Chapter 1

The Physical Chemistry of Polyphenols: Insights into the Activity of Polyphenols in Humans at the Molecular Level

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Abstract: This chapter reviews the following versatile physicochemical properties of polyphenols in relation with their potential activity in humans:

1) Interactions with proteins and lipid–water interfaces. These interactions must be qualified with respect to the current knowledge on polyphenol bioavailability and metabolism. They are expected to mediate most of the cell signaling activity of polyphenols.
2) A general reducing capacity that may be expressed in the gastrointestinal tract submitted to postprandial oxidative stress and also in cells, for example, by direct scavenging of reactive oxygen species, especially if preliminary deconjugation of metabolites takes place
3) The complex relationships with transition metal ions involving binding and/or electron transfer in close connection with the antioxidant versus pro-oxidant activity of polyphenols

Keywords: polyphenol, flavonoid, Health effectsbiological activity, mechanism, antioxidant, protein, membrane, metal ion, gastrointestinal tract, DFT methods.

1.1 Introduction

The activity, functions, and structural diversity of polyphenols in plants, food, and humans reflect the remarkable diversity of their physicochemical properties: UV–visible absorption, electron donation, affinity for metal ions, propensity to develop molecular interactions (van der Waals, hydrogen bonding) with proteins and lipid–water interfaces, and

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nucleophilicity. This chapter aims to exemplify how polyphenols act to promote health in humans at the molecular level. It rests on two common assumptions based on epidemiological evidence and food analysis (Manach *et al*., 2005; Crozier *et al*., 2010; Del Rio *et al*., 2013):

- The consumption of fruit and vegetables helps prevent chronic diseases and, in particular, favors cardiovascular health.
- Phenolic compounds, from the simple hydroxybenzoic and hydroxycinnamic acids to the complex condensed and hydrolyzable tannins, constitute the most abundant class of plant secondary metabolites in our diet and take part in this protection.

By contributing to the sensorial properties of food, for example, color and astringency, native polyphenols and their derivatives obtained after technological and domestic processing can directly influence the consumer's choice. Moreover, polyphenols undergo only minimal enzymatic conversion in the oral cavity and in the gastric compartment although their release from the food matrix (bioaccessibility) is an important issue. Thus, intact food polyphenols may directly promote health benefits in the upper digestive tract, in particular by fighting postprandial oxidative stress resulting from an unbalanced diet (Sies *et al*., 2005; Kanner *et al*., 2012). Beyond the gastric compartment, polyphenol bioavailability[1] (Fig. 1.1) must be considered as a priority to tackle any biological effects (Manach *et al*., 2005; Crozier *et al*., 2010; Del Rio *et al*., 2013). Indeed, even for polyphenols that can be partially absorbed in the upper intestinal tract (aglycones, glucosides), most of the dietary intake reaches the colon where extensive catabolism by the microbiota takes place: hydrolysis of glycosidic and ester bonds, release of flavanol monomers from proanthocyanidins, hydrogenation of the C═C double bond of hydroxycinnamic acids, deoxygenation of aromatic rings, cleavage of the central heterocycle of flavonoids, and so on. Conjugation of polyphenols and their bacterial metabolites in intestinal and liver cells eventually results in a complex mixture of circulating polyphenol *O*-β-D-glucuronides and *O*-sulfo forms (less rigorously called sulfates). When present, catechol groups are also partially methylated.

The concentration of circulating polyphenols is usually evaluated after treatment by a mixture of glucuronidases and sulfatases that release the aglycones and their *O*-methyl ethers. This concentration is usually quite low (barely higher than 0.1 μM) and much lower than that of typical plasma antioxidants such as ascorbate (> 30 μM). At first sight, this does not argue in favor of nonspecific biological effects, such as the antioxidant activity by radical scavenging or chelation of transition metal ions to form inert complexes. This seems all the more true that the catechol group, displayed by many common dietary polyphenols and which is a critical determinant of the electron-donating and metal-binding capacities, is generally either absent in the circulating metabolites (bacterial deoxygenation) or at least

[1]Bioavailability: the fraction of ingested polyphenol (native form + metabolites) that enters the general blood circulation and is thus potentially available for health effects.

Bioaccessibility: the first step of bioavailability, the fraction of ingested polyphenol (native form + metabolites) that is released from the food matrix and is thus potentially available for intestinal absorption.

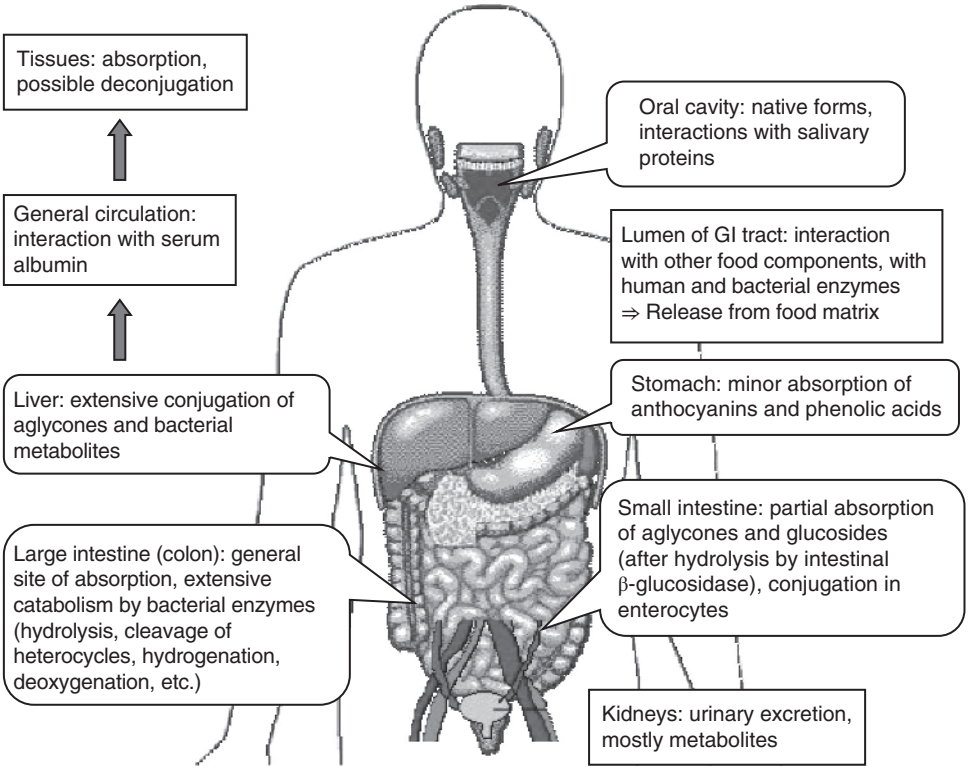

Fig. 1.1 A simplified view of polyphenol bioavailability. (*See insert for color representation of the figure*)

partially conjugated. However, the claim that *in vivo* polyphenol concentrations are low should be nuanced for the following reasons:

1) The complete assessment of polyphenol bioavailability must include the bacterial catabolites and their conjugates, some being much more abundant in the circulation than the parent phenol. A spectacular example can be found in the case of anthocyanins. Indeed, after consumption of blood orange juice, the total amount of native cyanidin 3-*O*-β-D-glucoside (C3G) in plasma is 0.02% of the ingested dose versus 44% for (unconjugated) protocatechuic acid (PCA), its main catabolite (Vitaglione *et al*., 2007). When the fecal content is also taken into account, PCA eventually represents ca. 73% of the metabolic fate of ingested C3G. Its absence in urine (unlike C3G) also suggests that it takes part in the antioxidant protection and is thus oxidized in tissues.
2) The circulating concentration and its time dependence say nothing concerning either the possibility of polyphenol metabolites accumulation at a much higher local concentration at specific sites of inflammation and oxidative stress or their deconjugation into more active forms.

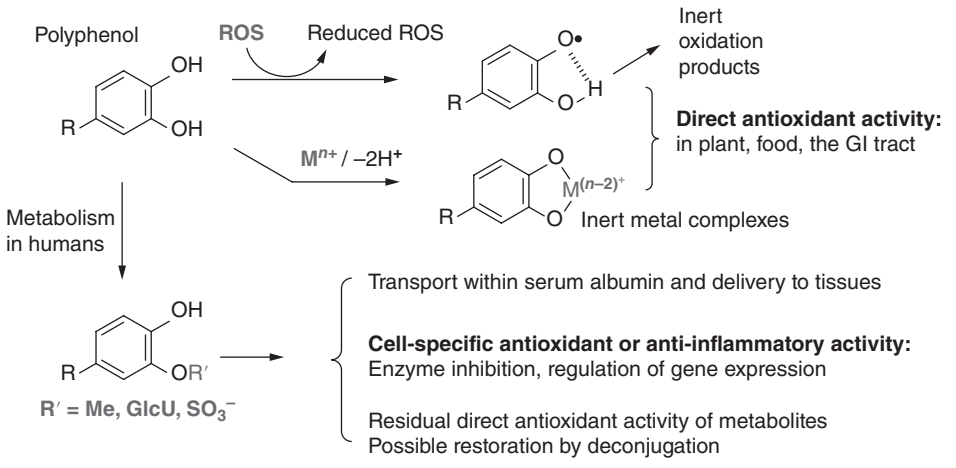

Fig. 1.2 Health effects expressed by polyphenols.

For instance, when quercetin is continuously perfused through the vascular wall of arteries, it rapidly undergoes oxidative degradation into PCA, whereas the fraction retained in the wall is much more stable and partially methylated (Menendez *et al*., 2011). By contrast, quercetin 3-*O*-β-D-glucuronide (Q3G), the main circulating metabolite, is not oxidized upon perfusion but slowly converted into quercetin. The kinetics of quercetin release parallels the inhibition in the contractile response of the artery. Thus, the biological effect can be ascribed to quercetin released from its glucuronide, which basically appears as a stable storage form. A schematic view for the bioactivity of polyphenols is summed up in Fig. 1.2.

1.2 Molecular complexation of polyphenols

The phenolic nucleus can be regarded as a benchmark chemical group for molecular interactions as it combines an acidic OH group liable to develop hydrogen bonds (both as a donor and as an acceptor) and an aromatic nucleus for dispersion interactions (the stabilizing component of van der Waals interactions).

1.2.1 Polyphenol–protein binding

Polyphenol–protein binding of nutritional relevance can be classified as follows:

- Binding processes within the gastrointestinal (GI) tract, that is, with food proteins, mucins, and the digestive enzymes, with an impact on the bioaccessibility of polyphenols and the digestibility of macronutrients
  - Interactions with plasma proteins, with an impact on transport and the rate of clearance from the general circulation
  - Interactions with specific cell proteins (enzymes, receptors, transcription factors, etc.) that would mediate the nonredox health effects of polyphenols

As the last two situations lie downstream the intestinal absorption and passage through the liver, they concern the circulating polyphenol metabolites. However, some exceptions may be found. For instance, epigallocatechin 3-*O*-gallate (EGCG), the major green tea flavanol, is a rare example of a polyphenol entering the blood circulation mostly in its initial (nonconjugated) form (Manach *et al*., 2005). No less remarkable, EGCG is also one of the rare polyphenols for which a specific receptor has been identified, namely the 67-kDa laminin receptor (67LR) that is expressed on the surface of various tumor cells (Umeda *et al*., 2008). EGCG-67LR binding leads to myosin phosphatase activation and actin cytoskeleton rearrangement, thus inhibiting cell growth. It provides a strong basis for interpreting the *in vivo* anticancer activity of EGCG and its anti-inflammatory activity in endothelial cells (Byun *et al*., 2014).

It is not the authors' purpose to provide the reader with an exhaustive updated report on polyphenol–protein binding processes (see Dangles and Dufour (2008) for a specific review on this topic). Only a few recent important examples will be discussed with an emphasis on works dealing with polyphenol metabolites.

