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UGOTAVLJANJE INTERAKCIJE ŽELEZA IN BAKRA S 3, 4-DIHIDROKSIFENILOCETNO KISLINO, METABOLITOM FLAVONOIDOV, Z UPORABO METODE DIREKTNE SPEKTROFOTOMETRIJE

EVALUATION OF INTERACTION OF 3, 4- DIHYDROXYPHENYLACETIC ACID, A METABOLITE OF FLAVONOIDS, WITH IRON AND COPPER BY USE OF DIRECT SPECTROPHOTOMETRICAL APPROACH

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I performed my master's thesis research work at the Department of Pharmacology and Toxicology of the Faculty of Pharmacy in Hradec Kralove, Charles University Prague, under the mentorship of Assoc. Prof. Premysl Mladenka, Pharm. D., Ph. D. and home mentorship of Prof. Marija Sollner Dolenc, Pharm. D., Ph. D.

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Author's statement

I, Anja Babič, hereby declare to be the author of this master's thesis which is a final result of my independent work under the mentorship of Prof. Marija Sollner Dolenc, Pharm. D., Ph. D. and co-mentorship of Assoc. Prof. Premysl Mladenka Pharm. D., Ph. D.

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KEY WORDS

3,4-dihydroxyphenylacetic acid, iron, copper, antioxidant, stoichiometry, complementary approach

LIST OF ABBREVIATIONS

ALDH- acetaldehyde dehydrogenase

AMI- acute myocardial infarction

BCS- bathocuproinedisulfonic acid disodium salt (indicator used in copper calibration)

CNS- central nervous system

Ctr1- copper transporter-1

DOPAL- 3,4-dihydroxyphenylacetaldehyde

DPPH- 2,2-diphenyl-1-picrylhydrazyl radical

HA- hydroxylamine

MAO- monoamine oxidase

PD- Parkinson’s disease

RNS- reactive nitrogen species

ROS- reactive oxygen species

TF- transferrin

TFRI- transferrin receptor 1

34DHPA- 3,4-dihydroxyphenylacetic acid

DOPAC-3,4-dihydroxyphenylacetic acid

POVZETEK

Železo in baker sta esencialna elementa, ključnega pomena za normalno delovanje celic, saj sta vključena v oksidacijsko-redukcijske reakcije, detoksifikacijo in mnoge druge celične procese. Železo se v organizmu pojavlja v obliki Fe^{2+} in Fe^{3+} ionov, ki sta najpogostejši obliki železa v pufrskih medijih. Predvsem je pomembno za nastajanje novih eritrocitov, medtem ko so primarni vir železa makrofagi v tkivih, ki fagocitirajo poškodovane eritrocite in posledično akumulirajo železo. Baker pa se v bioloških sistemih pojavlja kot Cu^{+} ali Cu^{2+} ion. Zaradi pomembne vloge bakra pri rasti, je predvsem ključna zadostna količina bakra ob rojstvu in začetnih tednih življenja. Kljub temu pa lahko presežna količina bakra v telesu povzroči nezadostno razvitost jeter in s tem ogroža zdravje človeka. Poleg tega so nepravilnosti pri metabolizmu bakra znane kot potencialne povzročiteljice kardiovaskularnih obolenj.

Čeprav sta oba elementa izrednega pomena, se moramo zavedati, da imata pozitivne lastnosti samo, kadar sta prisotna v optimalni koncentraciji. V kolikor je namreč železa ali bakra v organizmu preveč, se pojavijo neželeni učinki. Prevelike količine tovrstnih ionov v biološkem sistemu namreč spodbudijo nastanek toksičnih kisikovih in dušikovih reaktivnih zvrsti. Pri tem je najpomembnejša Haber-Weissova reakcija. Posledično lahko pride do oksidativnega stresa in lipidne peroksidacije, kar lahko vodi v prelom verig ali baz v DNA. Zaradi tovrstnih pogojev lahko pride do genetskih mutacij, celične smrti ali celo rakavih obolenj. Presežek železa ali bakra je torej tesno povezan s kroničnim vnetjem ali pojavom degeneracije tkiv in je zato eden izmed povzročiteljev kardiovaskularnih obolenj, bolezni centralnega živčnega sistema in rakavih obolenj. V primeru kardiovaskularnih obolenj je Fe znano kot povzročitelj reperfuzijskih poškodb med zdravljenjem srčnih infarktov in kapi. Prav tako presežek železa, kot tudi bakra, povzročata škodljive vplive na delovanje centralnega živčnega sistema. Akumulacija železa v možganih lahko pripelje do Parkinsonove ali Alzheimerjeve bolezni, medtem ko pomanjkanje vodi do hipomielinizacije, nepravilnosti metabolizma živčnih prenašalcev ter s tem povzroči motnje v motoriki. Tudi Wilsonova bolezen je eno izmed zdravstvenih stanj, ki je tesno povezano z metabolizmom bakra.

Zaradi velikega števila zaščitnih lastnosti polifenolnih spojin pred oksidativnim stresom so torej le te terapevtsko izredno zanimive. So kompleksna in številčna skupina spojin

prisotnih v veliko vrstah sadja in zelenjave in zato pogosta sestavina vsakodnevne prehrane ljudi. Širok spekter polifenolnih spojin z enakim osnovnim skeletom je rezultat omejenega števila metabolnih poti, po katerih le te nastajajo. Dve najpomembnejši sta poliketidna metabolna pot in metabolna pot šikimske kisline. 3,4-dihidroksifenilocetna kislina, na katero smo se osredotočili v magistrski nalogi, je eden izmed metabolitov kvercetina, enega najpogostejših polifenolov v sadju in zelenjavi. Glede na dejstvo, da je kvercetin eden izmed močnejših kelatorjev, bi lahko na podlagi tega sklepali, da je tudi 3,4-dihidroksifenilocetna kislina oz. DOPAC potencialen kelator železovih in bakrovih ionov. Strukturno je derivat fenilocetne kisline, kjer sta mesti fenilnega obroča na m- in p-mestu substituirani s hidroksilno skupino. Nastanek kompleksov kisline z železom/bakrom, potencialno zmanjša količino presežnega železa/bakra in s tem zmanjša toksičnost, povzročeno zaradi oksidativnih reakcij omenjenih kovin.

V magistrski nalogi smo ugotavljali vpliv različnih pH vrednosti na keliranje železa in bakra s 3,4-dihidroksifenilocetno kislino. Za ugotavljanje kelacije oz. nastanka kompleksov, smo uporabili UV- Vis spektroskopijo. Ob primerjavi absorpcijskih spektrov čiste spojine in absorpcijskih spektrov z dodatkom železa/bakra v presežku je bilo mogoče spremljanje in primerjava nastajanja spektrov pod različnimi pogoji. Za določanje stehiometrijskega razmerja med spojino in železom/bakrom smo uporabili komplementarni pristop, kjer se spreminja koncentracija testirane spojine, koncentracija dodanega železa/bakra pa ostaja enaka. Za primerjavo smo uporabili tudi Job-ov postopek, ki temelji na kontinuirnem spreminjanju razmerja molarnih koncentracij obeh reaktantov, medtem ko skupna molarna koncentracija ostaja enaka. Ko smo z omenjenimi metodami dobili vse rezultate, je sledila matematična analiza z uporabo 6 matematičnih metod. Prve štiri matematične metode so si zelo podobne. Skupna lastnost teh metod je vrednotenje maksimalne absorpcije (A) kompleksa, pomembna pa je tudi A nezreagirane snovi. Ključnega pomena pri vseh metodah pa je tudi Beer-Lambertov zakon, ki nam pomaga pri izračunu samega kelacijskega razmerja. Zadnji dve metodi, pa sta nekoliko različni. Metoda V je vezana na teoretične linije, ki posnemajo A najbolj verjetnih stehiometrij. Ker gre pri komplementarnem pristopu za stabilno molarno koncentracijo, je A rastla v odvisnosti od nastajanja kompleksov in je nadaljevala z rastjo dokler ni prišlo do porabe testirane substance. Primerjava izmerjene A s temi teoretičnimi linijami, nam pokaže iskano stehiometrijo pri različnih molarnih koncentracijskih razmerjih. Razlika pri metodi

VI je le, da temelji na predpostavki o nastanku različnih kompleksov z različnimi molarnimi absorpcijskimi koeficienti v presežku iona.

Po pregledu rezultatov spektrov in grafov, dobljenih na podlagi matematične analize, je sledila končna ocena stehiometrijskega razmerja med spojino in železom/bakrom. S primerjavo rezultatov uporabljenih metod smo lahko podali ocenjen približek rezultatov kelacijskega razmerja.

Uporaba različnih pH vrednosti je izrednega pomena, saj želimo čim bolj preučiti obnašanje spojine v podobnih pogojih, kot so v telesu. Tako smo izbrali najnižji uporabljen pH 4.5, ki ga lahko izmerimo npr. v želodcu in v lizosomih, ki so pomembni za transport železa. Pogoji z nizkim pH-jem so izrednega pomena tudi zaradi patološke acidoze, ki se pojavi pri vnetjih, kot tudi pri akutnem miokardnem infarktu in tumorjih. Nekoliko višji pH pogoji, kot so pH 5.5 in pH 6.8, pa so bili uporabljeni za oponašanje hude ali nekoliko blažje ishemije, kjer je znano, da železo sodeluje v poškodbi tkiv.

Rezultati so pokazali, da je preiskovana spojina zmožna kelirati Fe^{2+} v razmerju 1:1 pri pH 7.5, pri Fe^{3+} pa ni bilo zaznane kelacije spojine, ali pa je bila ta izredno nizka. V primerjavi z železom je do nastanka kompleksov prišlo v primeru Cu^{+}, v razmerju 2:1 pri pH 6.8, medtem ko je pri Cu^{2+} bila zaznana kelacija pri pH 6.8 (1:1) in 7.5 (2:1). Tako v primeru železa kot v primeru bakra pa ni bilo zaznane kelacije pri pH 4.5 in 5.5.

Vsekakor je potrebno več raziskav na tem področju, predvsem pri študijah stabilnosti vezave železa in bakra na DOPAC. Idealni kelator bi moral imeti visoko selektivnost za železo/baker in minimalen vpliv na kelacijo drugih biološko esencialnih ionov. Ključnega pomena se je tudi zavedati, da rezultati pridobljeni iz *in vitro* raziskav ne moremo popolnoma ekstrapolirati na pogoje *in vivo*, lahko jih jemljemo zgolj kot približek obnašanja spojine v fiziološko relevantnih pogojih.

ABSTRACT

Iron and copper are two essential elements that are necessary for all living cells. However, the optimal amount of both is crucial, as harmful effects may occur if copper or iron is present in excess. Generation of reactive oxygen and nitrogen species with subsequent lipid peroxidation is a common consequence of excess copper/iron. Their toxicity is based on the Haber-Weiss reaction and can additionally lead to DNA strand breakage or base breakage, both leading to possible genetic mutations or cell death. These conditions may cause chronic inflammation or tissue degeneration and have been implicated as an important factor in cardiovascular diseases, central nervous system diseases and cancer. As polyphenolic compounds possess functions as transient metal chelation, direct free radicals scavenging, regeneration of antioxidants and protection of cells against oxidative stress, they might be therapeutically very interesting. The studied compound in this case is DOPAC or, in other words, 3,4-dihydroxyphenylacetic acid. It is a microbial metabolite derived from the metabolic pathway of quercetin, which is known to be found in great quantities in fruits and vegetables.

The aim of the study was to determine the conditions where formation of complexes of DOPAC and copper/iron occurs. The method used was UV-Vis spectroscopy. In laboratory measurements, the complementary and Job's approach were used and the results obtained were precisely calculated with mathematical data analysis. 6 different mathematical methods were used, by which the determination of stoichiometric substance-iron ratio was summed up.

The results have shown that DOPAC is capable of chelation with Fe^{2+} at pH 7.5 in the ratio 1:1. However, in the case of Fe^{3+}, either no chelation was detected or the chelation was very low. In comparison with copper, the formation of complexes occurred with Cu^{+} in the ratio 2:1 at pH 6.8 and in the case of Cu^{2+}, where chelation was detected in ratios 1:1 and 2:1 at pH 6.8 and 7.5 respectively. However, in general no chelation was detected at pH 4.5 and 5.5, neither in the case of copper nor the case of iron.

1. INTRODUCTION

1.1. Physiological and Chemical Functions of Iron and Copper

Iron is a chemical element which is involved in numerous biological processes in mammalian cells. Iron is an essential element which is required by all mammalian cells. It is one of the components of hemoglobin, myoglobin and has a major role in redox reactions needed to produce energy, for the process of detoxification, and many others. Iron possesses incompletely filled d-orbitals so it can exist in various valences. Ferrous (Fe^{2+}) and ferric (Fe^{3+}) are the most common oxidation states of iron in aqueous media and the redox potential between them contributes to a wide range of metabolic pathways. Even though iron is an essential element and therefore needed for proper functioning of all living cells, in excess, it can be harmful. Excess of free iron promotes generation of toxic reactive oxygen species (ROS). ROS are responsible for causing damage to the cell components, as they attack cellular lipids, proteins and nucleic acids (1, 2, 3). Basically, the reason for harmful effects caused by iron is the Haber-Weiss reaction. In these reactions, catalytic amounts of iron are sufficient to generate hydroxyl radicals ($^{\cdot}OH$) from superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). These substances belong to the class of so called reactive oxygen intermediates and are inevitable byproducts of the process of aerobic respiration or enzymatic processes. However, redox active iron also catalyzes the generation of other ROS, for instance, peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$), thiyl ($RS^{\cdot}$) or thiyl-peroxyl ($RSOO^{\cdot}$) radicals. The onset of oxidative or nitrosative stress occurs when the body's antioxidant capacity is below the levels of ROS or reactive nitrogen species (RNS, e.g. peroxynitrite /$ONOO^{-}$/) increase beyond the antioxidant capacity of the organism, oxidative/nitrosative stress occurs. This condition is associated with several pathological processes, e.g. with chronic inflammation, ischemia-reperfusion injury or neurodegeneration. Therefore, excess amounts of redox active iron, as well as copper, are responsible for aggravating oxidative stress in the cell, which results in accelerated tissue degeneration (4). However, oxidative stress is a common factor in several diseases and may aggravate them, but its causative role has never been precisely documented. Nevertheless, many researchers are analyzing different compounds for its prevention in different pathologies, e.g. cancer, aging, neurodegenerative diseases such as Alzheimer's and Parkinson's disease as well as many cardiovascular diseases. (5).

The hydroxyl radical may be a result of breakdown of peroxynitrous acid or of the metal-mediated reduction of peroxides. As mentioned above, Fe^{2+} commonly reduces H_2O_2 and as a result $^{\cdot}OH$ is generated via the Fenton reaction (6). Similar is valid for cuprous ions. Thus through this reaction, Fe^{2+} and Cu^{+} are the reagents responsible for the transformation of the weak oxidant H_2O_2 into one of the most reactive compounds in nature. However, because of the conditions in the cell, Fe^{3+} or Cu^{2+} are reduced back to Fe^{2+} or Cu^{+} through non-enzymatic reactions. This occurs with the help of compounds known as reductants (such as ascorbate and reduced glutathione) and promotes a vicious cycle of hydroxyl radical production (7).

