Complexes of Natural Flavonoids with Transition Metals

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The Structure and the Role in Human Health

By

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I

INTRODUCTION

Metal cations play a vital role in multiple biochemical processes in living cells. The most abundant metals in the human body are calcium (Ca), potassium (K), sodium (Na), and magnesium (Mg). These are non-trace metals that contribute a little bit over 1 kg to the human body, the content of Ca being about 1 kg. (Jahnen-Dechent and Ketteler 2012, iii–iiii; Navarro and Vaquero 2016; Strazzullo and Leclercq 2014, 188) The following metals are less abundant than Ca, K, Na, and Mg, however, they are still important in everyday life: vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), and zinc (Zn). These d-block elements, from the fourth period of the periodic table, are trace metals, and their quantity in the average 70 kg adult does not exceed 7 g, the highest contribution coming from Fe and Zn (Maret 2016, 2), which is less than the least abundant non-trace element Mg (24 g). (Jahnen-Dechent and Ketteler 2012, iii) Even though trace metals are present in such a small quantity in the human body, they play a critical role in various biochemical processes, being principal constituents of hemoglobin, cytochromes, zinc finger proteins, copper proteins, manganese-dependent superoxide dismutase, and others. Recently, however, Cr stopped being classified as essential in humans. (Di Bona et al. 2011, 389; Vincent 2017, 2212–3) Molybdenum (Mo), a d-block element from the fifth period of the periodic table, is also known as essential in the human body, even though its content does not exceed 5 mg. (Kapp Jr. 2014) Mo is an indispensable component of xanthine oxidase and sulfite oxidase.

In the body, trace metals interact with organic molecules coming from food and thus play an important role in their metabolism and affect their accessibility to various organs. Recently, a lot of attention in scientific literature has been devoted to the discussion of special health-beneficial properties of vegetarian diets and various plant foods. It was even suggested that we should resume plant-based diets for which we are physiologically adapted, as the hunter-gatherer period characterized by harvesting wild food rather than cultivating crops occupied at least 90

percent of human history. (Lee and Daly 1999, 3; Glover and Martin 2012, R149) The major beneficial effect of fruits and vegetables consumed by people is attributed to a group of polyphenolic compounds that represent side products of primary metabolism in vascular plants, flavonoids. (Wen, Alseekh and Fernie 2020, 100–2) The spectrum of beneficial effects of flavonoids in human health is very broad and includes anticancer, antibacterial, antiviral, and anti-inflammatory roles. These molecules also stimulate cardioprotection, reduce adipocyte development thus inhibiting weight gain, and suppress the development of neurodegenerative diseases. Flavonoids by themselves have poor water solubility, moreover, they undergo metabolic transformations that change their properties. However, flavonoids' interactions with metal cations improve their stability, solubility, hydrophilicity, and bioavailability making them an attractive target for applications and studies.

Approximately 8,500 food molecular components and drug substances with flavonoid skeleton as a core structure have been combined into an integrated database of flavonoids. (Kinoshita et al. 2006, 181–2) Over 4,000 structurally distinctive flavonoids have been identified in plants. (Fink et al. 2007, 514) Based on their chemical structure, flavonoids can be divided into several principal subgroups: flavanones, flavones, isoflavones, flavonols, flavanols, flavanonols, and anthocyanidins. (Ramos 2007, 428) The key structural elements of flavonoids and the structures of flavonoids' subgroups are shown in Fig. 1. A unique polyphenolic structure of flavonoids that involves carbonyl and ether groups on the C ring along with multiple hydroxy groups provides the basis for various metal coordination modes such as those in which metal cation is attached to (1) 5-hydroxy and 4-keto groups, (2) 3-hydroxy and 4-keto groups, (3) two hydroxy groups in ortho orientation on the B ring (3' and 4'), (4) ortho hydroxy groups on the A ring (5 and 6, 6 and 7, or 7 and 8), and (5) 1-ether and 2'-hydroxy group. Fig. 2 shows various patterns of metal attachment to flavonoids using a model of hydroxylated derivatives of flavone. It is interesting to note that in the case of catechol-like binding (5-6 and 3'-4' modes in Fig. 2) and 3-4 binding, 5-membered rings involving metal cations are formed, while in the case of 4-5 and 1-2' binding (combining groups attached to different rings), 6-membered rings are produced with metal cations.

Multiple studies conducted on flavonoid-metal chelates show that besides the charge and electronic configuration of a metal cation, it is the overall electron density on the atom that significantly affects the strength of complex binding. Some experiments revealed that metal cations with more electrons tend to form stronger complexes. In the present book, a

series of d-block elements, metals with relatively high electron density, is examined. Besides their vital role in the human body, it is their relatively large size, compared with other life essential metals, and, consequently, high electron density that makes them an attractive object of study in connection with the formation of complexes with flavonoids.

This book is divided into eleven chapters, including the introduction and conclusions. Chapters 2 through 10 are devoted to individual trace metals Fe, Zn, Cu, Ni, Mn, Mo, Co, Cr, and V. For each metal: (1) the general role in the human body is discussed with a special accent on its coordination chemistry; and (2) the most significant and intriguing data on metal-flavonoid interactions collected over the previous ten years are described including the biological role of these interactions, the chemical structure of complexes, redox reactions, and the applications in health-related areas.

The materials collected in the book will be of interest to scientists with different expertise including nutritionists, pharmacologists, botanists, toxicologists, and organic chemists. The book will contribute to scientific research in a way that it will clarify and enlarge the notion of the role of metal-flavonoid interactions in human life at the system level. Particular emphasis is laid on the structure-activity relationship of metal-flavonoid complexes. This book will thus serve the health purpose, as it will provide rationales for designing strategies intended to maximize the health-beneficial effects of metal-flavonoid complexes with applications in medicine, toxicology, and environmental technology.

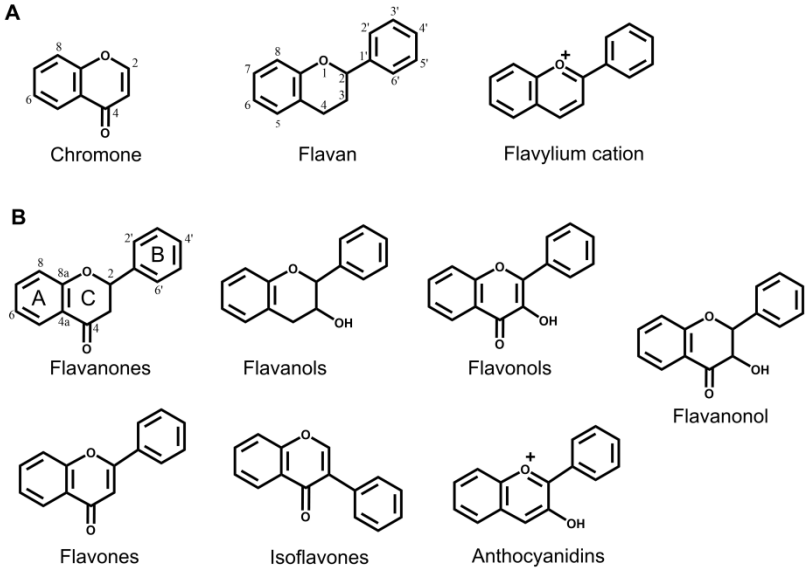

Figure 1. Structures of flavonoids. **A.** Core structural elements of flavonoids. **B.** Structures of seven flavonoids' subclasses.