1.2.1.1 Interactions in the digestive tract

In the postprandial phase, black tea drinking leads to vasorelaxation as evidenced by flow-mediated dilation experiments in humans and a strong increase in the activity of endothelial nitric oxide synthase (eNOS) (Lorenz *et al*., 2007). However, these effects are completely abolished when 10% milk is added to black tea. Experiments with isolated fractions of milk proteins show that caseins are actually responsible for this inhibition. It can thus be proposed that caseins bind and probably precipitate black tea polyphenols in the GI tract, thereby preventing their intestinal absorption. This is a spectacular example of how food proteins may sequester oligomeric polyphenols and cancel their bioaccessibility and downstream biological effects.

The binding between dietary polyphenols and the digestive enzymes is best evidenced with large polyphenols such as oligomeric proanthocyanidins (OPAs). For instance, OPAs inhibit pancreatic elastase, a serine protease, proportionally to their mean degree of polymerization (Bras *et al*., 2010). A K_i value of ca. 0.5 mM was estimated for a catechin tetramer. However, a mixture of *n*-mers ($n = 2$–6) rich in 3-*O*-galloyl flavanol units binds much more tightly ($K_i \approx 14\,\mu M$). Similar data were obtained with trypsin (Goncalves *et al*., 2007). By slowing down the digestion, such interactions could prolong the sensation of satiety and help fight weight gain and obesity. By contrast, simple phenols were shown to mildly enhance pepsin activity at pH 2 in the following order: resveratrol ≥ quercetin > EGCG > catechin (Tagliazucchi *et al*., 2005). Tannins are known to inhibit pancreatic lipase (McDougall *et al*., 2009), thereby possibly contributing to lowering fat intake. Polyphenol-rich berry extracts also inhibit pancreatic α-amylase (thus decreasing starch digestibility) and intestinal α-glucosidase, with tannins and anthocyanins being, respectively, the main contributors to the observed inhibition (McDougall *et al*., 2005). These mild inhibitory effects could help regulate the circulating D-glucose concentration.

1.2.1.2 Interactions beyond intestinal absorption

In the circulating blood, polyphenol metabolites likely travel in association with serum albumin, the most abundant plasma protein, which displays several binding sites for the transport of drugs, free fatty acids, and other nutrients. Our recent work (Khan *et al*., 2011) has shown that flavanone glucuronides (conjugation at the A- or B-ring) are moderate serum albumin ligands ($K_b = 3–6 \times 10^4\ M^{-1}$) that bind site 2 (subdomain IIIA), in contrast to the more planar flavones and flavonols, which bind site 1 (subdomain IIA).

Once delivered to tissues, polyphenol metabolites are expected to bind specific cell proteins to express their biological effects, in particular their well-documented anti-inflammatory activity (Pan *et al*., 2010; Spencer *et al*., 2012; Wu & Schauss, 2012). Inflammation is an adaptive response to deleterious stimuli, activating the immune system. What is at stake with dietary polyphenols is the inhibition of chronic low-grade inflammation (in contrast to acute inflammation following microbial infection) associated with the development of degenerative diseases, such as type 2 diabetes and cardiovascular disease. Indeed, this pathological state is deeply influenced by lifestyle and environmental factors, especially dietary habits.

At the cell level, inflammation involves complex signaling pathways and cascades (Fig. 1.3). In particular, mitogen-activated protein kinases (MAPKs, e.g., ERK, JNK, and

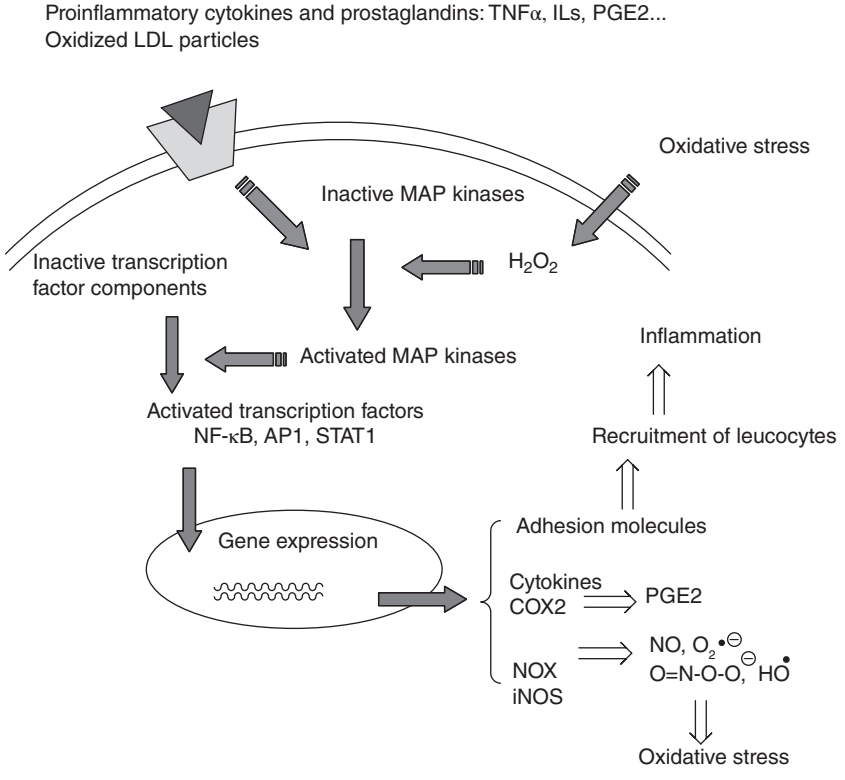

Fig. 1.3 Pathways of inflammation and oxidative stress in cells. Kinases, proinflammatory transcription factors, and pro-oxidant enzymes are possible target proteins for polyphenols and their metabolites.

p38) are important in the transduction of extracellular signals into cellular responses. When activated by oxidative stress or proinflammatory eicosanoids (prostaglandins, leukotrienes) and cytokines (e.g., TNFα, interleukins, and C-reactive protein), MAPKs phosphorylate both cytosolic and nuclear target proteins resulting in the assembly and translocation of transcription factors such as NF-κB, STAT1, and AP1. By upregulating the expression of inducible NO synthase (iNOS), cycloxygenase-2 (COX2), NADPH oxidase (NOX), cell adhesion molecules, cytokines, and cytokine receptors, these transcription factors trigger cell damage, inflammation, or apoptosis. MAPKs and the subsequently activated transcription factors (or their cytosolic components) are all potential targets of polyphenols and their metabolites, which rationalize their anti-inflammatory action. However, such mechanisms are subtle and not easy to track down to the highest level of resolution, that is, polyphenols interacting with specific proteins.

An additional difficulty also stems from the complex interplay between inflammation and oxidative stress. For instance, activated leucocytes (macrophages) produce reactive oxygen species (ROS) via the activity of NOX and iNOS. Conversely, NF-κB can be directly activated by ROS (Gloire *et al*., 2006). Indeed, H_2O_2 is known to inhibit Tyr phosphatases via oxidation of Cys residues in the catalytic domain, thereby triggering Tyr kinase activity and downstream signaling. Thus, the overall biological effects of polyphenols in cells may be a complex combination of anti-inflammatory and antioxidant activities.

The anti-inflammatory activity of polyphenols can develop through the following:

- The inhibition of the cycloxygenase (COX) and lipoxygenase (LOX) enzymes responsible for the production of the inflammatory mediators prostaglandins and leukotrienes from arachidonic acid, respectively
- The downregulation of proinflammatory genes

A few recent examples are reported as follows with an emphasis on the possible activity of polyphenol metabolites.

Among the two main circulating quercetin metabolites, namely quercetin 3-*O*-β-D-glucuronide (Q3G) and 3′-*O*-sulfoquercetin, only the latter is a potent 5-LOX inhibitor in activated monocytes reducing accumulation of LTB_4 by ca. 50% at 2 μM (Loke *et al*., 2008a). Unlike quercetin and 3′-*O*-methylquercetin, both metabolites were ineffective at inhibiting PGE_2 production. By contrast, with its free electron-rich catechol nucleus, Q3G is a much better inhibitor of LDL (low-density lipoprotein) peroxidation than 3′-*O*-sulfoquercetin.

NF-κB and STAT1 are important transcription factors for iNOS expression in macrophages. A structure–activity relationship with a series of flavonoid aglycones (Hamalainen *et al*., 2007) has shown that the inhibition of iNOS expression and NO production in activated macrophages is due to the inhibition of the nuclear translocation of either the sole transcription factor NF-κB (flavone, the flavanone naringenin, 3′-*O*-methylquercetin) or both NF-κB and STAT1 (the flavonols kaempferol and quercetin, the isoflavones genistein and daidzein). However, the inhibition is modest at low flavonoid concentration (10 μM) and abolished with the corresponding flavonoid glycosides. It is thus doubtful that the main

flavonoid circulating metabolites, that is, glucuronides, could exert a substantial anti-inflammatory activity via this mechanism, unless preliminary deconjugation takes place. A similar study in mouse microglia cells failed to demonstrate the anti-inflammatory activity of 3′-*O*-sulfoquercetin (Chen *et al*., 2005). More encouraging is a recent investigation dealing with the porcine isolated coronary artery instead of cultured cells (Al-Shalmani *et al*., 2011). In this study, it was shown that the lipopolysaccharide-induced alteration of the contractile response was significantly inhibited by low quercetin concentrations (0.1 μM) and higher concentrations (10 μM) of 3′-*O*-sulfoquercetin and Q3G. Moreover, NO production and iNOS expression were reduced. As the protection of the contractile response was abolished by an NF-κB inhibitor and persisted in endothelium-denuded segments, it can be proposed that quercetin and its metabolites act by inhibiting the NF-κB pathway in the vasculature, possibly by stabilizing the complex combining NF-κB and its cytosolic repressor IkB.

A direct binding between polyphenols and NF-κB proteins was suggested from experiments showing that procyanidin dimers B1 (epicatechin-β-4,8-catechin) and B2 (epicatechin-β-4,8-epicatechin), but not the more rigid A1 and A2, actually inhibit NF-κB-DNA binding (Mackenzie *et al*., 2009; Fraga *et al*., 2010). Docking experiments support a binding mode involving H-bonding between three phenolic OH groups of the dimers (the C3′-OH and C4′-OH of the terminal unit + the C7-OH of the extension unit) and two NF-κB Arg residues.

The anti-inflammatory activity of flavonoid metabolites in endothelial cells could also be mediated by their ability to inhibit the MAPK pathway. For instance, high D-glucose concentration is known to induce oxidative stress (evidenced by elevated H_2O_2 concentration) and subsequent activation of NOX and c-JUN N-terminal protein kinase (JNK) and caspase-3, which ultimately leads to apoptosis. Interestingly, D-glucose-induced JNK and caspase-3 activation and oxidative stress in endothelial cells are efficiently inhibited by physiological concentration (0.3 μM) of 3′-*O*-sulfoquercetin and Q3G (Chao *et al*., 2009).

Finally, the flavanone metabolites showing conjugation at the B-ring, namely 3′-*O*-sulfohesperetin, hesperetin 3′-*O*-β-D-glucuronide, and naringenin 4′-*O*-β-D-glucuronide, were also demonstrated to inhibit the adhesion of monocytes to TNFα-activated endothelial cells (ca. −20% at 2 μM) (Chanet *et al*., 2013). Gene expression analysis suggests that the protection involves the downregulation of genes coding for NF-κB, cell adhesion molecules, and cytoskeleton proteins.

Inhibition of pro-oxidant enzymes is also a mechanism for polyphenol metabolites to fight oxidative stress in cells. As an example, Q3G is a potent inhibitor of myeloperoxidase, which is secreted by neutrophils and macrophages at a site of inflammation and may be involved in LDL oxidation (Loke *et al*., 2008b; Shiba *et al*., 2008). Docking experiments suggest binding to a hydrophobic region of the enzyme with the B-ring pointing to the heme pocket.