$Fe^{2+} + H_2O_2 \Rightarrow Fe^{3+} + {}^{\cdot}OH + OH^{-}$ **Equation *1*: Fenton reaction (6)**

The formation of H_2O_2 occurs upon superoxide protonation in aqueous solution, which is described by the following equation (6).

$O_2^{\cdot -} + 2H^{+} \Rightarrow H_2O_2 + O_2$ **Equation *2*: Direct formation of H_2O_2 (6)**

$^{\cdot}OH$ is directly responsible for the oxidative damage, while $O_2^{\cdot -}$ is responsible indirectly; for example, from the oxidation of [4Fe-4S] iron-sulfur clusters to form H_2O_2 (6).

$[2Fe^{2+}\ 2Fe^{3+}\text{-}4S] + O_2^{\cdot -} + 2H^{+} \Rightarrow [Fe^{2+}\ 3Fe^{3+}\text{-}4S] + H_2O_2$ **Equation *3*: Indirect formation of H_2O_2 (6)**

$O_2^{\cdot -}$ also releases Fe^{2+} from enzymes, for instance ferritin, along with the [4Fe-4S]-containing dehydratases, by reducing Fe^{3+}. As a result, an unstable iron-sulfur complex is created and the release of free Fe^{2+} occurs (6).

$[Fe^{2+}\ 3Fe^{3+}\text{-}4S] \Rightarrow [3Fe^{3+}\text{-}4S] + Fe^{2+}$ **Equation *4*: Release of Fe^{2+} (6)**

Superoxide also has the ability to reduce aqueous Fe^{3+} or Cu^{2+} so these metal ions are able to react with H_2O_2 in a low-rate iron reduction. However, it is assumed that more abundant reductants such as NADH usually reduce cellular Fe^{3+} (6).

$O_2^{\cdot -} + Fe^{3+}$ or $Cu^{2+} \Rightarrow O_2 + Fe^{2+}$ or Cu^{+} **Equation *5*: Release of Fe^{2+} or Cu^{+} as a result of reduction (6)**

The summary of Fenton reaction and reduction of the catalyst, ferric iron or cupric ions is summarized in the Haber-Weiss reaction (6):

$O_2^{\cdot -} + H_2O_2 + H^{+} \overset{\text{»M«}}{\Rightarrow} O_2 + {}^{\cdot}OH + H_2O$ **Equation *6*: Haber-Weiss reaction (6)**

As the DNA backbone constitutes of negatively charged phosphate groups and electron rich nucleotide bases, metal ions interact with it. Transition metal ions, such as iron and copper also have the ability of creating a covalent bond with the nucleotide bases of DNA. The purpose of these metal ion interactions with the DNA backbone is stabilization for the purpose of balancing the charge of oxygen atoms of the phosphate backbone. The other possibility of balancing the charge is coordination according to electron pairs donated by nitrogen atoms of the bases, which is particularly common in guanine-rich sequences. However, when H_2O_2 is also present, redox active metal ions react with it, thus forming the highly reactive $^{\cdot}OH$. As a result, a strand breakage or base breakage may occur, both leading to possible genetic mutations, cancer or cell death (6).

Iron Physiology

A big source of the body's iron supply is present in hemoglobin in the red blood cells and their precursors, which represents almost a third of the total iron in a normal individual (Fig. 1). Daily, about 20-25 mg is needed for the production of new red blood cells. There are two normal paths where the body imports the required iron – one is through iron absorption, which is tightly controlled and limited (0.5- 2.0 mg daily), and the rest is recycled iron that was already present in the body. The primary source for this iron recycling process is tissue macrophages, which scavenge damaged red blood cells for iron in the process of phagocytosis. By phagocytosis, they harvest the iron that was in hemoglobin and then send it back into the circulation. However, there are many other iron storage spots in the human body, especially the cellular stores of iron in hepatocytes. Iron is unable to enter the cell through passive transport; therefore, it needs special carrier proteins. Special mechanisms are also required for the process of exportation of iron from the cell carrying it (1). Transferrin (TF) is an abundant plasma protein that is crucial for iron circulation. It keeps iron soluble and prevents Fe^{3+} ion from leaving aqueous plasma. It also prevents redox activity reactions with other molecules. By binding to a specific cell-surface receptor (TFR1), TF delivers the iron to the cells. The receptor is a homodimer that is capable of binding two molecules of TF. Wherever an unusually large demand for iron occurs, TFRI will be expressed at high levels. That occurs in cases of developing erythroid precursors, activated lymphocytes, placental syncytiotrophoblasts, as well as tumor cells (1, 4).

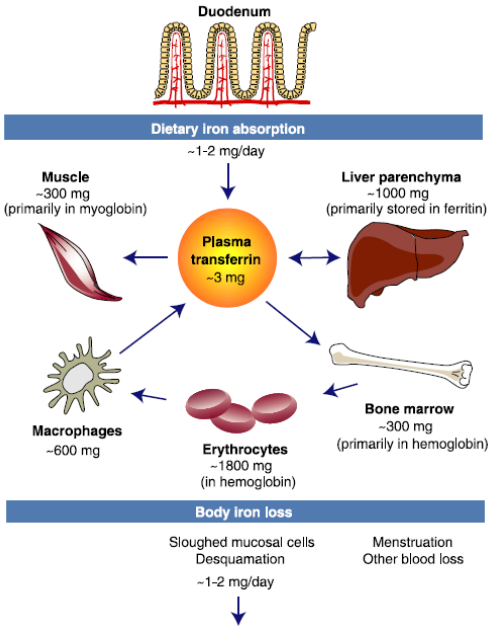

Figure 1: Iron distribution in the human body (4).

Copper Physiology

Copper is especially important in the period of infancy, as it is a crucial for rapid growth of the human body. Deficiency of copper is at that time a prominent concern, although a threat occurs in cases of immature liver function, resulting in an inability of handling greater copper levels, which would be exhibited as toxic effects (7).

In living organisms, copper has two oxidation states: cuprous (Cu^{+}), which is fairly soluble, and cupric (Cu^{2+}), with solubility in sub-micro molar range. The prevalent form in the biological system is Cu^{2+}, as it is oxidized by the presence of O_2 or other electron acceptors. However, the oxidation is reversible, as reductants such as ascorbate and reduced glutathione can give an electron to Cu^{+} and reduce it again. Because of the ability of copper to participate in one-electron reactions it has a major role as a redox reactant (7). Some of the main processes that copper is involved in are mitochondrial respiration, synthesis of melanin, dopamine metabolism, iron homeostasis, antioxidant defense, peptide amidation and others. Copper is also crucial in case of infections, because it is required by activated lymphocytic cells in order to produce interleukin-2 (7, 8).

Apically in the plasma membrane of intestinal epithelial cells, there are special transporters (Ctrl1), which carry out the uptake of dietary copper. In contrast to iron, copper can also get transported through enterocytes by passive diffusion. Once inside the cell, copper is

bound to one of several copper chaperones (Cox17, Cox11, ScoI, ScoII, ATOXI), ligand (glutathione) or to storage molecules. Similarly to iron, there is a very low cystolic concentration of unbound copper. Similarly, in blood plasma, copper is bound to a component called ceruloplasmin and also molecules such as albumins (9).

Defects in copper metabolism are known to impair cardiovascular health. Copper deficiency is implicated to be a defect of copper homeostasis that may lead to cardiac disease. There is yet another autosomal recessive inherited disorder of copper metabolism: Wilson disease. The disease is caused by exceeded amounts of copper which accumulates in many organs and tissues, therefore causing oxidative stress and cell destruction (8, 10).

1.2. Polyphenols

Polyphenolic compounds represent a large group of molecules present in most plants. They possess several important functions such as inhibition of pathogen development and microorganisms, protection against UV radiation and oxidative stress (11). In food, polyphenols contribute to color, flavor, odor, bitterness and oxidative stability. As polyphenols are naturally present in a variety of fruits, vegetables, cereals and beverages, they are a regular constituent of human food. Fruits like grapes, apple, pear, berries and cherries are polyphenol rich, as they contain up to 200 - 300 mg polyphenols per 100 g fresh weight (Tab. I). Therefore, since the fruits contain such significant amounts of polyphenols, also products derived from them are polyphenol rich (12).

A wide range of phenolic acids in plants have the same basic skeleton as a result of limited metabolic pathways though which such substances are formed. The two most important pathways from which the phenolic compounds derive are the polyketide and shikimic acid pathways (Fig. 2, 13).

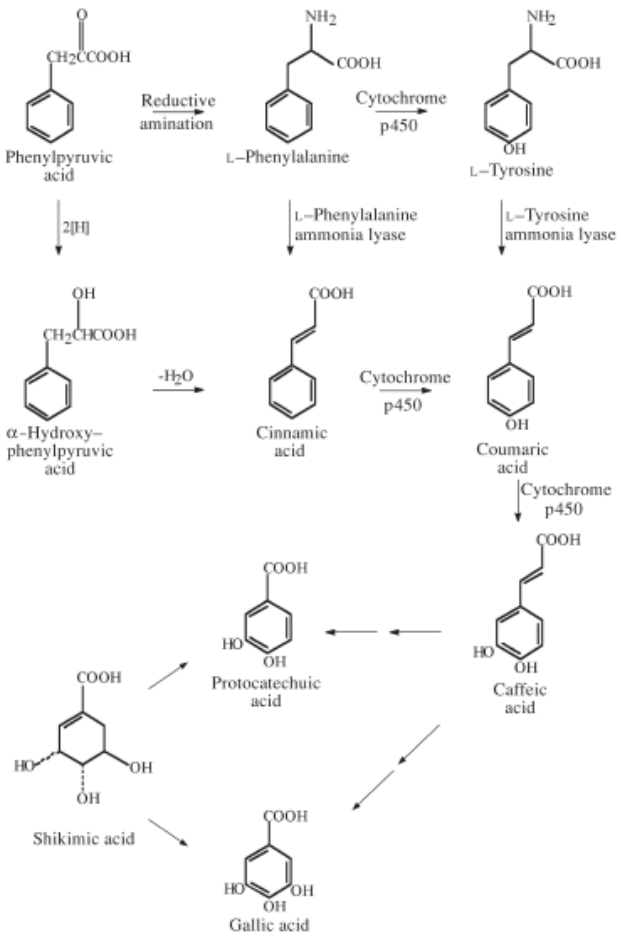

Figure 2: Main biosynthetic pathways for the formation of phenolic compounds (13).

There are different classes of polyphenols; some of these are phenolic acids, flavonoids, stilbenes, coumarins and lignans. The main criteria for the classification are the number of phenol rings they include and the key binding structural elements they possess. Phenolic acids are present in all plant material, however are found in greater amounts in acidic-tasting fruit. This class is further divided into hydroxyl benzoic and hydroxyl cinnamic acids. Flavonoids most abundantly occur in the human diet and their main characteristic is two aromatic rings bound together by three carbon atoms. More than 6000 different flavonoids occurring in plants have been described up to now (14). Thirdly, the group of stilbenes contains two phenyl moieties connected by a two carbon methylene bridge. Stilbens have an antifungal role in plants, as they are synthesized in response to injury or infection. The last class, lignans, contain a 2,3-dibenzylbutane structure. Several compounds from this group are considered to be phytoestrogens (12). All of the above described classes contain many different polyphenolic compounds; however, they share common functions. They are all reducing agents and the similar functions they possess are

scavenging of free radicals, participation in regeneration of antioxidants and protection of cells against oxidative damage (11). Some polyphenols also take part in modulation of gene expression, apoptosis and malignant transformation. Certain polyphenols may also prevent or reverse carcinogenesis by elimination of damaged cells (15).

1.2.1. Flavonoids and 3,4-dihydroxyphenylacetic Acid

Flavonoids are a class of secondary plant phenols widely distributed in leaves, seeds, bark and flowers of plants. Their structure consists of phenolic and pyrane rings and differs mostly in arrangements of hydroxyl, metoxyl and glycosidic side groups, as well as in the conjugation between the A and B rings. Some metabolic processes that may occur are an addition of hydroxyl groups, methylation, sulfatation and glucoronidation. In food, these compounds are primarily found as O-glycosides and polymers (16).

Table I: Dietary sources of flavonoids and phenolic acids (17).

Flavonoid	**Source**
catechins	tea, red wine
flavanones	citrus fruits
flavonols (e.g., quercetin)	onion, olives, tea, wine, apples
antocyanidins	cherries, strawberries, grapes, colored fruits
caffeic acid	grapes, wine, olives, coffee, apples, tomatoes, plums, cherries

DOPAC, or by its IUPAC name, 3,4-dihydroxyphenylacetic acid (Fig. 3), is a microbial metabolite of quercetin, which is one of the most frequently occurring polyphenols present in fruits and vegetables. Quercetin is thought to have many positive health effects; however, in the gastrointestinal tract the absorption rate is very low. Consequentially, it accumulates in the lumen where colonic microbiota degrades it. After degradation occurs, a large number of metabolites which can be absorbed and exert biological effects (antioxidant, antimicrobial and anti-inflammatory) are generated (18). They also include DOPAC which is also one of the most important metabolites of dopamine (Fig. 4). The structure of the molecule is an aromatic ring with an acetic side chain on one side and two vicinal hydroxyl groups in the other side. It may be considered a catechol (*m-*, *p-* dihydroxyphenyl) ring with an acidic side chain (19, 20). DOPAC is formed from dopamine by monoamine oxidase (MAO) and aldehyde dehydrogenase. There are two isoenzymes of MAO involved in its metabolism, MAO-A and MAO-B. The first one predominates in neural tissue, whereas both of them exist in non-neural tissue (21).

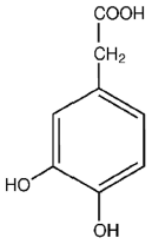

Figure 3: Structure of DOPAC (20).

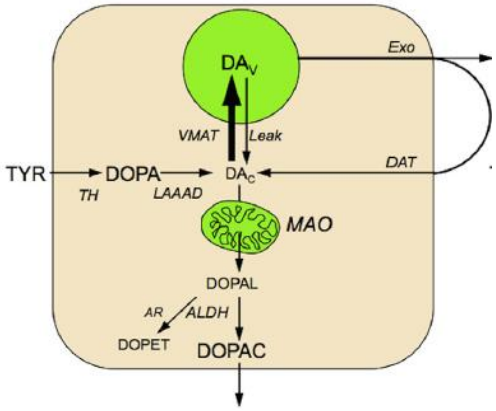

Figure 4: Synthesis and fate of dopamine (DA), which can either leak from vesicles (V) into the cytoplasm (C) or can be taken up via the cell membrane dopamine transporter (DAT). The vesicular monoamine transporter (VMAT) makes it possible to escape vesicular reuptake. Then DA is oxidatively deaminated by monoamine oxidase (MAO) which results in the formation of catecholaldehyde, dihydroxyphenylacetaldehyde (DOPAL). DOPAL is then further metabolized by aldehyde dehydrogenase (ALDH) to form dihydroxyphenylacetic acid (DOPAL) (22).