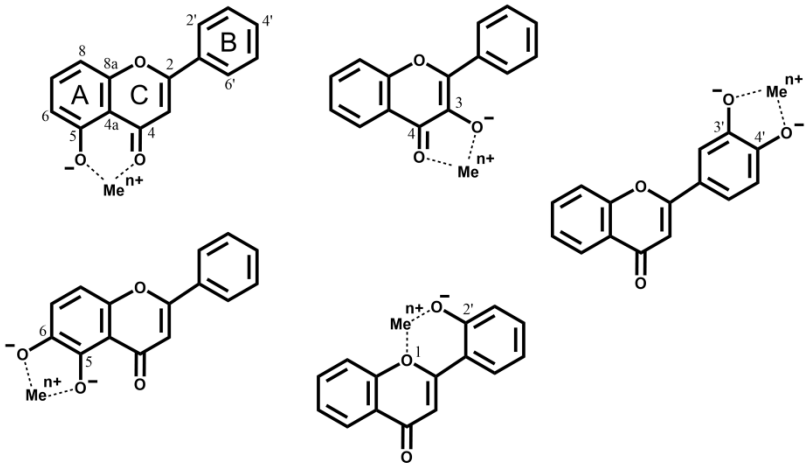

Figure 2. Structures of metal chelate complexes of flavone derivatives.

II

IRON (FE)

2.1. The role of Fe in human health

In the human body, Fe is the most abundant metal among d-block elements. Its content in the average 70 kg adult is about 4 g. (Emsley 2011, 255) Fe is both a fundamental nutrient and a potential toxicant to cells. Fe deficiency caused either by insufficient intake or low bioavailability may result in anemia, a disease characterized by a low level of red blood cells or less than normal hemoglobin. Remarkably, in developed countries, almost all cases with anemia are associated with Fe deficiency. People gain Fe through various foods. Beef, lamb, poultry, tuna, shrimp, clams, and other types of seafood contain heme-bound Fe (heme Fe). Plant foods such as cereal, bread, grains, beans, pulses, seeds, nuts, dark chocolate, spinach, cabbage, and potatoes are the sources of nonheme Fe. Heme Fe has high bioavailability, and its absorption by enterocytes (epithelial cells in the intestinal lining) in duodenum in the form of metalloporphyrin is not affected by other organic molecules. However, the absorption of non-heme Fe from the products of plant origin may be significantly influenced by redox reactions or chelation due to the presence of active molecules coming from various types of foods. Fe compounds in plants include ferrous sulfate, ferrous carbonate, ferrous gluconate, ferric citrate, ferric EDTA, ferric phytate, and others. (Nielsen, Tetens, and Mayer 2013, 3075) Unfortunately, there is no system-level algorithm that would allow us to predict the bioavailability of Fe in the human body as a result of the intake of certain products. However, it is well known that the presence of ascorbate and citrate increases the absorption of Fe. The beneficial effect of ascorbate is primarily associated with its ability to reduce Fe^{3+} (ferric state) to Fe^{2+} (ferrous state) increasing its solubility. The transmembrane protein in the duodenal enterocyte cytochrome b_{561} that possesses an ascorbate-binding motif catalyzes the redox reaction, facilitating the transfer of the electron from ascorbate in the cytoplasm to Fe^{3+} in the duodenal lumen. (Lane et al. 2015, 2279) Interestingly, it was found that dietary flavonoid quercetin also provides

electrons to cytochrome b_{561} in the process of the reduction of Fe^{3+}. The consumption of meat stimulates the absorption of non-heme Fe due to the presence of protein, especially the intake of beef. (Piskin et al. 2022, 20444) Similar to ascorbate and quercetin, the digestion products of the muscle tissue, cysteine and glutathione, can reduce Fe^{3+} to Fe^{2+}. Once Fe^{3+} is reduced to Fe^{2+}, it is transported across the apical membrane of enterocytes by divalent metal transporter (DMT1) from the lumen into the cytoplasm of the cells. Fe^{2+} cations are transported across the basolateral membrane of enterocytes from the cytoplasm into the bloodstream via a transmembrane protein ferroportin. (Meyron-Holtz et al. 2014, 2)

The slight chelating ability of both citrate and ascorbate may also assist in increasing the solubility of Fe^{3+} in the lumen. On the other hand, phytic acid, a major storage of phosphorous in plants, found in cereals and beans, suppresses the absorption of Fe, as it forms complexes with Fe^{3+} at the central position coordinated by six oxygen atoms. (Nielsen, Tetens, and Mayer 2013, 3077) The complexation with phytate hinders Fe penetration into enterocytes. The majority of polyphenols, tannic acid, egg proteins, and calcium inhibit Fe absorption.

Once Fe^{2+} is released into plasma from duodenum, it is oxidized back to Fe^{3+} by hephaestin and is captured by a protein transferrin that delivers Fe to the tissues of various organs. Upon cell penetration Fe^{3+} is reduced back again to Fe^{2+} by metalloreductase and is utilized for the synthesis of heme and iron-sulfur biogenesis in mitochondria. (Paul et al. 2017, 66) The excess Fe is stored in ferritin, primarily in hepatocytes (liver) and reticuloendothelial cells (immune system including spleen and bone marrow). Fe metabolism and bioavailability significantly affect the functioning of immune system. (Cronin et al. 2019, 6–9) Ferritin is released into plasma when there is a signal for more Fe requirement in the body. Ferritin thus operates as a buffer preventing Fe overload and Fe deficiency. Fe is stored in the ferric state inside ferritin and is released from the protein in the hydrated ferrous state.

Fe overload is a rare condition, as the quantity of this element in the body is regulated by ferritin and a complex of biochemical signals that controls its function. However, there exists a genetic condition named hemochromatosis. People with this condition tend to absorb too much Fe from food resulting in its overload in heart, pancreas, and liver, ultimately leading to heart diseases, cancer, and liver cirrhosis. Fe toxicity in general is associated with the tendency of Fe^{2+} to react with hydrogen peroxide (H_2O_2) leading to the formation of hydroxyl radicals $OH^{\bullet}$ in a Fenton reaction. $OH^{\bullet}$ is very reactive and is capable of binding to various molecules in cells, causing damage to DNA and proteins. Excessive

accumulation of Fe followed by the oxidation stress triggered by hydroxyl radicals may lead to the uncontrolled development of Alzheimer's disease (Zhao 2019, 83–4) and can mediate insulin deficiency and diabetes.

2.2. Fe in human proteins

Fe is a basic component of a large number of proteins. In hemeproteins representing a large class of metalloproteins, Fe is coordinated by four N atoms of porphyrin that acts as an equatorial ligand and one or two axial ligands. Hemeproteins could be classified based on their primary function. (1) Proteins performing oxygen transport in the human body are hemoglobin (red blood cells), myoglobin (muscle tissue), and neuroglobin (brain tissue, spinal cord tissue, and retina). (2) Hemeproteins catalyzing oxidation reactions (enzymes) in the human body are cytochrome P450 (CYP), catalase, and peroxidases. (3) Hemeproteins participating in electron transport occurring in mitochondria of eukaryotes are cytochrome a/a_3 (components of complex IV in respiratory chain), cytochrome b (a component of complex III in respiratory chain), and cytochrome c that transfers electrons from complex III to complex IV. Fe is permanently attached in these proteins and, therefore, is involved in their specific tasks such as capturing oxygen in lungs and carrying it to the tissues, scavenging reactive oxygen and nitrogen species, performing oxidative metabolism in cells, generating energy that fuels the synthesis of ATP, and other roles.

