehyde ...
Glu	Oxalic acid, pyruvic acid

The aldehydes produced during the lipid peroxidation can react with proteins residues and thus change their structure. This is not a direct reaction of ROS, but it can also cause serious damage of proteins.[29]

1.2.3. DNA

DNA (deoxyribonucleic acid) is a biological macromolecule bearing the genetic information in all living organisms. In eukaryotic cells, DNA is located inside cell nuclei. It codes all information concerning the function and structure of the organism. The DNA structure is composed by nucleotidic units (base + sugar + phosphate) arranged as a double helix. The double helix arrangement is formed by the characteristic pairs of bases which are connected either by two (adenine-thymine) or three (guanine-cytosine) H-bonds.[27] Due to its important role, DNA is strongly protected against changes which can cause serious change in the function of cells. Damages in structure may affect the right transcription of DNA and thus cause mutagenic changes. The reactions which contribute to DNA damage are oxidation, methylation, depurination or deamination.[2][3][30]

Generally, the reactive species are the most possible initiators for DNA changes. They cause mutation in base structure, deletion or insertion of chemical groups. The different reactive species have different effects on DNA structure. The most serious damage is caused by hydroxyl radical which can change all bases in DNA structure. Singlet oxygen reacts only with guanine and superoxide radical or hydrogen peroxide do not react.[2][3]

Other serious damages can be caused by condensation of aldehydes with adenine, guanine or cytosine. The most common is malondialdehyde, which is one of the major products of lipid peroxidation.[30]

As mentioned in the beginning, most of changes can have serious consequence. Therefore, the cells have sophisticated mechanism how repair such changes.[2][31]

1.3. Strategy of antioxidant defence

Antioxidant was defined by Gutteridge as the substance which, at a small concentration compared with the oxidizable substrate, considerably delays or inhibits the oxidation of the substrate.[15] It acts according to different mechanisms and at different stages:

→ to remove oxygen or to decrease local oxygen concentration

→ to remove metal ions catalysing ROS formation

→ to remove ROS (*e.g.*, $O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$)

→ to break the radical chain reaction

→ to regenerate endogenous antioxidants

→ to inhibit pro-oxidant enzymes

The defence mechanism of antioxidants involves three basic levels of protection – prevention, interception and reparation.

1.3.1. Prevention

In the organism, free metal ions, particularly iron and copper ions, catalyse the reactions that produce ROS (*e.g.*, Fenton reaction). Metal chelation would prevent their formation.

Proteins (*e.g.*, ferritin and transferrin) or enzymes (*e.g.*, gluthathione S-transferases) may regulate the production of ROS. Their activation or inactivation may prevent ROS overproduction.[1][32]

1.3.2. Interception

Beyond prevention, all organisms have also developed a system of efficient defence which directly eliminates the formed reactive species. This system is composed of enzymatic and non-enzymatic antioxidants.[1][32]

1.3.2.1. Enzymatic antioxidants

Three major enzyme groups participate in defence:

→ Superoxide dismutase (SOD)

→ Catalase

→ Gluthathione peroxidase (GPx)

Superoxide dismutase (SOD)

This enzyme is responsible for the removal of superoxide anion radical ($O_2^{\bullet -}$) by dismutation reaction to form hydrogen peroxide and dioxygen. It occurs in mitochondria as well as in cytosol. During one cycle, two superoxides are consumed.

$$2\ O_2^{\bullet -} + 2\ H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

There are two types of SOD enzymes, *i.e.* SOD enzyme containing zinc together with copper or containing manganese ions in their active site. The first one is mainly located in cytosol and the second one in mitochondria. The formed H_2O_2 is not as toxic as $O_2^{\bullet -}$, but it is a precursor of the formation of hydroxyl radicals. Therefore, the SOD system is followed by other mechanisms which suppress H_2O_2.

Catalase

Catalase is one of the enzymes which decomposes H_2O_2 to dioxygen and water. Its active site contains a HEME unit.

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} O_2 + 2\ H_2O$$

The function of this enzyme is limited by its low concentration in extracellular fluids. Therefore, the major role in the H_2O_2 elimination is played by gluthathione peroxidase.

Gluthathione peroxidase (GPx)

Gluthathione peroxidase is formed by four identical units, each of them containing one selenium atom in the active site. It catalyses the reaction of H_2O_2 with gluthathione (GSH) which is converted into gluthathion-disulfure. Gluthathione is a tripeptide containing cysteine.

$$2\ GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2\ H_2O$$

H_2O_2 is not only the substrate of peroxidase. It also converts lipid peroxides formed during lipid peroxidation to their alcohol form.

$$2\ GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$$

After the reaction, the gluthathion-disulfure has to be reversed back into its oxidized form. This function is catalysed by gluthathione reductase which requires cofactor, NADPH.

1.3.2.2. Non-enzymatic antioxidants

Antioxidants may *i)* intercept free radicals or *ii)* transfer free radicals from a sensitive to a less deleterious region. The most effective antioxidants combine both features. They first scavenge free radicals and then they are regenerated by interaction with water-soluble compounds which displace the free radicals away. The final product, after the deactivation process, is a non-radical molecule.

α-tocopherol (vitamin E)

Within the hydrophobic lipid membrane, the most effective antioxidant is α-tocopherol (vitamin E). α-tocopherol is a fat-soluble antioxidant which scavenges the radicals produced during the lipid peroxidation. Its efficiency is ascribed to its structure (Fig. 1.3). Two important characteristics help to understand its capacity to scavenge free radicals *i)* an OH group with an effective HAT capacity and *ii)* the capacity to incorporate the membrane with the hydrophobic chain acting as an anchor.[33]

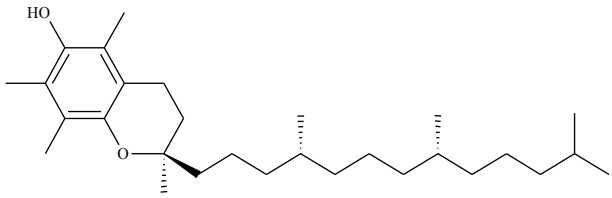


Figure 1.3: *Structure of α-tocopherol*

Carotenoids

Carotenoids are natural plant pigments. The chemical structure is a long unsaturated chain which connects two non-aromatic rings. Depending on the structure of these rings they are classified into several classes. The most famous form is β-carotene (Fig. 1.4). As liposoluble structures, they contribute to the protection of lipid membranes. They are very effective against singlet oxygen but their exact role in inhibition of the lipid peroxidation has not been very well described, yet.

They scavenge free radicals by another way than HAT from hydroxyl groups. The H-atom is abstracted from -CH_2- adjacent to double bonds and a carbon-centred radical is formed. The delocalization via the system of double bonds stabilizes this formed radical.[34]

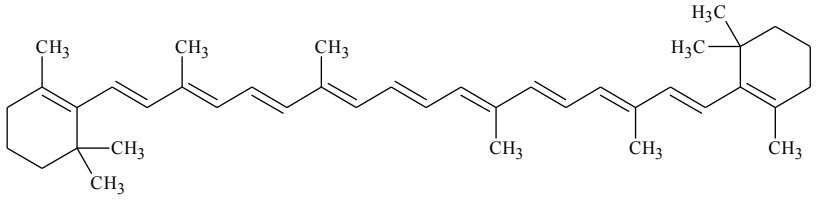


Figure 1.4: *Structure of β-carotene*

Ascorbic acid (vitamin C)

Ascorbic acid is another antioxidant agent in the organism. It is water-soluble and efficiently reacts with all types of reactive species (oxygen-, nitrogen- or sulphur-centred

radicals). The results of different studies show that ascorbic acid acts as a primary defence in blood. Due to its water-solubility, ascorbic acid may not suppress lipid peroxidation directly, but there exist evidences that it may reduce tocopherol radicals. Thus, this synergic reaction increases efficiency of tocopherol inside membrane.[35]

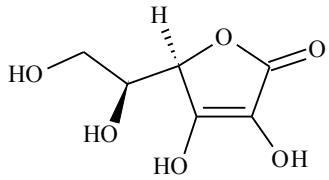


Figure 1.5: *Structure of ascorbic acid (vitamin C)*

Gluthatione (GSH)

Gluthatione is a tripeptide present in all mammalian cells where it plays many important roles (Fig. 1.6). It stores and transfers cysteine, it is important for cell proliferation and it is involved in cellular defence against ROS. GSH scavenges ROS *i)* non-enzymatically or *ii)* by electron transfer to reduce H_2O_2 in the reaction catalysed by gluthatione peroxidases. During both reactions, GSH is oxidized into its disulphide form (GSSG), but it can be regenerated from disulphide by gluthatione reductase. Gluthatione is also able to regenerate α-tocopherol. [36]

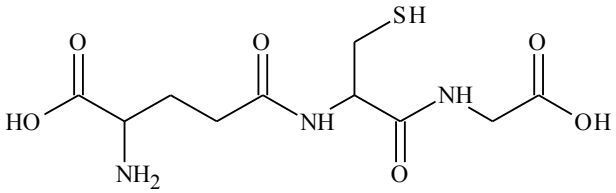


Figure 1.6: *Structure of gluthatione*

Note: The list of antioxidant compounds has not been completed. Other compounds may also act as antioxidants (*e.g.*, uric acid, bilirubin or polyphenols).

1.3.3. Reparation

When prevention and interception fail oxidative products are formed and accumulate in tissues. The cells possess enzymatic systems which can repair or eliminate such damages *i)* DNA repair, *ii)* degradation of the damaged proteins or *iii)* metabolization of lipid hydroperoxides formed during lipid peroxidation.[1][32]

1.4. Polyphenolic compounds

Polyphenolic compounds represent a big group of natural molecules. They are the most important group of secondary metabolites produced by plants. Secondary metabolites are specific to individual sort of plants and have different functions in their tissues. They are *i)* signalling molecules, *ii)* pigments&flavours to attract or repel animals, *iii)* protectors against viruses, bacteria, insects *etc.*

1.4.1. Chemical properties of phenolic compounds

Polyphenols are characterized by the presence of at least one benzene ring bearing at least one hydroxyl group (OH). Many properties of phenols are similar to those of alcohols but phenolic OH groups are influenced by the presence and character of the aromatic ring. The interaction between the OH groups and the aromatic ring affects their reactivity.

1.4.1.1. Acidity of phenolic compounds

Due to the presence of the aromatic ring, phenolic compounds belongs to weak acids. But depending on external conditions and chemical environment, the hydroxyl group located at aromatic ring may also have basic character. The hydroxyl proton in phenol structures is more acidic than hydroxyl proton located at aliphatic alcohols. Due to the presence of the

aromatic ring, the anion formed after proton release is delocalized over the benzene ring (Fig. 1.7). The acidity is also influenced by other substituents bonded to the ring.[37]

The acido-basic character of phenolic compounds is used for their separation.

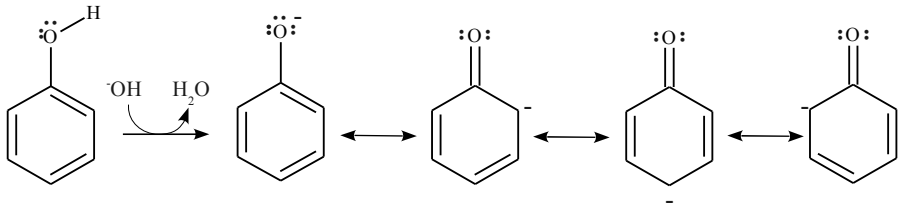


Figure 1.7: *Mesomeric structures of phenol after abstraction of proton*

1.4.1.2. Hydrogen bonds

The hydrogen bond is a weak interaction formed between H-atoms attached to electronegative element (O, N, …) and the free electron-pair of another strong electronegative atom (O, N, …). Even if it is a weak interaction, it strongly influences geometries and the physical properties of molecules.

Physical properties of phenols are influenced by the presence of OH groups which allow the formation of the intra and inter H-bonds (Fig. 1.8). H-bonds increase the melting point and the boiling point of phenols and decreases their solubility. It is also more difficult to purify them. Except of intermolecular H-bonds, phenolic compounds which contains *ortho*-substituted OH groups or oxo-group in *ortho*-arrangement with OH group may create intramolecular H-bonds (Fig. 1.8).

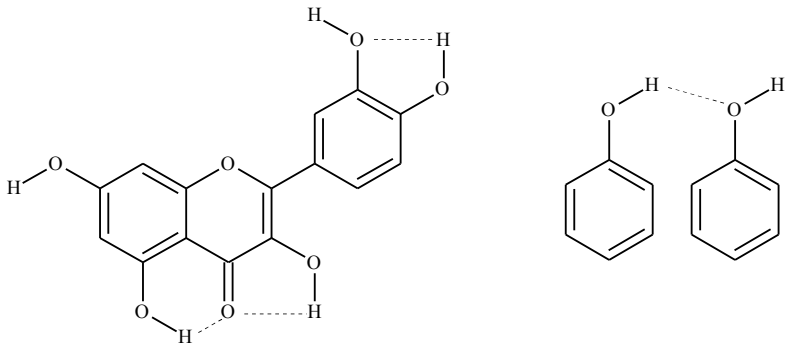


Figure 1.8: *Intramolecular H-bonds in quercetin and intermolecular H-bonds between molecules of phenol*

1.4.1.3. Ethers and glycosides of polyphenols

Both forms - ethers and glycosides - are widely distributed in nature. Ethers are produced by reaction between phenolic and alcoholic hydroxyl groups. The most common are ethers derived from methanol where the methoxy group is stable (Fig. 1.9).

In glycosides (Fig. 1.9), the sugar units are in ring-conformation (pyranose or furanose forms). The type of sugar influences the solubility of phenols and thus can help their absorption during digestion. In acidic conditions, the sugar part is easily hydrolysed.

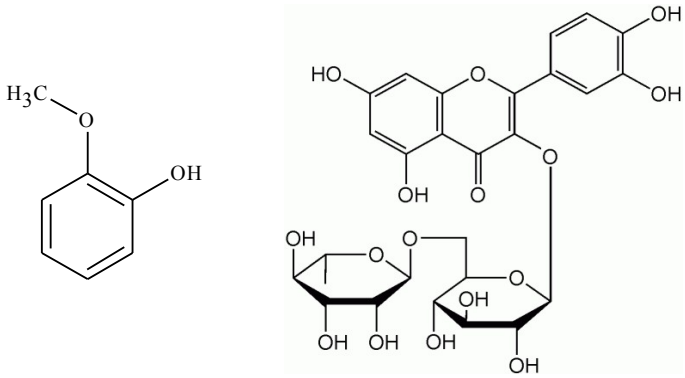


Figure 1.9: *Example of the ether and glycoside structure of polyphenolic compounds (the structures of guaiacol and rutin, respectively)*

1.4.1.4. Oxidation of phenols

The oxidation of phenols is the most important property and run more easily than with alcohols. This reaction is common in nature. One of the possible consequences is browning of tissues. During oxidative processes, a wide range of metabolites is formed. Some of them may be toxic for animals and plants but, on another hand, they may protect them from attacks of pathogenic micro-organisms. The capacity to oxidise natural polyphenols explains their capacity to scavenge free radicals and thus to stop oxidative damage.[38][39]

In vivo or *in vitro* oxidation could be initiated by enzymes (oxidoreductases, peroxidases, monophenol monooxygenases) or by one-electron abstracting agents (*e.g.,* ferric chloride, silver oxide, ferricyanide or free radicals). During the process, one electron together with one proton is transferred and the phenol radical is generated. This radical is stabilized by delocalization which can be described by several mesomeric structures (Fig. 1.10). The formed radical may be combined with other radicals to form dimers, oligomers *etc.* as in the case of catechols. Many biologically important compounds are synthesized by this mechanism in nature, for example lignins.[40] Other possible products are quinones which are also widely distributed in nature.[41]

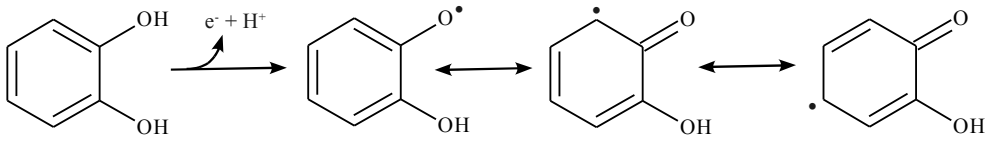


Figure 1.10: *Auto-oxidation of catechol*

1.4.2. Biosynthesis of phenolic compounds

Plant metabolism yields primary and secondary metabolites. The first group is necessary for living organisms, *e.g.*, essential amino acids, DNA, RNA, fats, carbohydrates. Secondary

metabolites are characteristic for classes of the organisms and specific enzymes are required for their biosynthesis. A wide range of secondary metabolites is produced by plants and micro-organisms. This huge number of compounds has gained the attention of chemists and biologists, allowing endless fields of research. Polyphenols belong to secondary metabolites formed by two possible pathways (acetate and Shikimate pathways).

The initial substrates for both pathways come from glycolysis or penthose phosphate metabolism.

1.4.2.1. Biosynthesis of phenolic compounds from acetate

More than one century ago, Collie[42] prepared aromatic compounds from polyacetic acid. This acid was formed by the 'head-to-tail' condensation (Claisen condensation) of the acetate units. Structural analysis of many natural polyphenols – (*e.g.*, orcinol and phloroglucinol) demonstrated that this mechanism also originates certain polyphenols.