1.3. Applicability of Phenolic Compounds in Treatment of Iron and Copper Associated Diseases

As already mentioned, several pathological conditions derive from iron and copper involvement in the generation of ROS; two of most important are heart disease and cancer. Chelation of copper and iron will lower the oxygen toxicity to cells. Binding of iron to the flavonoid antioxidants therefore means suppression of the accessibility of the iron to oxygen molecules and by that inhibition of oxygen damage (23).

The most important functional group for Fe-binding is the *p*-dihydroxyl group, i.e., molecules bearing catechol or galloyl groups. It is also assumed that the relation of number of hydroxyl groups and antioxidant activity of the compounds is linear (23, 24). The presence of these iron binding groups in food may, to some extent, explain the health protective role of specific phenolic compounds in the human diet (23).

DOPAC effects on human beings

There are many studies based on research of properties and characteristics of polyphenols, mostly carried out on flavonoids in their native form. These studies present the ability of flavonoids to interact with receptors, inhibit enzymes and to induce various responses in cultured cells. However, many effects observed in animal experiments or clinical studies may also be explained by their microbial metabolites, which have rarely been explored. As they are derived from phenolic compounds, it is predicted that these compounds also contribute to protection against oxidative stress. Another reported activity which these substances perform is also inhibition of platelet aggregation, in which DOPAC was proven to be even more active than its precursor quercetin (11). In comparison to other phenolic acids (e.g. 3,4,5-trihydroxybenzoic acid, 1,2,3-trihydroxybenzene, 3,4-dihydroxycinnamic acid…). DOPAC has been proven a weaker inhibitor of lipid peroxidation (56 %). Also a slightly lower elimination rate of H_2O_2 was reached with DOPAC, around 52 %. However, when scavenging for DPPH radical was tested, DOPAC was proven as the metabolite with the strongest scavenging activity. The percentage of radical elimination was reported to be around 70.8 %. While measuring such activities of 3,4-dihydroxybenzoic, 3,4-dihydroxycinnamic and 3,4-dihydroxyphenylacetic acids, it had been shown that an *m and p*- substitution of hydroxyls may be the most important factor in terms of characteristics and function of the compound. By relocating these groups, the anti-radical, anti-oxidant and hydrogen peroxide scavenging activities can be altered. In conclusion, the anti-radical, hydrogen peroxide scavenging activities and the ability to scavenge free radicals are positively correlated with the number of hydroxyl groups present on the aromatic ring (25).

In a study of Carrasco-Pozo, et al. (18), the protective effects of DOPAC were tested. The researchers wanted to observe whether DOPAC could have a protective role in case of apoptosis. They were also testing the function of mitochondria and the amount of oxidative stress which can be induced by the presence of cholesterol in pancreatic β cells. Based on the results of the study, it was proven that DOPAC protects against harmful effects of cholesterol, such as loss of cell viability. The study also revealed its protective effects as it prevents apoptosis induced by cholesterol. In apoptosis, the apoptotic enzymes called caspases occur, thus measuring the activation of these enzymes enabled to study the process itself. Cholesterol treatment resulted in a 133 % increase of caspase-3 and caspase-9 activities. However, such increases were prevented by the presence of DOPAC. In the

presence of DOPAC mitochondrial dysfunction as well as oxidative stress was concentration-dependently prevented (18). As DOPAC is also the major metabolite of DA in the CNS, most studies have been conducted on neuronal cell cultures. Both multiple system atrophy as well as Parkinson's disease (PD) involves stratial dopamine depletion. Recently, the post-mortem and in vivo data have indicated that in the putamen and heart of a patient with PD, a great decrease in vesicular sequestration of cytoplasmic cateholamines is present. Decreased vesicular sequestration could lead to a different fate of intra-neuronal dopamine towards enzymatic deamination by MAO. The immediate product of the deamination is the catecholamide 3,4-dihydroxyphenylacetaldehyde (DOPAL). This metabolite is toxic, as it cross-links with various proteins, which results in increased production of ROS. Normally DOPAL is detoxified by aldehyde dehydrogenase (ALDH) to form DOPAC, but in the case of PD, there is a reduction of the ALDH activity in putamen. Brain tissue DOPAC is remarkably decreased in the case of PD. The reason for this is the combination of depletion of vesicular dopamine stores and decreased ALDH activity. Brain tissue DOPAC is also correlated with CSF DOPAC, which is why DOPAC levels are used as a biomarker of Parkinsonism (22).

Cardiovascular Diseases

Cardiovascular diseases, which represent roughly 30-50 % of all human deaths, remain the most likely cause of death in developed and developing countries. All common cardiovascular diseases (atherosclerosis, coronary heart disease, arterial hypertension, heart failure) have the same background; the presence of oxidative stress (26, 27, 28). Problems involving iron mediated injury are of great importance in the cardiovascular system. Chronic myocardial dysfunction can be found as well in hemochromatosis (iron overload) and thalassemia (abnormal formation of hemoglobin) and represent common cause of death in these patients. Moreover, it has become apparent recently that iron-mediated processes can cause reperfusion injury during the treatment of heart attacks and strokes. Iron may also be involved in atherogenesis or vascular restenosis following angioplasty (27).

One of the important roles of iron is in reperfusion injury, which occurs in tissue that has been temporarily rendered ischemic. It takes place during myocardial infarctions or strokes treated with angioplasty or thrombolytic agents. Likewise, other similar procedures such as cardiopulmonary bypass or in organs removed for transplantation may be associated with

increased levels of free iron. Generation of ROS is proposed to be one of the mechanisms for reperfusion injury. That is why in these cases phenolic compounds are of interest as therapeutic agents (iron chelators) to prevent oxidative damage to myocardial cells. One of the possibilities is deferoxamine, which in animal studies was proven to prevent cytotoxicity due to H_2O_2 and limit ventricular dysfunction (27) and in vivo was protective in models of ischemia-reperfusion (3). However, it is important to keep in mind that iron chelators should reduce tissue iron levels, allow efficient transport and excretion without iron redistribution, prevent excessive iron accumulation in organs, and neutralize toxic labile iron pools. This is a so called iron paradox since iron is an essential element, so the chelator only needs to remove excess iron without interfering with iron homeostasis and iron-dependent enzymes (28).

Atherosclerosis, another cardiovascular condition connected with iron, may be reduced with the use of iron chelators, as they could prevent oxidation of LDL cholesterol. That way we may be able to influence endothelial dysfunction with increased platelet aggregation, which can result in formation of thrombosis that leads to an acute myocardial infarction (AMI). Heart failure and arrhythmias are a common consequence of AMI. Moreover, patients with cardiovascular problems characteristically have an increased production of ROS, and that is why iron chelators are of such interest in this field (15, 26, 27). A summary of proposed effects of flavonoids, some of them are associated with iron chelation, is presented in Tab. II.

Table II: Favorable effects of flavonoid compounds on cardiovascular disease (24).

Cardiovascular disease	Potentially positive influence due to flavonoids
Atherosclerosis and stable forms of coronary heart disease	↓ LDL oxidation by lowering the levels of oxidative stress as well as inflammation
Acute myocardial infarction	↓ ROS burst, inhibition of platelet aggregation
Heart failure	↓ oxidative stress, inhibition of metalloproteinases
Arrhythmias	↓ oxidative stress
Hypertension	Vasodilatory properties

Central Nervous System Diseases

In the brain, iron and copper are important co-factors for a large number of enzymes, including those involved in neurotransmitter synthesis and myelin formation. Excess as well as shortages of iron and copper will affect the brain. Transport of substances into the

brain is strictly regulated by the blood-brain barrier and the blood-cerebrospinal fluid barrier, which protect the brain environment. However, the uptake mechanisms of iron and copper to the central nervous system (CNS) are strongly connected. Iron deficiency, as well as iron overload, leads to altered copper homeostasis in the brain and vice versa. Furthermore, copper is required by many proteins that are of great importance in iron homeostasis, transportation and binding to TF receptors. Inside the brain, iron and copper are taken up by neurons and glia cells with various transporters (9).

The optimal amount of both elements is crucial for a normal activity of the CNS. Iron accumulation in brain occurs in many chronic neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's disease. On the other hand, iron deficiency leads to hypomyelination, abnormal cognition, changes in neurotransmitter metabolism and impaired motor function (4, 9).

Studies revealed that a larger amount of iron causes a larger amount of damage to neurons, hence clearly suggesting that chronic degeneration in the brain goes hand in hand with the accumulation of iron. One of the CNS diseases, PD, occurs as a result of dopaminergic neuronal death in the substantia nigra pars compacta. Excess of labile iron participates in the Fenton reaction and contributes to generation of oxidative stress, which causes degeneration of dopaminergic neurons. Many patients with this disease have an increased level of total iron in the substantia nigra. Furthermore, iron accumulates in neurons as their ability for iron reuse is reduced, resulting in a higher need for iron uptake. In case of PD, the ferroxidase activity mediated by ceruloplasmin is altered; this ends up lowering the capacity for iron export. There are also suggestions that iron accumulation may also originate from iron-containing monocytes and macrophages that migrate into the affected substantia nigra of the patients with PD (9). Another serious CNS condition which often initiates after the age of 65, Alzheimer's disease (AD), has also been connected to oxidative neuronal damage. It causes memory loss and is extremely debilitating, as the cause is selective disruption of neuronal cortico-cortical connections (9, 29).

One example of CNS disease, which is mostly connected to copper metabolism disorder, is Wilson's disease. It is an autosomal recessive inherited disorder which results in pathological accumulation of copper in many organs and tissues, but mostly in the liver and the brain. Some research outcomes suggest that initial treatment of the disease may include a chelating agent. Antioxidants are therefore compounds of interest as well. Copper

accumulation causes mitochondrial damage with alteration of lipid oxidation, which results in marked hepatic steatosis. When the liver capacity of copper storage is exhausted, copper is released into the circulation. However, copper toxicity mostly affects the CNS. Since it is not taken up by neurons, there is an increased amount of extracellular copper, which may explain the neuronal damage in Wilson's disease (8).

Cancer

Several *in vitro* studies and animal models suggest that polyphenols could inhibit the development of cancer, however it must be noted that concentrations used in some studies may substantially exceed those that may be achieved in tissue by dietary intake. Some of the mechanisms by which polyphenols exert anti-carcinogenic effects are antioxidative effects, modulation of enzyme activities associated with carcinogen activation and detoxification, flavonoid-mediated modulation of gene expression, apoptosis and malignant transformation, and many others. Carcinogenesis is a multi-stage process in which genetic changes affect proto-oncogenes or tumor suppressor genes in cells. Genetic alterations can occur when DNA is exposed to increased or persistent damage. ROS and RNS are potential carcinogens, as they can directly or indirectly induce structural alterations in DNA. Some polyphenols like quercetin, kaempferol and luteolin, show ability to inhibit oxidative DNA damage; therefore they are of possible therapeutical importance. Polyphenols may also modulate enzyme systems that metabolize carcinogens or pro-carcinogens to genotoxins. In this way, polyphenols can inhibit activation of carcinogens or convert them into a less reactive compound. Flavonoids also have the ability to down-regulate proto-oncogenes, which may be over-expressed in tumor cells. For example, the levels of the proto-oncogene Ki-ras (over-expressed in the human leukemia cell line) are reduced by the presence of quercetin. Down-regulation of proto-oncogenes results in inhibition of proliferation and also in an increase in apoptosis (15, 30).

2. RESEARCH OBJECTIVE

Iron and copper are essential nutrients that have a major role in several cellular processes, most importantly, enzymatic activation, and redox reactions. However, it is crucial to understand that these elements are beneficial only in optimum amounts. If these amounts are exceeded, harmful effects such as oxidative stress occur.

As polyphenols are abundant in the human diet and represent a source of therapeutically interesting compounds, the aim of this study is to determine the chelation of iron and copper by 3,4-dihydroxyphenylacetic acid, one of the known major metabolites of many flavonoids, including commonly consumed quercetin.

The experiments are based on employing UV-Vis spectrometry for the determination of substance-iron/copper complexes. A complementary approach method, as well as the standard Job's approach, will be used for preparation of samples containing different ratios of the substance and iron/copper, important for the prediction of stoichiometry. The experiments shall be performed at 4 different pathophysiological important pH values that may occur in our blood or tissue in the body (4.5, 5.5, 6.8, 7.5). After the gathering of experimental data, the mathematical analysis will follow, as the basis of the stoichiometry prediction.

For the prediction of results, the following hypotheses were made:

- It is predicted that DOPAC will not chelate in the lower pH conditions (4.5 and 5.5).

- The occurrence of complexes in the conditions of pH 6.8 and 7.5 in both cases with copper and with iron is predicted.

3. MATERIALS AND METHODS

3.1. Materials

- DOPAC: 3,4-dihydroxyphenylacetic acid; M_W = 168.15 g/mol, 98 %, Sigma-Aldrich, 850217
- Fe^{2+}- ferrous sulphate heptahydrate: $FeSO_4 \cdot 7H_2O$; ACS reagent, ≥ 99.0 %; M_W = 278.01 g/mol; Sigma-Aldrich, 215422
- Fe^{3+}- ferric chloride hexahydrate: $FeCl_3 \cdot 6H_2O$; reagent grade, ≥ 98 %, purified lumps; M_W = 270.30 g/mol; Sigma-Aldrich, F2877
- Fe^{3+}- ferric tartrate; M_W = 555.90 g/mol; Sigma-Aldrich, F1013
- Cu^{+}- cuprous chloride: CuCl; Sigma-Aldrich
- Cu^{2+}- cupric sulfate pentahydrate: $CuSO_4 \cdot 5\ H_2O$; Sigma-Aldrich
- Hydroxylamine hydrochloride (HA) ; ReagentPlus®, 99 %; M_W = 69.49 g/mol; Sigma-Aldrich, 159417
- Ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt); for spectrophotometric det. of Fe, ≥ 97.0 %; M_W = 492.46 g/mol; Sigma-Aldrich, 82950
- BCS (bathocuproinedisulfonic acid disodium salt)
- Ultrapure water (Milli-Q-RG, Merck Millipore, USA)
- Methanol (J.T. Baker, Avantor Performance Materials Inc., USA)
- Buffers
    - pH 4.5 , pH 5.5; 15 mM acetate buffer
    - pH 6.8 , pH 7.5; 15 mM HEPES buffer
- Semi-micro polystyrene (PS) cuvettes; 340-900 nm; VWR International, 634-0676
- Ultraviolet- transparent (UV) cuvettes
- Pipette and pipette tips (Eppendorf Research Plus, Eppendorf, Germany)
- Microtubes (1.5 mL- microtube Eppendorf PP 1.5 mL, Boettger, Germany)
- Tubes (15 mL- centrifuge tube 15 mL PP, Thermo Scientific)

3.2. Devices

- Spectrophotometer: UV-Vis spectrophotometer Helios Gamma (ThermoFisher Scientific Inc., USA) equipped with Software: VisionLite Software 2.2

- Analytical balance (Kern Alt-220 4NM, Kern, Germany)

3.3. Experimental Work

3.3.1. Preparation of Solutions

As a source of iron, ferrous sulphate heptahydrate (source of Fe^{2+} ions), ferric chloride hexahydrate (source of Fe^{3+} ions) and ferric tartrate (source of Fe^{3+} ions) were used. For the later, two different sources were used in the measurements because of low solubility of Fe^{3+} at higher pH levels. In the experimental work involving pH 4.5 and 5.5, ferric chloride hexahydrate was used in the case of iron chelation testing. In the case of higher pH values (6.8 and 7.5) the iron used for testing chelation was ferric tartrate.