Epicatechin glucuronides are even more potent than epicatechin at inhibiting NOX activity in stimulated endothelial cells (Steffen *et al*., 2008). Experiments with disintegrated cells showed that unlike epicatechin (which simply scavenges superoxide), the glucuronides are true NOX inhibitors (IC50 ≈ 5 μM). Similar observations were made with quercetin and its glucuronides.

1.2.2 Interactions with membranes

There is growing evidence that interaction of phenolic compounds with biomembranes is important to rationalize their beneficial effects and toxicity. Nowadays, experimental techniques tackling this issue (fluorescence spectroscopy and microscopy, solid-state NMR, surface plasmon resonance, atomic force microscopy, Langmuir–Blodgett trough) are elegantly supported by molecular dynamics simulations for a detailed description of the different aspects of polyphenol–membrane interaction (penetration, partitioning, positioning, crossing).

As a first approach, the partition coefficient (log*P*) of flavonoid aglycones, which measures the relative lipophilicity (e.g., flavones are more lipophilic than the corresponding flavanones), was shown to correlate with their antioxidant capacity to protect membranes (Saija *et al*., 1995). Nevertheless, log*P* does not reliably describe the amphiphilic character of polyphenols, a property that is of crucial importance to rationalize their membrane penetration and location.

Experimental (Hendrich *et al*., 2002; Ollila *et al*., 2002; Oteiza *et al*., 2005) and theoretical (Sinha *et al*., 2011; Kosinova *et al*., 2012) works have shown that many flavonoids (flavonols, flavones, flavanones, flavan-3-ols, isoflavonoids) can penetrate lipid bilayers and preferentially lie in the polar head-group region rather than being deeply buried within the lipid chains. The driving forces of interaction and penetration arise from the amphiphilic character of polyphenols. Aromatic rings provide the hydrophobic character for interactions with lipid chains while the phenolic OH groups mainly act as hydrogen bond donors to the polar head groups of phospholipids. Such intermolecular hydrogen bonds tend to maintain polyphenols just below membrane surface, thus slowing down membrane crossing (passive diffusion).

The importance of planarity has been suggested by comparing the capacity of various phenolic compounds to penetrate lipid bilayers (Areias *et al*., 2001; Lopez *et al*., 2014). However, this must be nuanced with flavonoids, as the torsion between the C- and B-rings is rather flexible. Indeed, catechin derivatives, which are nonplanar, penetrate membranes and lie at a similar location as quercetin derivatives. By favoring multiple H-bonding, 3-*O*-galloylation of catechins enhances membrane affinity but favors a more superficial contact (Sirk *et al*., 2008). Indeed, EGCG strongly binds through its B- and galloyl rings to the phosphodiester O-atoms and remains adsorbed on the bilayer surface. By contrast, EC mainly binds through its A-ring to the acyl O-atoms and is thus absorbed more deeply in the membrane.

The flavonolignan silybin locates at the interface of microsomal bilayers (Parasassi *et al*., 1984) as well as genistein and daidzein (Raghunathan *et al*., 2012), the former isoflavonoid being slightly more buried than the latter in agreement with its slightly higher lipophilicity (log*P* = 3.04 and 2.51, respectively). Interestingly, the lipophilic stilbenoid resveratrol (at physiological concentrations) appears more buried than most flavonoids and was shown to intercalate between phospholipid chains (Brittes *et al*., 2010; Olas & Holmsen, 2012). However, the resveratrol–membrane interactions depend on lipids (length of acyl chains, degree of unsaturation, nature of the head group). As another example, the relatively hydrophobic gallotannin 1,2,3,4,5-penta-*O*-galloyl-β-D-glucopyranose (log*P* = 2.0) inserts

more deeply into a lipid bilayer than the similar sized but much more hydrophilic catechin-α-4,8-catechin-α-4,8-catechin (log*P* = –0.92) (Yu *et al*., 2011).

Polyphenol penetration into membranes appears pH-dependent. For instance, quercetin displays pK_a values of 5.7, 7.1, 8.0 in water, corresponding to the three most acidic groups, namely C7-OH, C4′-OH, and C3-OH, respectively. At low pH, quercetin has a better capacity to penetrate lipid bilayers, whereas at neutral or basic pH, it locates closer to the polar domains, because of the repulsion between negative charges at the interface. Here, the experimental evidence (Movileanu *et al*., 2000) agrees with molecular dynamics simulations (Kosinova *et al.*, 2012).

Phenolic compounds are known to aggregate by π-stacking and H-bonding interactions. The aggregation of flavanols at the membrane surface slows down penetration, especially when the 3-*O*-galloyl moiety is present (Sirk *et al*., 2009). As a consequence, the partition coefficient of EGCG decreases with increasing concentration. The role of molecular size has also been suggested from molecular dynamics simulations. However, within the microsecond timescale, the difference in size between catechin and EGCG might weakly influence the penetration. Aggregation inside the lipid bilayer has also been indirectly evidenced with quercetin, due to segregation of the flavonol and clustering within microdomains (Movileanu *et al*., 2000). At relatively high concentration, curcumin aggregates within the lipid chains as well, which consequently decreases lipid ordering (Loverde, 2014). Quercetin and other flavonoids (rutin, naringenin, genistein) were also shown to stabilize membranes through a decrease in lipid fluidity (Arora *et al*., 2000). The authors suggested that this decrease in membrane fluidity might slow down free radical reactions. By contrast, resveratrol increases membrane fluidity. Its permeation of the membrane even in the gel phase confirms its high affinity to biomembranes.

In liposomes, flavonoids (e.g., quercetin) were proposed to inhibit lipid peroxidation by reducing the propagating lipid peroxyl radicals (Ioku *et al*., 1995). The location of flavonoids just below the polar head surface could be critical: if the compound is slightly more buried, it can inhibit the propagation stage. If it is closer to the polar head groups, its access to the lipid peroxyl radicals may be lost. Such slight changes may be driven by lipid composition, lipid phase, pH of the aqueous phase, and the polyphenol's pK_a and log*P* values. The specific location of flavonoids, that is, slightly less buried than vitamin E, is also ideal to enable regeneration of vitamin E.

As polyphenols are more likely to bind membranes as conjugates, it is quite relevant to compare aglycones with *O*-β-D-glucuronides and *O*-sulfo forms. In the case of quercetin, molecular dynamic simulations (Kosinova *et al.*, 2012) clearly show that the polar conjugates bind in a more superficial manner than the aglycone (Fig. 1.4). Thus, whereas quercetin lies below the interface, mostly parallel to the surface and with its 5-OH groups at 1.5 (±0.2) nm from the center of the DOPC membrane, Q3G is pulled to the surface by the glucuronyl moiety protruding in the aqueous phase, so that the conjugate lies in average at 1.8 (±0.2) nm from the center of the membrane. It can thus be anticipated that the quercetin conjugates are less efficient than quercetin at scavenging lipid peroxyl radicals, as suggested by the decrease in their ability to protect LDL (Loke *et al.*, 2008b). Similarly, the capacity of the metabolites at regenerating vitamin E in membranes is predicted to be lower than for quercetin. Again, deconjugation is

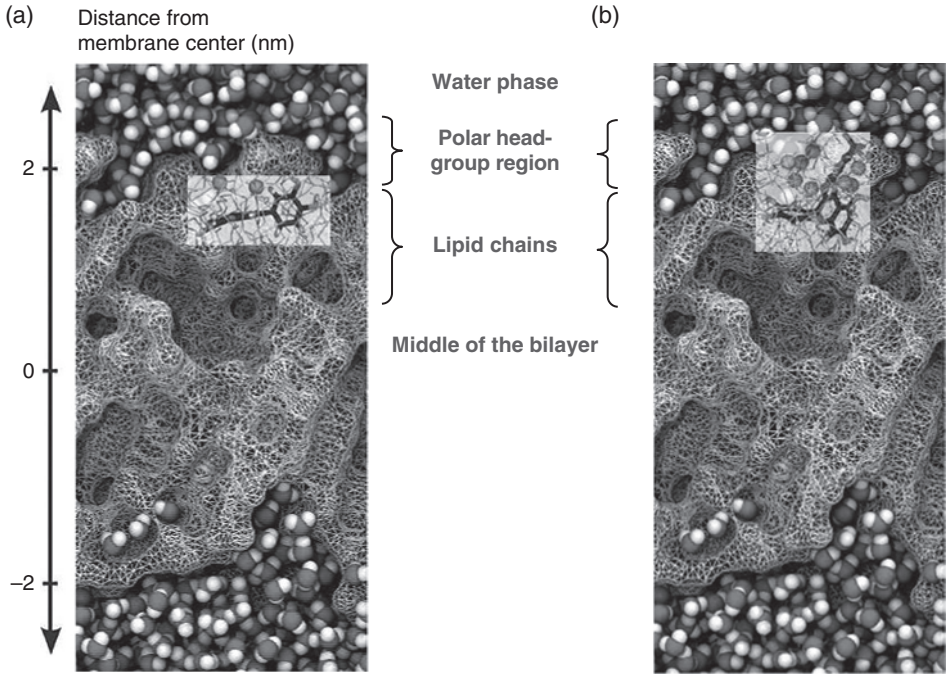

Fig. 1.4 Snapshots of the location of quercetin (a) and quercetin 3-*O*-β-D-glucuronide (b) in a 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine lipid bilayer. Spheres represent the phosphatidyl groups (Source: Adapted from Kosinova *et al*. 2012). (*See insert for color representation of the figure*)

expected to markedly increase the ability of polyphenols to protect membranes against oxidation, not only by restoring the redox activity but also by favoring their penetration into lipid bilayers.

Nonbioavailable oligomeric proanthocyanidins (OPAs) may also exert their bioactivity via direct interactions with the membrane of intestinal cells. Quite importantly, hexameric PAs were shown to specifically bind the lipid rafts of the Caco-2 cell membrane (Da Silva *et al*., 2012; Verstraeten *et al*., 2013), that is, more rigid domains rich in cholesterol, glycosphingolipids, and sphingomyelin and incorporating proteins involved in major cellular events. The interaction is cholesterol-dependent, results in a superficial decrease in membrane fluidity, and inhibits deoxycholate-induced cell permeabilization. Consequently, OPAs also inhibit the activation of MAP kinases and NOX in intestinal cells and could thus help fight chronic colonic inflammation and oncogenesis.

1.3 Polyphenols as electron donors

The catechol nucleus of many common polyphenols is a potent electron/H-atom donor for the reduction of the ROS involved in oxidative stress (Fig. 1.2). Given the limited intestinal absorption and extensive metabolism of dietary polyphenols in humans, this classic mechanism of antioxidant activity now seems especially relevant in the digestive tract where

dietary iron and hydroperoxides (H_2O_2, lipid hydroperoxides) may efficiently initiate the oxidation of dietary polyunsaturated lipids and eventually alter proteins (Dangles, 2012). As polyphenol metabolites are generally (i) much less reducing than native polyphenols and (ii) recovered in only low concentration in the blood circulation, the importance of their ROS-scavenging activity in cells largely depends on their possible accumulation and substantial deconjugation on the very site of oxidative stress. This possibility has actually been demonstrated with Q3G, which is accumulated in the macrophage-derived foam cells of human atherosclerotic lesions but not in the normal aorta (Kawai *et al*., 2008). Moreover, the metabolite is significantly taken up and deconjugated into quercetin in activated murine macrophages. Similarly, Q3G was found colocalized with macrophages and the pro-oxidant enzyme myeloperoxidase (MPO) in human atherosclerotic aorta (Shiba *et al*., 2008). Owing to its free catechol nucleus, this metabolite retains a strong reducing character and, for instance, efficiently inhibits LDL peroxidation (Kawai *et al*., 2008; Loke *et al*., 2008a). Thus, the accumulation of Q3G on a site of oxidative stress strongly suggests its possible ROS-scavenging activity *in vivo*.