The acetate is firstly activated by Coenzyme A. The formed acetyl-CoA is the precursor for several natural compounds *i.e.*, terpenes, fatty acids or polyphenolic compounds. To generate polyphenols, the several activated acetates are joined by Claisen condensation to form polyacetic acid, from which polyphenols are produced:

→ by Aldol condensation – phenolic acids

→ by Claisen condensation – acylphenols

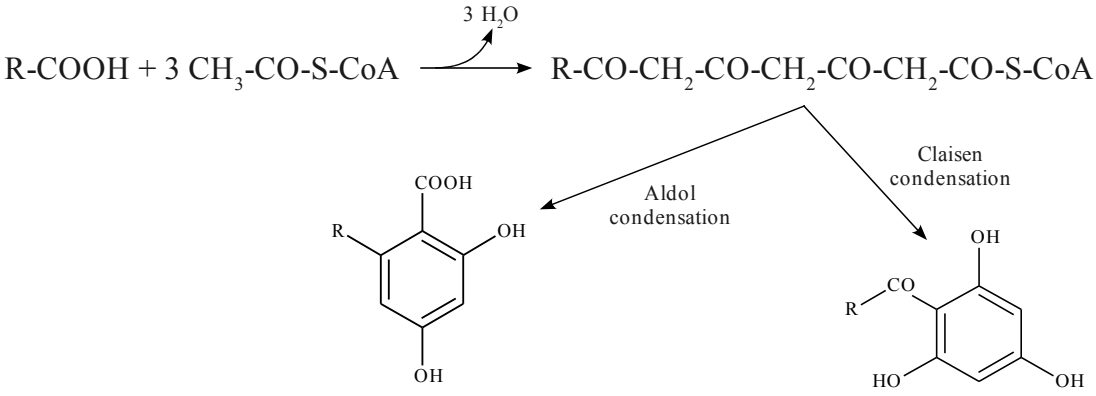


Figure 1.11: *Biosynthesis of phenolic compounds form acetate*

1.4.2.2. Biosynthesis of phenolic compounds by Shikimate pathway

The Shikimate pathway is the mechanism by which micro-organisms and plants prepare precursors for the essential aromatic amino acids – phenylalanine, tyrosine and tryptophan. This precursor is chorismate. The initial substrates of the Shikimate pathway are:

→ ***Phosphoenol-pyruvate*** which is one of intermediates from glycolysis

→ ***Erythrose-4-phosphate*** which comes from the penthose phosphate pathway

Chorismate synthesis is divided into seven steps which are catalysed by special enzymes. In the first step, phosphoenol-pyruvate and erythrose-4-phosphate are condensed into the saturated oxygenated heterocycle. The oxygen atom in heterocycle is exchanged by carbon in the second step. During the remaining five steps, two double bonds are inserted into the cyclohexane ring, which gives rise to chorismate.[43]

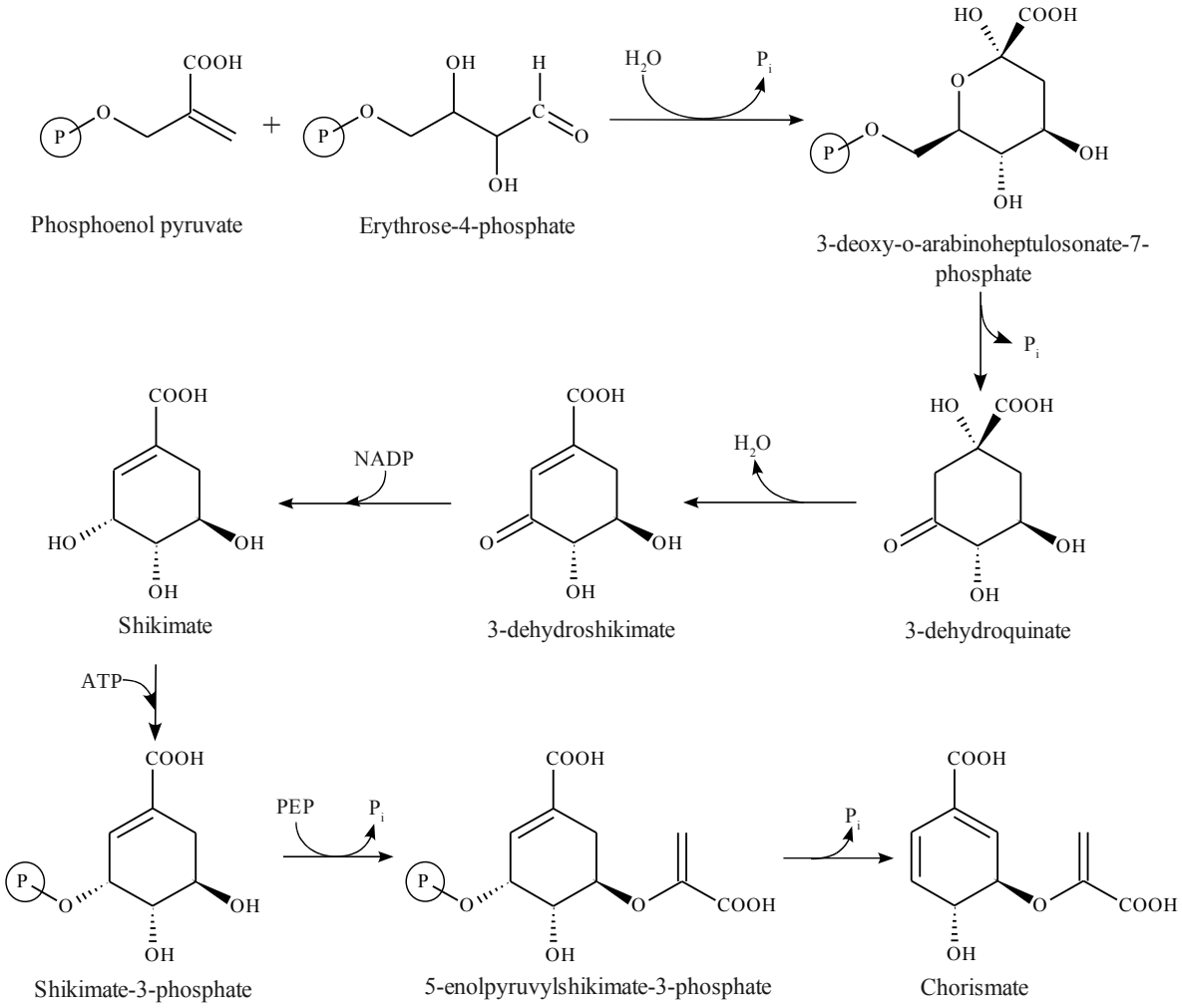


Figure 1.12: *Shikimate pathway (each step is catalysed by a specific enzyme)*

Chorismate is the substrate for at least five metabolic pathways which result in primary metabolites *i.e.*, *p*–hydroxylbenzoate, *p*–aminobenzoate and three aromatic amino acids (phenylalanine, tyrosine, tryptophan). Beyond their function as a structural units of proteins, the aromatic amino acids are the precursors for a large spectrum of the secondary metabolites including flavonoids and alkaloids.[44][45]

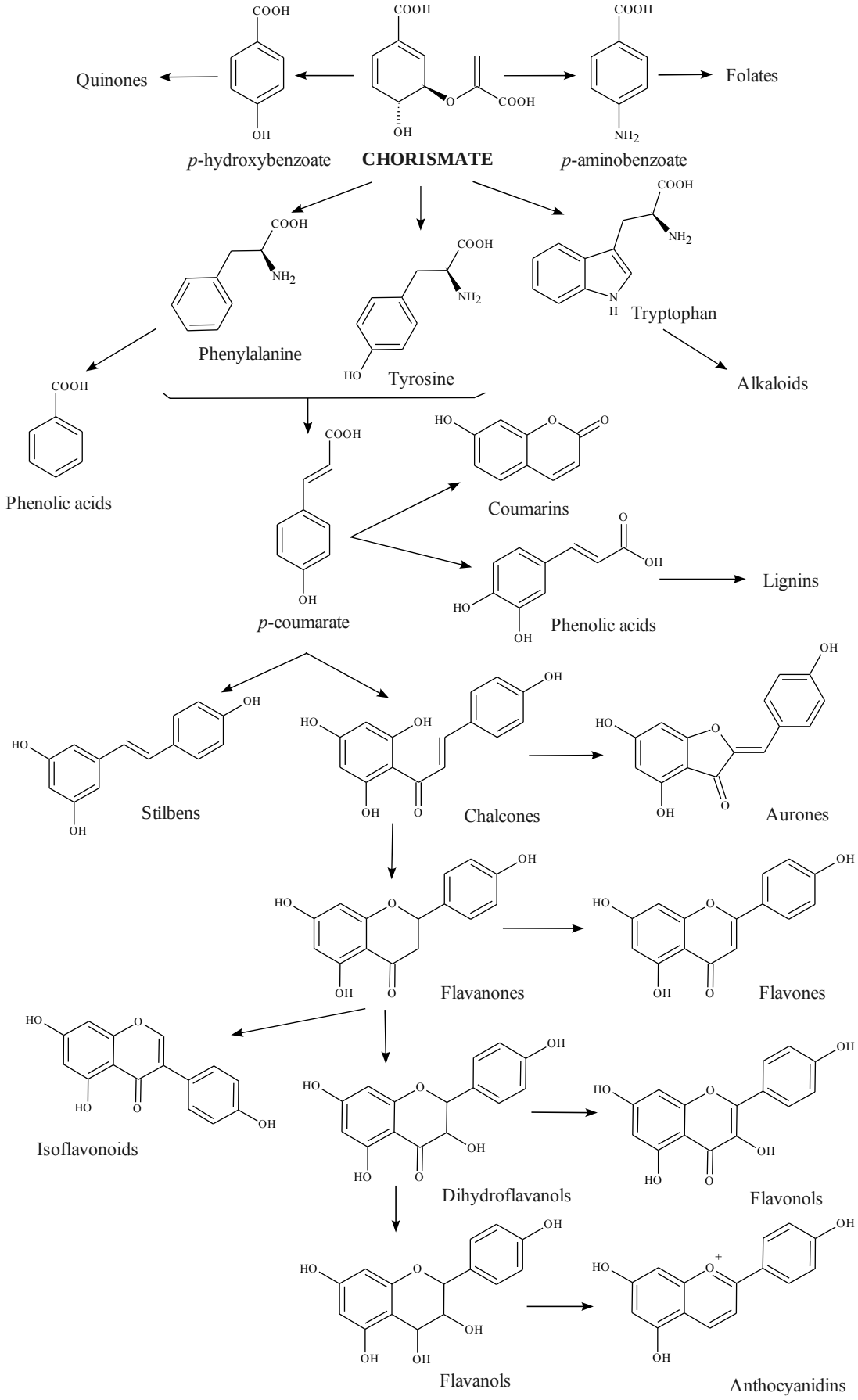
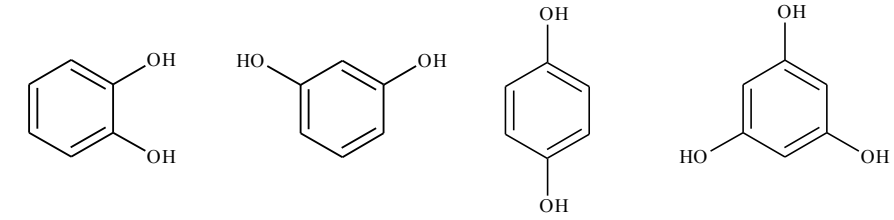


Figure 1.13: *Biosynthesis of different polyphenolic compounds*

1.4.3. Classification of polyphenols

1.4.3.1. Simple phenols

Simple phenols are built-up from one aromatic ring on which at least one OH group is bonded. The substituents may be in *ortho*- (1,2), *meta*- (1,3) or *para*-position (1,4). Natural simple phenols (Fig. 1.14) may be free or as glycosides or ether forms (methylethers).



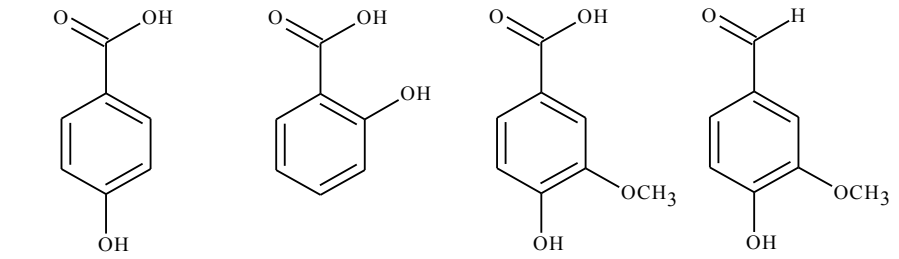
Benzen-1,2-diol (Catechol) | Benzen-1,3-diol (Resorcinol) | Benzen-1,4-diol (Hydroquinone) | Benzen-1,3,5-triol (phloroglucinol)

Figure 1.14: *Examples of simple phenols*

1.4.3.2. Phenolic acids

The phenolic acid class is divided into two subclasses. The first is derived from hydroxy-benzoic acid in which structure carboxyl group is directly bonded to the aromatic ring (Fig. 1.15).

They occur in free or in ester forms. They can be reduced to aldehydes or alcohols. Because of their properties, they have a wide range of applications. Salicylic acid is related to acetylsalicylic acid which is the active compound of aspirin. It is known for its antipyretic, analgetic and anti-inflammatory effects. Acetylsalicylic acid could be prepared from salicylic acid by the esterification of the OH group. Another compound widely used in food and cosmetic industries is vanillic acid. Its oxidized form is known as vanillin which is used instead of the natural vanilla extract.

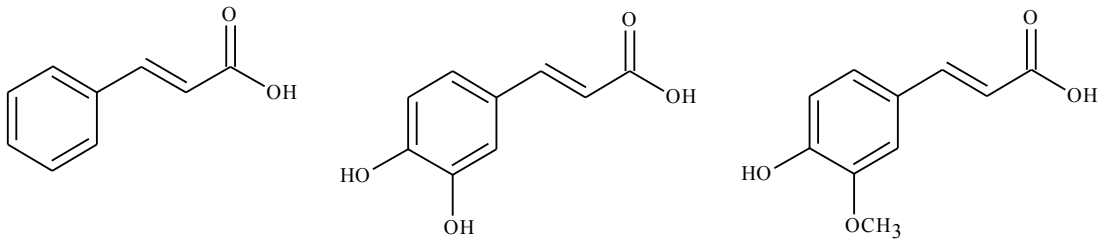


p-hydroxybenzoic acid | Salicylic acid | Vanillic acid | Vanillin

Figure 1.15: *Phenolic acids derived from hydroxybenzoic acid*

The second subclass is derived from cinnamic acid (Fig. 1.16). This group is more common than the previous one. It consists of cinnamic acid, caffeic acid, ferulic acid, sinapic acid and *etc.* The compounds are usually found as esters of quinic acid, shikimic acid or tartaric acid.

The properties of these compounds are also different. Caffeic acid is the main monomer for the synthesis of lignin. It is also the precursor for the synthesis of other acids (*e.g.,* ferulic acid and sinapic acid).[46] The dimers of ferulic acids form bridges between chains of hemicellulose.[47][48] Moreover, all compounds show anti-inflammatory, anti-cancer and other effects.



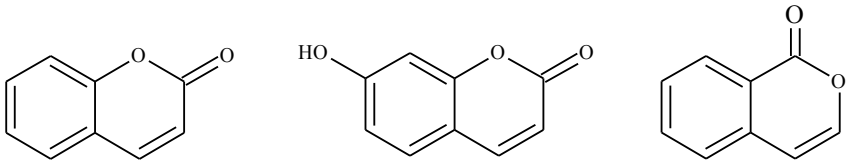
Cinnamic acid | Caffeic acid | Ferulic acid

Figure 1.16: *Phenolic acid derived from cinnamic acid*

1.4.3.3. Coumarins and isocoumarins

The structure of coumarins is derived from 1,2-benzopyrone. Their properties depend on substituents. The most notable physical property of most of natural coumarins is photosensitivity. Coumarin itself or 7-hydroxycoumarin (Fig. 1.17) are the most studied representatives of this group.[49]

Isocoumarins are structurally similar to coumarins (Fig. 1.17). Their structures are distinguished by the position of the hetero-oxygen and the carbonyl group. The most studied isocoumarin is phyroalexin which possesses antibiotic properties.



Coumarin 7-hydroxycoumarin Isocoumarin

Figure 1.17: *Structures of coumarins and isocoumarin*

1.4.3.4. Lignans

Lignans are dimers or oligomers of *p*-coumaryl alcohol, sinapyl alcohol or coniferyl alcohol (Fig. 1.18). The structures containing coniferyl alcohol are the most common in nature. They are generated by radical recombination after H-atom transfer from the hydroxyl group. The radical formed is delocalized over the conjugated structure. According to the type of bonds, they are divided into five major subgroups:[50][51][52]

→ *Lignans* are linked by the 8 – 8' bond.

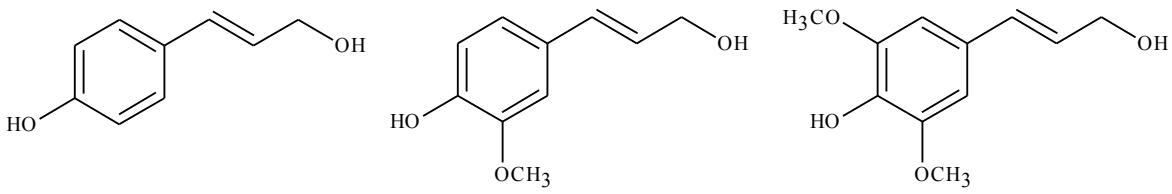
→ *Neolignans* are linked by other than the 8 – 8' linkage.

→ *Oxoneolignans* have units connected by the oxygen atom.

→ *Oligolignans*

→ *Norlignans* have a diphenylpentane carbon skeletons

Most lignans are optically active. They are found in wood and seeds which serve as insect deterrents and fungi prevention. They also have physiological properties, for example phytoestrogen activity and tumour inhibition.[50]



p-coumaryl alcohol Coniferyl alcohol Sinapyl alcohol

Figure 1.18: *The structural units of lignans*

1.4.3.5. Flavonoids

Flavonoids are the most widely distributed polyphenolic compounds. More than 4,000 different compounds have been identified. As secondary metabolites of plants, they exhibit a wide range of effects, *e.g.*, protection of *(i)* plant tissues against UV radiation and the consequent oxidative damage and *(ii)* against attacks by pathogens, insects or fungal parasites. [53] They are also responsible for the characteristic red, purple or blue colours of flowers, fruit and vegetables. They got attention for many beneficial effects on human health, for example their anti-cancer, anti-inflammation, antibacterial, hepatoprotective effects *etc.* These activities are often correlated to their capacity to scavenge free radicals.

Their structure is derived from phenylchroman (Fig. 1.19) containing a six-member heterocycle (C-ring). The compounds are formed by the condensation of three malonyl-CoA (A-ring) and *para*-coumaroyl-CoA.[43][45] They are divided into several subgroups according to the presence or absence of the 2,3 double bond and substitution on the C-ring.