The same procedure was used throughout the experiments. Firstly, a stock solution of DOPAC (5 mM) was prepared; 9.9 mg of DOPAC was weighed using an analytical balance and dissolved in methanol. For the following methods (absorption spectra, complementary approach and Job's method) 1 mM and 0.25 mM solutions of the compound were needed. These were prepared from 5 mM stock solution of DOPAC; 2 mL of 5 mM stock solution were diluted in 8 mL of methanol, resulting in a 1 mM DOPAC solution. For preparation of 0.25 mM solution, 2 mL of 1 mM solution of DOPAC was diluted in 6 mL of methanol.

Fresh solutions of ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) and ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) were prepared daily, as the solutions are poorly stabile. Ferric tartrate, on the other hand, was stored in the freezer and thawed prior to use. For 5 mM Fe^{2+} solution, 18.6 mg of $FeSO_4 \cdot 7H_2O$ were weighed and diluted in 13.380 mL of ultrapure water and the attenuations of the solution were prepared in the same ratio as described above. Fe^{3+} solution (5 mM) for measurements in pH 4.5 and 5.5 was prepared from 15.6 mg $FeCl_3 \cdot 6H_2O$ diluted in 11.543 mL of ultrapure water. The Fe^{3+} solution (5 mM) for measurements in pH 6.8 and 7.5 was prepared from 40 mg ferric tartrate dissolved in 14.391 mL of ultrapure water.

As the source of copper, cuprous chloride (source of Cu^{+}) and cupric sulfate pentahydrate (source of Cu^{2+}) were used, Cu^{+} dissolved in water solution of 0.1 M HCl and 1 M NaCl and Cu^{2+} in distilled water.

3.3.2. Iron and Copper Calibration

Calibration of the solutions (by measuring the absorption spectra) of ions was done daily. As a blank, 1.5 mL of ultrapure water was used and placed into the spectrometer in a PS cuvette. The spectrometer was set on reading absorbance from 550 – 570 nm (for iron) and 450 – 500 nm (for copper). The absorbance was read at 562 nm (for iron) and at 484 nm (for copper). The indicator used for calibration of iron was ferrozine (5 mM) and BCS (5 mM) for copper calibration. Ferrozine is a spectrophotometric reagent used for iron calibration, which forms magenta colored complexes with ferrous ions. Its absorption maximum is at 562 nm (30). In order to make the indicator solution, we weighed 369.35 mg of ferrozine and dissolved it in 15 mL of ultrapure water. As the solution was stable, it was stored in the fridge. Preparation of 10 mM hydroxylamine (HA) was also needed. 17.7 mg HA was dissolved in 25.471 mL of ultrapure water. In the case of copper, BCS indicator was used, which has an absorption maximum at 484 nm and forms orange colored complexes with cupric ions.

For calibration purposes, samples of different concentrations of ions were made, pipetted into Eppendorf tubes, thoroughly mixed, placed into PS cuvettes and measured in the spectrometer. The following samples were used for calibration (Tab. III, Tab. IV and Tab. V). An example of calibration curve is in the Fig. 5.

Table III: Iron (II) calibration- samples for calibration (the substances added as followed in the table). The slope of the calibration curve has to fall within the range of 0.025- 0.029 in order to be appropriate for further work.

Final concentration (Fe^{2+}) (µM)	ultrapure water (µL)	Fe^{2+} (µL)	ferrozine (µL)
100	970	30	500
70	979	21	500
40	988	12	500

Table IV: Iron (III) calibration- samples for calibration (the substances added as followed in the table). The slope of the calibration curve has to fall within the range of 0.025 – 0.029 for ferric chloride hexahydrate and 0.017 – 0.029 for ferric tartrate, in order to be appropriate for further work.

Final concentration (Fe^{3+}) (µM)	HA (10 mM) (µL)	ultrapure water (µL)	Fe^{3+} (µL)	ferrozine (µL)
100	500	470	30	500
70	500	479	21	500
40	500	488	12	500

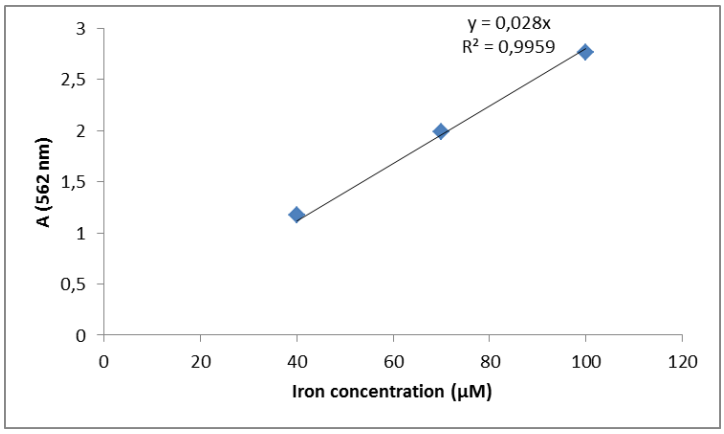

Figure 5: Example of a Fe^{2+} calibration curve.

Table V: Copper (II) calibration- samples for calibration (the substances added as followed in the table, pH 7.5). The slope of the calibration curve has to fall within the range of 0.011 – 0.016 in order to be appropriate for further work.

Final concentration (Cu^{2+}) (µM)	HA (10 mM) (µL)	HEPES buffer (µL)	Cu^{2+} (µL)	BCS (µL)
100	250	970	30	500
70	250	979	21	500
40	250	988	12	500

3.3.3. Determination of Chelating Capacity of DOPAC

Experiments for the determination of chelation of DOPAC with Fe^{2+}, Fe^{3+}, Cu^{+}, Cu^{2+} were performed at 4 different pathophysiological relevant pH values (pH 4.5, 5.5, 6.8 and 7.5). The buffers used were 15 mM; acetate for pH 4.5 and 5.5, HEPES for pH 6.8 and 7.5. All experiments were performed at room temperature. In the case of measuring the chelation activity of Fe^{2+} in buffer pH 7.5, HA (5 mM) was added with the purpose of prevention of Fe^{2+} oxidation (30). As Fe^{3+} would be reduced by HA, no HA was added when using buffer pH 7.5. However, while determining chelation of DOPAC and Cu^{+}, the addition of HA was necessary at every pH condition, as Cu^{+} oxidizes in the environment.

Measurements of absorption spectra of pure substance and of complexes were performed as shown in Tab. VI. Firstly, a blank sample was prepared from 500 µL of the solvent (methanol) and 1000 µL of the buffer at a suitable pH value and measured in the spectrophotometer as a baseline for the samples containing only substance. For samples containing substance and iron/copper, a blank sample was prepared from 500 µL solvent,

850 μL buffer and 150 μL iron/copper. The preparation of samples followed, as described in Tab. VI; for this part of the experiment, 0.25 mM solution of DOPAC and 5 mM solution of iron/copper were used. After having prepared each sample in an Eppendorf tube, the samples were thoroughly stirred. In the case of the samples with iron/copper added, because of formation of complexes, the sample needed to wait for 1 min before it could be measured. The first 4 samples didn't contain iron/copper, as we needed an insight to the characteristics of the compound itself, so we could later compare it to the characteristics of the complex. After the samples were ready for measurement, they were put in UV cuvettes and their absorption spectra were measured from wavelength 220 – 700 nm.

Table VI: Measurements of absorption spectra of DOPAC and of DOPAC in the presence of excess of iron/copper.

sample	input ratio substance : iron/copper	substance final concentration (mM)	iron/copper final concentration (mM)	solvent added volume (μL)	substance added volume (μL)	buffer added volume (μL)	iron/copper added volume (μL)
S1	2 : 0	0.020	0	530	120	850	0
S2	3 : 0	0.030	0	470	180	850	0
S3	4 : 0	0.040	0	410	240	850	0
S4	6 : 0	0.060	0	290	360	850	0
S5	1 : 6	0.083	0.5	0	500	850	150
S6	1 : 7.5	0.067	0.5	100	400	850	150
S7	1 : 10	0.050	0.5	200	300	850	150
S8	1 : 15	0.033	0.5	300	200	850	150

Following the first part of the experiment, the complementary approach was performed. Its aim was to help determine the complex stoichiometry. We varied the total molar concentration of DOPAC, while the molar concentration of the ions was kept constant.

In these experiments, 1 mM solution of DOPAC and 1 mM solution of iron/ copper were used. The samples were made as presented in Tab. VII. The blank consisted of 500 μL methanol and 1000 μL of appropriate buffer.

Table VII: Measurements determining the chelation capacity by the complementary approach.

sample	input ratio substance : iron/copper	substance final concentration (mM)	iron/copper final concentration (mM)	solvent added volume (µL)	substance added volume (µL)	buffer added volume (µL)	iron/copper added volume (µL)
C1	0.25 : 1	0.015	0.060	538	23	850	90
C2	0.5 : 1	0.030	0.060	515	45	850	90
C3	0.75 : 1	0.045	0.060	493	68	850	90
C4	1 : 1	0.060	0.060	470	90	850	90
C5	1.5 : 1	0.090	0.060	425	135	850	90
C6	2	0.120	0.060	380	180	850	90
C7	2.5 : 1	0.150	0.060	335	225	850	90
C8	3 : 1	0.180	0.060	290	270	850	90
C9	4 : 1	0.240	0.060	200	360	850	90
C10	6 : 1	0.360	0.060	20	540	850	90

In addition to the complementary method, another method was used- Job's approach. It is an analytical approach and is known as the method of continuous variation. It is useful for determination of stoichiometry of two interacting components. The total molar concentration of the two reactants is kept constant, while their molar concentration ratios are continuously varied through the series of samples (32).

In Job's approach (Tab. VIII and IX), 0.75 mM solution of DOPAC and 0.75 mM solution of iron were used. In the case of copper, 1 mM solution of DOPAC and 1 mM solution of copper were used. However, the molarity sum was 0.25 mM in both cases. As a baseline, we measured a blank containing 500 µL solvent and 1000 µL of appropriate buffer.

Table VIII: Job's approach for iron.

sample	input ratio substance : iron	substance final concentration (mM)	iron final concentration (mM)	solvent added volume (µL)	substance added volume (µL)	buffer added volume (µL)	iron added volume (µL)
J1	0.25 : 1	0.050	0.200	150	100	850	400
J2	0.5 : 1	0.083	0.167	150	167	850	333
J3	1 : 1	0.125	0.125	150	250	850	250
J4	1.5 : 1	0.150	0.100	150	300	850	200
J5	2 : 1	0.067	0.083	150	333	850	167
J6	3 : 1	0.188	0.063	150	375	850	125
J7	4 : 1	0.200	0.050	150	400	850	100
J8	5 : 1	0.208	0.042	150	417	850	83
J9	5.5 : 1	0.212	0.038	150	423	850	77
J10	6 : 1	0.214	0.036	150	429	850	71

Table IX: Job's approach for copper.

sample	input ratio substance : copper	substance final concentration (mM)	copper final concentration (mM)	solvent added volume (µL)	substance added volume (µL)	buffer added volume (µL)	copper added volume (µL)
J1	0.25 : 1	0.050	0.200	275	75	850	300
J2	0.5 : 1	0.083	0.167	275	125	850	250
J3	1 : 1	0.125	0.125	275	188	850	188
J4	1.5 : 1	0.150	0.100	275	225	850	150
J5	2 : 1	0.067	0.083	275	250	850	125
J6	3 : 1	0.188	0.063	275	281	850	94
J7	4 : 1	0.200	0.050	275	300	850	75
J8	5 : 1	0.208	0.042	275	313	850	63
J9	5.5 : 1	0.212	0.038	275	317	850	58
J10	6 : 1	0.214	0.036	275	321	850	54

3.3.4. Mathematical Data Analysis (32)

After establishing the absorption spectra of pure compound, complex and results from both the complementary approach as well as Job's approach, we were able to mathematically analyze the data. By the use of mathematical stoichiometric calculations, we were able to determine the stoichiometry of chelates.

Method I: Absorbance at the Absorption Maximum of the Complex

This method is based on a simple evaluation of A of samples at the wavelength of absorbance maximum of the complex λ_{cmax}.

Method II: Symmetry of the Absorption Maximum of the Complex

Method II is based on a theoretical assumption that the absorption maximum of a complex is symmetric if there are no interfering proximal absorption maxima (Fig. 6). The absorbance of the complex $A_{c\lambda sym1}$ was proposed to be the same as the A of the complex $A_{c\lambda sym2}$, at a similar distance from λ_{cmax} to the left (λ_{sym1}) or to the right (λ_{sym2}). This can be described using the following equations:

$$\lambda_{sym1} = \lambda_{cmax} - d$$

$$\lambda_{sym2} = \lambda_{cmax} + d$$

$$A_{c\lambda sym1} = A_{c\lambda sym2}$$

As the absorbance has an additive character, the measured A was composed from the A of the formed complex and the non-reacted compound at any wavelength. Furthermore, if the A of the complex is sufficient (> 0, 1) and the A of the substance $A_{s\lambda sym2}$ is zero, the measured absorbance $A_{\lambda sym2}$ should be equal to the A of the complex $A_{c\lambda sym2}$ (refer to the following equations).

$$A_{\lambda sym2} = A_{s\lambda sym2} + A_{c\lambda sym2}$$

$$A_{\lambda sym2} = A_{c\lambda sym2}$$

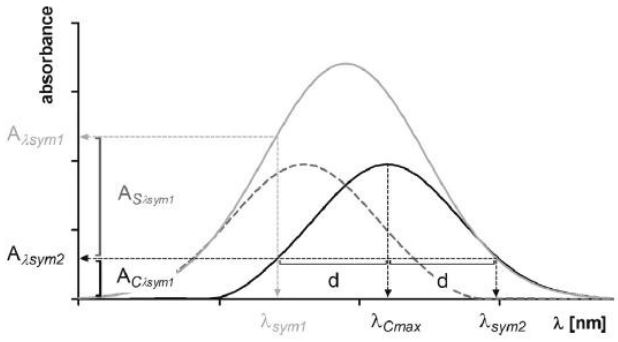

Figure 6: The method of the symmetry of A_{max} of the complex. The grey curve represents the measured A spectrum, which is the sum of the A spectrum of the non-reacted substance (dark grey curve) and the A spectrum of the formed complex (black curve) (32).