The most relevant ROS for scavenging by polyphenols are (Dangles, 2012) as follows:

- The superoxide radical anion produced by NOX, xanthine oxidase, or electron leakage from the mitochondrial inner membrane. The $O_2^{\bullet-}$ radical may then disproportionate (under SOD catalysis) to form hydrogen peroxide, or reduce Fe^{III} to Fe^{II}, or even combine with NO (produced by iNOS at inflammation sites) to form peroxynitrite.
- The hydroxyl radical produced by one-electron reduction of H_2O_2 by Fe^{II} (Fenton reaction) or by decomposition of peroxynitrite in acidic conditions
- The hypervalent Fe^{IV}═O species formed upon activation by hydroperoxides of heme proteins such as (met)myoglobin, myeloperoxidase, and COX.

1.3.1 The physicochemical bases of polyphenol-to-ROS electron transfer

1.3.1.1 Thermodynamics descriptors

The catechol but also the pyrogallol ring (e.g., the B-ring of several common flavonoids and phenolic acids, the galloyl residues of hydrolyzable tannins, and green tea flavanols) are particularly efficient electron/H-atom donors to scavenge free radicals. Their activity is enhanced if they are part of a long conjugation path (e.g., in hydroxycinnamic acids, flavones, and flavonols). The C3-OH group of flavonols and the guaiacol ring are also two moieties having efficient H-atom abstraction capacity (Goupy *et al.*, 2003; Trouillas *et al.*, 2006). These structure–activity relationships (SARs) related to free radical scavenging by antioxidants are well interpreted by the O─H bond dissociation enthalpy (BDE). Its evaluation is a complex experimental issue for polyphenol derivatives, while density functional theory (DFT) calculations are a powerful alternative to evaluate it, as the difference in standard enthalpy between the polyphenol (ArOH) and the aryloxyl radical ($ArO^{\bullet}$) obtained after H-atom abstraction.

$$\mathrm{BDE} = H^{\circ}_{(298\mathrm{K})}\left(\mathrm{ArO}^{\bullet}\right) + H^{\circ}_{(298\mathrm{K})}\left(\mathrm{H}^{\bullet}\right) - H^{\circ}_{(298\mathrm{K})}\left(\mathrm{ArOH}\right)$$

The computed O─H BDEs are particularly predictive. As a characteristic example, the relative H-donating capacity of the C3-, C3′-, C4′-, C5-, and C7-OH groups of quercetin is clearly confirmed by calculations. Indeed, the BDE values (in kcal mol^{-1}) are as follows: below 80 for the C3- and C4′-OH groups (very active); in the range 80–85 for C3′-OH (active); in the range 84–89 for C7-OH (poorly active)[2]; and higher than 90 for C5-OH (inactive). The SAR of DPPH$^{\bullet}$ scavenging is perfectly predicted by the sole BDE descriptor. When compared to other antioxidant assays (e.g., ABTS$^{+\bullet}$, ORAC, electrochemistry), required for a comprehensive antioxidant evaluation, BDE might not be sufficient and other minor descriptors are required for rationalization, which can be evaluated by quantum calculations as well (e.g., spin density distribution, electron transfer and deprotonation energies, number of "active" OH groups and H-bonds, frontier orbital energies and distribution).

Acting as an antioxidant, the polyphenol (ArOH) transfers a H-atom to the free radical (R$^{\bullet}$). The standard enthalpy of H-atom transfer (HAT) reaction from ArOH to R$^{\bullet}$ is given by

$$\Delta H^{\circ}_{298\mathrm{K}} = \mathrm{BDE}\left(\mathrm{ArOH}\right) - \mathrm{BDE}\left(\mathrm{RH}\right)$$

The reaction is exothermic if BDE (ArOH) < BDE (RH). The DPPH-H BDE is ca. 80 kcal mol^{-1} (Pratt *et al.*, 2004)[3]; therefore, the DPPH$^{\bullet}$ assay adequately discriminates active from nonactive phenolic compounds.

Concerning peroxyl radicals, BDE(ROO─H) is in the range 84–88 kcal mol^{-1} (Blanksby *et al.*, 2001; Ramond *et al.*, 2002), depending on the R substituent. This makes peroxyl radicals easier to scavenge than DPPH$^{\bullet}$. However, the SAR of DPPH$^{\bullet}$ and peroxyl scavenging are usually similar (Trouillas *et al.*, 2008). Of course, when peroxyl scavenging is measured in liposomes (R = hydrocarbon chain of fatty acid residues), the H-donating capacity of the phenol groups is not the only factor for consideration and its ability to penetrate the membrane must be considered as well (see Section 1.2.2).

The robustness of DFT methodologies has been repeatedly tested to accurately predict thermodynamic antioxidant descriptors on various phenolic compounds. A review of the huge amount of publications on BDE (DFT-based) calculations is far beyond the scope of this chapter (see, e.g., the review by Leopoldini *et al.*, 2011). The reader must be aware that care should be given to the choice of functionals, basis sets, and solvent models.[4]

[2]The C7-OH group is poorly active as pure H-atom donor. However, it is the most acidic group of many flavonoid derivatives, therefore being in some cases involved in the antioxidant action.

[3]The DPHH-H BDE is ca. 80.4 kcal mol^{-1} as obtained by IEFPCM-B3P86/6-31 + G(d,p).

[4]Solvent effects have to be accurately taken into account. The ideal treatment would be to include large numbers of solvent molecules around the antioxidant (solute) and simulate the entire system at the quantum mechanical level. Unfortunately, this is not possible due to constraints in computing power. Two different approaches can be adopted to alleviate this problem. Either a small number of solvent molecules are included and quantum mechanics calculations are performed using a continuum dielectric (hybrid approach), or a large number of solvent molecules are included and Monte Carlo or molecular dynamics simulations are performed. As an alternative to these sophisticated methods, the solvent can simply be treated as a continuum, for example, the polarizable continuum model (PCM). In this case, the antioxidant molecule sits in a shape-adapted cavity surrounded by the continuum solvent.

However, we believe that the 15-year background in this domain is now sufficient to calculate these major descriptors of the antioxidant action on large series of compounds.

1.3.1.2 Kinetics of hydrogen atom transfer

BDE and other thermodynamic descriptors correlate with static parameters (stoichiometry and EC50) obtained in antioxidant assays. However, under chemical and biological environments when several types of ROS are present, the impact of an antioxidant can be a matter of kinetics as well. Indeed, rate constants of ROS-scavenging may be very different from one ROS to another.

The hydroxyl radical is so reactive that it can react with any biomolecule with rate constants as high as 10^9–$10^{10}\,M^{-1}\,s^{-1}$. $HO^{\bullet}$ can initiate lipid peroxidation either by HAT or by addition on the PUFA (polyunsaturated fatty acid) carbon–carbon double bonds. In both cases, a carbon-centered radical is formed. This step exhibits high rate constants (e.g., $5\times10^8\,M^{-1}\,s^{-1}$ in lecithin bilayers) (Antunes *et al.*, 1996). The following step (O_2 addition to form peroxyl radicals) is also very fast, and the apparent rate constant for these two steps is ca. $10^9\,M^{-1}\,s^{-1}$ (Kamal-Eldin & Appelqvist, 1996). The rate constants of the propagation step (chain reaction in lipid bilayers) are much lower (10^1–$10^5\,M^{-1}\,s^{-1}$) (Tang *et al.*, 2000), and depend on the type of PUFA.

To inhibit the lipid peroxidation process, an antioxidant may scavenge radical initiators as well as peroxyl radicals formed in lipid bilayers. An effective antioxidant such as vitamin E can scavenge the peroxyl radicals with rate constants ranging from 10^3 to $10^6\,M^{-1}\,s^{-1}$, thus in competition against the propagation stage and acting by chain breaking in lipid bilayers (Antunes *et al.*, 1996). Interestingly, the scavenging of the *t*-butoxyl radical by α-tocopherol exhibits much higher rate constants of ca. $10^9\,M^{-1}\,s^{-1}$, when measured in solution (Evans *et al.*, 1992).

The rate constants for $DPPH^{\bullet}$ scavenging were measured for a series of phenolic acids and flavonoids (Goupy *et al.*, 2003; Roche *et al.*, 2005). Using a simple second-order kinetic scheme, the rate constants for the first (most labile) H-atom abstraction from the antioxidant were estimated as ca. 10^3, 10^3, and $2\times10^3\,M^{-1}\,s^{-1}$ for caffeic acid, catechin, and quercetin (catechol moiety), respectively, and $4\times10^3\,M^{-1}\,s^{-1}$ for epigallocatechin (pyrogallol moiety). Even if phenolic compounds react much faster with peroxyl radicals than with $DPPH^{\bullet}$, the rate constants to scavenge both radicals were correlated (Foti *et al.*, 2010). However, whereas α-tocopherol acts both as inhibitor of the initiation and propagation steps, phenolic compounds seem to inhibit mainly initiation. As described earlier, this strongly depends on the polyphenol chemical structure and the lipid composition of the membrane. Namely, slight changes of one of these parameters may bury more or less the compound in the bilayer, therefore modulating this conclusion.

The scavenging of the superoxide radical (in acid–base equilibrium with the perhydroxyl radical $HO_2^{\bullet}$) by active polyphenols is in the range 10^4–$10^7\,M^{-1}\,s^{-1}$ at pH 7. Again, the catechol and pyrogallol moieties provide fast superoxide scavenging. As an interesting example, theaflavin appears particularly efficient with a rate constant of $10^7\,M^{-1}\,s^{-1}$ compared to 6.4×10^4 and $7.3\times10^5\,M^{-1}\,s^{-1}$ for catechin and epigallocatechin gallate, respectively (Jovanovic & Simic, 2000). At pH 7, the following hierarchy was

obtained: catechin ~ epicatechin < epigallocatechin ~ epicatechin gallate < epigallocatechin gallate < theaflavin. At pH 10, one has galangin < kaempferol < catechin < quercetin ~ rutin, with variation in the rate constants of less than two orders of magnitude in this series. Phenolic compounds can also efficiently scavenge oxyl radicals (De Heer *et al.*, 2000) and singlet oxygen (Jovanovic & Simic, 2000; He *et al.*, 2009). The rate constants of $^1O_2$ scavenging by active flavonoids (Jovanovic & Simic, 2000; Mukai *et al.*, 2005) are in the range 10^6–$10^8\,M^{-1}\,s^{-1}$, that is, comparable to vitamin E (10^7–$10^8\,M^{-1}\,s^{-1}$) (Kamal-Eldin & Appelqvist, 1996) but lower than carotenoids ($10^{10}\,M^{-1}\,s^{-1}$) (Ouchi *et al.*, 2010).