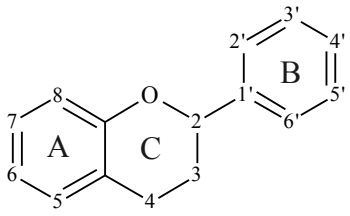
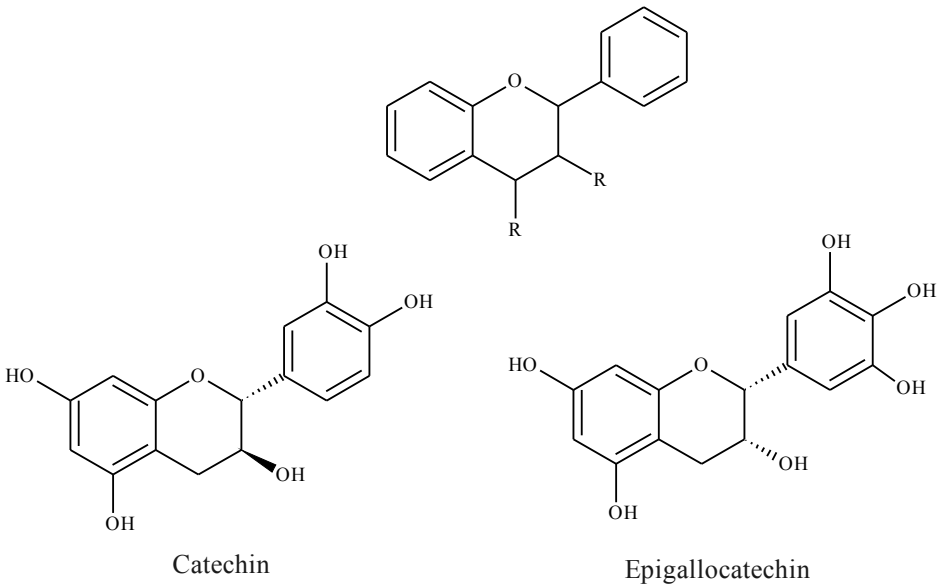


Figure 1.19: *2-phenylchroman - the structural pattern from which all flavanoids are derived*

The members of the individual subgroups have different substituents. The substitution by the hydroxyl group is the most common at positions C5, C7, C4' and C3'. In some cases, methyl ether are substituted instead of the OH group. Most of the natural compounds exist in plants as glycosides with glucose, rhamnose, galactose or arabinose as the sugar moiety. Some structures can also be found as esters of gallic acid or ferulic acid *etc.* Many studies focusing on their biological activities have been published.[54]

Flavan-3-ols, flavan-4-ols and flavan-3,4-diols

Flavanols are compounds with a saturated C-ring. Depending on type of flavanols, C3, C4 or both are substituted by an OH group (Fig. 1.20). The structure contains two asymmetric carbons which cause their optical activity. The most common compounds from this group are catechins or gallocatechins (Fig. 1.20) which are found, for example, in green tea. They are structural units for condensed tannins.



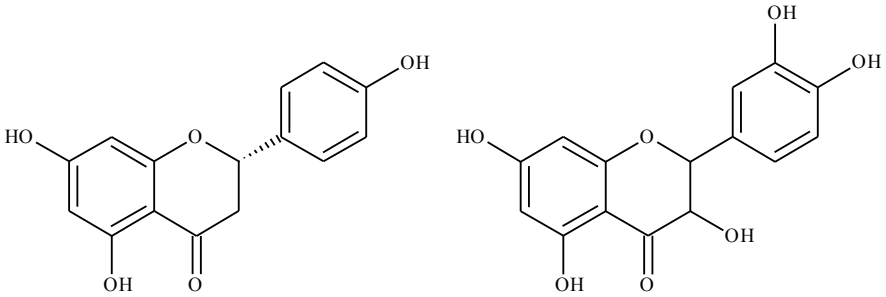
Catechin Epigallocatechin

Figure 1.20: *Basic structural pattern of flavanols (R= OH or H) and the most famous components of this group*

Flavanones and dihydroflavonols

The flavanone heterocycle is saturated with a carbonyl group bonded at C4. Their structure has also one and two asymmetric carbons for flavanones and dihydroflavonols, respectively. In nature, they are present as glycosides and aglycones. The most known flavanones are isolated from citrus fruit, for example naringetin (Fig. 1.21).[53]

Dihydroflavonols are characterized by the presence of the 3OH. The most studied representatives is taxifolin which is also known as dihydroquercetin (Fig. 1.21).



Naringetin Taxifolin

Figure 1.21: *Structures of naringetin (flavanone) and taxifolin (flavanonol)*

Flavones and flavonols

Flavones contain a carbonyl group at C4 and 2,3-double bond. This structure facilitates electron delocalization (Fig. 1.22).

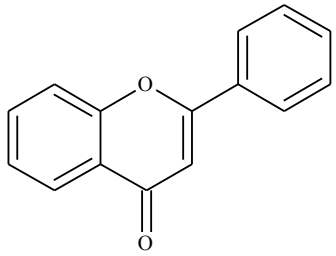


Figure 1.22: *Basic structure of flavones*

Flavonols are flavones with 3OH group. This subgroup contains many important compounds which are widely studied for their beneficial effects. The most widely distributed representatives are quercetin, kaempferol or myricetin (Fig. 1.23). Because of their structure, they are very strong antioxidants. Quercetin is the subject of many studies concerning its biological effects.[55] It is, together with its glycosides, a predominant flavonoid found in the human diet.

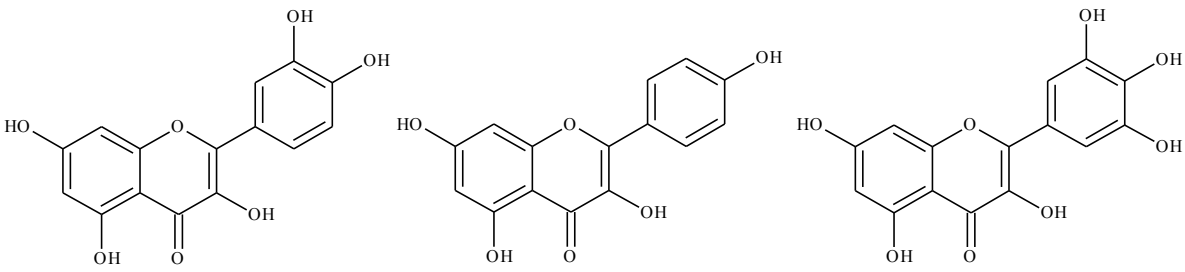
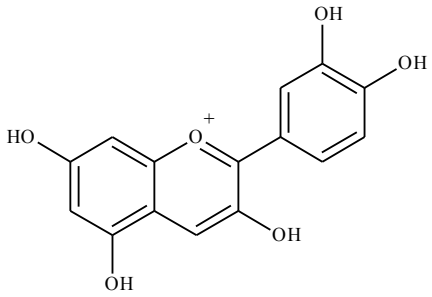


Figure 1.23: *The most common flavonols*

Anthocyanidins

Anthocyanidins are compounds with a pyrilium cation in their structure (Fig. 1.24). This cation has an aromatic character. The electron deficit is equilibrated by delocalization through the whole structure. In nature, they occur in glycoside forms in which the most common sugar unit is glucose. They are soluble in water and belong to pigments. Depending on pH, metal ions and sugar, they can colour flowers, fruit and vegetables from red to blue.[53]



Cyanidin

Figure 1.24: *Chemical structure of cyanidin*

Chalcones and aurones

Chalcones and related dihydrochalcones are subgroups of flavonoids with an open C-ring. Chalcones posses a 2,3-double bond while this bond is missing in dihydrochalcones (Fig. 1.25). Vast amounts of compounds are polyhydroxylated on different positions. The most common positions for hydroxyl groups are 5 and 7. Chalcones are intermediates in the biosynthesis of flavonoids and are associated with many biological activities.[56]

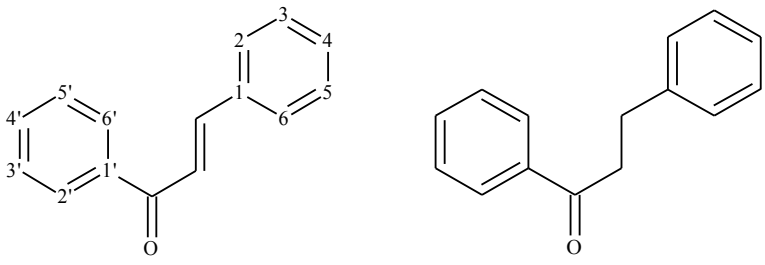


Figure 1.25: *Structural patterns of chalcone (with double bond) and dehydrochalcone (without double bond).*

Aurones are yellow pigments which can replace β-carotenes in plant tissues. They possess a five-member cycle which is formed by cyclization of chalcones (Fig. 1.26).

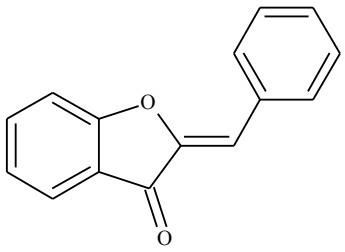


Figure 1.26: *Chemical structure of aurone*

Isoflavonoids and Neoflavonoids

These two groups differ from other flavonoids in the position of the B-ring. In isoflavonoids, the B-ring is bonded at C3 carbon and in neoflavonoids, it is at C4 (Fig. 1.27). As in the case of flavonoids, they can also be classified into several subgroups according to their sustituents.[57]

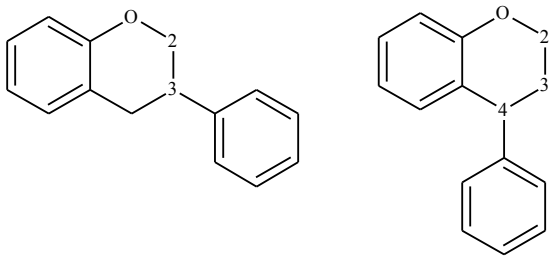


Figure 1.27: *3-phenylchroman and 4-phenylchroman - structural pattern from which isoflavanoids and neoflavanoids are derived.*

Flavonolignans

Flavonolignans are polyphenolic compounds which are formed by the condensation of one flavonoid unit and one lignan. From seeds of Milk Thistle, a mixture of flavonolignans (sylimarin extract) has been extracted. It contains different flavonolignans and flavonoids but

silybin (Fig. 1.28) is the main component. Its structure is derived from taxifolin and coniferyl alcohol. The extract has been used for more than two thousand years to treat liver problems and it also shows interesting anticancer activity.[58]

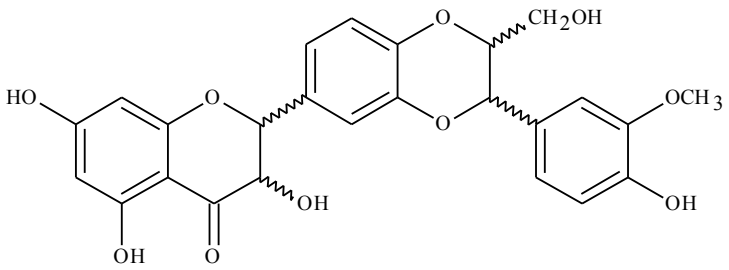


Figure 1.28: *Chemical structure of silybin*

Distribution of flavonoids

Flavonoids can be isolated from almost all plants. As secondary metabolites, they have several essential functions in plant tissues. Plants have been used in traditional medicine for a long time, and some beneficial activities are attributed to polyphenolic compounds. For humans, the main source of flavonoids is fruit, vegetables, spices, different beverages (*e.g.,* wine, tea, juice, cacao).

Catechins (*e.g.,* catechin, epicatechin, epigallocatechin *etc.*) are mainly found in green tea. By their oxidation (during fermentation), they are changed into numerous compounds including condensed tannins known as proanthocyanidins which give to black teas their characteristic colours and tastes. Condensed tannins can also be found in red wine.

Citrus fruits are rich in some flavonoids. We can find naringenin, quercetin, hesperidin and many others. Quercetin is one of the most distributed flavonoids (*e.g.*, onions, apples, tomatoes).

As mentioned above, flavonoids are also pigments responsible for different tones of red and blue in fruit and vegetables. The most important representatives are anthocyanins which

are found in all sorts of coloured fruit or vegetables, for example in cherries, raspberries, blueberries, strawberries, blood oranges, tomatoes, red cabbage, radish *etc.*

Tannins

Tannins are a group with a wide structural diversity. They are distributed in many plants (*e.g.,* chestnuts, oak). Their multiple hydroxyl phenols allow to form complexes with proteins, metal ions or polysaccharides. They are found in all plant tissues where they protect against infection. In natural medicines, they show anti-inflammatory and antiseptic features.[59] According to their structure, they are classified into three groups:

→ Condensed tannins

→ Hydrolysable tannins

→ Complex tannins

Condensed tannins or proanthocyanidins are oligomers and polymers derived from polyhydroxyl flavanols where the units are connected by the 4-8' or 4-6' C-C bonds. Their flavanol monomers have two asymmetric carbons on the C-ring giving them optical activity. Substituents have significant effects on reactivity. Some hydroxyl groups may be esterified with gallic acid.[60][61]

Hydrolysable tannins are characterized by the central saccharide unit which is esterified by gallic acid or hexahydroxydiphenic acids. According to the type of acid, they are divided into gallotannins and ellagitannins. This group has a large variability which comes from the number and location of the OH groups in their structure and linkage between individual acids. Acids could be linked by intra-ester bonds or inter-ester bonds. The latter thus enable them to form big polymer structures. The most common saccharide unit is D-glucopyranose.

Complex tannins or flavano-ellagitanins are compounds in which the structure is composed of ellagitannins combined with flavanols (catechin and epicatechin). These

complex tannins can be formed during the ripening of red wine and they show anti-cancer effects.

Note: This list of the polyphenol classes is not exhaustive but provides a general overview.

1.4.4. Biological activities of polyphenols

1.4.4.1. Protection against cardiovascular diseases

Cardiovascular diseases are usually associated with oxidation of low density lipoproteins (LDL). As in membranes, lipid peroxidation can modify LDL structure and the receptors responsible for their normal metabolism do not recognize them. The absorption of the oxidized-LDL by macrophages and T-lymphocytes produces cholesterol and other lipid products, which accumulate on the artery wall (in atheroma) to induce atherosclerosis.[62]

As lipid peroxidation inhibitors, polyphenols may prevent atherosclerosis. In the literature concerning polyphenolic compounds, the 'French paradox' is often used as example for their protective effects against cardiovascular diseases.[63] People from south-west of France were eating meals full of the saturated fat, nevertheless, the risk of cardiovascular diseases was lower than that is in other countries. This has been correlated with a higher consumption of red wine containing high concentration of polyphenols.

In another study, the diet of people living in regions around the Mediterranean Sea was compared with other regions of Europe. The people living in south regions have also a lower risk of cardiovascular diseases. This maybe consequence of high consumption of olive oil together with diets rich in vegetable.

1.4.4.2. Protection against cancer

The carcinogenesis is a long cellular process. In many case, the development of tumour is associated with our living-style, *e.g.*, living in polluted conditions, smoking, prolonged UV light irradiation. Most of these deleterious external conditions lead to the overproduction of ROS which may cause serious changes in DNA. Usually, DNA modifications are immediately repaired by specific enzymes. However, the reparation may not be completed and changes may initiate mutagenesis.

Many studies confirmed that diet rich in fruit and vegetables has protective effects against certain types of cancers (*e.g.,* breast, lung, stomach, pancreas, colon, prostate, *etc.*). This effect is associated with the presence of antioxidants including polyphenols.[64][65][66]

1.4.4.3. Anti-inflammatory effect

Inflammation is defined as a pathophysiological process which is characterized by redness, edema, fever and pain. Polyphenols are anti-inflammatory compounds. This effect has been known and used for a long time in traditional Chinese medicine and in cosmetic industry. Polyphenols contribute to the regulation of cellular activities of inflammation-related cells – mast cells, macrophages, lymphocytes and others. They also inhibit the enzymes responsible for the production of prostaglandins, leukotrienes (COX, LOX) which are crucial mediators of inflammation.[67]

This list of positive effects is not exhaustive. Polyphenols also protect against all effects and diseases associated with the oxidative stress. They are also antiseptic, antiviral, hepatoprotective *etc.*

The exact mechanism of action is not clearly established for all compounds and biological activities, mainly *in vivo*. In the case of living organisms, it must be stressed that absorption, metabolism and bioavailability are also crucial factors.

1.4.4.4. Antioxidant properties of polyphenolic compounds

As an antioxidant, they may:

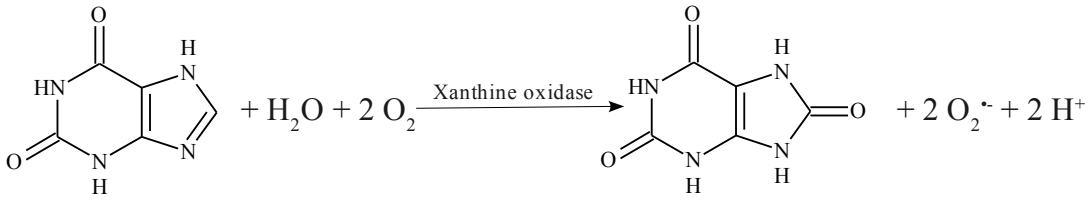
→ inhibit enzymes which are associated with the production of ROS (*e.g.*, xanthine oxidase, cyclooxygenases or lipoxygenases)

→ deactivate free metal ions (*e.g.*, Fe^{2+}, Cu^{2+}, *etc.*) which can catalyse decomposition of hydroxyl peroxide into high reactive $^{\bullet}OH$ free radicals

→ directly scavenge ROS

Inhibition of enzymes

Xanthine oxidase is involved in metabolism of xanthine into uric acid during which the superoxide radical is generated:



Depending on their chemical structures, flavonoids are xanthine oxidase inhibitors.[68] Three structural properties are mandatory for this activity:

→ the presence of the 5OH and 7OH groups

→ the presence of the 2,3-double bond

→ the absence of the 3OH group

Luteolin, chrysin and apigenin are the most active inhibitors (with IC_{50}* factor equal 0.55 ± 0.04, 0.84 ± 0.13 and 0.70 ± 0.23 μM).[68] These compounds exhibit all the three mentioned conditions to effectively inhibit xanthine oxidase.

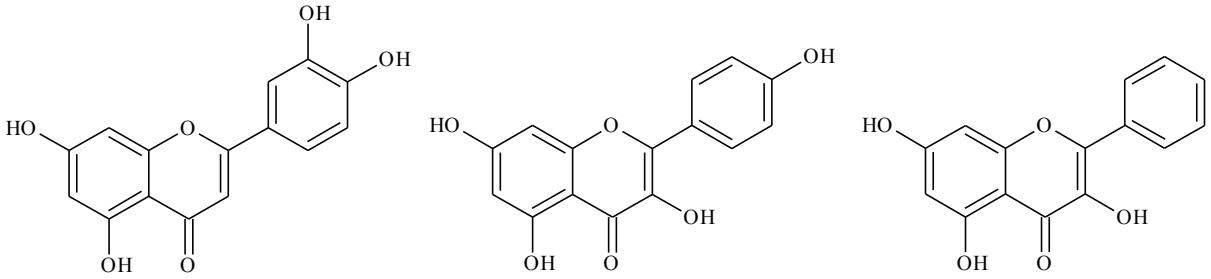


Figure 1.29: *Structures of the most effective inhibitors of xanthine oxidase*

Polyphenols also inhibit lipoxygenases (LOXs) and cyclooxygenases (COX) enzymes which catalyse oxidation of fatty acids:

$$\text{fatty acid} + O_2 \xrightarrow{\text{LOX or COX}} \text{fatty acid peroxide}$$

Free radical generation is coupled with LOX and COX-driven reactions. Both enzymes are included in the arachidonic acid cascade which is the source of the powerful bioregulators such as prostaglandins, leukotrienes *etc.*[69] All those products are involved in the immune system, that is why, both enzymes regulate inflammation, atherosclerosis, asthma and other diseases.