The measured $A_{\lambda sym1}$ was directly used for the assessment of the molar concentration of the non-reacted substance.

$$A_{\lambda sym1} = A_{s\lambda sym1} + A_{c\lambda sym1}$$

As said before, the following equation needs to be taken into account:

$$A_{\lambda sym1} = A_{s\lambda sym1} + A_{\lambda sym2}$$

At this point we need to consider the Lambert-Beer law in order to calculate the molar concentration of the non-reacted substance (c_s) in the equation. The l is the known width of the cuvette and $\varepsilon_{s\lambda sym1}$ is the molar absorption coefficient of the substance at λ_{sym1}.

$$A_{s\lambda sym1} = c_s \cdot \varepsilon_{s\lambda sym1} \cdot l$$

$$c_s = (A_{\lambda sym1} - A_{\lambda sym2}) / (\varepsilon_{s\lambda sym1} \cdot l)$$

We are able to calculate the chelation ratio (X) as described in the following equation; c_{s0} is the initial molar concentration of the substance and c_{Fe} is the final molar concentration of iron in the sample.

$$X = (c_{s0} - c_s) / c_{Fe}$$

Method III: Calculation Using the Absorption Maximum of the Substance

Determination of the molar concentration of the non-reacted substance (c_s) is crucial for the calculation of the stoichiometry using the absorption maximum of the substance. However, it must be noted that the A at the wavelength of absorption maximum of the substance ($A_{\lambda smax}$) was used. $A_{\lambda smax}$ is the sum of the A of the non-reacted substance and the formed complex. Considering the Lambert-Beer law, the molar concentration of the non-reacted substance (c_s) is therefore calculated by the equation below.

$$A_{\lambda smax} = (c_s \cdot \varepsilon_{s\lambda smax} \cdot l) + (c_c \cdot \varepsilon_{c\lambda smax} \cdot l)$$

In the equation, $\varepsilon_{s\lambda smax}$ and $\varepsilon_{c\lambda smax}$ are the molar absorption coefficients of the substance and the formed complex, at the wavelength of absorption maximum of the substance (λ_{smax}). By conversion of the molar concentration equivalents of the substance, the unknown molar concentration of the complex (c_c) is calculated.

$c_c + c_s = c_{s0}$

The concentration of the non-reacted substance is:

$c_s = ((A_{\lambda smax} / l) - (\varepsilon_{csmax} \cdot c_{s0})) / (\varepsilon_{s\lambda smax} - \varepsilon_{c\lambda smax})$

Afterwards, the calculation of stoichiometry is again accomplished by the following equation as is the previous method.

$X = (c_{s0} - c_s) / c_{Fe}$

Method IV: Calculation Using the Absorption Maximum of the Complex

This method is analogous to Method III, with the exception that A is measured at the wavelength of absorption maximum of the complex (λ_{cmax}).

Method V: Theoretical Determination of Absorbance of the Complex at the Wavelength of its Absorption Maximum

Method V (Fig. 7) is based on theoretical lines, established to mimic absorbance of the most probable stoichiometries. As the molar concentration was stable throughout the complementary approach, A was at first rising dependently on the formation of complex, and it lasted as long as all added substance reacted with iron and formed the complex.

$A_{\lambda cmax} = A_{c\lambda cmax}$

$A_{c\lambda cmax} = c_{s0} \cdot \varepsilon_{c\lambda cmax} \cdot l$

When all iron was exhausted, the absorbance $A_{\lambda cmax}$ rose only dependently on the A of the further added non-reacted substance $A_{s\lambda cmax}$.

$A_{\lambda cmax} = A_{c\lambda cmax} + A_{s\lambda cmax}$

$A_{\lambda cmax} = (c_{eq} \cdot \varepsilon_{c\lambda cmax} \cdot l) + ((c_{s0} - c_{eq}) \cdot \varepsilon_{s\lambda cmax} \cdot l)$

For stoichiometry 1:1, the molar concentration equilibrium is at $c_{s0} = c_{Fe}$, for stoichiometry 2:1, $c_{s0} = 2 \cdot c_{Fe}$, etc. The comparison between the measured A with these theoretical lines reveals the searched stoichiometry or even the reaction stoichiometry at different molar

concentration ratios. The identical approach was used at the wavelength of absorption maximum of the substance.

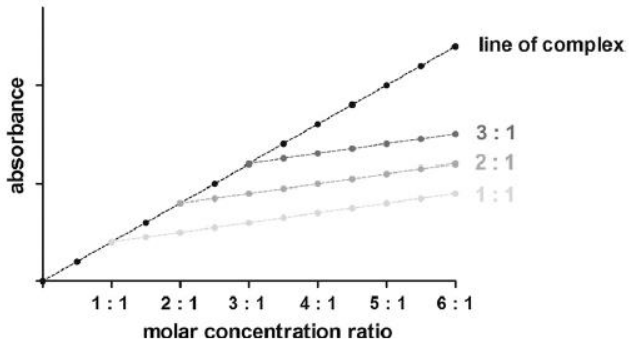

Figure 7: Theoretical determination of A using method V. The black line corresponds to the A of the formed complex at the excess of iron. The light grey line represents the A at the stoichiometry 1:1, the grey line at 2:1 and the dark grey line at 3:1 (32).

Method VI: Theoretical Determination of the Sum of Absorbance of the Non-reacted Substance and the Complex at the Absorption Maximum of the Substance

Method VI has many similarities with the Method V approach. The principle is the same; the absorbance depended on the complex occurrence until the point where all of the iron was consumed for the complex formation. Afterwards, the only thing changing the absorbance was the added non-reacted substance. In this method, the assumption is that different types of complexes occur, which consequentially means that these complexes have different molar absorption coefficients. That is why the absorbance was not rising linearly up to the concentration equilibrium. The lines depending only on the non-reacted substance were constructed directly, based on the measured A at most probable chelation ratios. An identical approach was used at the wavelength of the absorption maximum of the complex.

4. RESULTS AND DISCUSSION

Phenolic compounds are a very versatile group of secondary plant metabolites. They vary in chemical structure, molecular weight and biological properties. Many of them are regularly consumed as a part of the human diet. As phenolic compounds have antioxidant activity and are able to scavenge free radicals, they are of a clear importance for further research of their influence on cardiovascular diseases, neurodegenerative diseases, cancer and others. DOPAC is one of the metabolites of quercetin, which is one of the most abundant phenolic compounds in our diet.

The aim of this thesis research project was to determine the effect of 3,4-dihydroxyphenylacetic acid on iron and copper chelation, as we were trying to determine the antioxidant activity of DOPAC.

4.1. Interaction with Iron

The following graphs present absorption spectra of the substance with the excess iron in both oxidation states, Fe^{2+} and Fe^{3+}, for the detection of complex formation, the assessment of molar absorption coefficients (Fig. 8-13, 15, 16, 18-25) and measurements aimed at analysis of complex stoichiometry (Fig. 14, 17). In excess iron experiments, the pure substance was tested in 4 different molar concentrations: 20 μM, 30 μM, 40 μM and 60 μM, while the compound with iron was tested in the following ratios: 1:6, 1:7.5, 1:10, 1:15. However, in the complementary approach for complex stoichiometry analysis, the concentration of iron was 90 μM, but the concentration of DOPAC was changing from 23 μM to 540 μM. Graphical depiction of complex stoichiometry is shown in corresponding graphs (Fig. 14, 17).

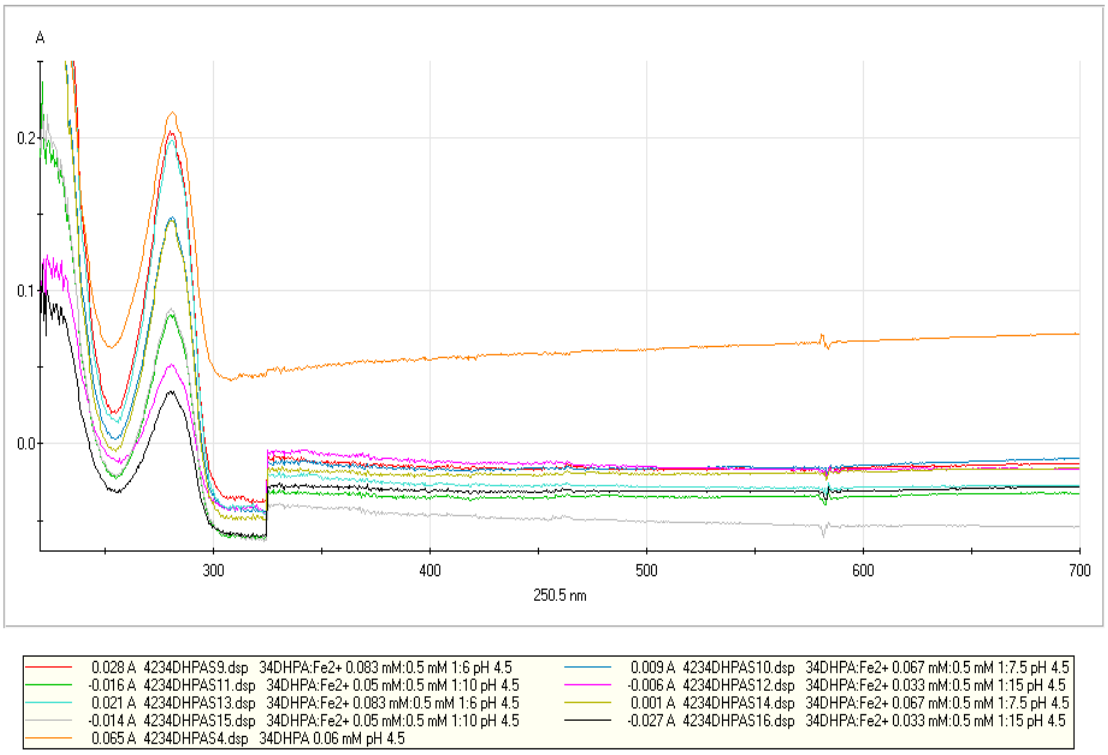

Figure 8: Absorption spectra of DOPAC and its mixture with Fe^{2+} at pH 4.5. Apparently, addition of iron did not modify the spectrum.

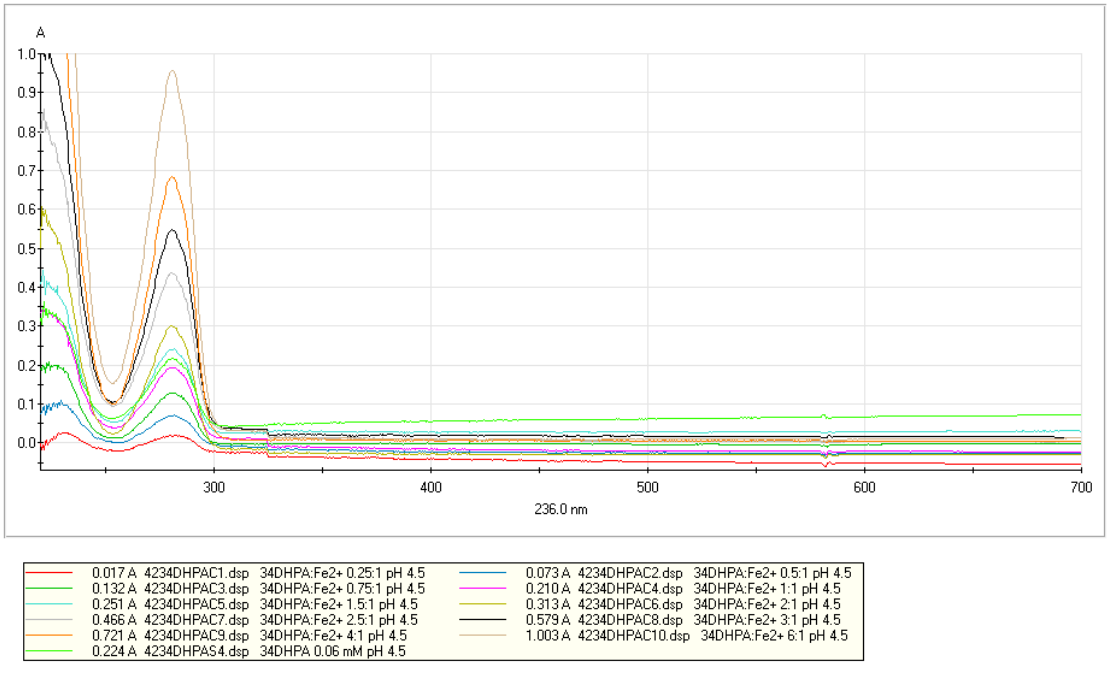

Figure 9: Complementary approach spectra of DOPAC compared to DOPAC with Fe^{2+} at pH 4.5. As expected, addition of iron did not modify the spectrum.

As we see in Fig. 8 and 9, there is no shift in the absorption spectra when comparing the spectra of pure substance and the spectra of complex with Fe^{2+} at pH 4.5. The explanation of this phenomenon likely lays in pH conditions. Based on the pKa of the catechol moiety (34), it is known that DOPAC does not chelate in low pH conditions, such as 4.5. That is due to the catechol ring not having chelating properties at physiological conditions, but having low chelating activity at a lower pH (33).

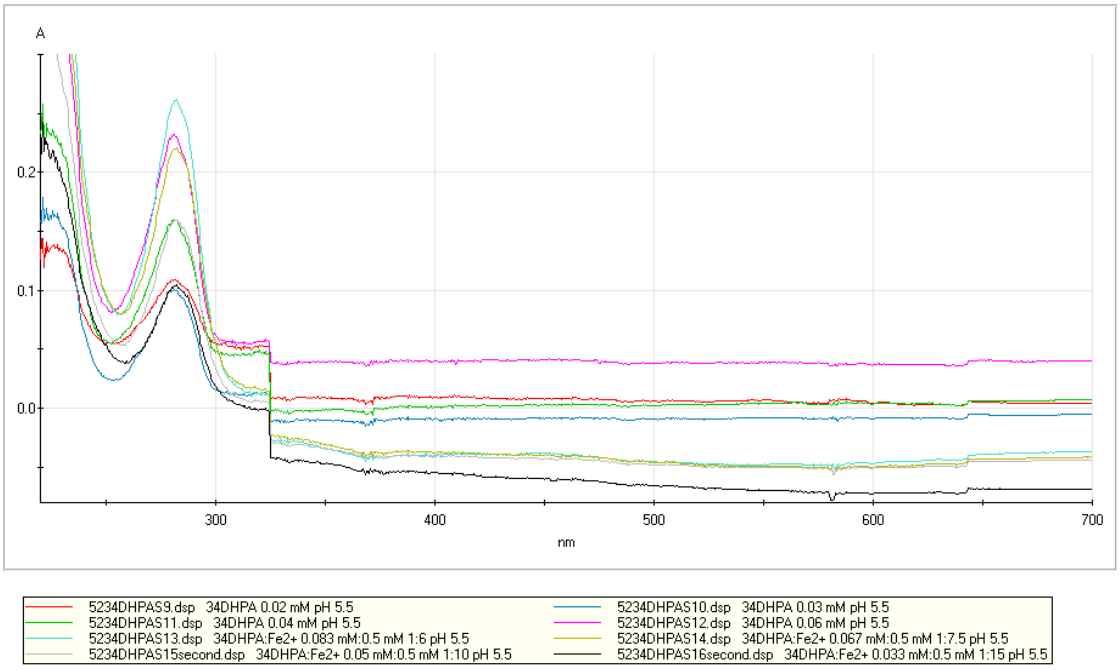

Figure 10: Absorption spectra of DOPAC and its mixture with Fe^{2+} at pH 5.5. Similarly to pH 4.5, addition of iron did not modify the spectrum.