1.3.1.3 Kinetics and mechanisms

Rate constants are related to BDEs (Foti *et al.*, 2010; Mayer, 2011) but not strictly correlated. Small variations in BDE may be associated with huge variations in rate constants. Therefore, BDE is only a primary descriptor that provides basic understanding and rough estimate of kinetics. To properly estimate kinetic parameters, a thorough knowledge of the scavenging mechanism is required. Four mechanisms are usually proposed for free radical ($R^\bullet$) scavenging by phenolic compounds (ArOH):

HAT (H-atom transfer) and/or PCET (proton-coupled electron transfer)

$$\mathrm{ArO}-\mathbf{H}+\mathrm{R}^\bullet \rightarrow \mathrm{ArO}^\bullet+\mathrm{R}-\mathbf{H} \tag{1}$$

SET–PT (sequential electron transfer–proton transfer)

$$\mathrm{ArO}-\mathbf{H}+\mathrm{R}^\bullet \rightarrow \mathrm{ArOH}^{\bullet+}+\mathrm{R}^- \rightarrow \mathrm{ArO}^\bullet+\mathrm{R}-\mathbf{H} \tag{2}$$

SPLET (sequential proton loss–electron transfer)

$$\mathrm{ArO}-\mathbf{H} \rightarrow \mathrm{ArO}^-+\mathrm{H}^+;\ \mathrm{ArO}^-+\mathrm{R}^\bullet \rightarrow \mathrm{ArO}^\bullet+\mathrm{R}^-;\ \mathrm{R}^-+\mathrm{H}^+ \rightarrow \mathrm{R}-\mathbf{H} \tag{3}$$

AF (adduct formation)

$$\mathrm{ArO}-\mathbf{H}+\mathrm{R}^\bullet \rightarrow \left[\mathrm{ArOH}-\mathrm{R}\right]^\bullet \rightarrow \mathrm{ArO}^\bullet+\mathrm{R}-\mathbf{H}\ \text{and/or other oxidation products} \tag{4}$$

Reaction (1) is the direct HAT, which corresponds to the homolytic dissociation of the O─H bond of any active (low BDE) phenolic OH group. The HAT mechanism should refer to all processes in which electron and proton are transferred in one kinetic step. The PCET (Huynh & Meyer, 2007) terminology is often used to distinguish a specific HAT, according to the number of molecular orbitals or electrons that are involved in the process (Fig. 1.5). In PCET, even if being transferred in the same kinetic step, electron and proton follow different routes. Namely, in the case of a phenolic compound scavenging an oxygen-centered free radical, the proton is transferred from the OH group of the former to the oxygen lone pair of the latter, in principle across the hydrogen bond pre-established between both reactants (Fig. 1.5a). This proton transfer occurs in the plane defined by the aromatic ring.

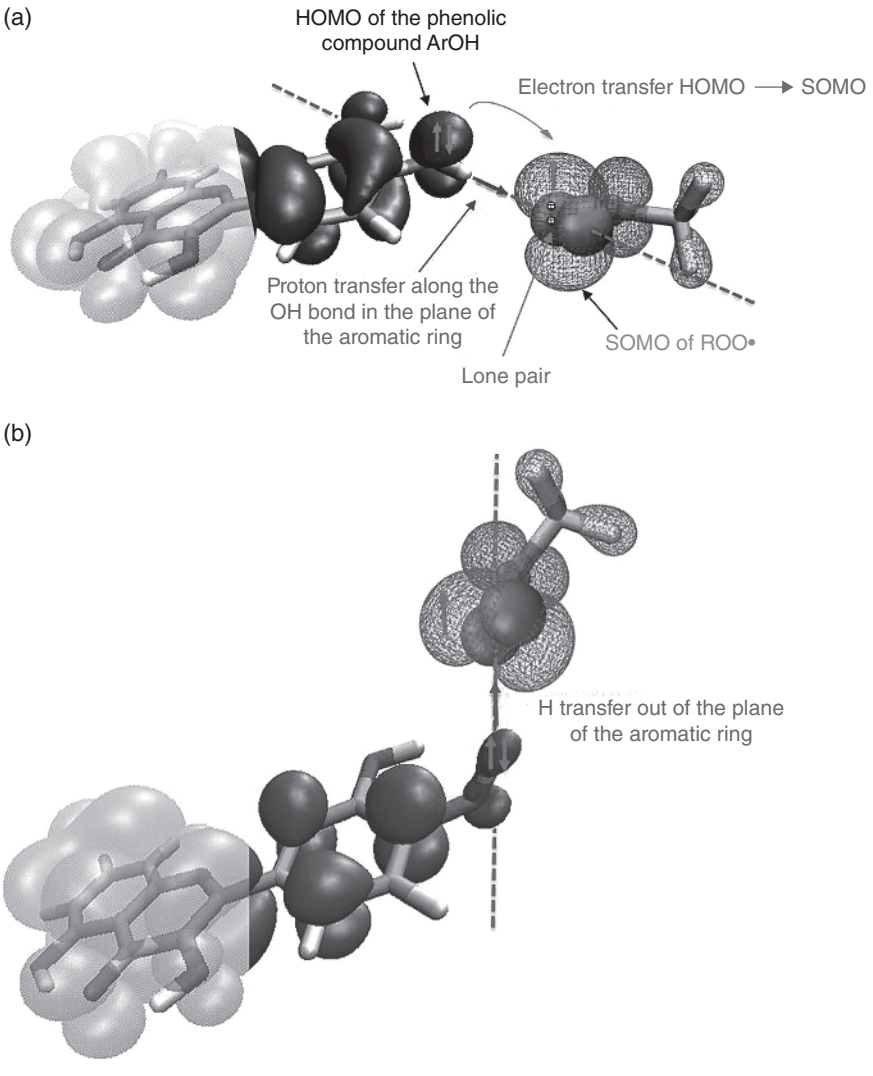

Fig. 1.5 Schematic description of (a) PCET involving five electrons and (b) HAT. (*See insert for color representation of the figure*)

Concomitantly, the electron is transferred from the π-type HOMO (highest occupied molecular orbital) of ArOH to the π-type SOMO (singly occupied molecular orbital) of the free radical, both MOs being perpendicular to the plane in which the proton transfer occurs. In HAT (Fig. 1.5b), both proton and electron are transferred through the same σ-type MO.

The prereaction complexes are of crucial importance to determine which mechanism of action proceeds and its effectiveness (Di Meo *et al.*, 2013). Depending on the phenolic antioxidant and the free radical, various noncovalent arrangements are possible according to the type of interactions (e.g., H-bonding, XH–π interaction, lone pair–π interaction, or π–π stacking, see Fig. 1.6) (DiLabio & Johnson, 2007; Foti *et al.*, 2010; Inagaki

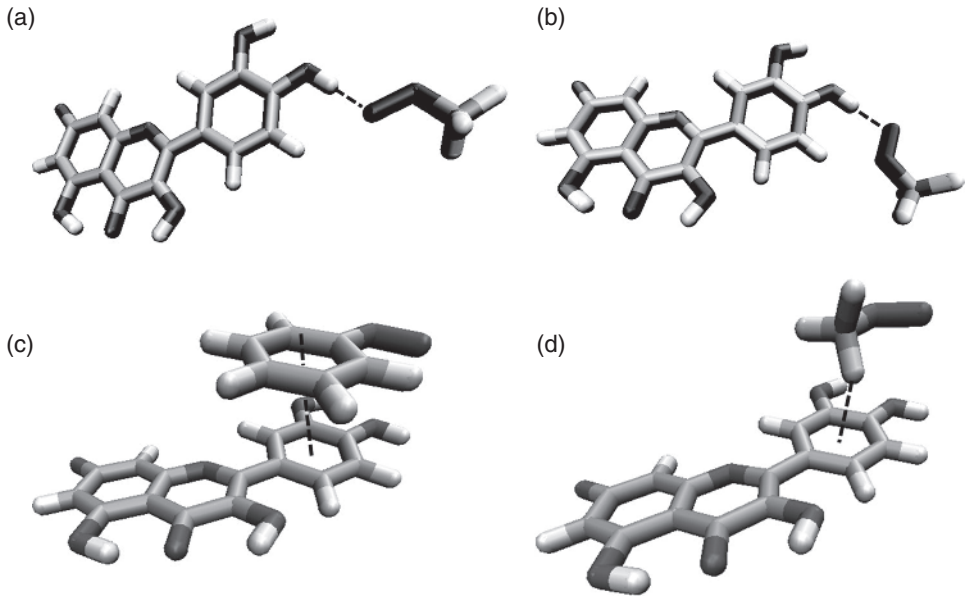

Fig. 1.6 Various geometries of noncovalent ArOH---ROO• arrangements. (a) *transoid* H-bonding, (b) *cisoid* H-bonding, (c) π–π stacking, and (d) XH–π stacking complexes. (*See insert for color representation of the figure*)

et al., 2011). According to the molecular arrangements in the prereaction complexes, there are many cases with phenols in which reaction (1) is neither a pure PCET nor a pure HAT, therefore being a mixed quantum process.

Theoretical studies have evaluated the rate constants of free radical scavenging by phenolic compounds with a reasonable agreement with experiment (Lingwood *et al.*, 2006; Tejero *et al.*, 2007; Chiodo *et al.*, 2010; Galano *et al.*, 2012; Di Meo *et al.*, 2013; Garzon *et al.*, 2014). These studies were based on the transition state theory, requiring calculation of the HAT/PCET transition states. Again, DFT provides a relevant compromise between accuracy and reasonable computational time. However, a careful choice of the functional is even more critical for kinetics than for thermodynamics. The HAT/PCET mechanism may be better described by MPWB1K (Zhao & Truhlar, 2004; Lingwood *et al.*, 2006), knowing that classical hybrid functionals usually underestimate Gibbs energy of activation of HAT processes. However, in the case of peroxyl radical scavenging by polyphenols, B3LYP also provided reasonable agreement with experimental values (DiLabio & Johnson, 2007). Other functionals such as MO5-2X also seem adapted to describe those transition states (Galano *et al.*, 2012). In any event, even if we believe that methodological refinements are still needed, DFT-based rate constants are particularly accurate at predicting (i) SAR and (ii) competition between PCET and electron transfer processes (Di Meo *et al.*, 2013). Interestingly, most of these studies have highlighted the necessity to account for tunneling effect, which, depending on the system, may contribute to rate constant values by up to five orders of magnitude.

The PCET mechanism is known to exhibit kinetic solvent effects (KSEs). PCM provides a perfect description of nonpolar solvents. Concerning polar solvents, PCM-type solvation

properly takes polarizable effects into account but not intermolecular hydrogen bonding effects. Quantum calculations with explicit solvation obtained from molecular dynamics simulations would be the method of choice to accurately evaluate KSEs. However, such calculations are still time-consuming and not adapted for large series of compounds. Classical models have been developed, which stress significant decreased rate constants (Nielsen & Ingold, 2006; Litwinienko & Ingold, 2007).

Depending on the ROS reacting with ArOH, the second mechanism (SET–PT) could occur as a secondary and minor contribution. In this case, the electron transfer (first step) forms the radical cation $ArOH^{\bullet+}$, which is characterized by the ionization potential (IP). This mechanism can be formed and followed by pulse radiolysis (lifetimes = 0.1–1 µs). Its first-order decay reflects deprotonation (Joshi *et al.*, 2004), thus leading to the same final products as PCET (i.e., $ArO^{\bullet} + RH$). However, it is unlikely with polyphenols because $ArOH^{\bullet+}$ is highly unstable (Trouillas *et al.*, 2006; Di Meo *et al.*, 2013), except under certain conditions similarly with the very reactive free radicals $HO^{\bullet}$ or with various bases (Markovic *et al.*, 2013).

Many polyphenols exhibit pK_a values within the physiological pH range and thus are partially found in their phenolate forms. In such situations, electron transfer is favored, the rate constants of SPLET being much higher than in HAT/PCET (Di Meo *et al.*, 2013 and references herein).

From a kinetics point of view, the process is advantageously analyzed within the Marcus theory. In its original formulation, the semiclassical Marcus equation defines the activation barrier as the sum of the Gibbs energy ΔG° for the ET reaction and the reorganization energy λ of the system along the reaction coordinate corresponding to ET (Huynh & Meyer, 2007; Borrelli & Peluso, 2013).

$$k_{ET} = k_A \exp\left(\frac{\lambda + \Delta G^{\circ}}{4\lambda k_B T}\right)$$

The multiplicative coefficient k_A corresponds to preassociation.[5] Near the diffusion-controlled limit, 10^9–$10^{11}\,M^{-1}\,s^{-1}$, both rate constants for diffusion (k_D) and electron transfer (k_{ET}) are comparable and kinetically coupled according to (Huynh & Meyer, 2007) the following:

$$\frac{1}{k_{obs}} = \frac{1}{k_D} + \frac{1}{k_{ET}}$$

The reorganization energy λ can be separated into two components corresponding to the energy barriers for the geometrical relaxation along the ET reaction coordinate of solute (λ_i) and solvent (λ_S) molecules, respectively (Fig. 1.7a). The internal contribution λ_i can also be described as the average of the two terms λ_{i1} and λ_{i2}[6] (Fig. 1.7b). The classical

[5]It depends on the internuclear separation distance, Avogadro's number, and electrostatic energy of association.