Several studies focused on inhibitor efficiency of different polyphenolic compounds.[70] [71][72][73] Robak et al. tested thirty nine different flavonoids on the activity of both enzymes. Concerning the inhibition of LOX the aglycones were the most active compounds. The esterification at 3OH and 7OH avoid the activity. The most active compounds are luteoline, 6-hydroxy-luteoline, patuletin and myricetin which possess a pyrogallol structure in A-ring or B-ring.[72]

*IC_{50} corresponds to the concentration of compound required to inhibit 50% activity of enzyme activity; the lower the IC_{50}, the higher the antioxidant activity.

Formation of the chelate complexes with free metal ions

The presence of the metals (*e.g.*, copper, iron, zinc) in active sites is essential for the normal function of many enzymes and they are also responsible for transport of oxygen molecules in human body. Usually, the metal ions are safety bonded in proteins where they could not cause any problem. However, ions can be released from proteins at low pH or as a result of protein damage caused by peroxides or by reactive species. Low pH can appear only locally, for example, during phagocytosis, inflammation or during ischemia injury.

Released metal ions may catalyse the Fenton reaction during which the highly reactive $^{\bullet}OH$ free radicals are formed by the decomposition of H_2O_2:[18]

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$$

A few detailed studies focusing on the ability of polyphenols to form chelate complexes with metal ions have been published.[74][75][76][77][78][79][80] The formation of chelate complexes inactivates the action of metal ions and potentially inhibits the metal-dependent processes.

The capacity of polyphenols to make complex with ion strongly depends on their structure. Generally, the metal chelating ability is related to the presence of the *ortho*-dihydroxy pattern in structure. Moreover, chelation is also possible when the OH adjoins with carbonyl group as is illustrated in Figure 1.30.[74] Metal chelation was recognized by some authors as a minor mechanism.[81]

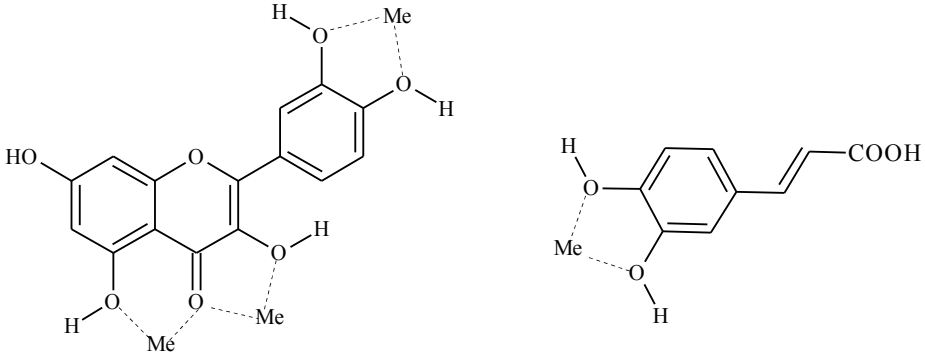


Figure 1.30: *Metal chelation for quercetin and caffeic acid.*

Direct scavenging of free radicals

The most important way, how polyphenols can protect the cells against the injury caused by free radicals, is the direct scavenging. The capacity to effectively scavenge free radicals is associated with:

i) the capacity of the compound to donate electron or proton to free radicals

ii) the capacity of the new radical to be stabilized by π-electron delocalization

Over the past years, the free radical scavenging property of many polyphenols (ArO–**H**) has been extensively studied and four different mechanisms have been proposed. The first three are HAT mechanisms following:

$$ArO–\mathbf{H} + R^{\bullet} \rightarrow ArO^{\bullet} + R–\mathbf{H}$$

i) ***Coupled proton–electron transfer (CPET)*** is characterized by the homolytic cleavage of O-H bonds:[82][83]

$$ArO–\mathbf{H} + R^{\bullet} \rightarrow ArO^{\bullet} + R–\mathbf{H}$$

ii) ***Electron transfer–proton transfer (ET-PT)*** is a two-step approach. The first step produces a radical cation ($ArOH^{\bullet+}$), which is unstable and easily dissociates by a heterolytic cleavage:[84][85][86]

$$ArO–\mathbf{H} + R^{\bullet} \rightarrow ArO\mathbf{H}^{\bullet+} + R^{-} \rightarrow ArO^{\bullet} + R–\mathbf{H}$$

iii) ***Sequential proton loss-electron transfer (SPET)*** is a three-step mechanism. The whole process is initiated by a proton loss under alkaline conditions. This step is followed by an electron transfer from the anion to the free radical. The whole process is terminated by the formation of the same products as in the first two approaches.[87]

$$ArO–\mathbf{H} \rightarrow ArO^{-} + H^{+}$$

$$ArO^{-} + R^{\bullet} \rightarrow ArO^{\bullet} + R^{-}$$

$$R^{-} + H^{+} \rightarrow R–\mathbf{H}$$

All these three mechanisms exhibit the same thermodynamical balance since reactants and products are similar. Only kinetics will determine the most favourable process.

iv) The forth possible mechanism is the ***formation of adducts***:[88]

$$ArOH + R^{\bullet} \rightarrow [ArOH\text{-}R]^{\bullet}$$

This mechanism is relatively specific for polyphenols, *e.g.,* •OH may add on double bonds and aromatic rings.

Many studies rationalized the structure activity relationship for HAT:[81][89]

→ presence of an *ortho*-dihydroxy moiety in the B-ring.

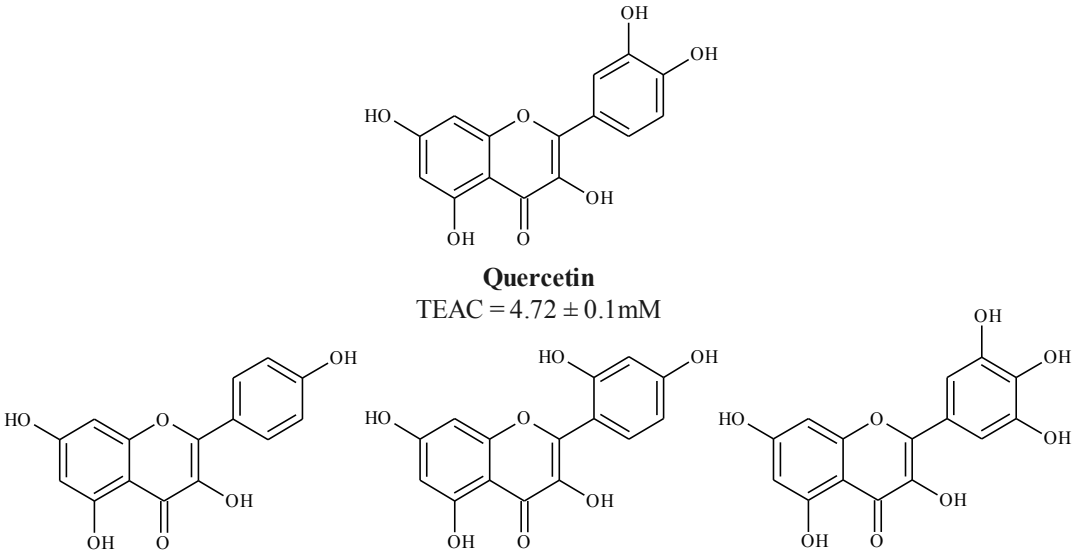
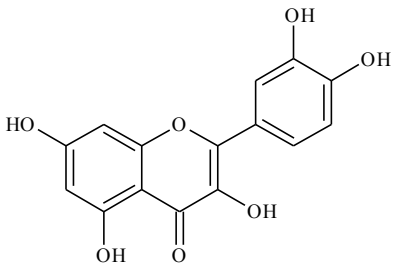
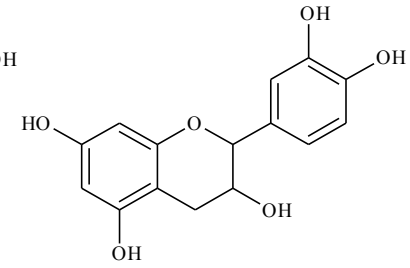


Figure 1.31: *Importance of ortho-arrangement in B-ring for activity of flavonoids (TEAC = trolox equivalent antioxidant capacity (the higher value of TEAC, the higher the antioxidant activity))*

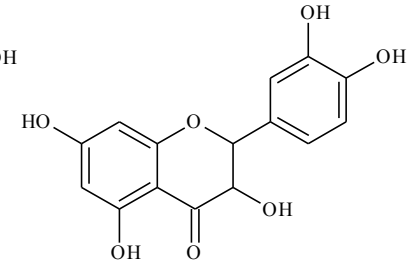
→ the presence of the 2,3-double bond. This enhances π-electron delocalization over the molecule.



Quercetin
TEAC = 4.72 ± 0.1 mM



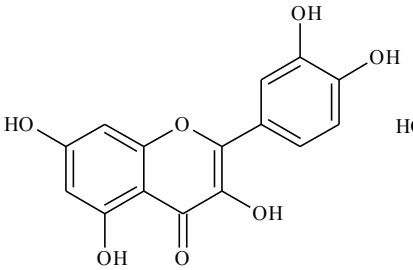
Catechin
TEAC = 2.40 ± 0.05mM



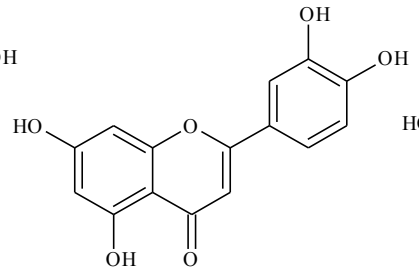
Taxifolin
TEAC = 1.9 ± 0.03 mM

Figure 1.32: *Influence of the 2,3-double bond and the keto group at C4 (TEAC = trolox equivalent antioxidant capacity (the higher value of TEAC, the higher the antioxidant activity))*

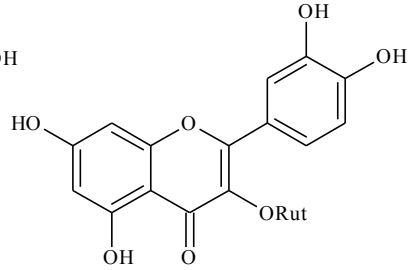
→ the presence of the 3OH group. If this group is blocked by a saccharide moiety, the antioxidant activity significantly decreases.



Quercetin
TEAC = 4.72 ± 0.1 mM



Luteolin
TEAC = 2.1 ± 0.05mM



Rutin
TEAC = 2.4 ± 0.06 mM

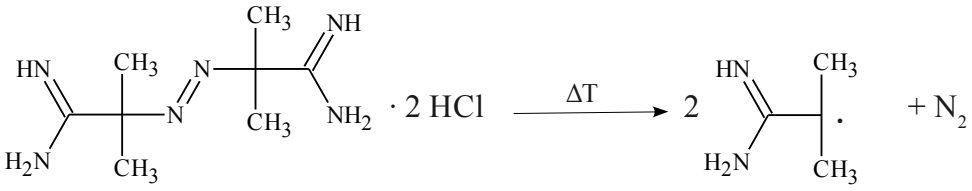
Figure 1.33: *Influence of the 3OH group in the flavonoid strucutre (TEAC = trolox equivalent antioxidant capacity (the higher value of TEAC, the higher the antioxidant activity))*

Moreover the number of the OH groups is often correlated to the free radical scavenging capacity.[90] Quercetin satisfies all these criteria and it is an antioxidant of reference.

Measurement of antioxidant activity

In living organisms, the measurement of the antioxidant activity is relatively difficult since numerous side reactions may influence the reactivity. There exist different *in vitro* tests to evaluate free radical scavenging capacity. This helps to screen the antioxidant capacity of molecules and extracts.

In 1985, Wayner et al. published the first approach – the total radical-trapping antioxidant parameter also known as TRAP.[91] This method was based on the scavenging of peroxyl radicals generated by the thermal decomposition of 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH):



$$2\ R^{\bullet} + 2\ O_2 \rightarrow 2\ RO_2^{\bullet}$$

The formed peroxyl radicals are more or less scavenged by the studied compounds. The reaction is monitored by fluorescence. The measured parameter is the concentration of antioxidant required to scavenge 50% of AAPH radicals (IC_{50} or EC_{50}); the lower IC_{50}, the more effective the antioxidant.

Another resulting parameter may be measured, the 'trolox equivalent antioxidant capacity' (TEAC). It corresponds to the concentration of trolox with the same capacity to scavenge AAPH as 1 mM of the studied antioxidant. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxyl acid) is a water soluble derivative of vitamin E (Fig. 1.34).

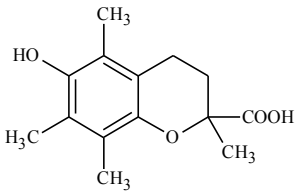


Figure 1.34: *Structural pattern of trolox*

Other radicals are used to measure the total antioxidant activity:

→ ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) – This compound is oxidized into its radical-cation form ($ABTS^{\bullet+}$) which is scavenged by antioxidants. Since this radical-cation is coloured, the test is followed by UV/visible spectroscopy.

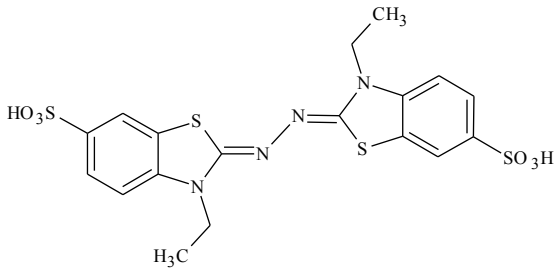


Figure 1.35: *Structure of ABTS*

→ DPPH (1,1-diphenyl-2-picryl-hydrazyl) – This radical is stable and violet in solution. When DPPH is reduced, the solution turns from violet to yellow. The DPPH decrease can also be followed by electron spin resonance (ESR).[92]

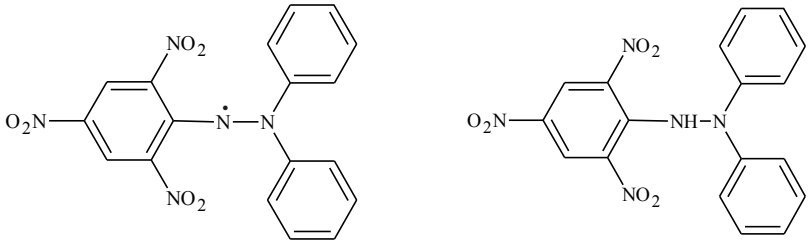
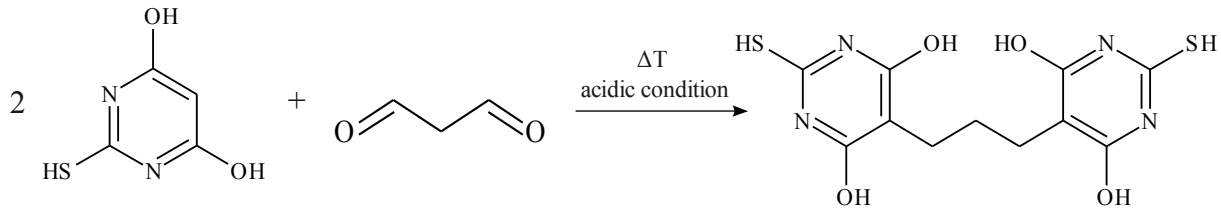


Figure 1.36: *Structures of the stable free radical DPPH and its reduced form*

→ Superoxide and hydroxyl radical are usually produced by water radiolysis, preferentially the pulse radiolysis. The detection is monitored by fast spectroscopy. It can also be followed ESR using spin traps to attach and stabilize the free radicals (*e.g.,* 5,5-dimethyl-pyrroline N-oxide (DMPO)). The formed adduct is still a radical but with a longer life time, thus detectable after irradiation by ESR.

A particular attention is given to assays of the lipid peroxidation inhibition. They are performed on biological systems including erythrocytes, lipoproteins (LDL or HDL), fraction of mitochondrial membranes, liposomes, but also lipid emulsions, oils *etc.*

To study lipid peroxidation, several methods have been described in the literature. The lipid membrane is firstly stressed by the Fenton reaction, γ-ray, AAPH and ABTS. This yields oxidative products that are detected, *e.g.*, by spectroscopy (*e.g.*, conjugated dienes and malondialdehyde (MDA)).



TBA MDA MDA-$(TBA)_2$

Chapter 2

Theoretical part

2. Methods of computational chemistry

The term "computational chemistry" corresponds to the description of chemical properties of compounds obtained by mathematical treatments performed on computers. Due to improvements of computational facilities, computational chemistry has become a very useful tool for Science nowadays, even for large biological systems.

2.1. Schrödinger equation and quantum mechanics

In the end of the 19th century, Max Karl Ernst Ludwig Planck postulated that the energy of light is emitted according to discrete quanta. Albert Einstein generalized this theory when he explained the photoelectric effect. These results imply that light can be viewed as a flow of photons, which are particles without mass but with kinetic energy, and also as an electromagnetic waves (wave/corpuscular duality).

In the beginning of the last century, Louis de Broglie demonstrated that particles with mass also exhibit wave properties. This was the basis of quantum mechanics formulated by Erwin Schrödinger in 1926:

$$\boldsymbol{H}\,\Psi = -it\,\frac{d\,\Psi}{d\,t}$$

where E is the energy of the system, Ψ is the wave function containing all the information of the system. It depends on the coordinates of all particles $\vec{x}_i$ and time:

$$\Psi = \psi\left(\vec{x}_1, \vec{x}_2, .., \vec{x}_N, t\right)$$

The wave function itself has no physical meaning. However, $|\psi|^2$ (or product $\bar{\psi}\psi$ if Complex numbers are considered) corresponds to the probability of finding the system at a given time in a given space.

To solve the Schrödinger equation, the wave function can be split into its spatial and time parts:

$$\psi(\vec{x}_i, t) = \psi(\vec{x}_i) T(t)$$

In this text I will concentrate only the solvation of the time-independent Schrödinger equation in which the wave function is written only in its spatial form.