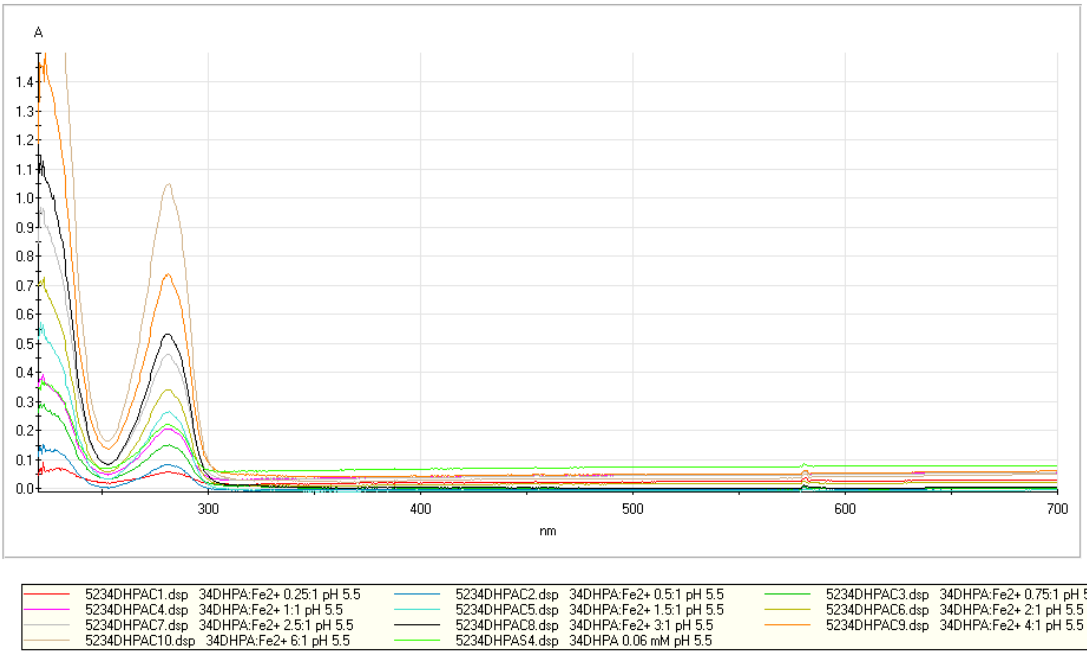

Figure 11: Complementary approach spectra of DOPAC compared to DOPAC with Fe^{2+} at pH 5.5. In line with Fig. 10, the spectrum was not modified after addition of iron.

As with the previous conditions, we can confirm that in the tested samples with Fe^{2+} and DOPAC at pH 5.5 (Fig. 10 and 11) no chelation reactions are taking place. The reason is likely the same as with pH 4.5.

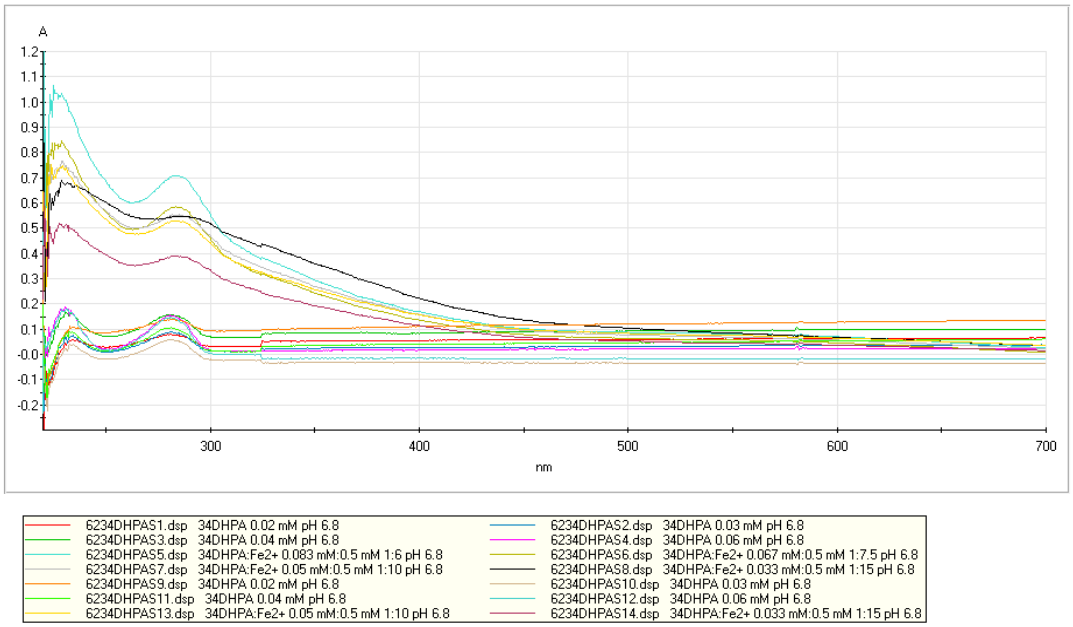

Figure 12: Absorption spectra of DOPAC and its complex with Fe^{2+} at pH 6.8. A slight bathochromic shift of the DOPAC spectra is clearly seen after addition of Fe^{2+} ions (the black line in particular).

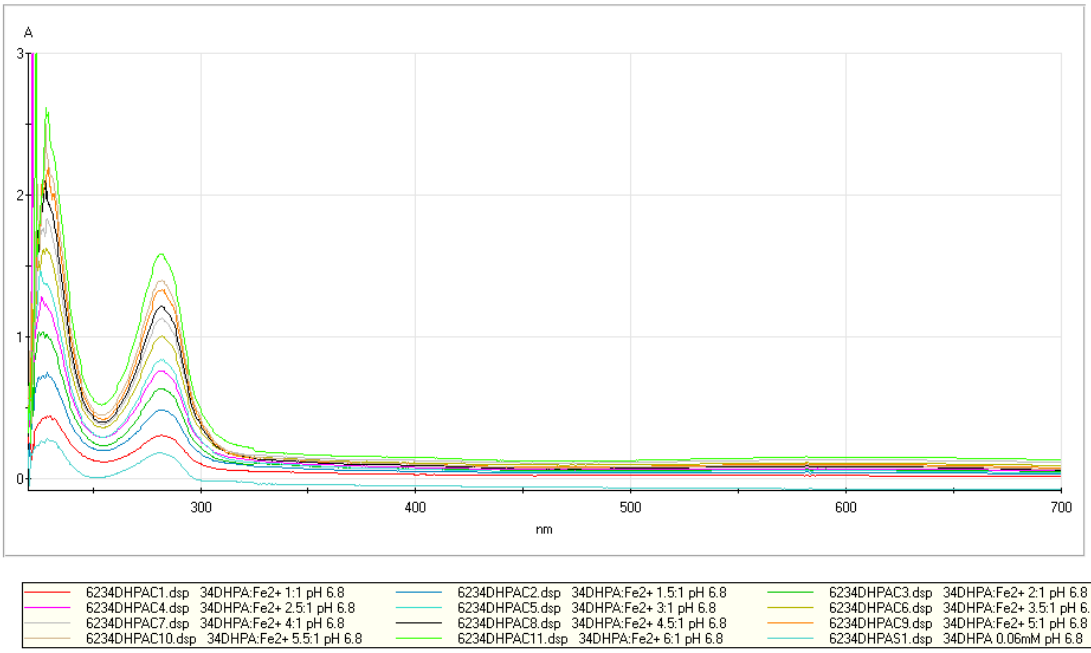

Figure 13: Complementary approach spectra of DOPAC compared to DOPAC with Fe^{2+} at pH 6.8. The addition of iron does not seem to alter the spectra, but the calculation was performed nonetheless, based on complex formation from Fig. 12.

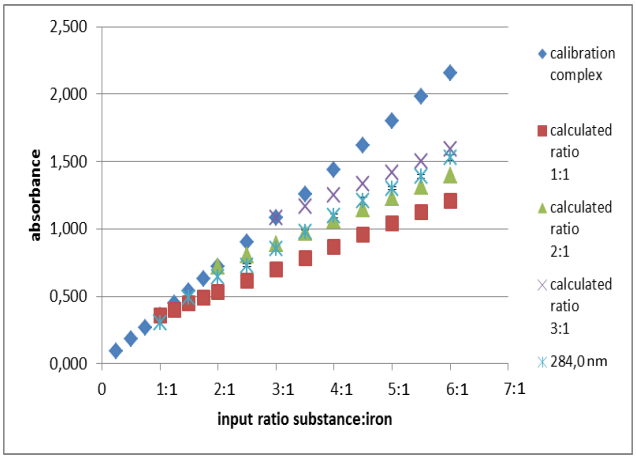

Figure 14: Results of a mathematical analysis with method V, Fe^{2+} pH 6.8. The measured data fits clearly into the stoichiometric ratio 2:1 at least up to the input ratio 4:1. At higher ratios, the result is not fully convincing and stoichiometry 3:1 cannot be fully excluded.

At pH 6.8, the absorption spectra of DOPAC were clearly changed after addition of ferrous ions (Fig. 12). The data from complementary approach (Fig. 13) was analyzed by several methodologies. The best resolution can be seen with method V in Fig. 14. A chelation ratio of 2:1 changing into 3:1 was detected by method V. Similarly, chelation of ferrous ions occurred at pH 7.5 as well (Fig. 15). The interpretation from spectra (Fig. 16) was much easier. The results of method V show chelation ratio of 1:1.

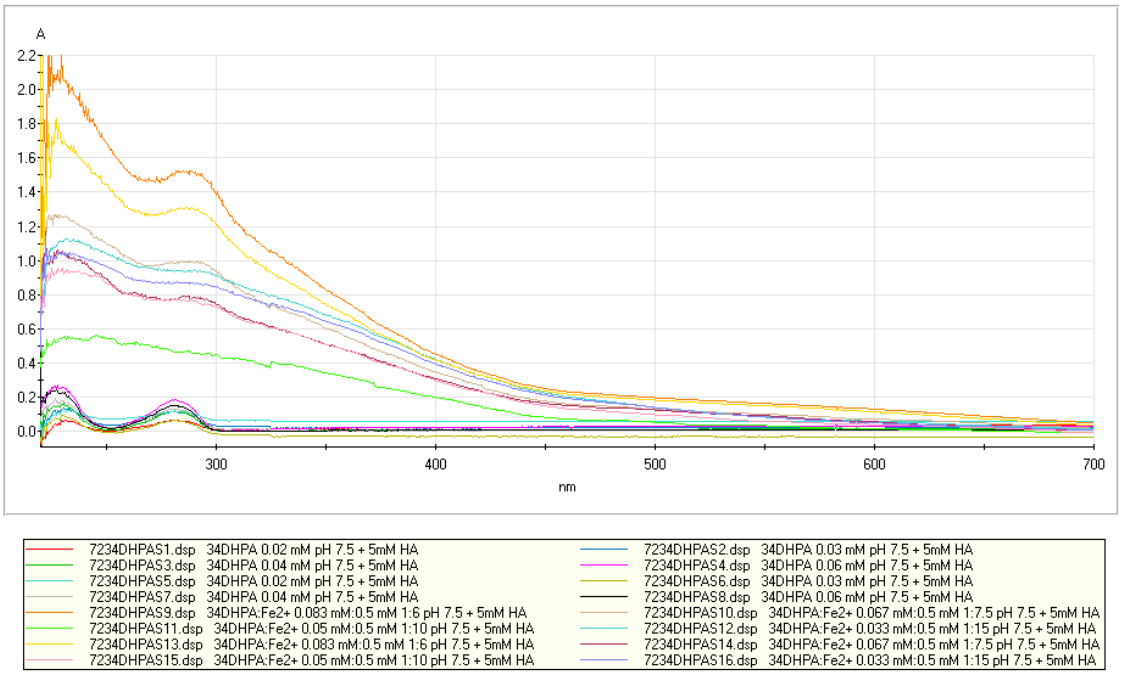

Figure 15: Absorption spectra of DOPAC and its complex with Fe^{2+} at pH 7.5.

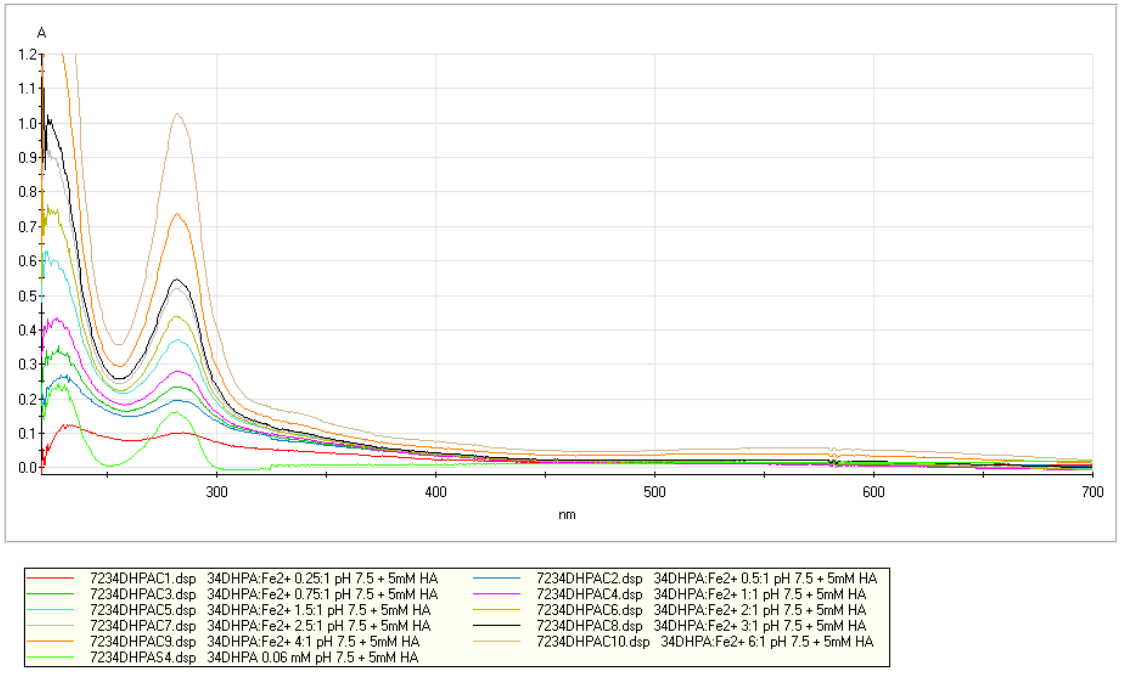

Figure 16: Complementary approach spectra of DOPAC compared to DOPAC with Fe^{2+} at pH 7.5. In low ratios of iron and DOPAC (red line), the complex with iron clearly has different spectral characteristics from the DOPAC.