[6]λ_{i1} is the difference between the energy of the reactants in the geometry characteristic of the products and that in their equilibrium geometry; λ_{i2} corresponds to the difference between the energy of the products in the geometry characteristic of the reactants.

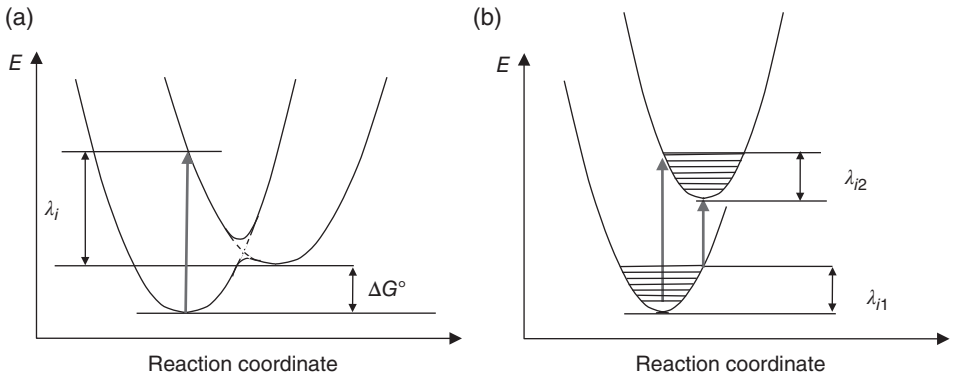

Fig. 1.7 Schematic potential energy surfaces with respect to the reaction coordinate (a) corresponding to the electron transfer and (b) defining the internal reorganization energies.

dielectric continuum model, as initially developed by Marcus for ET between spherical ions in solution, can be used to estimate λ_S (Huynh & Meyer, 2007). The kinetic description of electron transfer is described as nonadiabatic, that is, geometries do not have time to evolve on the ET timescale. For a comprehensive description, the ET probability should be treated within a full quantum approach, including at least the vibrational modes of importance along the reaction coordinate (Borrelli & Peluso, 2013). The Marcus–Levich–Jortner formulation of the ET process introduces electronic coupling between the antioxidant HOMO and the free radical SOMO in the geometry of the prereaction complexes. Within this formalism, the tunneling effect has been added. The type and stability of noncovalent prereaction complexes determine MO overlap and thus k_{ET} (Di Meo *et al.*, 2013). A comprehensive evaluation of all noncovalent prereaction associations is mandatory.

Both HAT/PCET and SPLET mechanisms are in competition to scavenge DPPH$^{\bullet}$, peroxyl, hydroxyl, oxyl, and superoxide radicals. The latter mechanism is faster but is only favored in polar solvents and under neutral to basic conditions, according to the pK_a of the phenolic antioxidant. As said earlier, SET–PT is unlikely with polyphenols and the AF mechanism more specific to HO$^{\bullet}$. ROS scavenging of polyphenols should actually be seen as a complex process following various regimes. The four mechanisms exhibit the same thermodynamic balance since reactants and products are the same (see reactions 1–4). Consequently, the global process is well described by BDE values.

It is also noteworthy that polyphenols have been described to react by HAT with the aryloxyl radical of vitamin E, thereby enabling its regeneration.

$$\mathrm{VitE-OH + R^{\bullet} \rightarrow VitE-O^{\bullet} + RH;\ ArOH + VitE-O^{\bullet} \rightarrow ArO^{\bullet} + VitE-OH} \qquad (5)$$

Among other examples, catechin and epicatechin are as effective as vitamin C at regenerating vitamin E within lipid bilayers (Mukai *et al.*, 2005). Electron spin resonance measurements in micelles also showed that caffeic acid can reduce the aryloxyl radical of vitamin E, while vitamin C in turn can reduce the semiquinone radical derived from caffeic acid (Laranjinha & Cadenas, 1999). This sequence of electron/H-atom transfers from the

aqueous phase (vitamin C as the ultimate electron donor) to the lipid phase (vitamin E) with caffeic acid at the interface to connect the two vitamins may explain the strong synergism observed between the three antioxidants in the inhibition of LDL peroxidation.

Singlet oxygen quenching proceeds either by a physical mechanism that converts $^1O_2$ into $^3O_2$ or by a chemical mechanism that generates various oxidation products. The former process is usually favored with compounds having extended π-conjugated systems (He *et al.*, 2009; Ouchi *et al.*, 2010). Noncovalent interactions between polyphenols and $^1O_2$ may also favor physical quenching via a charge transfer exciplex (Mukai *et al.*, 2005), which may undergo intersystem crossing (change in spin state).

1.3.2 ROS scavenging by polyphenols in the gastrointestinal tract

From both *in vitro* and *in vivo* studies, it is becoming clear that advanced lipid oxidation end products (ALEs) can accumulate in the stomach under postprandial conditions (Kanner *et al.*, 2012). The most efficient route to ALEs is probably the heme-induced autoxidation of PUFA residues in dietary triacylglycerols, phospholipids, and cholesterol esters. The process is especially fast in the gastric compartment due to the presence of O_2 in nonlimiting concentration and to acidic conditions making heme iron (the main dietary iron form, typically brought by muscle meat) particularly pro-oxidant. It is triggered by the heme-induced homolytic cleavage of hydroperoxide traces inevitably contaminating lipid-rich food. This reaction is the first step of a Heme-Fe^{III}/Heme-Fe^{IV} redox cycle yielding propagating lipid peroxyl radicals ($LOO^{\bullet}$) through which lipid hydroperoxides (LOOHs) are formed. Thus, lipid hydroperoxides are both consumed during initiation and formed during propagation, so that their net accumulation is highly variable. For instance, when minipigs were fed with a meal based on sunflower oil (major PUFA = linoleic acid, C18 : 2) and red meat, LOOHs detected by their conjugated diene (CD) moiety were only moderately accumulated in the stomach (a maximum of +35% over ca. 3 hours), whereas malondialdehyde increased by a factor 5 over 4 hours (Gobert *et al.*, 2014). Even ALEs such as malondialdehyde and 4-hydroxyalkenals may be underestimated because of the fast reaction of these strong electrophiles with nucleophilic protein residues (Lys, His, Cys). After digestion of the protein-bound fraction, ALEs may be absorbed in the intestinal cells and express cytotoxic effects. For instance, there is strong evidence suggesting that dietary 4-hydroxyalkenals are central in the relationship between excessive red meat consumption and the risk of colon cancer (Pierre *et al.*, 2007). Moreover, modification of LDL by dietary ALEs is a likely mechanism leading to modified LDL particles with increased susceptibility to oxidation and atherogenic character (Kanner *et al.*, 2012). Hence, fighting dietary PUFA autoxidation in the stomach is one of the most important antioxidant mechanisms for polyphenols. Indeed, native forms possessing a free catechol group (caffeic acid, (epi)catechin, quercetin, and their derivatives) are abundant in the diet and, with the possible exception of flavanol polymers (highly polymerized proanthocyanidins), are readily released from the food matrix upon gastric digestion (Bouayed *et al.*, 2011). For instance, fruit and vegetables were found as efficient as their polyphenol-rich extracts at inhibiting malondialdehyde accumulation in minipigs (Gobert *et al.*, 2014).

Based on *in vitro* experiments, the mechanism of heme-induced lipid peroxidation in the stomach is expected to be sensitive to pH variations and the action of the digestive enzymes. *In vivo* measurements showed that the gastric pH quickly increases from a basal value of 2 to ca. 5 after food ingestion, then slowly decays back to its basal value along the gastric emptying (Gobert *et al*., 2014). Model experiments in micelles and emulsions showed that lipid peroxidation induced by metmyoglobin (CD monitoring) at pH 5–6 is fast and accompanied by a gradual degradation of the heme (Goupy *et al*. 2007). At pH 4, the heme cofactor (hematin) is quickly dissociated from the protein moiety (globin) and its degradation with release of free iron is faster than at higher pH. Although those changes do not markedly affect the overall rate of CD accumulation in emulsions, they strongly alter the performance of the phenolic antioxidants (Lorrain *et al*., 2012). Indeed, common dietary flavonoids and hydroxycinnamic acids are potent inhibitors of metmyoglobin-induced lipid peroxidation in emulsions of sunflower oil stabilized by proteins or phospholipids at pH 5–6, whereas they are much less active at pH 4. On the other hand, polyphenols can efficiently reduce ferrylmyoglobin ($MbFe^{IV}$═O), prepared by reacting metmyoglobin ($MbFe^{III}$─OH) with H_2O_2. It is thus proposed that polyphenols inhibit metmyoglobin-induced lipid peroxidation by reducing hypervalent heme species formed by reaction of $MbFe^{III}$─OH with lipid hydroperoxides (Lorrain *et al*., 2010). This mechanism (inhibition of initiation) requires an intact metmyoglobin particle and is much less efficient at pH 4 when hematin is released followed by free iron. Indeed, at pH 4, polyphenols are unlikely to bind free iron and quench its pro-oxidant activity. Unlike polyphenols, α-tocopherol does not reduce $MbFe^{IV}$═O and acts by reducing the lipid peroxyl radicals (inhibition of propagation). However, both polyphenols and α-tocopherol can protect the heme from degradation (Goupy *et al*., 2007). The mechanism by which phenolic antioxidants can fight heme-induced lipid peroxidation in the stomach is summarized in Fig. 1.8.

The gastric protease pepsin and the gastric lipase could influence both lipid peroxidation in the stomach and its inhibition by polyphenols. For instance, pepsin could modify the structure of the interface by partial hydrolysis of adsorbed proteins. Moreover, mild pepsin-catalyzed proteolysis is known to convert metmyoglobin into more efficient inducers of lipid peroxidation (Carlsen & Skibsted, 2004). Gastric lipase can release free fatty acids and diacylglycerols within emulsions of triacylglycerols, thereby contribution to 5–30% of lipid digestion. However, addition of gastric fluid did not alter the rate of lipid peroxidation in sunflower emulsions but strongly attenuated the inhibitory capacity of quercetin (Lorrain *et al*., 2012).

1.4 Polyphenols as ligands for metal ions

Polyphenols bearing a catechol group can bind a variety of metal ions, including aluminum, iron, and copper ions. Flavonoids may also display additional binding moieties, such as 3-hydroxy-4-oxo and 5-hydroxy-4-oxo substitutions (Engelmann *et al*., 2005). The pH-dependent displacement of protons from those groups by metal ions yields chelates, and this binding can have important consequences for the polyphenols' redox activity (stability, pro- and antioxidant activity) (Perron & Brumaghim, 2009) and bioavailability.