H is the so-called Hamilton operator (Hamiltonian). It describes the total energy of the system, so its form is composed of all the contributions to the energy. The general expression is:

$$\boldsymbol{H}_{tot} = \boldsymbol{T}_n + \boldsymbol{T}_e + \boldsymbol{V}_{nn} + \boldsymbol{V}_{ee} + \boldsymbol{V}_{ne}$$

The first two members correspond to the kinetic energies of electrons ($\mathbf{T_e}$) and nuclei ($\mathbf{T_n}$). They are written as the sum of the operators describing the motion of electron *i* or nuclei *a* and they depend on their coordinates.

$$\boldsymbol{T}_e = \sum_{i=1}^{N} -\frac{1}{2}\nabla_i^2$$

$$\boldsymbol{T}_n = \sum_{a=1}^{A} -\frac{1}{2\mathrm{M}_a}\nabla_a^2$$

The next two members describe electrostatic repulsion between nuclei ($\mathbf{V_{nn}}$) and electrons ($\mathbf{V_{ee}}$). This is written as the sum of all the possible electron–electron and nuclei–nuclei interactions:

$$\boldsymbol{V}_{nn} = \sum_{a=1}^{A} \sum_{b>a}^{A} \frac{Z_a Z_b}{|R_{ab}|}$$

$$\boldsymbol{V}_{ee} = \sum_{i=1}^{N} \sum_{j>i}^{N} \frac{1}{|r_{ij}|}$$

The last member ($\mathbf{V_{ne}}$) of the equation describes the attractive interaction between nuclei and electrons.

$$\boldsymbol{V}_{ne} = \sum_{i=1}^{N} \sum_{a=1}^{A} -\frac{Z_a}{|R_{ai}|}$$

The Schrödinger equation is a partial differential equation, in which the Hamiltonian acts on the wave function and it returns the same wave function multiplied by a scalar (E). We know that this form of the equation has solution only for some values of E, which correspond to the energies of the system. Such energy values are called eigenvalues, which correspond to eigenfunctions ψ.

2.1.1. The Born-Oppenheimer approximation

The exact solution of the Schrödinger equation is only found for the one-electron systems. Due to difficulties associated with the many-particle equation, approximations are used. The first approximation was proposed by Born and Oppenheimer.[93]

In molecular system, the mass of nuclei is much higher than the mass of electrons, so the speed of nucleus is much lower than that of electrons. As a consequence, the kinetic energy of nuclei is equal to zero when electrons are moving in a field of fixed nuclei. This idea enables us to divide the total wave function of the system into its electronic and nuclear components:

$$\Psi_{total} = \psi_{electron}\,\psi_{nuclei}$$

The total energy is equal to the sum of both contributions – the nuclear and the electronic energies. When the Born-Oppenheimer approximation is applied, the electronic Schrödinger equation is solved for a given conformation of nuclei. The corresponding Hamiltonian is written as:

$$\boldsymbol{H} = \boldsymbol{H}_e + \boldsymbol{V}_{nn} = \boldsymbol{T}_e + \boldsymbol{V}_{ne} + \boldsymbol{V}_{ee} + \boldsymbol{V}_{nn}$$

where $\mathbf{V_{nn}}$ is constant for a given conformation.

The Born-Oppenheimer approximation allows to separate *(i)* quantum mechanics, where energy is calculated from the electronic Schrödinger equation and *(ii)* molecular mechanics, where energy is calculated by using Newton's laws (motion of nuclei). The methods of molecular mechanics will be discussed in the following part.

As mentioned above, the exact solution of Schrödinger equation is possible only for one-electron systems, *e.g.,* the hydrogen atom. The solution of the Schrödinger equation is a set of energies E_n with the corresponding wave functions (ψ_{nlm}). The three indexes are the quantum numbers:

Quantum number	Range of quantum number	Notation	Characteristic
n	From 1 to n	Principal quantum number	Energetic level of electrons
l	From 0 to (n-1)	Orbital quantum number	Moment of motion of electrons
m_l	From -l to +l	Magnetic quantum number	Specific of the orbital cloud

The spin of electron is viewed as the moment of the electron rotation on itself, but this is a pure quantum property. It cannot be measured directly. The electrons are fermions, therefore projection of moment onto z-axis is equal to ± ½. Each electron in a poly-electronic system is described by its coordinates and its spin. The one-electron wave function can be split into its spatial (Φ) and its spin (s) parts. The wave function is called 'spinorbital' and it is denoted χ:

$$\psi_i = \chi_i = \phi(x, y, z)s$$

The electrons are indistinguishable, which is important for poly-electronic system. Since they are fermions, the sign of the wave function changes when two electrons are exchanged. This is called the antisymmetry of the wave function.

The most convenient form of the wave function which satisfies antisymmetry is a Slater determinant:

$$\Psi = \frac{1}{\sqrt{N!}} \begin{vmatrix} \chi_1(1) & \chi_2(1) & \dots & \chi_N(1) \\ \chi_1(2) & \chi_2(2) & \dots & \chi_N(2) \\ \dots & \dots & \dots & \dots \\ \chi_1(N) & \chi_2(N) & \dots & \chi_N(N) \end{vmatrix}$$

In this determinant, χ_i is the individual spinorbital characterized by the coordinates and the spin of electron *i*. The factor $1/\sqrt{N!}$ is included to normalize the function. The columns are formed from electron orbitals, while the rows describe the electron coordinates. If two lines are exchanged (*i.e.*, exchange of two electrons), the sign of the determinant changes (antisymmetry). If there are two identical lines (identical electrons), the determinant equals zero, which correspond to the Pauli principle (two electrons cannot have the same spin and spatial location).

2.1.2. Energy of poly-electronic systems

The energy of the system is given by:

$$E = \frac{\int_{-\infty}^{\infty} \bar{\Psi} \boldsymbol{H} \Psi \, dr}{\int_{-\infty}^{\infty} \bar{\Psi} \Psi \, dr}$$

Since orthonormal spinorbitals are considered, the denominator equals 1. The numerator is a product of numerous integrals. Some of them equal zero and the other contribute to the energy. Integrals may be divided into three energetic contributions:

→ Kinetic energy and potential energy of single electrons moving in a field of nuclei – this is denoted as the core energy and it is the sum of contributions of the individual spinorbitals χ_i:

$$E_{total}^{core} = \sum_{i=1}^{N} \boldsymbol{H}_{ii}^{core}$$

$$\boldsymbol{H}_{ii}^{core} = \int \chi_i(1) \left(\frac{-1}{2} \nabla_i^2 - \sum_{a=1}^{A} \frac{Z_a}{|R_{ai}|} \right) \chi_i(1) \, d\tau_1$$

→ Classic Coulomb electrostatic repulsion between two electrons – the total electrostatic contribution is the sum electrostatic interactions between the electron in a spinorbital χ_i and the other N-1 electrons:

$$E_{total}^{Coulomb} = \sum_{i=1}^{N} E_i^{Coulomb} = \sum_{i=1}^{N} \sum_{j>i}^{N} \boldsymbol{J_{ij}}$$

$$\boldsymbol{J_{ij}} = \iint \chi_i(1)\chi_j(2)\left(\frac{1}{|r_{ij}|}\right)\chi_i(1)\chi_j(2)\,d\tau_1\,d\tau_2$$

→ Exchange interaction which is a non-classical contribution. It arises from the correlation of the motion between electrons with parallel spin. This correlation comes from the Pauli principle which explains that the probability to find two electrons with the same spin at the same place equals zero.

$$E_{total}^{exchange} = \sum_{i=1}^{N} E_i^{exchange} = \sum_{i=1}^{N} \sum_{j>i}^{N} \boldsymbol{K_{ij}}$$

$$\boldsymbol{K_{ij}} = \iint \chi_i(1)\chi_j(2)\left(\frac{1}{|r_{ij}|}\right)\chi_i(2)\chi_j(1)\,d\tau_1\,d\tau_2$$

Generally, the exchange energy is a small contribution to the total energy of molecular systems, but it is necessary for an accurate description of the electronic structures.

2.1.3. The Hartree-Fock approximation

Even within the Born-Oppenheimer approximation, the exact solution of the Schrödinger equation remains inaccessible. The Hartree-Fock approximation simplifies the problem.

In many-electron systems, all electrons interact together and their motions are correlated. Hartree proposed to exchange the influence of the other electrons on a single electron by a potential field. Thus, a given electron moves in a field formed by nuclei and other electrons. Each electron is described by its own wave function ψ_i, which is the solution of the one-electron Schrödinger equation:

$$h_i\psi_i = \epsilon_i\psi_i$$

In the Hartree approximation, the many-electron Hamiltonian is defined as the sum of the one-electron Hamiltonians, h_i:

$$\boldsymbol{H} = \sum_{i=1}^{N} h_i = \sum_{i=1}^{N} \left[-\frac{1}{2} \nabla_i^2 - \sum_{a=1}^{A} \frac{Z_a}{|R_{ai}|} + V_i \right]$$

The many-electron wave function is the product of the one-electron wave functions:

$$\Psi_{HP} = \psi_1 \psi_2 \ldots \psi_N$$

Form of the many-electron wave function, which was defined by Hartree, is not antisymmetric. To improve this approach, Fock used the wave function as a Slater determinant, which is antisymmetric. After the substitution of this determinant, the Hartree-Fock equations are obtained:

$$\left[\boldsymbol{H}^{core}(1) + \sum_{j \neq i}^{N} \boldsymbol{J}_j(1) - \sum_{j \neq i}^{N} \boldsymbol{K}_j(1) \right] \chi_i(1) = \sum_j \varepsilon_{ij} \chi_j(1)$$

where $\boldsymbol{H}_{core}$ is the core potential for electron i, $\boldsymbol{J}_j$ is Coulomb repulsion between electrons and $\boldsymbol{K}_j$ is the exchange energy. The term in square brackets is also called the Fock operator (f_i) and the one-electron equation is:

$$f_i \chi_i = \sum_j \varepsilon_{ij} \chi_j$$

A mathematical treatment allows to formulate this equation as an eigenvalue equation:

$$f_i \chi_i = \varepsilon_i \chi_i$$

Due to the antisymmetrical character of the Slater determinant, the Hartree-Fock equations contain the exchange contribution.

The form of the Hartree-Fock equations requires to use a self-consistent field procedure for solvation. It starts with a guess wave function that is introduced into the equations to find energies. These energies give a new set of wave functions which are used for a new cycle. This is repeated until convergence, *e.g.*, no change in energy.

The variational principle states that the total energy obtained from the Hartree-Fock approximation is always higher than the energy of the exact solution of the Schrödinger equation. This is due to many parameters including the correlation that exist between antiparallel electrons. This contribution is called the electron correlation energy, which is not described within the HF approach.

2.1.4. Roothaan-Hall equations

The Hartree-Fock approximation allows to solve the Schrödinger equation but this is not practical for the description of molecules. The most popular strategy for molecules is to write each molecular orbital as a linear combination of atomic orbitals (LCAO-MO).

$$\chi_i = \sum_{\nu=1}^{K} c_{\nu i} \phi_\nu$$

Roothaan and Hall firstly introduced the atomic orbitals into Hartree-Fock equations which then gain the following form:

$$f_i \sum_{\nu=1}^{K} c_{\nu i} \phi_\nu = \epsilon_i \sum_{\nu=1}^{K} c_{\nu i} \phi_\nu$$

A mathematical rearrangement of these equations leads to a matrix form:

$$\sum_{\nu=1}^{K} c_{\nu i} \int \phi_\mu f_i \phi_\nu dr = \epsilon_i \sum_{\nu=1}^{K} c_{\nu i} \int \phi_\mu \phi_\nu dr$$

where the integral on the right side describes the overlap between two atomic orbitals μ,ν and it is denoted S. This overlap does not necessary equal zero because the orbitals may be located on different atoms. The integral on the left side is the matrix form of the Fock operator. Then, the Roothaan-Hall equations can be abbreviated into the following matrix form:

$$FC = SCE$$

Matrix C contains coefficients c_{vi} and E is a diagonal matrix whose elements are orbital energies. This form of the Roothaan-Hall equations is not an eigenvalue formulation ($FC = CE$) which requires to use the self-consistent field methods. Fortunately, a mathematical transformation converts this equation into the required form and an iterative procedure can be used, as described above. The result is a set of K molecular orbitals.

2.1.5. Basis sets

The solution of the Roothaan-Hall equations strongly depends on the choice of the mathematical shape used for atomic orbitals. The most famous are Slater-type orbitals written as follows:

$$f_{STO} = P(x, y, z)e^{-\alpha r}$$

Such a function decays exponentially with the distance from nuclei, but their calculation is difficult. In computational chemistry, it is common to replace Slater-type orbitals by Gaussian functions:

$$f_{GTO} = P(x, y, z)e^{-\alpha r^2}$$

The difference between both functions is only in the exponential term, but this change makes integral calculation much easier. It was also found that replacing Slater orbitals by single Gaussian function is not relevant and at least three Gaussian functions are required. Therefore, each orbital is replaced by a linear combination of several Gaussian functions:

$$\chi_\mu = \sum_{i=1}^{M} d_i \phi_i$$

where M is the number of Gaussian functions Φ_i, and d_i are the corresponding coefficients. The accuracy often increases with the number of Gaussian functions (Fig. 2.1).

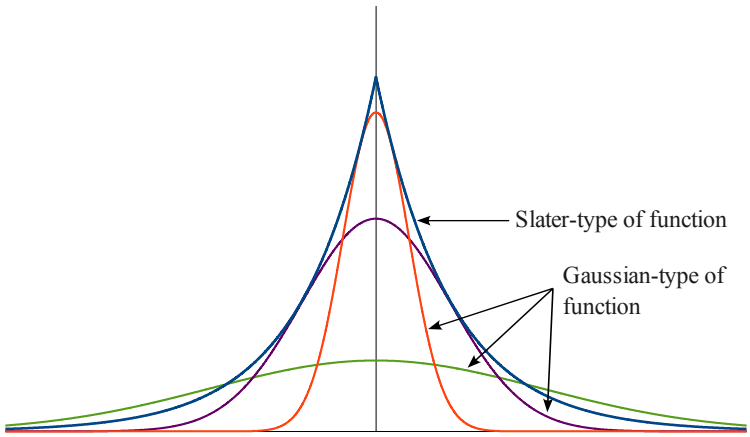


Figure 2.1: *Approximation of Slater-type of orbitals by Gaussian functions*

The set of Gaussian functions is called the basis set and are the Pople-type basis set since Pople developed the use of linear combination of n Gaussian functions (STO-nG).[94]

The minimal basis set contains only occupied atomic orbitals and does not give very accurate results. To improve the description, other non-occupied orbitals (virtual orbitals) are used. When the number of functions is doubled the basis set is a double-ζ. By the same way, triple- or quadruple-ζ exist. Generally, functions are added till the results do change.

Pople and co-workers also developed 'split valence' basis sets (*k-lmnG*). The term *k* indicates the number of Gaussian functions used for the description of core electrons. Due to high energy of the core electrons, they have to be described accurately so more functions are used. The *lmn* term indicates the number of valence orbitals and Gaussian functions that are used for their description (Fig. 2.2).

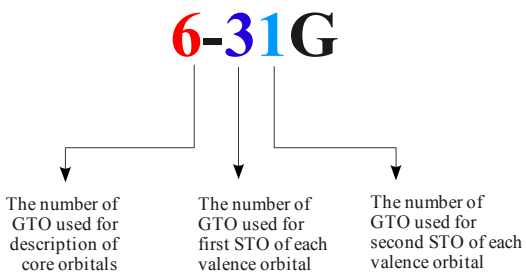


Figure 2.2: *Pople's split valence basis set*

To improve the description of asymmetry in electron distribution, polarization functions are used, by adding d- or f-orbitals for heavy atoms or p-orbitals for hydrogen atoms. The polarisation functions are written in bracket after G and they are separately described for the heavy atoms and for the hydrogen atoms (notations with asterisks are also used) (Fig. 2.3).

Bond polarization and electron delocalization can also be described by the use of diffuse functions which are denoted by '+'. One '+' means that the diffuse functions are added for heavy atoms and '++' means that diffuse functions are added to the hydrogen atoms (Fig. 2.3).

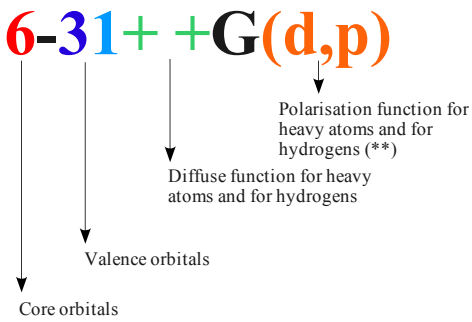


Figure 2.3: *Pople's split valence basis set with diffuse function and polarisation function*

Except of Pople's basis sets, there exist natural orbital basis set or correlation consistent basis sets (Dunning-type).[95] There are several sizes of these basis sets, *i.e.*, cc-pVDZ, cc-pVTZ and so on. The *cc* in the beginning introduce correlation consistent basis sets, *p* denotes polarization function and the last three letters describe valence electrons (valence double-ζ or triple-ζ ...). Diffuse function can also be added, indicated by the prefix 'aug'.

2.1.6. Post Hartree-Fock methods

Even with infinite basis sets the exact solution of the Schrödinger equation cannot be achieved by this way. The difference between Hartree-Fock and the exact energies is the electron correlation energy (E_C). This energy mainly contains the correlation of the antiparallel electrons.

The Hartree-Fock theory describes the electron correlation for parallel electrons. Even if small, the correlation of antiparallel electrons may strongly influence the description of certain chemical properties.

Theoretically, the exact energy can be obtained by adding the electron correlation to Hartree-Fock energy:

$$E = E_{HF} + E_{cor}$$

There exist several methods proposed to improve Hartree-Fock calculations which are known as the post HF methods that we will not develop in this manuscript:

→ Methods of configuration interaction (CI)

→ Methods of coupled clusters (CC)

→ Perturbation theory (MP)

2.1.7. Semi-empirical methods

Due to the number of two-electron integrals in the Hartree-Fock method, the calculation of small molecules may be long and difficult. To simplify the calculation, semi-empirical methods have been developed, in which some integrals are approximated or neglected (*e.g.* core electrons are not included, minimal basis set is used, two-electron integrals are neglected or parametrized from experimental data).

The main advantage of semi-empirical methods is the decrease of the computational time and the possibility to work on bigger systems. However, due to the number of approximations and parametrizations they can fail in the description of some properties, *e.g.,* van der Waals interaction. Generally, the semi-empirical methods are relatively good for the prediction of molecule geometry.

Since 60's, many new methods have been developed and some of them are parametrized for the description of certain properties. The most common methods are CNDO (Complete

neglect of differential overlap), INDO (Incomplete neglect of differential overlap), MNDO (Medium neglect of differential overlap), AM1 (Austin model 1), PM3 (Third parametrization of MNDO).

2.2. Density functional theory

From the beginning of quantum mechanics, the physicists tried to replace the wave function (which does not have any physical meaning and depends on all the coordinates and the spins of electrons) by an interpretable. Density Functional Theory (DFT) proposes to replace the wave function by the electron density.

The electron density is the function described by only three coordinates and it corresponds to the probability that electron is located in a specific place. The energy of the system is thus a function of a function (the electron density), which is called a 'functional'.

Thomas and Fermi proposed to write the total energy as a function of the electron density $\rho(r)$.