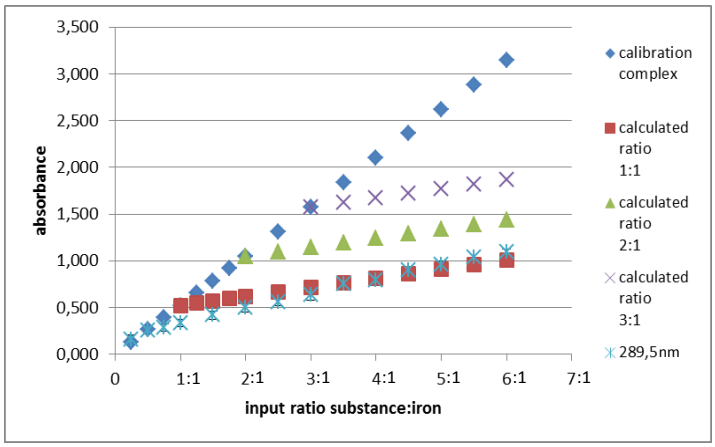

Figure 17: Results of mathematical analysis with method V, Fe^{2+} pH 7.5. These results clearly suggest the 1:1 stoichiometry (3 repetitions of measurements).

The 3,4-dihydroxyl group has a predicted pKa of 6.7. It is therefore sensitive to deprotonation at physiological pH conditions (34). As seen from the results, chelation of DOPAC occurs both at pH 6.8 and 7.5 when Fe^{2+} is present. As metal ions such as Fe^{2+} and Fe^{3+} prefer octahedral geometry, they are able to coordinate up to three catecholate groups.

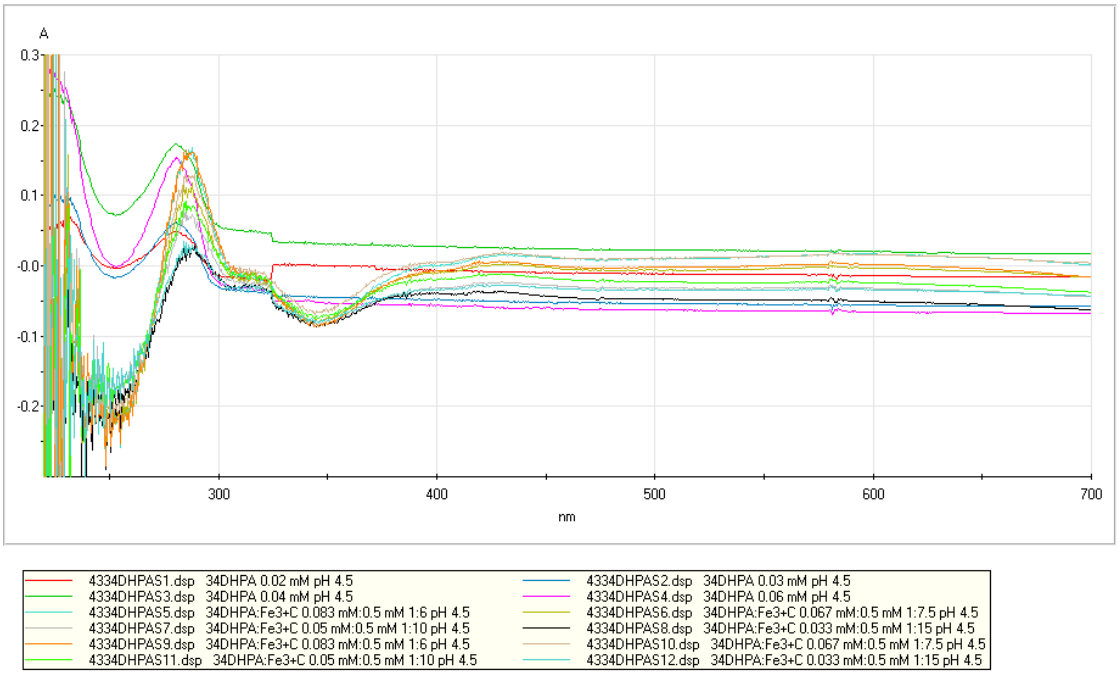

Figure 18: Absorption spectra of DOPAC and its mixture with Fe^{3+} at pH 4.5. The negative values occurred because we had used a ferric ions solution as a blank in these sets of experiments.

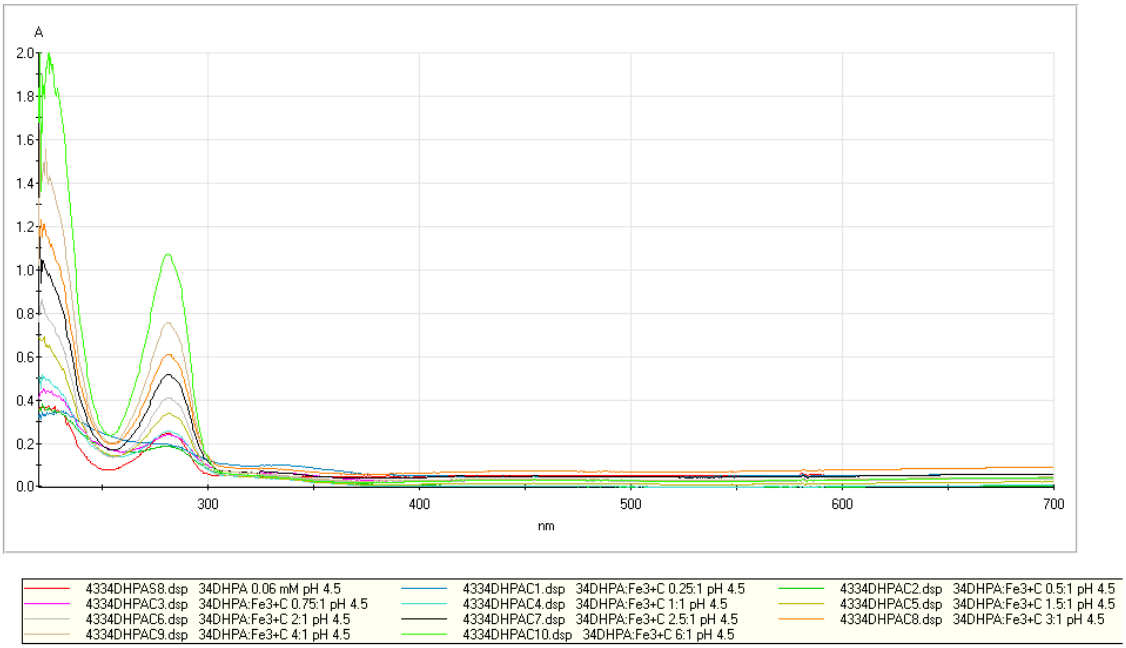

Figure 19: Complementary approach spectra of DOPAC compared to DOPAC with Fe^{3+} at pH 4.5.

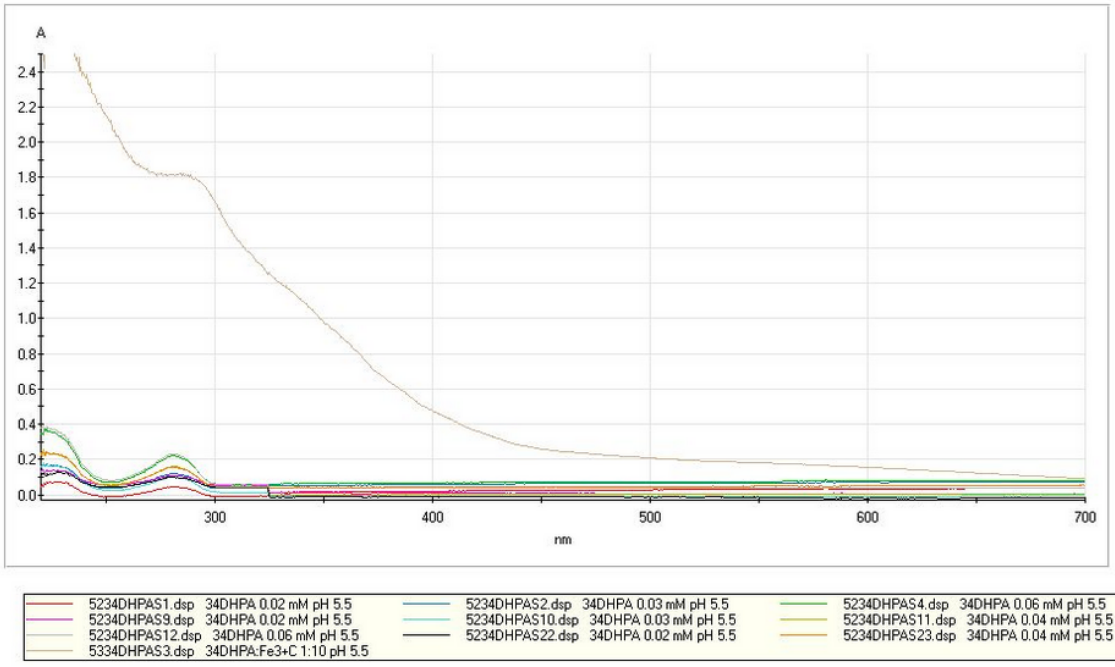

Figure 20: Absorption spectra of DOPAC and its mixture with Fe^{3+} at pH 5.5.

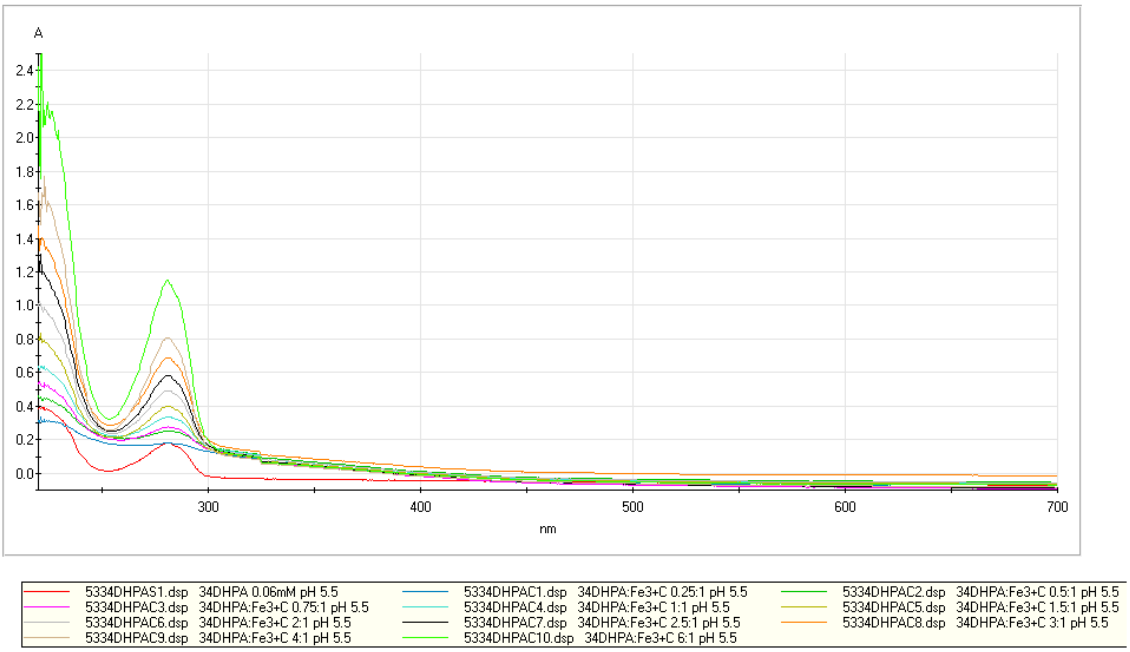

Figure 21: Complementary approach spectra of DOPAC compared to DOPAC with Fe^{3+} at pH 5.5.

Similarly to ferrous ions, there was no complex formation with ferric ions at acidic pHs (Fig. 18- 21). Interestingly, it appears that DOPAC is not able to chelate ferric ions at neither of the other two selected pHs (Fig. 22- 25) in contrast to ferrous ones (Fig. 8- 11).

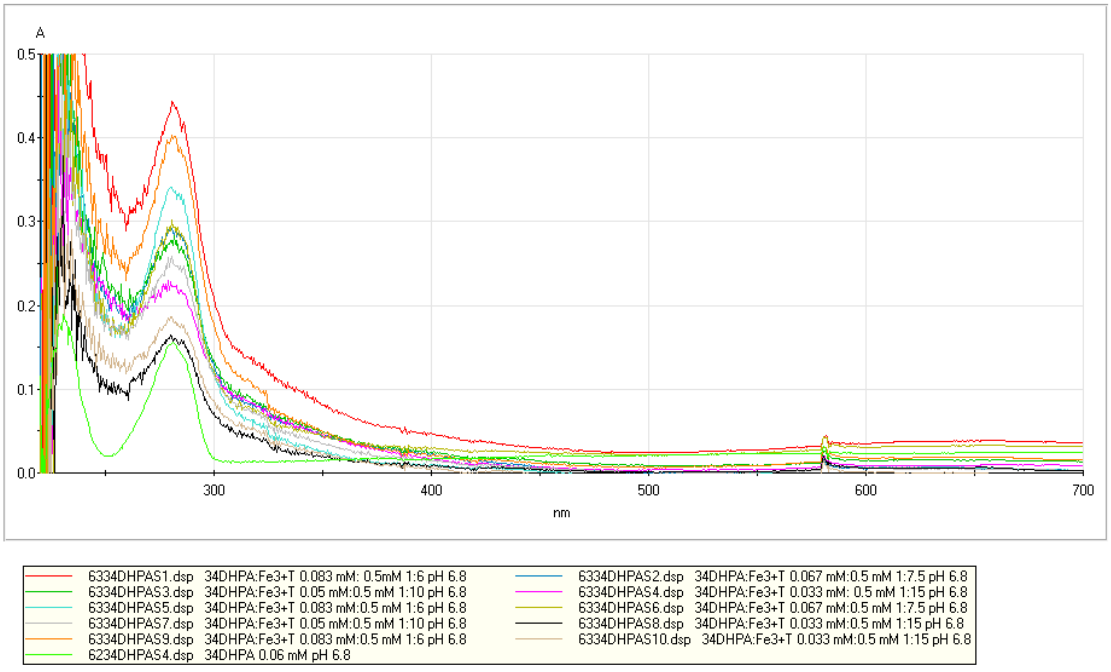

Figure 22: Absorption spectra of DOPAC and its mixture with Fe^{3+} at pH 6.8. Once again we can sum up that there is no chelation of DOPAC and Fe^{3+} at pH 6.8, as there seems to be no shift in the absorption spectra.