Besides hemeproteins, Fe is present in iron-sulfur proteins, another class of metalloproteins. These proteins contain clusters that consist of Fe atoms in different oxidation states linked by sulfur (S) bridges and attached to apo-protein via cysteine residues or sometimes histidine residues. The simplest catalytic center of iron-sulfur protein contains one Fe atom coordinated by four S atoms of cysteine residues. The other catalytic centers may contain two Fe atoms and two S-links (2Fe–2S) or four Fe atoms and four S atoms (4Fe–4S, cubane-type cluster). In these structures, Fe atoms are linked together via S-bridges, while the cluster itself is attached to the protein via S-atoms on cysteine residues. There also exist 3Fe-4S clusters and even more complex polymetallic systems containing 7 or 8 Fe atoms. Iron-sulfur proteins are actively involved in biochemical reactions constituting metabolic pathways. Their ability to delocalize electrons over different Fe and S atoms and thus facilitate electron transport defines their major role in biology. In addition to their active involvement in oxidative phosphorylation in mitochondria, regulation of enzymatic activity, control of protein conformation,

catalysis, disulfide reduction, and Fe storage, iron-sulfur proteins are also known to play a significant role in DNA replication, DNA damage recognition, and repair. (Fuss et al. 2015, 1255–7; Puig et al. 2017, 1486–8)

Besides hemeproteins and iron-sulfur proteins, there are multiple enzymes performing numerous biological functions where Fe in is coordinated by different ligands in the catalytic center, typically amino-acid residues such as histidines, aspartate, and asparagine. Examples include oxygenases, hydrohylases, demethylases, fatty acid desaturases, Fe superoxide dismutases (Fe-SOD), ribonucleotide reductases, and others. Fe-dependent proteins with their diverse structural features of coordination complexes in the catalytic center represent a broad area of research that attracts attention of scientists specializing in organometallic chemistry, physical chemistry, and biochemistry.

Oxygenases are a group of enzymes that catalyze dioxygenation, namely the addition of an oxygen molecule into an organic molecule. In this process, both atoms of the molecular oxygen are incorporated into the substrate which prompts some scientists to call this group of enzymes dioxygenases. An example of dioxygenase is lipoxygenase, a family of Fe dependent enzymes that catalyze the dioxygenation of polyunsaturated fatty acids, leading to the formation of fatty acid hydroperoxides. In these enzymes, for example human 5-lipoxygenase, the non-heme catalytic Fe is coordinated by N atoms of three conserved histidine residues and on O atom of the main chain carboxylic group of the C-terminus. (Gilbert et al. 2011, 218) As opposed to dioxygenation, hydroxylases function by facilitating the incorporation of only one O atom from molecular oxygen into the substrate, while the second O atom is reduced to water. For this reason, hydroxylases are sometimes called mono-oxygenases. Examples include aromatic amino-acid hydroxylases such as tryptophan, tyrosine, and phenylalanine hydroxylases. These enzymes catalyze Fe-mediated addition of one O atom into the respective amino-acids with a concurrent conversion of a second O atom into water molecule with the use of tetrahydrobiopterin (BH_4) as an electron donor. Phenylalanine hydroxylase catalyzes the conversion of phenylalanine to tyrosine. The hydroxylation of tyrosine and tryptophane represent the first steps in the biosynthesis of neurotransmitters, catecholamine and serotonin, respectively. (Roberts and Fitzpatrick 2013, 351–3; Szigetvari et al. 2019, 292)

Demethylases catalyze the removal of methyl groups from DNA, RNA, proteins, specifically histones, and other biomolecules. Methylation of DNA plays an important role in the regulation of gene expression. Histones' methylation also causes changes in transcription turning the

genes in DNA “on” and “off” by modulating the accessibility of transcription factors to DNA, as DNA in the cell nucleus is wound around histones. Methylation of mRNA regulates the expression of proteins. An example is N6-methyladenine demethylase that catalyzes the demethylation of N6-methyladenine in RNA. In this enzyme, the catalytic Fe(II) is coordinated octahedrally in a motif comprising two histidine residues, aspartate residue, and three water molecules. The Fe atom is utilized as a cofactor, while α-ketoglutarate and oxygen molecules are used as co-substrates in the reaction of demethylation. (Aik et al. 2014, 4742)

Fatty acid desaturases catalyze the formation of double bonds in molecules by removing two H atoms from two neighboring saturated C atoms. Desaturases are characterized by the presence of histidine clusters around di-iron centers. For example, in stearoyl-CoA desaturase, nine conserved histidine resides comprise the cluster in the catalytic center. Two Fe ions are coordinated by N atoms of these histidine residues, and the carbonyl group on asparagine molecule attaches to the di-iron center via water molecule. The catalytic center is bordering the bend in the substrate binding burrow. (Nagao, Murakami, and Umeda 2019, 328) Stearoyl-CoA desaturase is utilized by the body for the synthesis of oleic acid from stearic acid. Besides being a ubiquitous component of all cells, oleic acid also exhibits health beneficial effects in the human body as it decreases cholesterol and reduces inflammation thus improving heart conditions.

Superoxide dismutase (SOD) catalyzes the reaction of disproportionation, in which a harmful reactive species superoxide anion $O_2^{\bullet-}$ is converted to a less harmful hydrogen peroxide, H_2O_2, and an oxygen molecule, O_2. In Fe-SOD, an active site contains a single Fe ion at the center of trigonal bipyramid. While two histidine and one aspartate residues occupy equatorial positions, one histidine residue and a water molecule (or hydroxide anion OH^-) occupy axial positions. There also exist SOD enzymes that depend on Mn, Cu, and Zn at the metal center. In terms of the set of amino-acids that coordinate the metal ion as well as the secondary and tertiary structure, Fe-SOD is similar to Mn-SOD, while CuZn-SOD is different. (Sheng et al. 2014, 3868–9)

Ribonucleotide reductase is an enzyme that catalyzes the formation of deoxyribonucleotides from ribonucleotides. This enzyme is responsible for reducing all four ribonucleotides, the building blocks of DNA. In humans, a beta subunit of ribonucleotide reductase contains an oxygen-bridged di-iron center (Fe-O-Fe) and a stable tyrosyl radical. Since inaccurate DNA replication may lead to the development of tumors, ribonucleotide

reductase and its cofactor containing Fe play a vital role in carcinogenesis. (Aye et al. 2015, 1–4)

25% of the body's Fe is contained in ferritin, whose role is to store Fe. 60% of Fe is complexed with hemoglobin in the red blood cells. Only 15% of Fe is bound to myoglobin in muscle tissue and other proteins responsible for various functions, described above.

The interactions of Fe with biomolecules capable of forming chelate complexes and taken into organism with food, specifically flavonoids coming from fruits, vegetables and other plant products, play an essential role in Fe homeostasis. Since Fe is a central atom of coordination complexes in the catalytic centers of multiple enzymes, the presence of flavonoids forming high affinity complexes with Fe may significantly affect the activity of these enzymes. It is known that metabolism and health beneficial properties of flavonoids are strongly affected by their interactions with Fe. Moreover, flavonoids' complexation with Fe^{2+} or Fe^{3+} represents one of the major events in the network of reactions defining their biological activity. The following subchapters describe the structure of Fe-flavonoid complexes and the biological role of Fe-flavonoid interactions.

2.3. The structure of Fe-flavonoid complexes

Natural flavonoids possess specific structural features that facilitate the formation of coordination compounds involving metal ions and are often characterized by significant metal-binding affinities. These structural elements include hydroxy groups attached to neighboring C atoms (catechol motifs), and carbonyl groups together with hydroxy groups located on nearby C atoms. O atoms provide electron pairs that extend to the d-orbitals of metal cation forming coordinate covalent bonds. Flavonoids typically form bidentate ligands thus attaching to the metal cation by O atoms. Metal-flavonoid chelates have been studied for several decades, and out of all metal ions capable of coordinating to polyphenolic compounds Fe cations, Fe^{2+} and Fe^{3+}, are most commonly presented in contemporary scientific research.