Generally, the total energy of system can be written as a sum of the potential and the kinetic energies. The potential term can be directly formulated as a function of the electron density, where the interaction between nuclei and electrons ($\mathbf{V_{ne}}$) and electron repulsion ($\mathbf{V_{ee}}$) have a classical form:

$$V_{ne}[\rho(r)] = \sum_a \int \frac{Z_a \rho(r)}{|R_a - r|} dr$$

$$V_{ee}[\rho(r)] = \frac{1}{2} \iint \frac{\rho(r_1)\rho(r_2)}{|r_{12}|} dr_1 dr_2$$

To describe kinetic energy, Thomas and Fermi introduced the non-interacting uniform gas of electrons (with a non-zero electron density) in a field of positive charge (the jellium). This assumption allows to describe the kinetic energy as:

$$T_{TF}[\rho(r)] = \frac{3}{10}(3\pi^2)^{\frac{2}{3}} \int \rho^{\frac{5}{3}}(r) dr$$

The total energy, according to Thomas and Fermi, is written as the sum of all the contributions:

$$\boldsymbol{E}[\rho]=\boldsymbol{T}_{TF}[\rho]+\boldsymbol{V}_{ne}[\rho]+\boldsymbol{V}_{ee}[\rho]$$

Therefore, the electron density depends only on three space coordinates. This approach reduces the main problem of the wave function – the number of variables.

It had been used for solid physics, but it was not adapted for most of chemical properties since the interactions between particles are not explicitly considered. The situation changed in 60's, when Hohenberg and Kohn proposed two theorems which allow the achievement of DFT.

2.2.1. The first theorem of Hohenberg and Kohn

The first theorem states that the electron density can be used to express all properties. "The external potential $\mathbf{V}_{ext}$ is determined, within a trivial additive constant, by the electron density."[96] This means that the electron density carries the information about the whole system (*e.g.,* number of atoms, their proton number *etc.*) from which the Hamiltonian can be obtained and thus the wave function, the energy and all other properties of the system. The total energy of the system is:

$$\boldsymbol{E}[\rho]=\boldsymbol{V}_{\mathrm{exp}}[\rho]+\boldsymbol{T}[\rho]+\boldsymbol{V}_{ee}[\rho]+\boldsymbol{E}_{ncl}[\rho]$$

→ $\mathbf{V}_{ext}[\rho]$ is the external potential energy. This contribution depends on the studied system and in absence of any external field, it corresponds to the interaction between nuclei and electrons, $\mathbf{V}_{ne}[\rho]$.

$$\boldsymbol{V}_{ext}[\rho]=\boldsymbol{V}_{ne}[\rho]=-\sum_{A=1}^{K}\sum_{i=1}^{N}\frac{Z_A}{r_{Ai}}$$

→ **T**[ρ] and $\mathbf{E}_{ee}[\rho]$ are the kinetic energy and the potential of the electron-electron interaction, respectively. They are general for all systems. The exact form for both functionals is a mystery. The electron-electron interaction is separated into the classical Coulomb interaction, $\mathbf{V}_{ee}[\rho]$, and the non-classical contribution, $\mathbf{E}_{ncl}[\rho]$:

$$E_{ee}[\rho] = V_{ee}[\rho] + E_{ncl}[\rho]$$

$\mathbf{E}_{ncl}[\rho]$ contains corrections which are not involved in $\mathbf{V}_{ee}$. This member does not have any exact expression.

2.2.2. The second theorem of Hohenberg and Kohn

This theorem is the variational principle for DFT. The electron density of the ground state, ρ_0, corresponds to the minimal energy E_0. In other words, for a given ρ the corresponding energy E is such as:

$$E_0 \leqslant E$$

2.2.3. Kohn – Sham orbitals

The Hohenberg-Kohn theorems are the pillars of DFT and allow to use the electron density as a fundamental quantity. The electron density uniquely determines the external potential, which determines the Hamiltonian, which in turn determines the wave function and thus the total energy. However, the expression of the kinetic energy, **T**[ρ], and the non-classical contributions for electron-electron interaction, $\mathbf{E}_{ncl}[\rho]$ are not known.

Kohn and Sham proposed a genial solution to this problem. They proposed to describe the kinetic energy by an indirect way. They suggested to use a non-interacting electrons system, for which the kinetic energy is:

$$T_S = -\frac{1}{2}\sum_{i}^{N} \langle \varphi_i | \nabla^2 | \varphi_i \rangle$$

This expression does not give the exact value of the kinetic energy of the interacting system but it contains the major part. The correction to the kinetic energy ($\mathbf{T_C}[\rho]$) is added into the expression of the total energy functional:

$$\boldsymbol{E}[\rho]=\boldsymbol{T_S}[\rho]+\boldsymbol{V_{ne}}[\rho]+\boldsymbol{V_{ee}}[\rho]+\boldsymbol{T_C}[\rho]+\boldsymbol{E_{ncl}}[\rho]$$

where the last two members are combined in one common functional, $\mathbf{E_{XC}}$. This term is known as the exchange-correlation energy and describes everything that is not exactly known, *i.e.* correction to the kinetic energy and non-classical effects of the electron-electron interaction.

$$\boldsymbol{E_{XC}}[\rho]=\boldsymbol{T_C}[\rho]+\boldsymbol{E_{ncl}}[\rho]=(\boldsymbol{T}[\rho]-\boldsymbol{T_S}[\rho])+(\boldsymbol{E_{ee}}[\rho]-\boldsymbol{V_{ee}}[\rho])$$

For the non-interacting system, the equations with an one-electron operator h_S^{KS} are:

$$h_S^{KS}\varphi_i=\varepsilon_i\varphi_i$$

$$h_S^{KS}=-\frac{1}{2}\nabla^2+V_{eff}(r)$$

$$\boldsymbol{V_{eff}}(r)=\boldsymbol{V_{ne}}(r)+\int\frac{\rho(r_2)}{|r_{12}|}dr_2+\boldsymbol{V_{XC}}(r)$$

where $\mathbf{V_{XC}}$ is the exchange-correlation potential. In these equations φ_i (when orthonormal) are the Kohn-Sham molecular orbitals such as:

$$\rho(r)=\sum_1^N|\varphi_i(r)|^2$$

As for the Hartree-Fock method, these equations are iteratively solved.

2.2.4. The exchange – correlation functional

All the energetic terms of the equation are explicitly known except the exchange-correlation functional $\mathbf{E_{XC}}$. It contains the non-classical part of the electron-electron interaction, $\mathbf{E_{ncl}}$, and the correction to the kinetic energy, $\mathbf{T_C}$. Over the past years, several approaches were proposed to describe this functional.

2.2.4.1. Local density approximation

One of the most famous description was built with the concept of the uniform gas of electrons, in which the electron density is constant over the whole space. The corresponding exchange–correlation energy is given by:

$$\boldsymbol{E}_{\boldsymbol{XC}}[\rho] = \int \rho(r)\,\varepsilon_{XC}(\rho(r))\,dr$$

where $\varepsilon_{XC}[\rho]$ is the exchange–correlation energy computed per electron. This energy is weighted with the probability $\rho(r)$. Due to the local character of this function, this approach is called the local density approximation (LDA). The equation is usually separated into the exchange and the correlation contributions:

$$\varepsilon_{XC}(\rho(r)) = \varepsilon_{X}(\rho(r)) + \varepsilon_{C}(\rho(r))$$

The most widely used functional is SVWN. Its exchange part was described by the Slater exchange (obtained from the HF approximation) and the correlation part was proposed by Vosko, Wilk and Nusair.

If we consider two density functional (ρ_α, ρ_β) instead of one, this may lead to a better description of the system, *e.g.*, in an unrestricted scheme this is the local spin-density approximation (LSDA), for which the energy is:

$$\boldsymbol{E}_{\boldsymbol{XC}}[\rho_\alpha, \rho_\beta] = \int \rho(r)\,\varepsilon_{XC}(\rho_\alpha(r), \rho_\beta(r))\,dr$$

2.2.4.2. Generalized gradient approximation

The LDA approach gives a relatively good description for solid-state systems, but it is insufficient for most of the applications in chemistry. One way to improve this method is to include the gradient of the electron density (first derivative), thus breaking the non-realistic uniformity. The first attempt of such functionals is the gradient expansion approximation (GEA). Unfortunately, GEA did not improve the accuracy.

The next approach, was the generalized gradient approximation (GGA). These functionals are generally written as:

$$\boldsymbol{E}_{XC}^{GGA}[\rho_\alpha, \rho_\beta] = \int f(\rho_\alpha, \rho_\beta, \nabla\rho_\alpha, \nabla\rho_\beta)\, dr$$

In practice, functionals are split into individual contributions, which are usually constructed mathematically. The exchange part of $\boldsymbol{E}_X^{GGA}$ is written as:

$$\boldsymbol{E}_X^{GGA} = \boldsymbol{E}_X^{LDA} - \sum_\sigma \int F(s_\sigma)\rho_\sigma^{\frac{4}{3}}(r)\, dr$$

where the argument of the function F is the reduced density gradient for spin σ:

$$s_\sigma(r) = \frac{|\nabla\rho_\sigma(r)|}{\rho_\sigma^{4/3}(r)}$$

According to the description of F, we can classify exchange part into two basic classes. The first one was developed by Becke (its acronym is B). Becke uses only one parameter (β = *0.0042*):

$$F^B = \frac{\beta s_\sigma^2}{1 + 6\beta s_\sigma \sinh^{-1} s_\sigma}$$

The functionals, which are related to this approach, are FT97 (Filatov, Thiel – 1997), CAMs (Handy et al. - 1993), PW91 (Burke, Pendew, Wang), *etc.*

The second class uses for F a rational function of the reduced density gradient. The most representatives are B86 (Becke - 1986), P86 (Perdew – 1986) or PBE (Perdew, Burke, Ernzerhof - 1996).

The correlation part has more complicated forms and it may not have any physical meaning. The most representatives of correlation counterparts are PW91 (Perdew, Wang – 1991), LYP (Lee, Yang, Parr – 1988). The LYP functional is, for example, derived from the correlation energy of helium and it contains only one empirical parameter.

Theoretically, it is possible to combine all exchange parts with all correlation counterparts, *e.g.*, BP86 or BLYP.

The next improvement of methods is the use of the second derivative of the electron density, which corresponds to the kinetic energy density: meta-GGA functionals.

2.2.4.3. Hybrid functionals

With the previous methods, the exchange contribution may be overestimated while HF gives the exact exchange energy. One can calculate the exchange contribution by HF theory by:

$$\boldsymbol{E}_X^{HF} = \iint \varphi_i(r_1)\,\bar{\varphi}_j(r_1)\frac{1}{r_{ij}}\varphi_j(r_2)\,\bar{\varphi}_i(r_2)\,dr_1\,dr_2$$

and the correlation counterpart is mathematically approximated.

$$\boldsymbol{E}_{XC} = \boldsymbol{E}_X^{exact} + \boldsymbol{E}_C$$

This approach provides relatively good results for atoms, but when applied to molecules it fails. A good compromise to solve this problem is to include only a part of the exact HF exchange.

$$\boldsymbol{E}_{XC} = a\,\boldsymbol{E}_X^{HF} + (1-a)\,\boldsymbol{E}_{XC}^{DFT}$$

When *a* equals 0.5, the functional is called half-and-half (HandH), *i.e.*, the combination of half 'exact' exchange and half exchange-correlation from DFT.

Currently, there are many functionals which are built by the combination of different exchange and correlation parts with different number of parameters. One of the most used was proposed by Becke (1993) and it contains three parameters describing contributions of individual members:

$$\boldsymbol{E}_{XC}^{B3} = \boldsymbol{E}_{XC}^{LSD} + a\,(\boldsymbol{E}_{XC}^{HF} - \boldsymbol{E}_X^{LSD}) + b\,\boldsymbol{E}_X^{B} + c\,\boldsymbol{E}_C^{PW91}$$

where $a = 0.20$, $b = 0.72$ and $c = 0.81$.

Actually, there are many different functionals and many other are under study. The results obtained with the new functionals are, in principal, compared with experimental data and may describe specific properties. Some are suitable to describe delocalized systems, others accurately describe kinetics.

Advantages of DFT

→ It is based on the electron density, which has a physical meaning and depends only on three spacial coordinates.

→ It intrinsicly includes the electron correlation

→ Certain DFT functionals give results comparable to the MP2 post-HF methods with a much smaller computational effort.

→ They are less time-consuming.

Disadvantages of DFT

→ We do not know the exact exchange-correlation functional. It has to be approximated.

→ DFT provides a poor description of the long-range interactions (new functionals are developed for this description, e.g., DFT-d, CAM-B3LYP).

→ Self-interaction must be corrected.

2.3. Method of molecular mechanics

The main limitation of QM and DFT methods is the size of the molecular system. Only system of a few hundreds atoms may be treated. For larger systems as proteins, membranes or nucleic acids other approximations must be used.

Within the Born-Oppenheimer approximation, the motion of nuclei may be treated separately. Atoms are treated as balls characterized by mass and van der Waals radius, while

bonds are springs described by force constants. To distinguish the different atoms and bonding, different atom types are defined, *e.g.,* carbon sp^3, sp^2, aromatic sp^2 *etc.* For such modelled systems Newton mechanics is used. The methods based on this approach are the molecular mechanic methods.

The aim of these methods is mainly to estimate the ground state geometry for a given system. The big biomolecules can adopt many conformations with different values of energies. This can be modelled by the potential energy surface (PES) giving the energy as a function of geometrical coordinates. The minima on PES correspond to more stable conformations. They are separated from each other by high or small energy barriers.

The energy in molecular mechanics is written as the sum of terms describing the individual energetic contributions, such as bond stretching ($\mathbf{E_{bond}}$), angle bending ($\mathbf{E_{angle}}$), torsion rotation ($\mathbf{E_{torsion}}$) and non-covalent interactions ($\mathbf{E_{noncov}}$):

$$\boldsymbol{E = E_{bond} + E_{angle} + E_{torsion} + E_{noncov}}$$

Bond stretching

The most precise function for description of bond potential was proposed by Morse:

$$\boldsymbol{E_{bond}} = D_e\left[1 - e^{-a(l-l_0)}\right]^2$$

Since this potential is difficult to calculate and it also contains three parameters, it is not usual to use it in molecular modelling. Therefore, this curve is approximated by simpler forms.

For bond stretching, the simplest expression is the Hooke harmonic potential:

$$\boldsymbol{E_{bond}} = \frac{k}{2}\left(l - l_0\right)^2$$

This term contains only two parameters. k is the force constant which defines the force required to deviate bond from its equilibrium. The second parameter l_0 is the equilibrium bond length. This harmonic potential is a very good approximation for equilibrated structures,

however, it is not the best when the molecules are farther from the energy minimum. In this case, the description using a Taylor series is better-adapted:

$$E_{bond} = \frac{k}{2}(l - l_0)^2\left[1 - k_1(l - l_0) - k_2(l - l_0)^2 - \ldots\right]$$

This approach improves the description of bond potential but more parameters are required and the calculation is subsequently more time consuming.

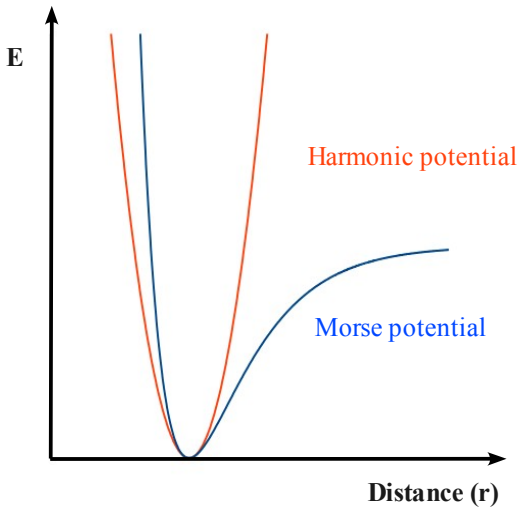


Figure 2.4: *Morse and Hooke's harmonic potential describing bond stretching*

Angle bending

The energy related to deviation of angles can also be written as a Hooke's harmonic potential:

$$E_{angle} = \frac{k}{2}(\Theta - \Theta_0)^2$$

where k and Θ_0 are the force constant and the equilibrium angle, respectively. To make the description more accurate, higher orders of a Taylor series maybe included.

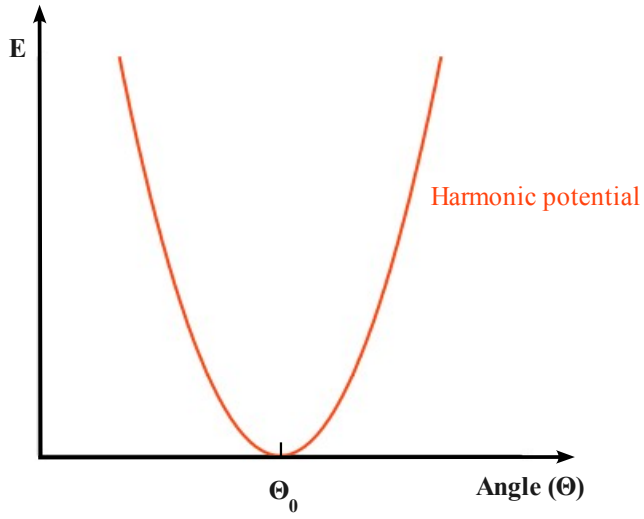


Figure 2.5: *Harmonic potential describing angle bending*

Rotation of dihedral angle

The torsion angle is defined as angle between two planes. In molecule, it corresponds to the rotation around bond and it is determined by four atoms. Together with the non-covalent interactions, this is the most important contribution responsible for structural changes. Therefore, their description has to be as precise as possible.

The torsion potential is commonly written as a series of cosine terms which reflect the periodicity of bond rotation:

$$E_{torsion} = \sum_{n=0}^{N} \frac{V_n}{2}\left[1+\cos(n\omega-\gamma)\right]$$

This expression requires three types of parameters. V_n gives qualitative values of the barrier amplitudes. n is the multiplicity which determines the number of potential minima over 360°. γ is a shift of the cosine curve.

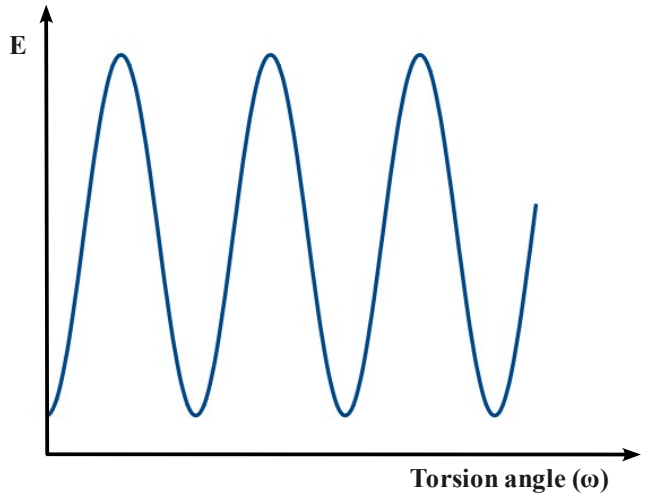


Figure 2.6: *Periodic function describing the torsion angle*

The non-covalent interactions

The non-covalent interactions represent energies between non-bonded atoms. As mentioned above, they significantly contribute to the geometry. They are separated into two contributions – the electrostatic and van der Waals interactions.