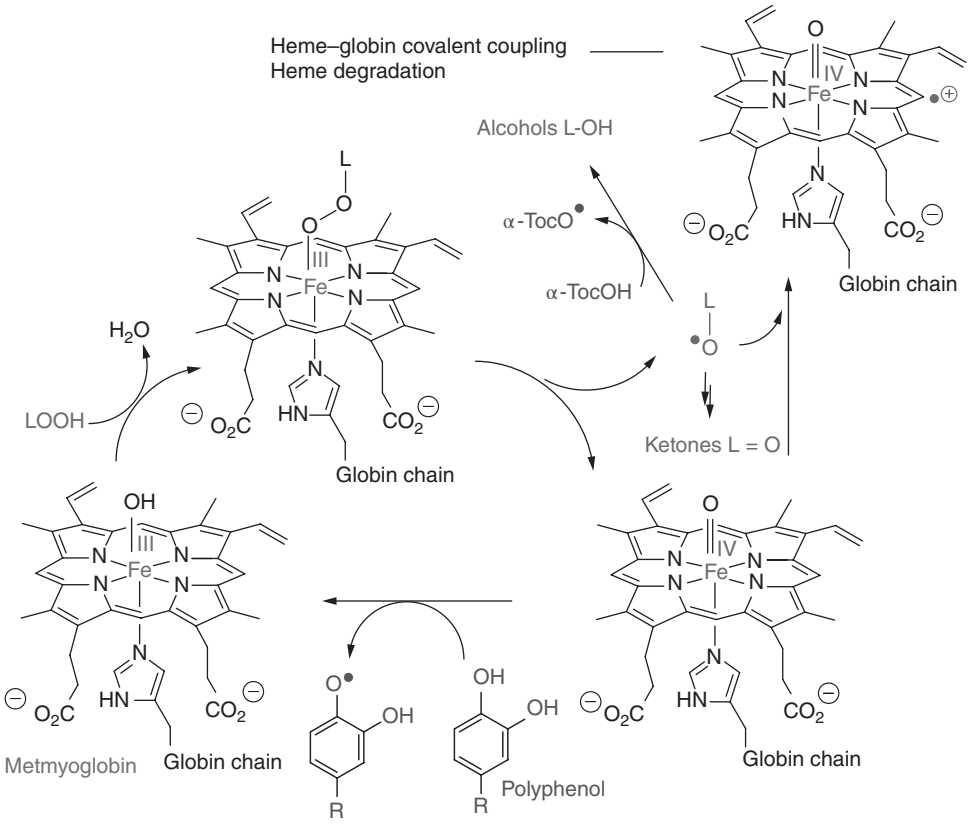

Fig. 1.8 Activation of heme iron (metmyoglobin) by lipid hydroperoxides and the role of phenolic antioxidants.

For instance, iron–flavonoid complexes present in colored beans are not absorbed through the intestinal barrier (Hu *et al*., 2006).

1.4.1 Interactions of polyphenols with iron and copper ions

As iron is the most abundant transition metal ion in human diet and in tissues, iron–polyphenol interactions have been thoroughly investigated. Depending on pH and the presence of other competing ligands (e.g., buffer components), such interactions may be coupled with different redox phenomena (Fig. 1.9):

- In strongly acidic aqueous solution or water–MeOH mixture (pH 1–3), despite the strong competition between protons and iron ions for the binding site, fast Fe^{III} binding can be observed with flavonols at the 3-hydroxy-4-oxo group (as for quercetin, in which additional binding at the catechol group is possible) and with flavanols and hydroxycinnamic acids at the catechol group. The latter binding mode is evidenced by the weak phenol-to-Fe^{III} charge transfer absorption appearing at 700 nm. In such acidic conditions, Fe^{III} sufficiently oxidizes, so phenol-to-iron electron transfers take place, thereby resulting

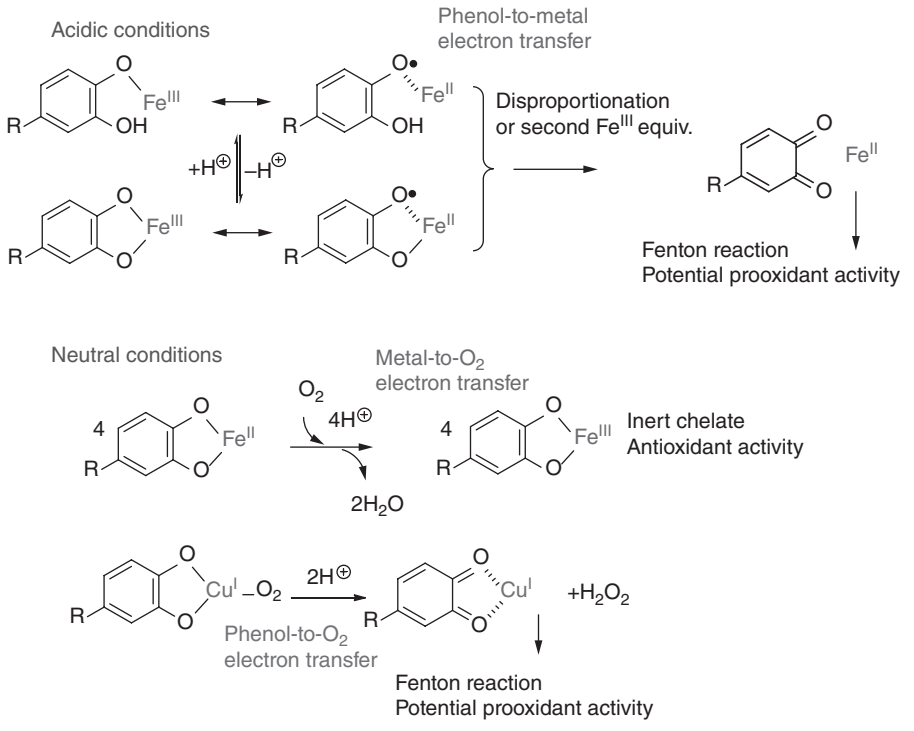

Fig. 1.9 Polyphenols and transition metal ions. Acidic conditions: iron binding and subsequent electron transfer. Neutral conditions: binding and autoxidation.

in the reduction of several Fe^{III} equivalents. By contrast, with the flavanone naringin, binding at the 5-hydroxy-4-oxo group is not followed by electron transfer (Hynes & O'Coinceanainn, 2004; El Hajji *et al*., 2006; Ryan & Hynes, 2007, 2008). The pH dependence of the observed rate constant of electron transfer suggests that the reaction preferentially involves a monoprotonated catecholate ligand. With ferulic and sinapic acids, electron transfer takes place with no evidence of an intermediate Fe^{III}–phenol complex (outer sphere mechanism).

- In mildly acidic to neutral solution, Fe^{III} becomes less oxidizing and Fe^{II} more prone to autoxidation. In a (1 : 1) MeOH–water mixture, high-spin Fe^{III}–quercetin complexes are observed by ESR as well as its partial conversion into a high-spin (ESR-silent) Fe^{II} complex. The formation of Fe^{II}–quercetin complexes is also confirmed by MS (Mira *et al*., 2002; Guo *et al*., 2007). Similar observations were reported for phenolic acids bearing a catechol group in neutral N-containing buffers susceptible to stabilize Fe^{II} (Chvatalova *et al*., 2008). However, the Fe^{III}-to-Fe^{II} conversion is slow and remains limited (typically, < 10% after 1 hour). By contrast, catechol-bearing phenolic acids and flavonoids efficiently accelerate the reverse process, that is, the autoxidation of Fe^{II} into Fe^{III} (Chvatalova *et al*., 2008; Perron *et al*., 2010). Consistently, in a neutral phosphate buffer, Fe^{II}–catechol binding is fast and accompanied by a rapid autoxidation of Fe^{II} into Fe^{III} (confirmed by the ferrozine test and the development of catechol-to-Fe^{III} charge transfer

UV-vis absorption bands) with negligible ligand oxidation (El Hajji *et al*., 2006; Nkhili *et al*., 2014). By contrast, the Fe^{III}–phenol binding is slow (because of phosphate ions competing for the metal ion) and the reduction of Fe^{III} into Fe^{II} is even slower (manifested by a weak consumption of O_2 featuring the fast autoxidation of Fe^{II} back to Fe^{III}). Overall, at neutrality, Fe^{II}/Fe^{III}–phenol interactions converge toward the formation of relatively stable Fe^{III}–phenol complexes, in agreement with the general view that iron–phenol binding inhibits the redox activity of iron and is thus a mechanism for the antioxidant activity of phenolic compounds. For instance, the iron–quercetin binding totally inhibits the accumulation of malondialdehyde from 2-deoxyribose by the Fenton reaction in a neutral phosphate buffer (Guo *et al*., 2007). When the reaction is carried out in the presence of EDTA, the iron–quercetin binding is abolished, thus significantly decreasing the antioxidant action, ascribed here to the radical-scavenging activity of the flavonol. Similar conclusions were drawn from a study showing polyphenols inhibiting DNA damage induced by the Fenton reaction (Perron *et al*., 2008).

By contrast, in acidic conditions, the lower affinity of phenols for iron ions (especially in the presence of competing ligands such as buffer components and surfactants) and their capacity to reduce Fe^{III} into Fe^{II} underlie their relative inefficiency at inhibiting (nonheme) iron-induced lipid oxidation (Lorrain *et al*., 2012) or even their pro-oxidant activity (Sorensen *et al*., 2008; Kristinova *et al*., 2009).

Unlike Fe^{III}, copper ions are not hard enough to form complexes with polyphenols in acidic conditions. However, the binding is fast and quasi-irreversible in neutral conditions even in phosphate buffer (El Hajji *et al*., 2006; Nkhili *et al*., 2014). Remarkably, copper–polyphenol complexes, especially involving Cu^{I}, are quite labile and quickly evolve toward oxidation products with concomitant O_2 consumption, H_2O_2 production, and maintenance of copper at the low-valence level + I. Thus, Cu^{I}–phenol complexes transfer electrons from the ligand (instead of the metal center) to the Cu^{I}-bound O_2. The persistence of Cu^{I} (even when starting from the Cu^{II}–phenol complexes) and the accumulation of H_2O_2 favor the onset of the Fenton reaction. It is thus no surprise that polyphenols are less efficient at inhibiting copper-induced than iron-induced biologically relevant oxidation processes and may even behave as pro-oxidants in the presence of copper (Perron *et al*., 2011). For instance, green tea flavanols display contrasted influences on DNA damage induced by Cu^{I}–H_2O_2: while EC and EGC are pro-oxidant throughout the entire concentration range (0.1–3000 µM), EGCG behaves as a weak antioxidant and ECG as both pro-oxidant and antioxidant at low and high concentrations, respectively.

Interactions of polyphenols with transition metal ions, even as unidentified metal traces, can mediate polyphenol autoxidation and the concomitant production of H_2O_2 and electrophilic *o*-quinones or *p*-quinone methides. Interestingly, this phenomenon can occur in cell culture with subsequent activation of genes of the electrophile-responsive element (EpRE) that governs the expression of several phase II detoxifying enzymes such as NAD(P)H–quinone oxidoreductase and enzymes involved in glutathione biosynthesis and transfer (Erlank *et al*., 2011). Indeed, H_2O_2 and electrophiles derived from polyphenol oxidation could modify critical Cys residues of Keap1, a repressor protein bound to EpRE transcription factor Nrf2 in the cytosol. The subsequent dissociation of Keap1 from Nrf2 triggers the

translocation of Nrf2 into the nucleus and the onset of gene expression. Thus, the mild oxidative stress caused by polyphenol autoxidation stimulates the cellular antioxidant defense. The ability of flavonoids to upregulate EpRE genes is correlated to their electron-donating capacity expressed by the HOMO energy (Lee-Hilz *et al*., 2006). The enol group of flavonols seems more important that the catechol group as shown by the variations of induction factor: quercetin (IF = 10) > 3′-*O*-methylquercetin (IF = 7.8) > kaempferol (IF = 7.1) > luteolin (IF = 3). Thus, if flavonoid metabolites (glucuronides and sulfates) undergo partial hydrolysis in cells, the concomitant restoration of the reducing capacity could be beneficial, not only because of the enhanced antioxidant (ROS-scavenging) activity but also because of the enhanced pro-oxidant activity leading to increased detoxifying capacity.