Flavonol quercetin (Fig. 3A) represents an effectual model for studying the structural and physicochemical properties of Fe-flavonoid complexes, as it possesses three different metal binding sites. Moreover, quercetin is often used as a point of reference in the studies of other flavonoids in relation to the stabilities of their complexes with metals, metal binding sites, stoichiometry, and metal reduction potency. Quercetin is found in various fruits, leafy vegetables, and especially onion. It is one of the most

thoroughly studied natural flavonoids that exhibit cardiovascular protection and anticancer effects.

There have been published a lot of contradictory data on the structure of Fe-quercetin coordination compounds, specifically on the binding site of metal cation and the stoichiometry. The discrepancy in conclusions could arise from different conditions employed in the studies. However, some common structural characteristics could be discerned, when analyzing multiple literature sources on Fe binding to quercetin. The d^5 configuration of Fe^{3+}, in which electrons are unpaired, facilitates the formation of complexes with octahedral orientation of ligands. Thus, Fe^{3+}-quercetin chelate also adopts an octahedral configuration, stabilizing a ferric state. As for the binding site in quercetin, out of three possibilities, namely C3'-hydroxy-C4'-hydroxy (3'-4'; catechol-like coordination, Fig. 3B), C3-hydroxy-C4-carbonyl (3-4; maltol-like coordination, Fig. 3C), and C4-carbonyl-C5-hydroxy (4-5; acetylacetone-like coordination, Fig. 3D), the catechol motif on the B ring seems to be the most plausible scenario. The structure of the quercetin molecule and the potential Fe binding sites are depicted in Fig. 3.

Papan et al. employed NMR, UV-Vis and FTIR technologies in the study of quercetin-Fe^{3+} complex and reported a 1:2 (metal:ligand) stoichiometry at pH 7.4. (Papan et al. 2020, 8) In this structure, the central atom Fe^{3+} is attached to two bidentate ligands through the deprotonated O atoms on quercetin molecules and two water molecules, thus forming an octahedral complex. Based on the analysis of FTIR spectra, the authors excluded the possibility of Fe^{3+} binding to quercetin via 4-5 moiety and reported the coordination of Fe^{3+} to quercetin via the catechol group (ortho-dihydroxy) on the B ring and 3-4 moiety. (Papan et al. 2020, 6) Mixed binding sites were proposed in the same complex. (Fig. 3E–G)

Remarkably, in an alternative work by Corrente et. al., the authors also determined the unfeasibility of Fe^{3+} coordination to quercetin via 4-5 moiety in quercetin. The conclusion was made based on the combined analysis of experimental and computational NMR. (Corrente et. al. 2021, 10) The preferred binding site in the Fe^{3+}-quercetin complex involved catechol motif on the B ring (Corrente et. al. 2021, 6), which had been also confirmed by the earlier studies conducted in acidic and neutral media. The complex formed between catechol moiety and Fe^{3+} cation possesses certain stability, since Fe^{3+} is classified as a hard Lewis acid, while catechol is known to be a hard Lewis base. The reaction between a hard base and a hard acid is typically fast and leads to the production of strong complexes. Moreover, the presence of Fe^{3+} in the system reduces the effective pKa of catechol hydroxy groups, bringing their values from 6.4

(C4') and 11.5 (C3') (Papan et. al. 2020, 6) to the apparent values of about 5–8 for pure catechol (Bijlsma et al. 2020, 2) and even lower. This results in a more stabilized deprotonated state of quercetin.

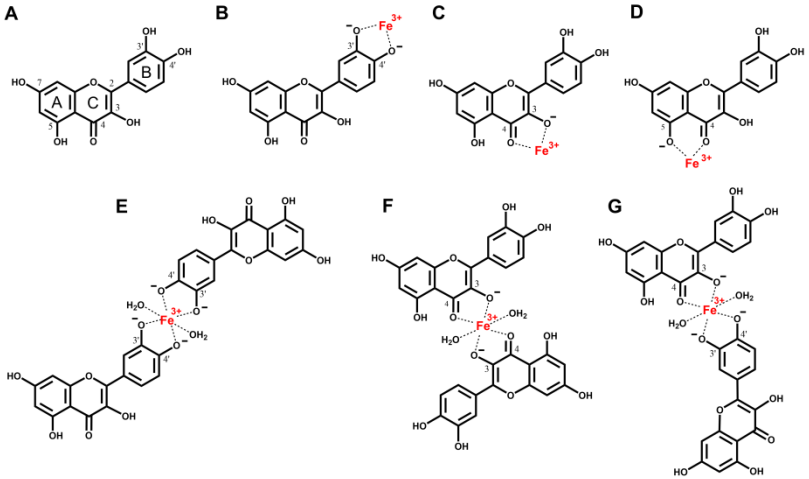

Figure 3. Structures of quercetin and Fe-quercetin chelates. **A.** Quercetin. **B.** Quercetin bound to Fe with 3'-4' site. **C.** Quercetin bound to Fe with 3-4 site. **D.** Quercetin bound to Fe with 4-5 site. **E.** Structure of Fe-quercetin chelate showing two quercetin bidentate ligands bound with 3'-4' sites. **F.** Structure of chelate having two ligands bound to Fe with 3-4 sites. **G.** Structure of chelate having two quercetin ligands bound to Fe with 3-4 and 3'-4' sites.

A commonly-known stoichiometric trend expected in metal-flavonoid chelates is that the number of flavonoid molecules attached to the metal cation increases with pH, since at higher pH more OH group tend to lose protons. However, the exceptions of this general rule are observed, which are due to the complexity of the flavonoid structure and possibly the involvement of mesomeric effects in the molecule. Thus, Corrente et al. observed a 1:2 (metal:ligand) complex at pH value of about 3 and a mixture of compounds having two quercetin molecules (1:2) and one quercetin molecule (1:1) attached to Fe^{3+} at pH greater than 3. (Corrente et. al. 2021, 4) In both stoichiometric cases of Fe^{3+}-quercetin complex, H_2O and OH^- coordinate to the central atom fulfilling the octahedral coordination geometry.

The studies involving Fe chelate complexes of quercetin derivatives provide additional insights into the stoichiometry and the preferable Fe coordination sites in flavonoid molecules. In the gastrointestinal tract and

in the liver, quercetin is metabolized forming isorhamnetin and tamarixetin. Isorhamnetin and tamarixetin are 3'-O- and 4'-O-methylated derivatives of quercetin, respectively. (Fig. 4) Since both derivatives lack catechol motif on the B ring, which is expected to be the most favorable spot for Fe coordination, they are unable to form chelates at this position. However, the experiments show that both methyl derivatives do form chelates with the stoichiometry of 1:2 (metal:ligand) at pH 6.8 and 7.5. In these complexes, the Fe cation is attached to a 3-4 site located on the C ring. (Lomozová et al. 2021, 5931) Remarkably, isorhamnetin exhibits stronger Fe^{2+} chelation potency than tamarixetin at pH of 7.5, even though the coordination site seems to be identical and no alternative metal-binding sites are available in the molecules, since 4-5 was proven to be inactive with respect to metal coordination, as discussed earlier. (Lomozová et al. 2021, 5929) This difference could be explained by considering resonance structures that show the delocalization of the negative charge as a result of the electron donating effect of hydroxy groups located on the B ring of flavonoids. Since the hydroxy group exhibits a stronger electron donating properties when attached to a benzene ring than the methoxy group, the charge delocalization is analyzed for hydroxy groups rather than methoxy groups, and for simplicity the ionized molecules are considered (Fig. 4). It could be seen from Fig. 4 that because of the different locations of hydroxy groups on isorhamnetin and tamarixetin (C4' and C3' positions, respectively), the delocalization of the negative charge reaches into the C ring in isorhamnetin, while in the case of tamarixetin, the negative charge stays spread out only over the B ring. This probably results in a stronger stabilization effect in the case of the positively charged metal, namely Fe^{2+}, coordinating at the binding site located on the C ring. Little difference between the stability constants was observed for isorhamnetin and tamarixetin in the case of Fe^{3+} chelation. The affinities to Fe^{3+} were higher than the affinities to Fe^{2+}. (Lomozová et al. 2021, 5932)