The electrostatic interaction is related to the differences in charge distribution. The charges used may be determined experimentally or by quantum calculations. There exist several way how electrostatics may be described. The first and easiest one is to use the partial atomic charges located on atomic nuclei. This conception allows to write the electrostatic interactions by classical Coubomb's expression for charged particles:

$$E_{el} = \sum_{i=1}^{N_a} \sum_{j=1}^{N_b} \frac{q_i q_j}{4\pi\epsilon_0 r_{ij}}$$

Other approaches do not focus only on atomic charges, but also on the influence of the different multipoles – the dipoles, the quadrupoles *etc.* The multipoles are calculated by methods of quantum mechanics or they are obtained from experimental measurement.

The van der Waals interactions are usually given by a Lennard-Jones 6-12 potential:

$$E_{vdW} = \frac{C_1}{R^{12}} - \frac{C_2}{R^6}$$

C_1 and C_2 are constants describing collision distance and minima of curve. The first and second terms are the repulsion and attractive interactions between atoms, respectively. Both depend on the distance between atoms.

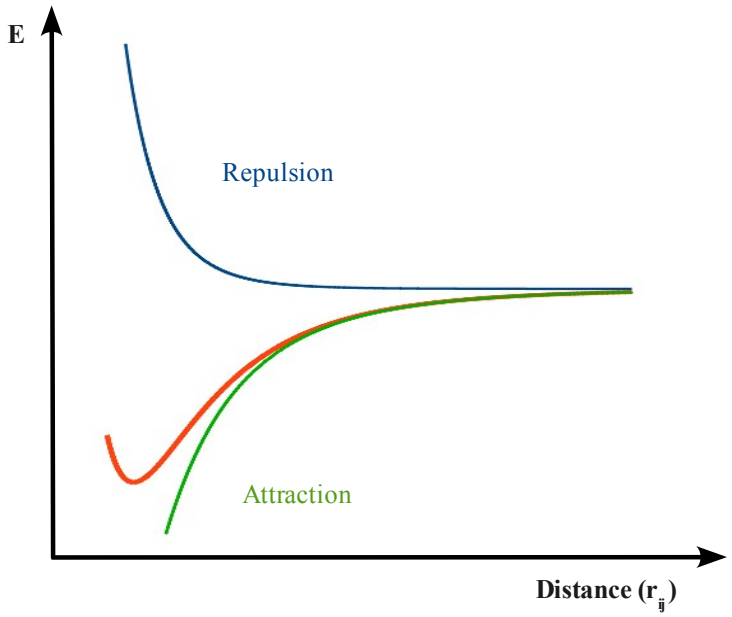


Figure 2.7: *Lennard-Jones 6 -12 potential. The blue curve corresponds to repulsion, the green corresponds to attraction and the orange is sum of both contributions.*

The parameters and the set of terms which are used in the expression of energy are denoted as a force field. Each atom type is thus characterized by specific set of constants. The set of given terms for individual contributions and the sets of constants determined the final geometry and properties of the studied molecular system. The parameters are obtained experimentally or they can be calculated by the correlated *ab initio* methods.

All above mentioned contributions are basically implemented in all the force fields, and the corresponding potential energy is well-approximated. However, for some properties (*e.g.*, vibrational spectra), more accurate potential forms and other contributions are required (*e.g.*, cross terms, $E_{cross,}$ which reflect the coupling between internal coordinates).

Molecular mechanics can treat a wide range of molecular systems from small molecules to big macromolecules. Since each type of system needs a specific set of parameters, many different force fields have been developed. Some of them are dedicated, *e.g.,* AMBER is designed to study proteins or DNA. There also exist universal force fields which can be used for a larger spectrum of molecules but results must be carefully checked.

Some examples of existing force fields are summarized in the following table.

Acronym	Name	Range
AMBER[97][98]	Assisted model building with energy refinement	Organic molecules and biomolecules (proteins or DNA)
CHARMM[99]	Chemistry at Harward macromolecular mechanics	Biomolecules (proteins and DNA)
GROMOS[100]	Groniger molecular simulation	Biomolecules
OPLS[101][102]	Optimized potentials for liquid simulation	Biomolecules and some organic molecules
MMx		Organic molecules
Yeti[103]		Docking of small molecules

For calculation of large macromolecular systems, united atoms are used. Instead of three balls of CH_2 group, only one is used, with a bigger van der Waals radius to include hydrogen atoms. Using united atoms reduces the number of parameters so the calculation is faster. To improve the description of H-bonding, hydrogen atoms are explicitly described on polar atoms.

Advantages and disadvantages of molecular mechanics:

→ Due to the number of atoms, biomolecules (proteins, nucleic acids or membrane) can only be treated by molecular mechanics and the parametrizations are often well adapted.

→ Nonetheless, since the atoms are substituted by balls, many properties associated with the electronic structure of molecules are not described, *e.g.,* mainly reactivity.

→ Another limiting point of MM appears when considering unusual molecules which must be properly re-parametrized

2.3.1. Molecular dynamic simulations

Molecular mechanics allows to predict the geometry with the minimal energy, but molecules are dynamic systems *vs* time. To simulate geometrical reorganization two types of methods exist:

→ Molecular dynamic simulations

→ Monte Carlo simulations

Molecular dynamics (MD) directly simulates the time-dependent behaviour of the molecular systems. From an initial geometry, MD uses the equations of motion to generate many other geometries and their corresponding coordinates, velocities *etc.* (trajectory).

The motion of the system can be described by several ways. The most widely distributed approaches are based on the Newton's second law ($F = ma$), which can be written in its differential form:

$$\frac{-dV}{d\mathbf{r}} = m\frac{d^2\mathbf{r}}{dt^2}$$

where $\frac{-dV}{d\mathbf{r}}$ corresponds to the force acting on the particle of mass m, r is a vector containing the particle coordinates and V is the potential energy of the system.

There exist several models which are treated by the Newton's laws. The most simple (firstly used in 1957) uses hard-spheres. It assumes that spheres move with a constant velocity which changes when a collision with another ball occurs. This collision is considered elastic, *i.e.*, when velocities are recalculated, the principle of conservation of the linear moment has to be taken into account. Usually, this model is not very useful for real systems but it can be used for simulation of fluids.

In real models, the force acting on one particle depends on its position and also on the position of the surrounding particles. In this case, the motion of all particles is coupled and it cannot be calculated by analytical methods. To describe the motion of such systems, the finite difference method is used for the integration of the equations of motion. The principle is to divide the whole simulation into many small time-steps in which the forces acting on particles remain constant. Starting from time *t*, those forces determine the positions and velocities of particles for a following instant ($t+\delta t$). The time interval δt is usually around a few femtoseconds and a whole MD simulation may not be longer than several nanoseconds.

There are several algorithms which perform the integration of the motion equations. All of them describes the dynamical properties and new positions of the system by Taylor series expansion:

$$\boldsymbol{r}(t+\delta t)=\boldsymbol{r}(t)+\delta t\,\boldsymbol{v}(t)+\frac{1}{2}\delta t^2\boldsymbol{a}(t)+\frac{1}{6}\delta t^3\boldsymbol{b}(t)+\ldots$$

$$\boldsymbol{v}(t+\delta t)=\boldsymbol{v}(t)+\delta t\,\boldsymbol{a}(t)+\frac{1}{2}\delta t^2\boldsymbol{b}(t)+\frac{1}{6}\delta t^3\boldsymbol{c}(t)+\ldots$$

$$\boldsymbol{a}(t+\delta t)=\boldsymbol{a}(t)+\delta t\,\boldsymbol{b}(t)+\frac{1}{2}\delta t^2\boldsymbol{c}(t)+\ldots$$

where *r* are the coordinates of the system, *v* is the velocity which is obtained as the first derivative of *r, a* is the acceleration (the second derivative of *r*) and *b* is the third derivative *etc.*

The most common example of the integration methods, in which Taylor series are applied, is the Verlet algorithm,[104] *e.g.*, to describe the position at step ($t + \delta t$), the algorithm requires the acceleration at *t* , *a(t)*, and the positions at $t - \delta t$ and *t:*

$$\left.\begin{aligned}\boldsymbol{r}(t+\delta t)&=\boldsymbol{r}(t)+\delta t\,\boldsymbol{v}(t)+\frac{1}{2}\delta t^2\boldsymbol{a}(t)+\frac{1}{6}\delta t^3\boldsymbol{b}(t)+\ldots\\ \boldsymbol{r}(t-\delta t)&=\boldsymbol{r}(t)-\delta t\,\boldsymbol{v}(t)+\frac{1}{2}\delta t^2\boldsymbol{a}(t)-\frac{1}{6}\delta t^3\boldsymbol{b}(t)+\ldots\end{aligned}\right\}\ \boldsymbol{r}(t+\delta t)=2\boldsymbol{r}(t)-\boldsymbol{r}(t-\delta t)+\delta t^2\boldsymbol{a}(t)+\ldots$$

This approach has two main disadvantages. First, the velocities are not explicitly expressed in equations and must be computed *a posteriori*. Second, the Verlet algorithm is not self-starting since the new position is obtained from two previous positions. So, at $t = 0$, one set of position is missing and must be recalculated from Taylor series. Other algorithms which arise from the Verlet approach try and solve these problems, *e.g.*, the leap-frog algorithms,[105] velocity Verlet[106] *etc.*

As mentioned above, all information (coordinates, velocities …) are contained in the trajectory. The thermodynamic properties of the system can be recalculated from this trajectory as an average value over time:

$$\langle A \rangle = \frac{1}{M} \sum_{1}^{M} A(\boldsymbol{p}^N, \boldsymbol{r}^N)$$

where A is the thermodynamic property described at $\boldsymbol{r}$ and for the momentum $\boldsymbol{p}$ at a given time. M is the number of time steps. Most of processes are running in solution in which interactions between the studied system (solute) and solvent affect the properties of molecules, *e.g.*, their geometry, total energy, charge distribution *etc.* Therefore, the solvent is usually introduced into simulation. There exist two ways – the solvent may be introduced implicitly or explicitly.

Implicit solvent models, also known as continuum solvent models, replace the individual molecules of solvent by a field which is characterized by its dielectric constant. The studied system is located in the cavity which is surrounded by the dielectric continuum. The cavity can have different shapes and sizes. The simplest is a sphere but the cavity may be shape-adapted, *e.g.*, a combination of van der Waals spheres centred on each individual atom. The dielectric field polarizes the molecular system which in turn polarizes the electric field. The resultant polarization is well described by a distribution of punctual charges on the cavity surface. The difference in energy of the solute in vacuum and in solvent is the solvation

energy (ΔG_{sol}). It usually contains three contributions, *i.e.,* the electrostatic component (ΔG_{el}), the van der Waals interaction (ΔG_{vdW}) and the cavity formation (ΔG_{cav}):

$$\Delta G_{sol} = \Delta G_{el} + \Delta G_{vdW} + \Delta G_{cav}$$

A wide variety of the implicit solvent models have been developed. They differ by the construction of the cavity and the expression of the individual contributions. Although these models do not incorporate solvation effects at the atomic level, they usually provide a consistent description of solvation.

Explicit solvent models include the individual molecules of solvent, according to the real density, in the surrounding of the molecular system. The solvent is described by an appropriate force field. The most used solvent is a water for which, due to its specific properties, many models have been proposed (*e.g.*, the TIPnP[107] and the SPC/E[108] models are practically the most applied). Explicit solvent is the most appropriate approach for the description of solute-solvent interactions and is often used in MD simulations. These models are computationally time-consuming since numerous atoms and interactions must be calculated.

To reduce the number of molecules, there are confined in a spatial box shape-adapted. The most typical boxes are cubic, but hexagonal prism and dodecahedron are also used. Simply placing the solute into the box of solvent can lead to artefact coming from different position of solvent molecules. Some molecules are located close to the solute, some are in the solvent bulk and some are located at the limits of the box, *i.e.*, next to vacuum. To be consistent with reality, periodic boundary conditions are considered. In such conditions, the box is replicated in all directions to obtained an infinite system and thus, the particles can virtually move without any boundary limitation. To keep a constant number of particles inside the box, a leaving particle is replaced by another particle which enters at the opposite side (Fig. 2.8).

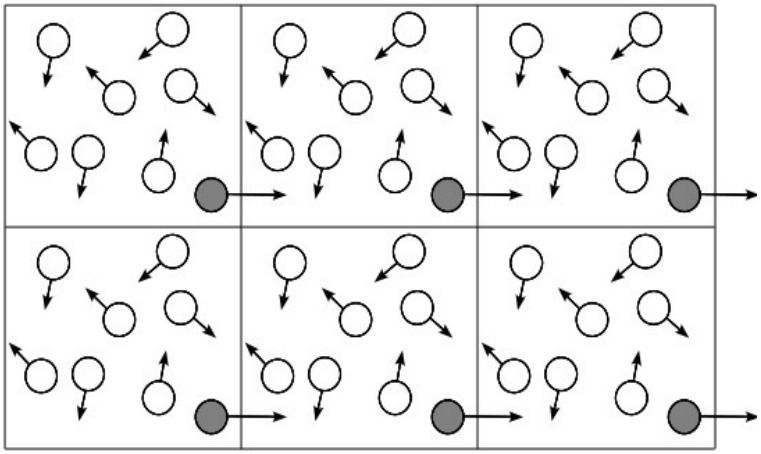


Figure 2.8: *Periodic boundary conditions*

When periodic boundary conditions are applied, the box must verify two main conditions:

→ The total charge inside the box equals zero (to avoid infinite charge). When the total charge is different from zero, counterions are added to compensate.

→ The appropriate box size is defined. In the case of a too small box, interactions between the neighbouring solutes may introduce errors. Besides for too large boxes the computational cost dramatically increases.

For long-range and short-range interactions, when no limits are defined all pairs are taken into account. This also dramatically increase the computational time. To reduce the number of computed interactions a cut-off is usually applied for non-bounded interactions. The energy of interactions between atoms separated by a distance higher than the cut-off equals zero. This is particularly adapted for Lennard-Jones potential, since in this case energy falls down quickly. Nevertheless, for long-range interactions, the definition of a non-adapted cut-off introduces errors.

When the initial geometry is prepared and all parameters are defined, the system must be equilibrated. To avoid abnormal contacts and overlaps between atoms, mainly due to solvation, the initial structure is firstly optimized (using MM). During minimization, *(i)* the solute and solvent adopt a conformation according to polarity and *(ii)* solvent fills up the

empty bubbles in the bulk formed during the solvation. The thermodynamic data obtained from MD simulations are analysed within statistical physics. The thermodynamic properties are described for an ensemble of particles defined under particular physical conditions. Different ensembles exist.

The NVE ensemble (or adiabatic or microcanonical ensemble) describes a system in which the number of molecules (*N*), volume (*V*) and total energy (*E*) are constants. In order to keep the total energy constant, the particles of the system can interact together but not with outward particles. The energy is calculated as the sum of the potential energy, calculated from position, and the kinetic energy, which is determined from velocities. The temperature is estimated from an average of the kinetic energy:

$$\langle E_{kin} \rangle = \frac{3 n k_B T}{2}$$

where n is the number of degrees of freedom and k_B is Boltzmann's constant.

Another system widely used in MD is the NVT (or canonical) ensemble in which number of particles, volume and temperature (T) are constants. These conditions are well-adapted to follow the behaviour of the system *vs.* temperature. These calculations are performed using algorithms allowing temperature control (so-called thermostat algorithms).

The Berendsen thermostat algorithm[109] considers that the system is coupled to an external heat bath (*e.g.*, a source of thermal energy) fixed at a given temperature. The temperature of the system is corrected according to:

$$\frac{dT}{dt} = \frac{T_0 - T}{\tau}$$

where τ is a time constant and T_0 is the reference temperature. This thermostat algorithm does not generate rigorous canonical ensemble, but it is well-adapted for relaxing system.

The thermostat based on external system approach was defined by Nóse and it was modified by Hoover.[110][111] Hoover introduced the heat bath as an integral part of the system. Within the Nosé-Hoover thermostat the Hamiltonian of the system is extended by friction terms. The friction parameter, ξ, is a fully dynamic quantity with its own equation of motion:

$$\frac{d\xi}{dt} = \frac{T - T_0}{Q}$$

where T_0 is the reference temperature and Q determines the coupling between reservoir and the real system, it is so-called the mass parameter. This parameter strongly depends on T_0 and it controls the flow of energy between the heat bath and the system.

Beyond the conditions defined for temperature, the pressure can also be kept constant. The isothermal and isobaric systems correspond to many experiments. MD simulations under these conditions are required to accurately compare the predicted results to experimental data. The system keeps the pressure constant by changing the volume of the simulation box.

As in case of isothermal system, the pressure is controlled by barostats algorithms. Most of them are conceptually analogous to thermostats algorithm. *E.g.,* in the Berendsen barostat, [109] pressure changes as:

$$\frac{d\boldsymbol{P}}{dt} = \frac{(\boldsymbol{P}_0 - \boldsymbol{P})}{\tau_p}$$

where P_0 is the reference pressure. The volume of the box is scaled by λ factor which is given:

$$\lambda = 1 - \kappa \frac{\delta t}{\tau_p}(P - P_0)$$

where κ is the isothermal compressibility determining the volume fluctuation. The new positions of particles are then described:

$$\boldsymbol{r}_i' = \lambda^{1/3} \boldsymbol{r}_i$$

With an optimized system, a short simulation is performed to heat up the system from 0 K to a defined temperature (*e.g.,* 300 K). The temperature and pressure slowly increase and

the system has enough time to relax according to changing in external conditions. To be sure that the equilibrium state is reached, some properties are monitored (temperature and pressure, kinetic and potential energies, volume of box, density). During the heating, both potential and kinetic energies increase. When the required temperature is reached, the kinetic energy is stabilized and the potential energy reflects the relaxation of the system. If no change is recorded in the potential energy surface, the studied system is equilibrated. The remains monitored properties are also stabilized.

To see the changes in structure the root mean square deviation (RMSd) is calculated, from the initial structure. Theoretically, heating causes big changes in conformation, so, the final RMSd is relatively high, but in an equilibrated system the parameters only slightly fluctuate.

When the system is equilibrated the sheer simulation can start.