1.4.2 A preliminary theoretical study of iron–polyphenol binding

Various theoretical studies have rationalized metal–phenol binding in active sites of non-heme iron-containing enzymes such as dioxygenases (Nakatani *et al.*, 2009), whereas less theoretical studies directly deal in details with metal–polyphenol binding in aqueous solution (Furia *et al.*, 2014 and references herein), especially to rationalize oxidative processes. In this case, the influence of pH is of crucial importance and must be investigated. This section provides some highlights based on theoretical calculations performed with DFT[7] on catechol, chosen as an adequate polyphenol prototype (C. Tonnelé & P. Trouillas, unpublished results). Calculations performed on 3-*O*-methylquercetin (as a model of the common 3-*O*-glycosides of quercetin) revealed similar electronic features (data not shown), thus confirming that catechol (mono- and bi-deprotonated) is a relevant model to tackle metal–polyphenol binding and the further redox processes related to their antioxidant/pro-oxidant effects.

1.4.2.1 Charge states, spin states, and geometries

Metal–polyphenol binding requires a thorough analysis of charge and spin states. Complexation of Fe^{III} with the catecholate monoanion leads to the $[Fe^{III}(Cat^{1-})(H_2O)_5]^{2+}$ (coordination of the catechol ligand through the sole deprotonated O-atom, i.e., monodentate mode) and $[Fe^{III}(Cat^{1-})$

[7] A proper and reliable description of iron complex spin states constitutes a very challenging issue that can hardly be tackled by the most accurate quantum methods but rather by the less computational costly DFT approach. However, the results highly depend on the functional chosen. Among all the investigated DFT functionals, OPBE, which combines Handy's and Cohen's optimized exchange OPTX with PBE correlation, was shown to be one of the most performing. Thus, in the present work, all geometries were optimized at the OPBE/6-31 + g(d,p) level, and the LANL2DZ pseudopotential/basis set was used to describe the iron ion. Solvent effects were accounted using a polarizable continuum model within its IEFPCM formalism; explicit solvent molecules were also included in the coordination sphere of the iron ion. The Gibbs free energies were obtained by summing (i) thermal correction to free energies as obtained on OPBE/6-31 + g(d,p)-LANL2DZ geometries to (ii) electronic energy, as obtained by a single-point calculations performed with OPBE/6-311 g(3df,3pd)-LANL2DZ. All calculations were performed with the Gaussian09 package.

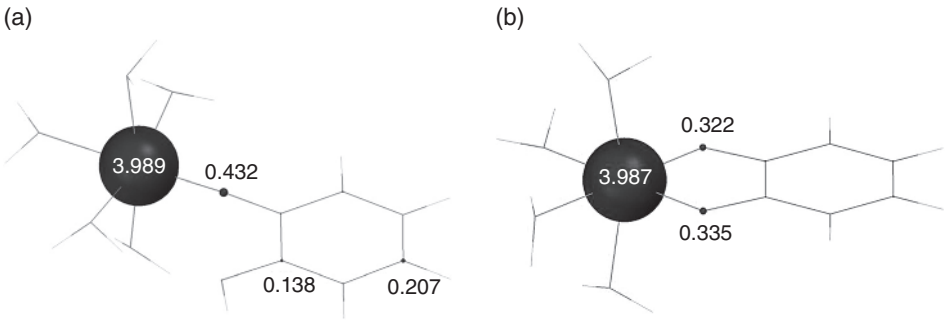

Fig. 1.10 Spin density distribution: (a) $[Fe^{III}(Cat^{1-})(H_2O)_5]^{+2}$ and (b) $[Fe^{III}(Cat^{2-})(H_2O)_4]^{+1}$.

$(H_2O)_4]^{2+}$ (coordination of the catechol ligand through both O atoms, i.e., bidentate mode) complexes.[8] A clear high-spin octahedral coordination is observed with both complexes. In $[Fe^{III}(Cat^{1-})(H_2O)_5]^{2+}$, the spin propagation occurs via the iron–oxygen bond and further develops into the aromatic ring (Fig. 1.10a). The charge and spin features support the hypothesis of a Fe^{II}–semiquinone radical character, in agreement with ligand-to-Fe^{III} ET (Fig. 1.11). Interestingly, $[Fe^{III}(Cat^{1-})(H_2O)_4]^{2+}$ did not exhibit any significant spin delocalization on the catechol ligand. This complex is most probably an intermediate toward a second deprotonation of the catechol moiety, thus yielding the corresponding bideprotonated complex $[Fe^{III}(Cat^{2-})(H_2O)_4]^{+1}$. This complex is more stable in its high-spin state (sextet) by 40.0 and 36.5 kcal mol^{-1} compared to the lower-spin states (quartet and doublet), respectively. Interestingly, it only exhibits a slight spin density propagation over the ligand (Fig. 1.10b), which suggests that ligand-to-Fe^{III} ET within this complex is less favorable than within $[Fe^{III}(Cat^{1-})(H_2O)_5]^{2+}$, in agreement with the pH dependence of the experimental rate constant of electron transfer (Hynes & O'Coinceanainn, 2004). The bideprotonated catechol can also bind to ferrous ions, forming the $[Fe^{II}(Cat^{2-})(H_2O)_4]^{0}$ complex also stabilized in its high-spin state, that is, quintet.

According to the metal/polyphenol molar ratio, one can also expect the formation of multicatechol complexes. The 1 : 3 stoichiometry provides the most stable complex (binding energy of −202.0 kcal mol^{-1}), while the two 1 : 2 complexes (equatorial–equatorial and axial–equatorial arrangements) exhibit similar binding energies of −180.5 and −181.6 kcal mol^{-1}, respectively. For comparison, the binding energy for 1 : 1 stoichiometry is expectedly the highest (less stable complex) and amounts to −129.1 kcal mol^{-1}.[9]

1.4.2.2 Oxidation of the bideprotonated catechol

The charge distribution in $[Fe^{III}(Cat^{2-})(H_2O)_4]^{+1}$ and its weak Fe^{II}–semiquinonate radical character in solution suggest the possibility of ligand-to-Fe^{III} ET (Fig. 1.11). On the basis of this assumption, we propose a mechanism involving metal-induced disproportionation

[8] In this case, the conformational rearrangement suggested an incomplete coordination of the iron ion, leading to less stable complex.

[9] It is worth noting that the absolute values are hardly comparable to related experimental data; however, the relative energies are considered reliable.

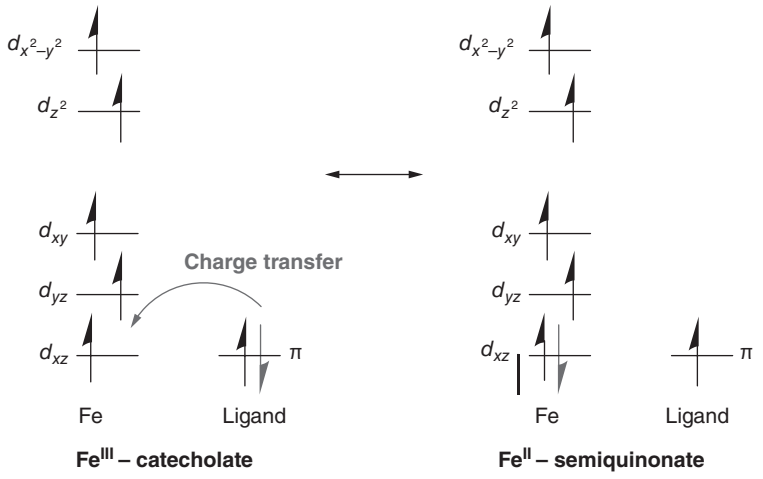

Fig. 1.11 Electronic structure of the Fe^{III}–Catecholate complex in its high spin (sextet) state, showing the two possible electronic configurations.

of the complex giving products **1** and **2** (Fig. 1.12). Compound **1** is the reduced product and corresponds to the $[Fe^{II}(Cat^{2-})(H_2O)_4]^0$ complex. Compound **2** is the oxidized product, in which the catechol moiety has been oxidized to the *o*-quinone form ($[Fe^{II}(Quinone)(H_2O)_4]^{+2}$).

In complex **2**, the ligand exhibits features of a quinone form with localized π-bonds, with reduced C─O bond lengths (1.26 Å), and a pattern of alternating single and double bonds, together with a total charge close to 0.

1.5 Conclusions

Whereas clinical studies have established causal relationships between diets rich in polyphenols and health, biochemical investigations in cells including the true circulating metabolites are a critical step toward understanding the molecular mechanisms at work. Among polyphenol metabolites, the simplified structures delivered by the colonic microbiota and their conjugates by the intestinal and liver enzymes probably deserve special attention as the large intestine is the major site of absorption for dietary polyphenols. Considering the global pool of metabolites, polyphenols are more bioavailable than we once believed. For instance, it was estimated that ca. 70% of green tea flavanol intake was recovered in the urine of human volunteers (Del Rio *et al*., 2013). As polyphenols may exert a direct antioxidant action by ROS scavenging in the digestive tract, the possible accumulation in this site of polyphenol oxidation products also deserves specific investigation as nothing is known about the bioavailability and potential bioactivity of such compounds. It must also be kept in mind that the low concentrations of individual circulating metabolites typically observed after a meal rich in polyphenols do not exclude accumulation of

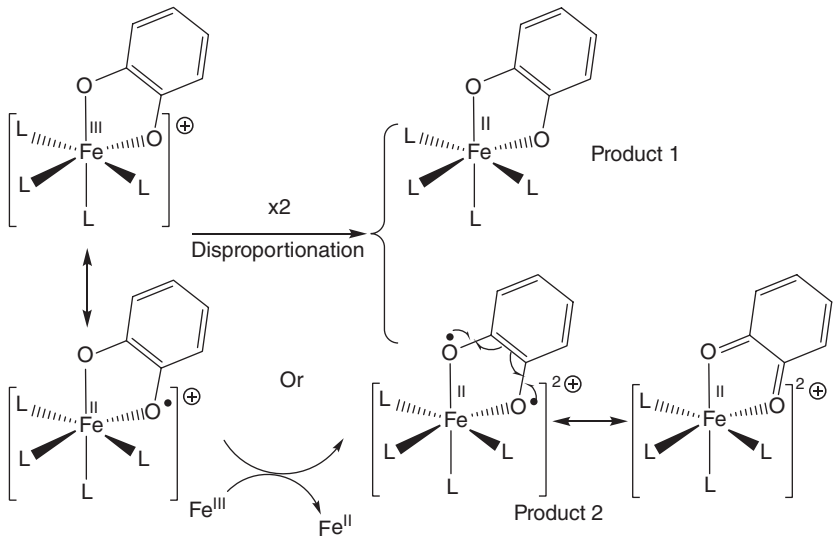

Fig. 1.12 Proposed mechanism for the Fe^{III}-induced oxidation of catechol (L = H_2O).

much higher concentration at specific inflammation sites and subsequent deconjugation into more active forms. Finally, besides their activity in human cells, dietary polyphenols may also contribute to human health by modulating the activity of the colonic microbiota (Anhe *et al*., 2015).

Experimental and computational physical chemistry may be of great help in understanding the health effects of polyphenols at the molecular level. For investigating the activity in the GI tract, approaches combining simple chemical models with experiments in dynamic digesters including physiological parameters deduced from studies in humans and animals could be quite rewarding. After intestinal absorption, the ongoing identification of potential targets, including cytosolic and membrane proteins but also specific membrane lipid domains, will foster further binding and molecular modeling investigations to bring the knowledge to the highest resolution of intra- and intermolecular hydrogen bonding, van der Waals and ionic interactions, and electron transfer within defined supramolecular assemblies. It is thought that these multidisciplinary approaches will efficiently contribute to establishing ever more firmly the important role of dietary polyphenols in maintaining human health.

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