The studies of isoquercetin, 3-*O*-glucoside of quercetin (Fig. 4), found in multiple fruits and vegetables and utilized in food supplements, shed more light into the structure of flavonoid complexes with Fe. Experiments conducted at pH ranging between 4.5 and 7.5 revealed the stoichiometry of 1:1 (metal:ligans) for isoquercetin complexes with both Fe^{3+} and Fe^{2+}, which is different from the 1:2 ratio observed in Fe complexes of quercetin, isorhamnetin, and tamarixetin. The difference is most likely due to the steric hindrance constituted by glucose at C3 position resulting in the presence of only one bidentate ligand bound to Fe cation. The potential of isoquercetin to form Fe complexes increased with pH, supporting the commonly observed phenomenon that the deprotonated ligands have a

higher tendency to bind metal cations than the fully protonated ones. (Catapano et al. 2017, 7) The comparison of the affinity to Fe between isoquercetin and quercetin revealed a stronger binding in case of the latter, suggesting that the 3-4 site plays an important role in chelation, since it is not available in isoquercetin (Fig. 4), while both flavonoids have a catechol binding site on the B ring. (Catapano et al. 2017, 10)

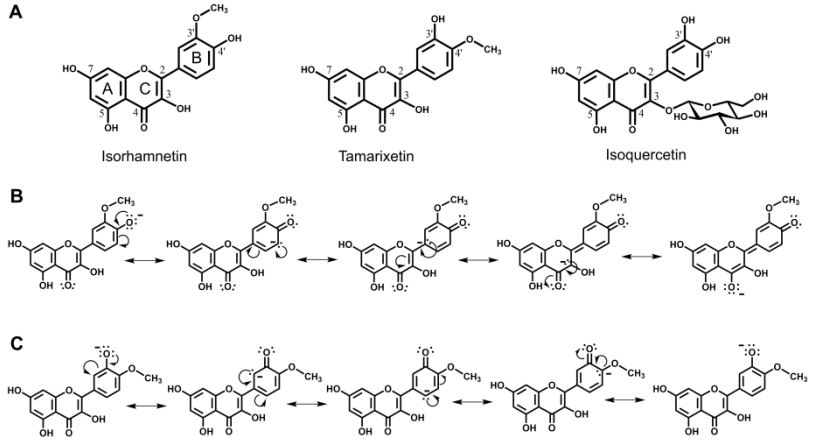

Figure 4. Methylated derivatives of quercetin. **A.** Structures of isorhamnetin, tamarixetin, and isoquercetin. **B.** Resonance structures of isorhamnetin deprotonated at C4'. **C.** Resonance structures of tamarixetin deprotonated at C3'. Curving arrows in **B** and **C** represent transfer of electrons in the formation of a new resonance structure when moving from left to right in a row of structures.

Silymarin is an active ingredient in milk thistle (*Silybum marianum* L.), an ancient medicinal plant named for the white veins in its leaves. Milk thistle possesses antioxidative properties. It is used to treat various maladies and in our days could be purchased as an oral capsule or liquid extract. Silymarin is extracted from the plant's seeds and represents a mixture of flavonolignans. These compounds may serve as a model for structure-activity studies of flavonoids' metal chelation. Tvrdý et al. performed comparative studies of several selected silymarin flavonolignans shown in Fig. 5 and correlated the results with the measurements of quercetin. The authors found a striking difference between planar molecules having a double bond between C2 and C3 atoms and non-planar structures with saturated C atoms at the same positions. Specifically, both 2,3-dehydrosilybin (DHS) molecules (DHS A and DHS B) formed stable chelate Fe complexes with the stoichiometry of 1:2 or 1:3

(metal:ligand), (Tvrdý et al. 2018, 119) while the other flavonolignans, silybin A, silybin B, and silychristin, did not form coordination compounds with Fe at all or produced very weak complexes. (Tvrdý et al. 2018, 121) Since all flavonolignans in the study lack the catechol moiety on the B and A rings, the only sites accessible for chelation are 3-4 and 4-5. The 4-5 site was proven to be hugely ineffective, so the only available and potentially active site is 3-4, which appears to be a very weak ligand in non-planar molecule, possibly because of the weakened acidity of the hydroxy group at C3 position in sylibin compared with that in DHS. In DHS, a π-conjugated system which is spread all over A, B, and C rings, stabilizes the anion formed when 3OH group is deprotonated, while in silybins this π-conjugated system is disrupted because of the lack of a 2-3 double bond, which explains a weak acidity. Interestingly, no difference between the Fe chelation potency was detected for the two enantiomers, DHS A and DHS B, (Tvrdý et al. 2018, 119) suggesting that the Fe binding site is remote from the chiral centers, represented by C11 atoms in the molecules. (Fig. 5) The comparison of DHS with quercetin (Fig. 3A) showed a stronger activity of the latter in the formation of chelates with Fe at pH 6.8 and 7.5, revealing the involvement of the catechol moiety on the B ring of quercetin. At lower pH values, however, no difference between DHS and quercetin was observed. (Tvrdý et al. 2018, 121)

In an alternative work by Mladěnka et al., the activity of a 3'-4' (catechol) site towards coordination of Fe was also revealed through the analysis of 26 flavonoids from different subclasses. The chelating potency of the catechol motif was substantial at higher pH and dropped significantly at lower pH, consistent with the studies of Tvrdý et al., and others. (Mladěnka et al. 2011, 695–6) The authors also demonstrated the significant activity of a 3-4 site in combination with a 2-3 double bond. The maltol-like binding site on the C ring (3-4) was stronger than the catechol moiety on the B ring. Remarkably, the C6-hydroxy-C7-hydroxy (6-7) site on the A ring in baicalein appeared to be even more powerful than 3-4 and 3'-4'. (Fig. 6) The chelating activities of flavonoids were also correlated with deferoxamine (Fig. 6), a well-known Fe-chelating agent used under Fe overdose. Some flavonoids involving a catechol motif on the B ring and a 3-4 site with a 2-3 double bond on the C ring and the stoichiometry of 1:1 did exhibit Fe binding potency similar to that of deferoxamine, however, this was only the case at physiological pH. The examples include quercetin (Fig. 3A) and myricetin (Fig. 6). When tested at lower pH, the Fe binding activity of these flavonoids was essentially weaker than that of deferoxamine. Remarkably, the affinity of baicalein to Fe remained at the same level with that of deferoxamine at all pH values

ranging between 4.5 and 7.5, indicating an exclusive Fe coordination power of the catechol motif on the A ring. (Mladěnka et al. 2011, 697)