Chapter 3

Oxidation of polyphenolic compounds and their dimerization

3. Oxidation of polyphenolic compounds and their dimerization

3.1. H-Atom acceptor capacity of free radicals used in antioxidant measurements

(This article was published in International Journal of Quantum Chemistry in 2010

Pavlína Košinová, Florent Di Meo, El Hassane Anouar, Jean-Luc Duroux, Patrick Trouillas

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3.2. Dimerization process of flavonolignans: A theoretical highlighting

(This part correspond to a manuscript under revision for European Journal of Chemical Physics and Physical Chemistry)

3.3. Dimerization process with guaiacol moieties

(This study is a part of an article published in Journal of Physical Chemistry A in 2009)

3.4. Dimerization of phenolic acid

(This study is a part of an article published in Physical Chemistry Chemical Physics in 2009)[47]

3.5. Dimerization of quercetin, Diels-Alder *vs* Radical-coupling-approach: a theoretical study

(This is an article under preparation)

Chapter 4

Interaction between polyphenols and lipid membranes

4. Interaction between polyphenols and lipid membranes

4.1. Molecular interaction between quercetin & quercetin metabolites and lipid bilayer membranes

(This section correspond to collaborative article under preparation with Karel Berka and Michal Otyepka)

4.2. Interaction of argenteane and substituted biphenyl with lipid membrane

Conclusion:

As demonstrated for silybin, dehydrosilybin and quercetin, the dimerization of phenoxyl radicals is divided into two steps. First, the bond between both radicals is formed. The product of this step is a *keto*-form dimer in which the planarity of aromatic rings is broken. This explains, why this step is not thermodynamically favourable. To allow dimerization, this step must be followed by stabilizing step either *(i)* tautomerization during which a *enol*-form of dimer is formed, or *(ii)* formation of ring, as quercetin. If this second step is not allowed, the dimerization reaction is an unfavourable process. This process is useful to synthesize new compounds regioselectively but it may also occur in the organism.

In the second part of our work, we were interested in the interaction of polyphenols with lipid membranes. The influence on the location and orientation of substituents was investigated. This helps to rationalize the difference in the antioxidant activity observed between, *e.g.,* quercetin and its metabolites.

It is known, that flavonoids are effective inhibitors of lipid peroxidation when *(i)* it has the capacity to scavenge free radicals and *(ii)* it is soluble in apolar conditions. The results from our simulations confirmed this and we also added the third condition – appropriate position and orientation inside the membrane.

All the studied polyphenols were located inside the membrane. The orientation and deepness of incorporation are strongly dependent on the type, position and charge of substituents. If the active hydroxyl groups are located close to region of the double bonds (this part is more sensitive to lipid peroxidation damage), the polyphenol will be an effective inhibitor of lipid peroxidation. According to our results, the glucuronide part and sulphate

groups shifted the molecule toward the polar region. Charged groups are always located out of membrane, that is why, it also keeps the rest of molecule close to polar surface.

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Annexes 1

Table A: BDEs of HOO-**H**, CH_3OO-**H** and CH_3CH_2OO-**H** obtained with different methods of calculation in water phase using the 6-31+G(d,p) basis set.

	Methods	BDE (kcal/mol)		
		HOO–H	**CH_3OO–H**	**CH_3CH_2OO–H**
Pure DFT functionals	HCTH	86.2	86.2	85.3
	HCTH147	86.8	86.8	85.9
	HCTH407	86.2	86.2	85.3
	HCTH93	86.2	86.2	85.3
	VSXC	88.3	88.6	88
	SVWN	99.9	100.2	99.5
	BLYP	87.7	87.6	86.7
	BP86	90	90.2	89.3
	B1B95	88.4	88.7	88
Hybride DFT functionals	B1LYP	88.1	88.3	87.5
	B3LYP	89.3	89.4	88.6
	O3LYP	88.9	88.8	87.9
	B3P86	91.7	92	91.2
	B3PWP91	88	88.3	87.5
	B971	87.8	88	87.3
	B972	85.1	85.2	84.5
	B98	88.9	89.1	88.4
	BHandH	93.8	94.9	94.3
	BHandHLYP	88.3	88.9	88.3
	BMK	89.9	90.1	89.5
	MPW1K	87.6	88.2	87.6
	MPW1PW91	87.4	87.8	87
	PBE1PBE	87.4	92.8	87.1
HF	HF	65.5	66.8	66.3
	MP2	91.7	-	92.2
	CCD	89.8	-	90.2
	CCSD	86.2	-	85.9
G-methods	G1	-	-	-
	G2MP2	-	-	-
	G3	-	-	-

Table B: Influence of basis set on BDEs of HOO-**H**, CH_3OO-**H** and CH_3CH_2OO-**H** at the DFT level in water with (3a) B3P86 and (3b) BHandH.

Basis set	BDE (kcal/mol)		
	HOO–H	CH_3OO–H	CH_3CH_2OO–H
3a			
6-31G	87.8	88.5	87.7
6-31G(d)	86.1	87.2	86.6
6-31G(d,p)	89.2	90.3	89.7
6-31+G(d,p)	91.7	92.0	91.2
6-31++G(d,p)	91.1	91.3	90.6
6-311G	87.5	88.1	87.4
6-311G(d)	85.6	86.6	86.0
6-311G(d,p)	89.5	90.4	89.8
6-311+G(d,p)	91.2	91.4	90.6
6-311++G(d,p)	91.2	91.3	90.6
6-311+G(d,2p)	91.9	92.0	91.2
6-311++G(d,2p)	91.9	91.9	91.2
6-311+G(2d,3p)	91.7	91.7	91.0
6-311++G(2d,3p)	91.6	91.7	91.0
6-311+G(2d,3pd)	91.9	92.1	91.3
6-311++G(2d,3pd)	92.0	92.0	91.3
3b			
6-31G	90.2	91.6	91.0
6-31G(d)	88.1	89.9	89.5
6-31G(d,p)	91.4	93.2	92.8
6-31+G(d,p)	93.8	94.9	94.3
6-31++G(d,p)	93.1	94.1	93.5
6-311G	89.5	90.9	90.4
6-311G(d)	87.4	89.1	88.7
6-311G(d,p)	91.5	93.0	92.7
6-311+G(d,p)	93.1	94.0	93.4
6-311++G(d,p)	93.0	93.9	93.4
6-311+G(d,2p)	93.8	94.6	94.0
6-311++G(d,2p)	93.7	94.5	94.0
6-311+G(2d,3p)	93.5	94.3	93.8
6-311++G(2d,3p)	93.5	94.3	93.8
6-311+G(2d,3pd)	93.8	94.7	94.2
6-311++G(2d,3pd)	93.9	94.7	94.2

Annexes 2

Influence of the prototypes on conformation

In the most stable conformers of ***[C20-O-C21']$_{proto}$*** and ***[C21-C21']$_{proto,}$*** the values of the relevant dihedral angles were τ1 (C21-C20-O-C21') = -14°, τ2 (C20-O-C21'-C20') = -72° and τ3 (C20-C21-C21'-C20') = 136°. As expected, the two aromatic rings were strongly tilted with respect to each other, as a result of steric hindrance. The effect of solvent on geometries was very small except for those three torsion angles which were slightly shifted to 0°, -87° and 128°, respectively. The geometries obtained for the ***[C20-O-C21']$_{proto}$*** and ***[C21-C21']$_{proto}$*** were used to build the silybin intermediate-prototype dimers (***[C20-O-C21']$_{inter}$*** and ***[C21-C21']$_{inter}$***) and the whole structures (C20-O-C21' and C21-C21'). Due to steric hindrance and H-bonding, the torsion angles were modified after optimisation (see the *"Influence of silybin-prototype size on dimerization thermodynamics"* section, later in text, for a detailed description).

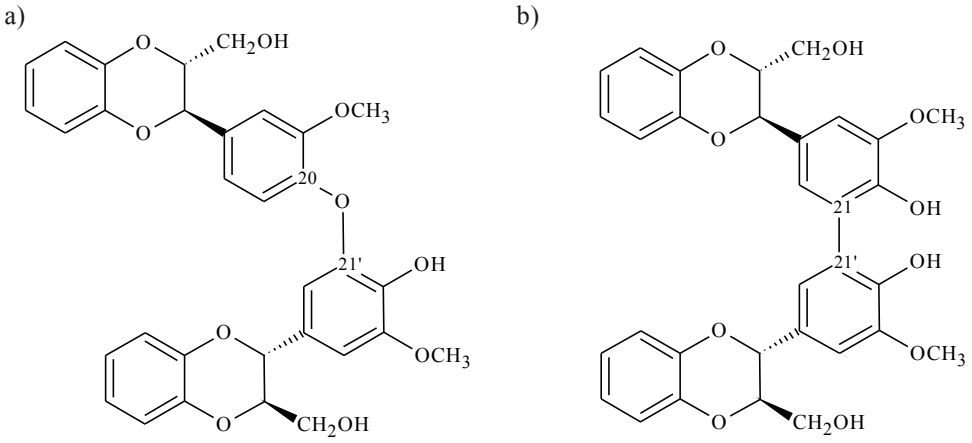

Figure A: *Structures of (a)* ***[C20-O-C21']$_{inter}$*** *and (b)* ***[C21-C21']$_{inter}$***

Influence of silybin-prototype size on dimerization thermodynamics

The influence of system size and substitution on geometries, ΔH, ΔG was examined first with the ***[C20-O-C21']$_{inter}$*** and ***[C21-C21']$_{inter}$*** (Fig. A) and then with the structures ***C20-O-C21'*** and ***C21-C21'*** (Fig. 3.7). ΔH and ΔG were significantly influenced when increasing system size (Table A). Nevertheless, the hierarchy in terms of stability between the different dimers was not influenced compared to the ***[C20-O-C21']$_{proto}$*** and ***[C21-C21']$_{proto}$***. As a matter of fact the ***C21-C21'*** dimers were preferentially formed, which was in good agreement with the experimental observations.

Table A: Thermodynamics for the dimerization of silybin intermediate-prototypes. ΔH and ΔG (kcal/mol) are the differences in enthalpy and Gibbs free energy, respectively between reactants and *keto*- or *enol*- form dimers. Calculations were performed in the gas phase (B3P86/6-31+G(d,p)//B3P86/6-31G(d)).

	***Keto*-form**		***Enol*-form**	
	ΔH	**ΔG**	**ΔH**	**ΔG**
[C20-O-C21']$_{inter}$	-14.9	0.6	-38.7	-23.3
[C21-C21']$_{inter}$	-0.6	11.0	-46.8	-34.6

The differences observed between the prototypes and the whole system essentially come from competition between intra-molecular stabilizing interactions (mainly H-bonds), π-electron conjugation and steric hindrance; the larger the structure, the higher the possibilities. It must be stressed that the whole ***C20-O-C21'*** and ***C21-C21'*** were not totally conjugated (*i.e.*, HOMO is not delocalized over the entire molecule). This makes the whole system *a priori* relatively less stable than the small prototypes (***[C20-O-C21']$_{proto}$*** and ***[C21-C21']$_{proto}$***), which are totally conjugated. Besides intra-H bonding may stabilize the system.

As an example ***C21-C21'*** showed a double H-bonding interaction, allowing the formation of a stabilized pseudo-ring (Fig. 3.9). The ***C21-C21'*** *axial* compound was favoured

as compared to ***C21-C21'*** *equatorial*. As can be seen in Figure 3.9, the latter structure is destabilized by the steric repulsion between the two O-atoms of the E-rings. This repulsion induced a change in the torsion angle between the two E-rings, from 140° in ***C21-C21'*** *axial* to 60° in ***C21-C21'*** *equatorial*. Absent in ***C21-C21'*** *axial*, this repulsive interaction appeared after a molecular re-arrangement in ***C21-C21'*** *equatorial* that is required to form the stabilized pseudo-ring. For ***C21-C21'*** *equatorial*, the steric interaction slightly counteracted the stabilizing effect of the H-bonds, by about 3.5 kcal/mol in the solvent.

Concerning dimers formed by a C-O-C bond, ***C20-O-C21'*** *equatorial* was clearly favoured as compared to ***C20-O-C21'*** *axial.* Again, this comes from a stabilizing intra-H bonding that can be observed in ***C20-O-C21'*** *equatorial*. The ***C20-O-C21'*** *axial* structure was relatively linear thus avoiding any intramolecular interactions, especially H-bonds.

Annexes 3

Table A: Kinetics ($\Delta G^{\#}$ in kcal/mol) of the dimerization process by a) the Diels-Alder (DA) mechanism, b) the radical-coupling-approach (RCA) (second steps) at DFT level MPWB1K/6-31+G(d,p)//MPWB1K/6-31G(d).

a)

	Non-polar		**Polar**	
	$\Delta G_{DA}^{\#}$	**k**	**$\Delta G_{DA}^{\#}$**	**k**
C2-O-C4'*/C3-O-C3'*	37.9	$2.5x10^{-17}$	40.7	$9.1x10^{-18}$
C2-O-C3'*/C3-O-C4'*	36.6	$2.1x10^{-16}$	40.4	$1.4x10^{-17}$

b)

	Non-polar		**Polar**	
	$\Delta G2_{RCA}^{\#}$	k	**$\Delta G2_{RCA}^{\#}$**	**k**
C2-O-C4'*/C3-O-C3'*	34.9	$3.5x10^{-15}$	35.5	$5.1x10^{-14}$

Table B: Kinetics ($\Delta G^{\#}$ in kcal/mol) of the dimerization process by a) the Diels-Alder (DA) mechanism, b) the radical-coupling-approach (RCA) (second steps) for prototypes and whole dimers at DFT level B3P86/6-31+G(d,p)//B3P86/6-31G(d).

a)

	Non-polar		**Polar**	
	$\Delta G_{DA}^{\#}$	**k**	**$\Delta G_{DA}^{\#}$**	**k**
C2-O-C4'*/C3-O-C3'*$_{(proto)}$	32.7	$1.6x10^{-13}$	38.3	$5.1x10^{-16}$
C2-O-C3'*/C3-O-C4'*$_{(proto)}$	32.7	$1.6x10^{-13}$	38.4	$3.8x10^{-16}$
C2-O-C4'*/C3-O-C3'*	29.6	$3.1x10^{-11}$	-	-
C2-O-C3'*/C3-O-C4'*	30	$1.4x10^{-11}$	35.3	$7.9x10^{-14}$

b)

	Non-polar		Polar	
	$\Delta G2_{RCA}^{\#}$	k	**$\Delta G2_{RCA}^{\#}$**	**k**
C2-O-C4'*/C3-O-C3'*$_{(proto)}$	30.9	3.2×10^{-12}	32.7	6.0×10^{-12}
C2-O-C4'*/C3-O-C3'*	30.2	1.1×10^{-11}	-	-

Annexes 4

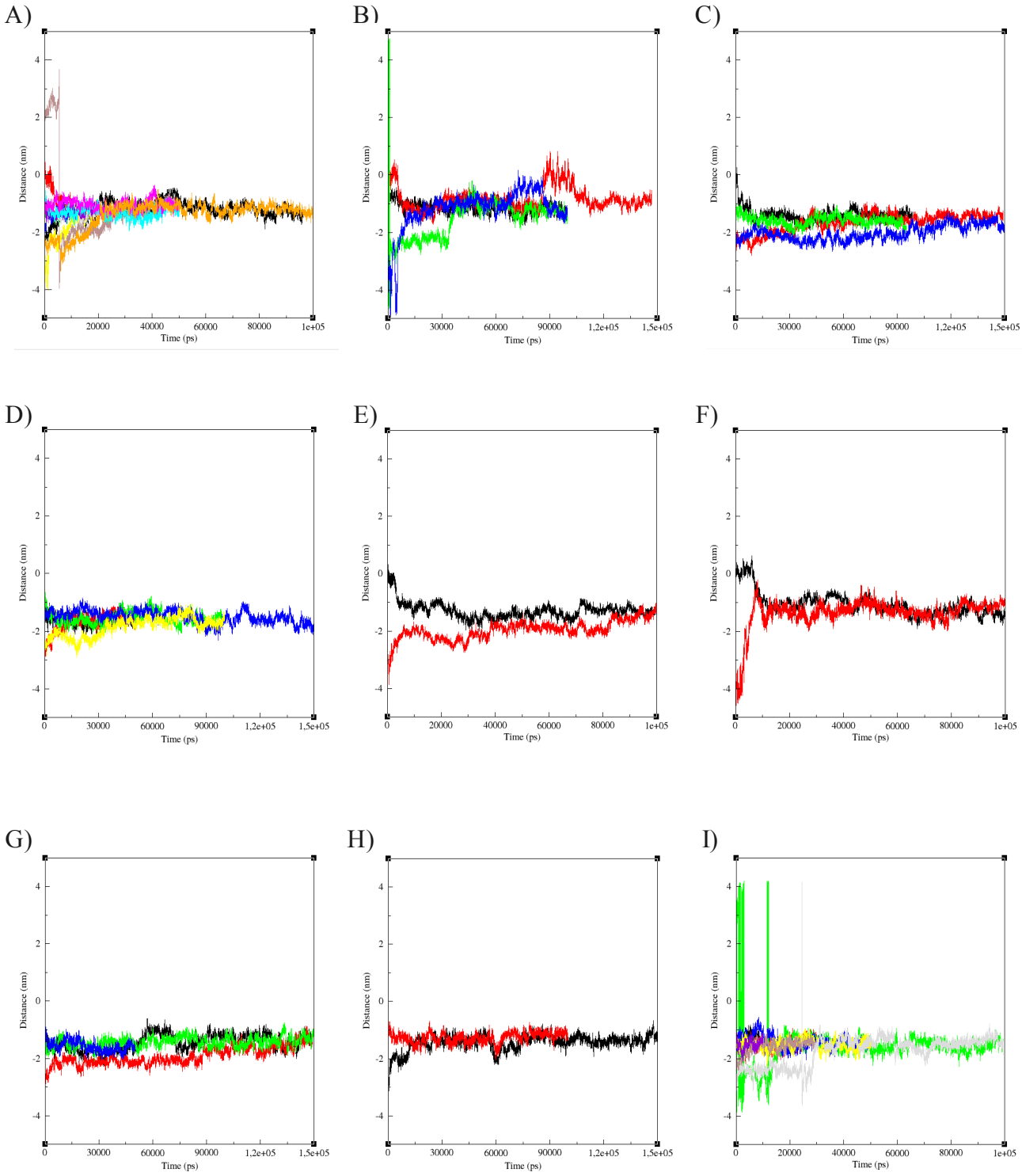

Figure B: *Position of the centre of mass (COM) along the MD simulation from different initial positions for (**A**) quercetin, (**B**) 3'-O-methyl-quercetin, (**C**) protonated form of 3-quercetin-sulphate, (**D**) deprotonated form of 3-quercetin-sulphate, (**E**) protonated form of 3'-quercetin-sulphate, (**F**), protonated form of quercetin-3-glucoronide (**G**), deprotonated form of quercetin-3- glucoronide(**H**) protonated form of caffeic acid and (**I**) deprotonated form of caffeic acid. The position of the COM always converged into the same place.*