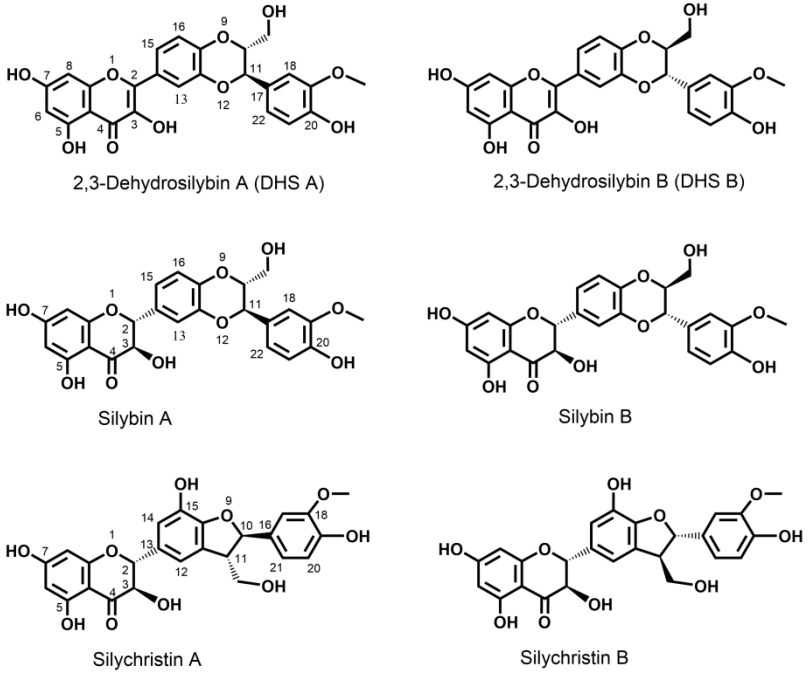

Figure 5. Structures of flavonolignans constituting silymarin.

In a comprehensive analysis performed by Mladěnka et al. who studied 26 flavonoids of various structure, the authors concluded that the 4-5 site located on A and C rings is less efficient in Fe binding than the three sites described above (3'-4', 3-4, and 6-7). This conclusion was in line with that made by various other researchers who conducted similar studies. However, in a recent study involving thorough experimental and computational approaches on luteolin and its complexes with metals, Malacaria et al. revealed a preferable binding of Fe^{3+} to the 4-5 site of the flavonoid. The authors stated that these complexes, having OH groups attached to Fe^{3+} in addition to luteolin, were 2.8 kcal/mol (in case of one OH group) and 1.4 kcal/mol (in case of two OH groups) more stable than the same species with the catechol (3'-4') binding mode. (Malacaria et al. 2022, 6) This result seems to contradict multiple works where the weakness of the 4-5 binding site was reported. However, in a lot of cases,

where the 4-5 site was studied, a hydroxy group at the C3 position was also present, examples including quercetin (Fig. 3A) and myricetin (Fig. 6). In these molecules, the 3-4 site is available along with 4-5, possibly creating a competition for Fe binding. In flavone luteolin studied by Malacaria et al., only the 4-5 site is available for metal coordination in addition to the catechol moiety on the B ring, (Fig. 6) ultimately resulting in speciation profiles showing preferred 4-5 binding, which contradicts the conclusions reported by Papan et al. and Corrente et al. about the weak chelation activity of the 4-5 site.

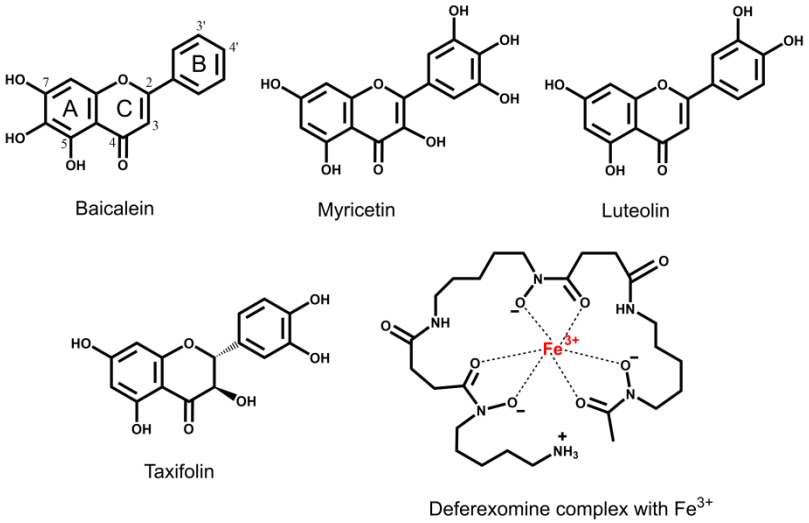

Figure 6. Structures of baicalein, myricetin, luteolin, taxifolin, and Fe^{3+}-deferexomine complex. Hexadentate ligand deferoxamine forms six bonds with a central atom, making Fe^{3+} inactive.

In the studies of luteolin, the authors reported a 1:1 (metal:ligand) stoichiometry and an octahedral coordination around Fe^{3+}, where the remaining spots were occupied by water molecules. (Malacaria et al. 2022, 6) The octahedral geometry of Fe^{3+}-flavonoid complexes is in agreement with the works by Papan et al. and Corrente et al. discussed above. The Fe^{3+}-luteolin complex exhibited remarkable antioxidant activities as revealed by 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) test. DPPH$^{\bullet}$ is a dark crystal made of stable free radical that has a strong absorption band centered at 517 nm. The decrease in the absorbance is a signature of the neutralization of the radical caused by a flavonoid or its metal complex. In

a DPPH$^{\bullet}$ assay, Fe^{3+}-luteolin turned out to be a more powerful radical scavenger than Cu^{2+}-luteolin, Al^{3+}-luteolin, and free luteolin. (Malacaria et al. 2022, 9) Additionally, the Fe^{3+}-luteolin complex appeared to be powerful in suppressing the levels of interleukin, a group of proteins facilitating communication between cells and principally essential in stimulating immune responses. The level of interleukin was evaluated in macrophages derived from white blood cells monocytes in response to lipopolysaccharides, endotoxins that induce inflammation and fever. In higher organisms, a reaction to lipopolysaccharides may result in anaphylactic shock and even death. Therefore, the level of interleukin in this assay is a measure of health protection effect caused by luteolin and its complexes. Remarkably, the Fe^{3+}-luteolin complex turned out to be the most efficient interleukin inhibitor in a series of tested complexes that included Al^{3+}-luteolin, Cu^{2+}-luteolin, and free luteolin. (Malacaria et al. 2022, 10)

The studies of Fe binding to isoflavones provide further insights into the mechanisms of the formation of coordination compounds. Karlíčková et al. tested 13 isoflavones with different structures. All compounds lacked catechol motifs, so exclusively the role of the 4-5 (acetylacetone-like) binding site in chelate formation, as well as the influence of the other groups on A and B rings such as hydroxy, methoxy, and glycosyl entities on the chelation potency of 4-5 were evaluated. It was found that only four isoflavones, specifically those having 4-5 site formed chelates with Fe^{2+} and Fe^{3+}: biochanin A, prunetin, genistein, and genistin. (Fig 7) The binding potency for Fe^{2+} was weak, especially at low pH, and increased at physiological pH. (Karlíčková et al. 2015, 3) The difference in the binding strength between different isoflavones was distinctly seen when the total Fe (Fe^{3+} and Fe^{2+}) was tested at pH 4.5, especially at a 10:1 (isoflavone:metal) ratio. At these conditions, biochanin A was a lot stronger than the other three isoflavones. Prunetin and genistin exhibited similar binding affinities, which was approximately 25% lower than that of biochanin A, while genistein was almost 90% less efficient than biochanin A. (Karlíčková et al. 2015, Suppl. 2) This result is surprising, since biochanin A and genistein are very close in chemical structure, the only difference being the hydroxy group at the C4' position in genistein and the methoxy group at the same position in biochanin A (Fig. 7) The drastic difference in the affinity of the 4-5 site to Fe cations must be primarily associated with the acidity of the hydroxy group at the C5 position. The pKa values of hydroxy groups in genistein were determined to be 7.2 (C7), 10.0 (C4'), and 13.1 (C5). (Zielonka, Gebicki, and Grynkiewicz 2003, 961) In alternative works, pKa values in the order of

11 for hydroxy group at C5 position were reported suggesting a stronger acidity. However, the presence of the other two hydroxy groups in genistein molecules, makes the deprotonation energy of 5-OH especially high, since in this case the proton is detached from a molecule that already has a charge of "-2". This explains a low chelation activity of 4-5 site in genistein compared with the other isoflavones having only two hydroxy groups.

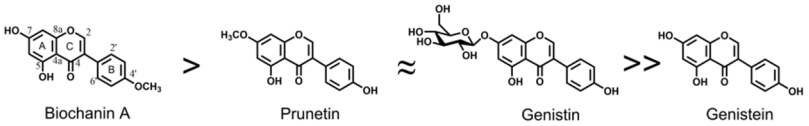

Figure 7. Structures of four isoflavones arranged in order of decreasing their Fe binding strength tested at pH 4.5 (Karlíčková et al. 2015, 3).

2.4. The redox processes associated with the formation of Fe-flavonoid complexes

All flavonols discussed above including quercetin, its glycosyl derivative isoquercetin, and both methyl metabolites isorhamnetin and tamarixetin reduce ferric Fe (Fe^{3+}) to ferrous Fe (Fe^{2+}). The mechanism of the reduction of ferric cations by flavonoids involves several steps. In the first step, an electron is transferred from a flavonoid to a complexed Fe^{3+} followed by the formation of semiquinone type radical and free Fe^{2+}. In the second step, the radical donates a second electron to a free Fe^{3+} cation in the solution leading to the formation of quinone and a second Fe^{2+} cation. Further steps of flavonoid transformation involve degradation, oxidative coupling, and polymerization resulting in the formation of various products giving a brown color to the solution (Bijlsma et al. 2020, 2)

Even though a number of experiments revealed a significant activity of multiple flavonols in the reduction of Fe^{3+}, their reducing power significantly varies within this subgroup of flavonoids depending on the number, location, and the nature of substituents positioned at the flavonol core skeleton. For example, isorhamnetin depicted in Fig. 4 exhibits a greater Fe reducing activity than tamarixetin. (Lomozová et al. 2021, 5932) It is reasonable to hypothesize that a flavonol with a lower pKa (stronger acid) of the hydroxy group on the B ring will donate electrons easier to the oxidizing agent, than a flavonol with higher pKa, due to a higher H-dissociation degree of the former. The hydroxy group on C4' of

isorhamnetin is more deprotonated than the hydroxy group on C3' of tamarixetin, since 4'-OH has the lowest pKa values out of all hydroxy groups in quercetin (Papan et al. 2020, 6), which explains a greater Fe^{3+} reducing potency of isorhamnetin compared with that of tamarixetin. However, there is evidence indicating a reverse trend in activity. Thus Catapano et al. reported an increase of Fe^{3+} reduction capacity of isoquercetin at lower pH or more protonated molecule. (Catapano et al. 2017, 10) This trend was opposite to Cu, suggesting a more complicated mechanism behind flavonol oxidation that possibly involves other hidden reactions specifically dependent on the type of a metal.

The analysis of the Fe reduction activity of silymarin components depicted in Fig. 5 provides additional insight into the structure-activity relationship in regard to Fe reduction. DHS proved more powerful Fe reducer than silybin A and silybin B, (Tvrdý et al. 2018, 122) which is in line with the conclusions found in other studies about flavonols being more active reducers than the other flavonoid subclasses, as flavonolignans DHS A and DHS B have a 2-3 double bond and a hydroxy group at C3 position similar to flavonols. However, another silymarin component, silychristin (Fig. 5), exhibited a much higher reducing activity than both DHS and silybins, (Tvrdý et al. 2018, 122) which could be attributed to the presence of hydroxy group at C15 position in the molecule (Fig. 5). This result suggests that there is no direct and straightforward correlation between chelation potency and reduction activity of flavonoids, since silychristin did not exhibit a noticeable chelation activity towards Fe, and the presence of hydroxy group at C15 position cannot contribute to the formation of coordination compounds with metal. The Fe reduction potency of silychristin was however weaker than that of flavonol quercetin (Fig. 3A) and flavanonol taxifolin (Fig. 6).

While flavonols overall exhibit significant Fe reducing activity, isoflavones proved extremely weak reducers despite the presence of hydroxy groups on the B and A rings. In the work performed by Karlíčková et al., out of the 13 isoflavones tested in the Fe reduction assays even at the 1:20 (metal:isoflavone) ratios, none of the molecules reduced Fe^{3+} by more than 10%. (Karlíčková et al. 2015, 4)

The reducing effect of flavonoids has also been demonstrated for heme Fe in cytochrome c (cyt c), a protein located at the inner membrane of mitochondria, which is responsible for electron transfer in oxidative phosphorylation. Additionally, cyt c is involved in intrinsic apoptosis and acts as lipid peroxidase when bound to cardiolipin. Lipid peroxidation precedes the detachment of cyt c from the membrane in the early stages of apoptosis. In a comprehensive study involving 17 flavonoids of different

structure, Rice et al. revealed that isoflavones were very weak reducers of the heme Fe, while flavonols such as myricetin, kaempferol, and fisetin exhibited a considerable reduction activity, (Rice et al. 2021, 7) consistent with the results of other experiments involving non-heme Fe. (Lomozová et al. 2021, 5931; Karlíčková et al. 2015, 4) Cyt c reduction activities of flavonoids correlated with their antioxidant potential, or more specifically, with their ability to inhibit the peroxidase activity of a cyt c- cardiolipin complex. (Rice et al. 2021, 13) The antioxidant activity of flavonoids, in addition to their cyt c reduction potency, depended on their membrane permeability and hydrophilicity.

The potency of flavonols and other polyphenolic compounds to reduce Fe^{3+} plays an important role in the generation of ROS, since the resulting Fe^{2+} may react with hydrogen peroxide, H_2O_2, in an oxidation process leading to the formation of hydroxyl radicals, hydroxide ions, and Fe^{3+}: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$. This oxidation process of Fe^{2+} by H_2O_2 is called Fenton reaction. It is believed that the strong chelators of both Fe^{2+} and Fe^{3+} impede the Fenton reaction, since the formation of the coordination layer around Fe^{2+} hinders its interaction with H_2O_2, while complexation with Fe^{3+} protects the cation from its reduction to Fe^{2+}, therefore, preventing the formation of hydroxyl radicals. Thus, for the same flavonoid, multiple outcomes are expected in reagard to promoting or inhibiting the formation of ROS, and the ultimate result strongly depends on the concentrations of flavonoid and metal, the pH of the system, the solvent, the source of Fe, and the type of an assay. Thus, Lomozová et al. observed that at large metal to ligand ratios (predominance of Fe^{3+} over isorhamnetin), a significant Fe reduction was observed, while at small ratios (prevalence of isorhamnetin), no detectable Fe reduction was detected, and chelation process dominated over reduction. Skalski et al. observed the antioxidant activity of isorhamnetin in human plasma treated with H_2O_2 and Fe by employing the measurements of lipid peroxidation, the carbonyl group measurement, and the measurement of thiol groups. (Skalski et al. 2019, 617)

Despite inconsistencies encountered in different research works, overall, the studies of redox processes involving various flavonoids and Fe cations converge to similar conclusions. Nkhili et al. reported that the reduction of Fe^{3+} by polyphenolic compounds is very slow, especially if compared with the reduction of Cu^{2+}. (Nkhili et al. 2014, 1190) A similar result was observed for quercetin. (Hajji et al. 2006, 317) The kinetic studies of metal binding to various phenolic compounds revealed much faster chelation of Fe^{2+} than Fe^{3+}, which could also contribute to the impediment of Fenton reaction thus resulting in an overall antioxidant

effect. (Nkhili et al. 2014, 1186; Hajji et al. 2006, 317) Additionally, autoxidation of phenolic compounds, a process of spontaneous reaction with O_2 resulting in the production of H_2O_2, is mildly hindered under the complexation with Fe, as was shown in different independent works for quercetin, rutin, catechin, and other polyphenols. For this reason, the formation of coordination complexes contributes to antioxidant effect as well. (Nkhili et al. 2014, 1192; Najji et al. 2006, 312) Remarkably, autoxidation kinetics of quercetin does not depend on the oxidation state of Fe, suggesting that Fe^{2+} bound to flavonoid is converted to Fe^{3+} in the complex.

2.5. The effect of Fe-flavonoid complexes on the properties of biological systems

A lot of health beneficial effects resulting from the intake of fruits and vegetables are attributed to flavonoids. To understand the mechanisms underlying the positive role these compounds play in the human body, a lot of in vivo and in vitro studies have been performed, where various biological properties were evaluated in the presence of the flavonoids and compared with control experiments. A notable conclusion arising from these studies is that the flavonoids' affinity to Fe happens to be as crucial for their metabolism and the general role in the human body, as their antioxidant activity, hydrophobicity, membrane permeability, pK_a values, and other chemico-biological properties. Moreover, numerous experiments showed that flavonoids bound to Fe cations exhibit enhanced activity in various biochemical processes compared with their activity in a free state. On the other hand, the behavior of Fe cations also alters due to their complexation with flavonoids. For example, penetration of the charged particles (Fe^{3+} or Fe^{2+}) through hydrophobic entities, such as membranes, could be facilitated due to their chelation to flavonoids, which provides partial charge covering. This complexation with polyphenolic compounds ultimately affects overall Fe homeostasis in the human body.

Flavonoids interactions with Fe cations may significantly influence the structural features and the dynamics of biochemical processes occurring in lipid bilayers, a building material of biological membranes in organisms. The formation of chelates with Fe^{2+} at the surface of the membranes not only inhibits Fenton reactions but also impedes the propagation of lipid peroxidation. Besides a significant antioxidative effect triggered at the surface of the membrane, flavonoids also affect the biophysical properties of lipid bilayers, for example, they decrease the fluidity of the membrane thus making the bilayer more rigid and shielding it from the flow of

harmful agents. It was shown that flavonoids in a Fe-bound state exhibit higher lipophilicity than in a free state. Free flavonoid at physiological pH is more or less deprotonated depending on the structure and position of a given hydroxy group on the core skeleton. The negative charges concentrated on O atoms make the molecule even more impermeable through the lipid bilayer than in a protonated state. When Fe^{2+} or Fe^{3+} is coordinated by a flavonoid, which typically occurs with two hydroxy groups on the B ring (3'-4' binding site) or hydroxy and carboxy groups on the C ring (3-4 binding site) as depicted in Fig. 3 for quercetin, the negative charges neutralize, increaing the lipophilicity of the molecule.

When a Fe cation is attached to two or three flavonoid molecules, the positive charge is buried inside the complex, potentially inhancing cation's permeability through the membrane. These complexes may cause aggregation and fusion of lipid bilayers, in which a hydrophilic part of the coordination compound, containing Fe cation and O atoms attached to it, produces a bridge exposed to an aqueous phase, while hydrophobic parts of the molecule are embedded into the bilayers. As a result, huge liposomes are produced. These lipid aggregates are also stabilized by Ca^{2+} cations that form bridges between phosphates located on the surface of liposomes, enhancing the adhesion forces between bilayer domains. (Tarahovsky et al. 2014, 1241) Thus Fe-flavonoid complexes could be utilized in the production of liposomal drug delivery tools.

The interactions of Fe-flavonoid complexes with biomembranes are especially significant in the regions of lipid rafts, the domains within the membranes that are typically thicker, as they have more ordered acyl side chains than the rest of the bilayer and contain cholesterol, sphingmyelin, and anchored proteins. The interactions of Fe-flavonoid chelates having 1:2 and 1:3 stoichiometry with lipid rafts affect the arrangement of membrane proteins modulating the functioning of receptors and signal transducers. It is suggested that in lipid rafts, proteins also provide sites for the attachment of Fe-flavonoid coordination compounds. As a result of these interactions: (1) proteins cluster in membranes initiating the formation of the massive lipid aggregate, (2) the adsorption of the protein to the membrane is facilitated, and (3) proteins accumulate together and form water-soluble fibers outside of the membrane. (Tarahovsky et al. 2014, 1242)

The interactions of Fe cations with flavonoids are of critical concern in the process of Fe penetration into enterocytes, absorptive cells in duodenum, the first part in the small intestine. Fe homeostasis in the human body is heavily dependent on its absorption. The apical membrane (or luminal membrane) separates enterocytes from the lumen of duodenum

and represents the first barrier in Fe transfer to the blood. When in the lumen, Fe^{3+} could be reduced to Fe^{2+} by flavonoids, which facilitates Fe transfer through the membrane since the solubility of Fe^{2+} is greater than that of Fe^{3+}. The chelation of Fe^{2+} by flavonoids can improve Fe permeability through the membrane, as lipophilicity of a complex containing Fe^{2+} buried in a molecular aggregate is better than that of free Fe^{2+}, however, most experiments that revealed this fact were conducted in vitro, while the penetration of Fe^{2+} or its complexes through the real apical membrane in the body is inextricably linked to additional processes such as the involvement of metal transporters, the interactions of flavonoids with the transporters, and other factors that may not be taken into account in vitro.

Lesjak et al. studied the effect of quercetin (Fig. 3A) on Fe absorption in rats' duodenal mucosa. The acute exposure of the cells to quercetin resulted in (1) an enhanced transfer of Fe through the apical membrane into the cell and (2) a decreased transfer of Fe through the basolateral membrane (from the cell into the bloodstream). Interestingly, the exposure of mucosa cells to methylated derivatives of quercetin such as 3-methyl-quercetin and 3,4'-dimethyl-quercetin and penta-methyl-quercetin resulted in a significant suppression of the Fe transfer across the apical membrane, compared with quercetin itself, while the experiments with 4'-methyl-quercetin led to only a slight decrease in Fe uptake. (Lesjak et al. 2014, 2–3) This result indicates that the enhancement of Fe uptake across the apical membrane stimulated by quercetin is associated with chelation of Fe^{2+}, and 3-4 is the major chelation site responsible for the binding. (Fig. 3B) The experiments testing the export of Fe to the blood from enterocytes revealed a similar methylation effect, as the exposure of the cells to derivatives containing methyl group at 3C position of quercetin led to a significant increase of Fe transfer across the basolateral membrane compared with the effect of parental quercetin, (Lesjak et al. 2014, 2–3) again suggesting the role of Fe chelation at the 3-4 site.

The researchers observed the suppressed expression of ferroportin, a protein responsible for the transportation of Fe^{2+} cations across the basolateral membrane, as a result of the rats' exposure to quercetin. (Lesjak et al. 2014, 3) Additional experiments were performed with Caco-2 cells. This cell line is derived from colon carcinoma, however, it is often used as a model to study molecular transfer across the intestinal epithelial layer, as these cells possess absorptive properties similar to those of duodenal enterocytes. The studies with Caco-2 cells revealed that the exposure to quercetin suppressed the expression level of ferroportin and, consequently, decreased the efflux of Fe into the blood. The repression of