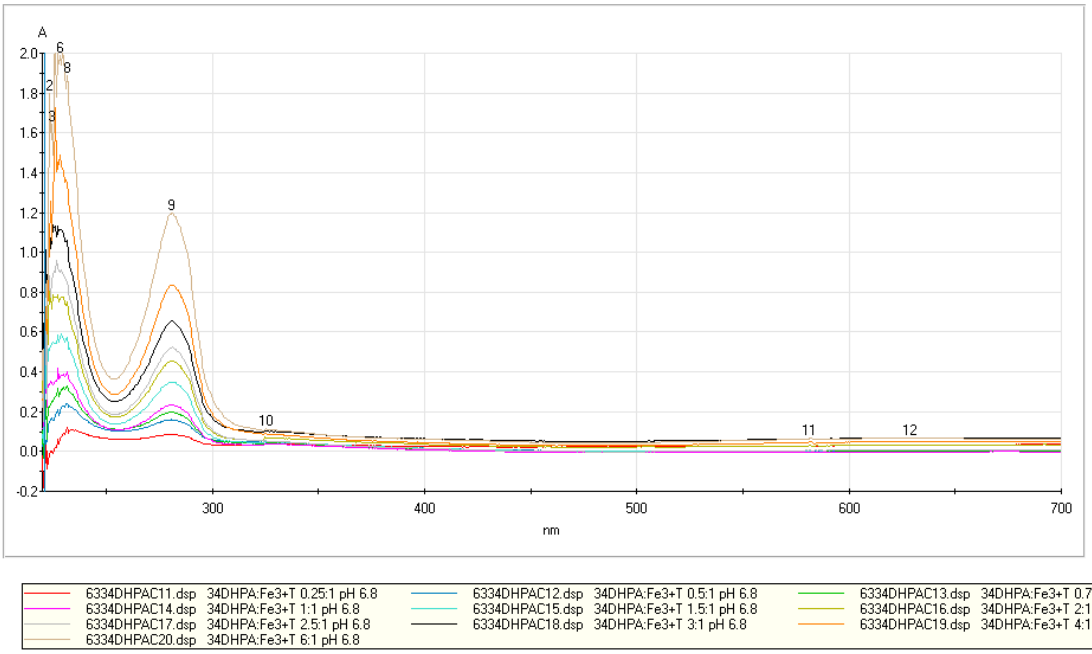

Figure 23 : Complementary approach spectra of DOPAC compared to DOPAC with Fe^{3+} at pH 6.8.

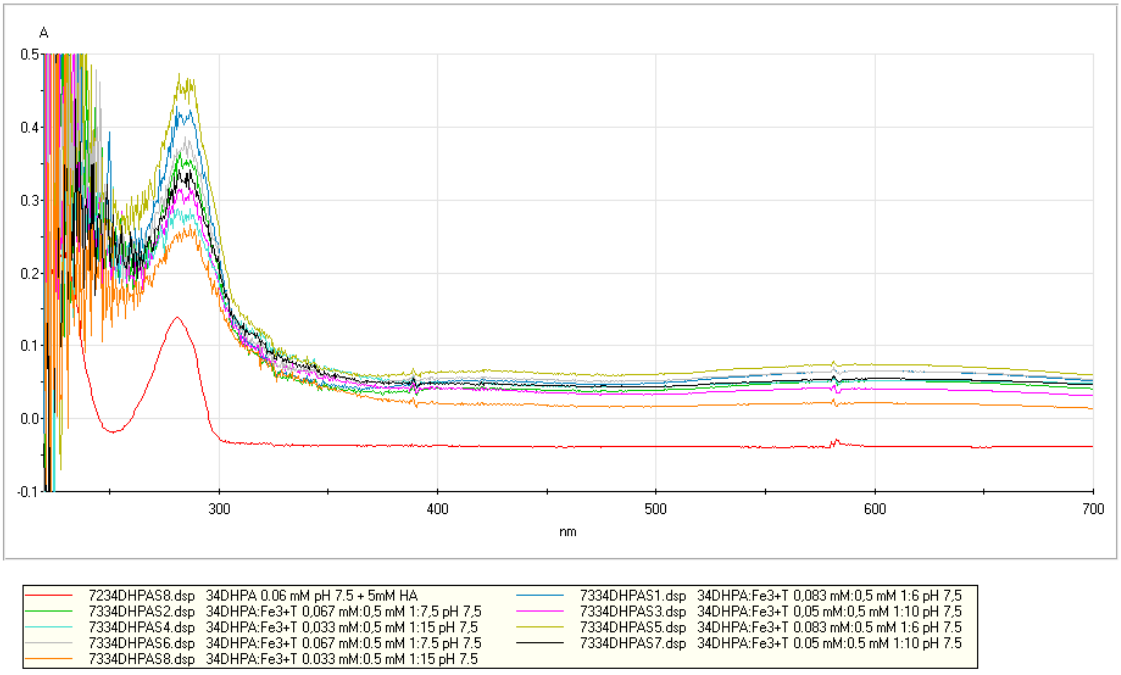

Figure 24: Absorption spectra of DOPAC and its mixture with Fe^{3+} at pH 7.5.

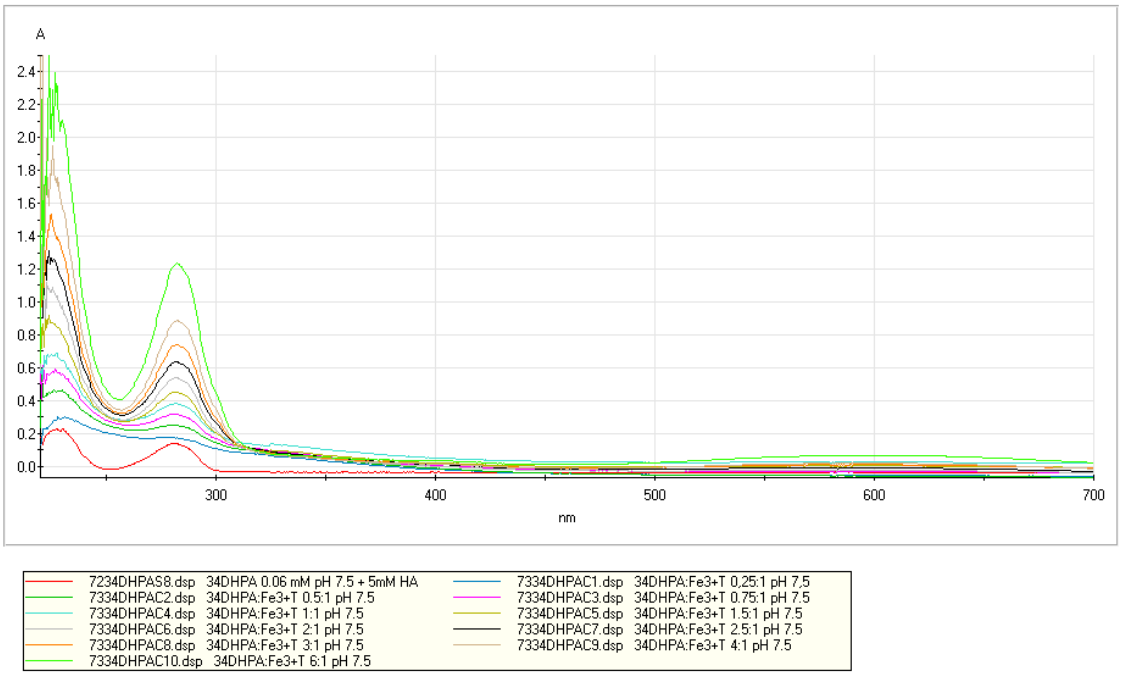

Figure 25: Complementary approach spectra of DOPAC compared to DOPAC with Fe^{3+} at pH 7.5.

As seen from the results above, we cannot clearly state the prediction of any complexes between DOPAC and Fe^{3+} at pH 6.8 and 7.5, as the differences between the absorption maxima of the substance and of the mixture are minimal.

4.2. Interaction with Copper

The following graphs present absorption spectra of the substance with excess copper in both oxidation states, Cu^{+} and Cu^{2+}, for the detection of complex formation and the assessment of molar absorption coefficients (Fig. 26-31, 33, 34, 36-41, 43, 45-47) and measurements aimed at analysis of complex stoichiometry (Fig. 32, 35, 42, 44, 48, 49). As in the case of iron, in copper excess experiments the pure substance was tested in 4 different molar concentrations (20 µM, 30 µM, 40 µM and 60 µM). The compound with copper was tested in the ratios: 1:6, 1:7.5, 1:10, and 1:15. However, in the complementary approach for complex stoichiometry analysis the concentration of copper was 90 µM, but the concentration of DOPAC was changing from 23 µM to 540 µM. Graphical depiction of complex stoichiometry is shown in corresponding graphs (Fig. 32, 35, 42, 44, 48, 49).

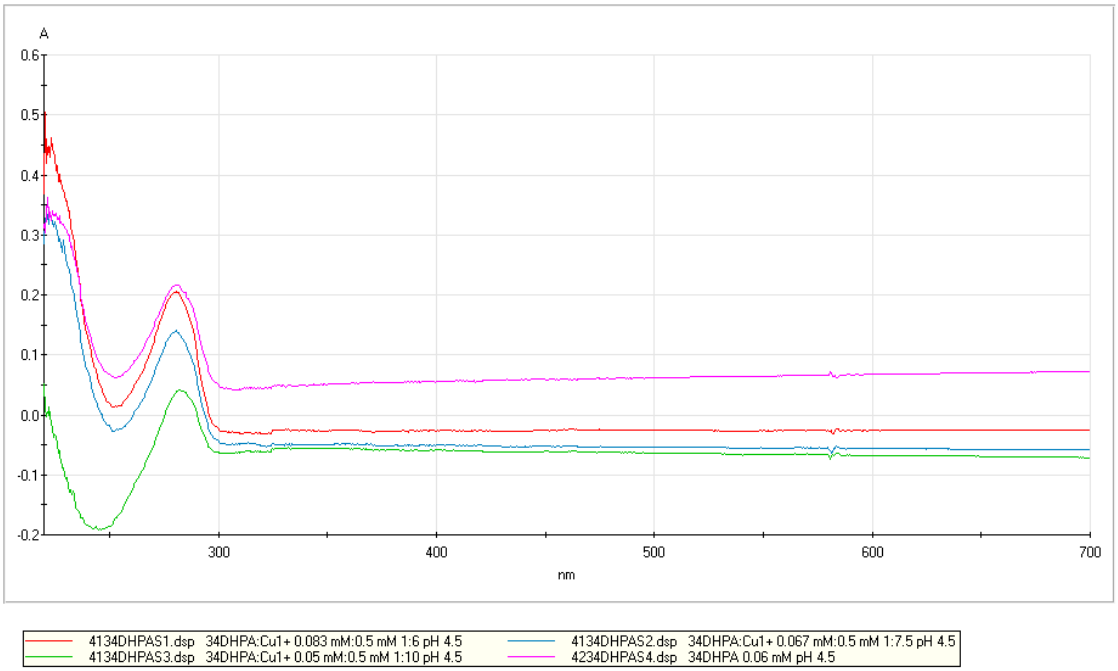

Figure 26: Absorption spectra of DOPAC and its mixture with Cu^+ at pH 4.5. There is no difference between the spectra of DOPAC and the mixture of DOPAC with Cu. This suggests that no complex is formed.

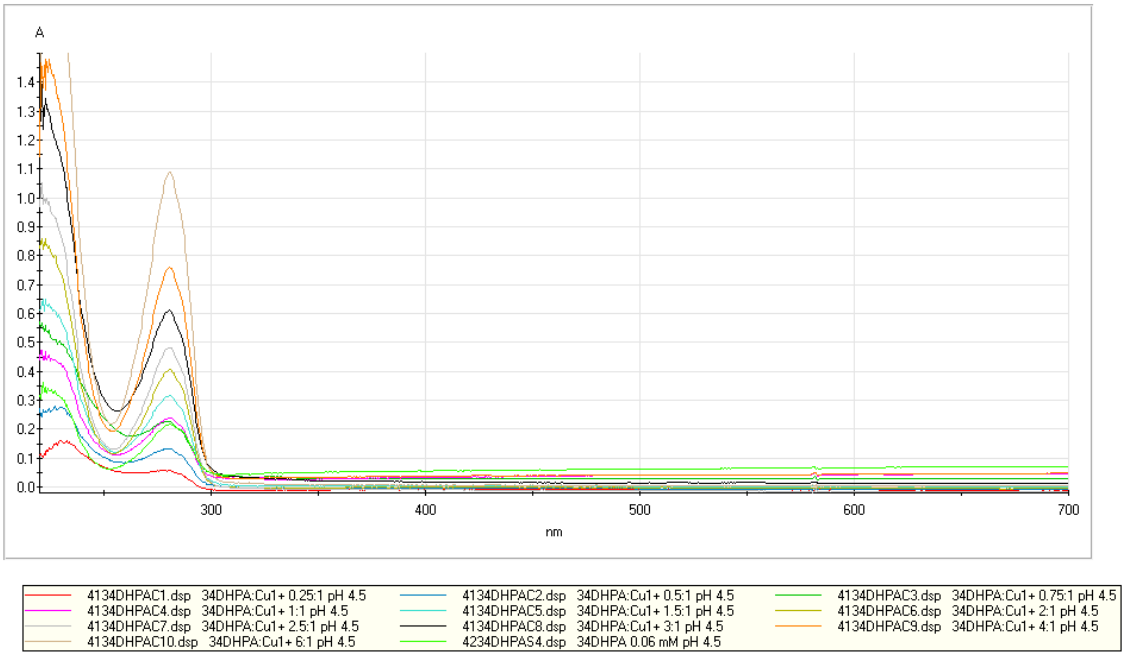

Figure 27: Complementary approach spectra of DOPAC compared to DOPAC with Cu^+ at pH 4.5.

In the tested samples with Cu^+ at pH 4.5, no formation of complexes was detected (Fig. 26, 27) as there was no visible shift of absorption spectra. However, it is commonly known

that in low pH conditions, DOPAC doesn't chelate, which has been confirmed by these results.

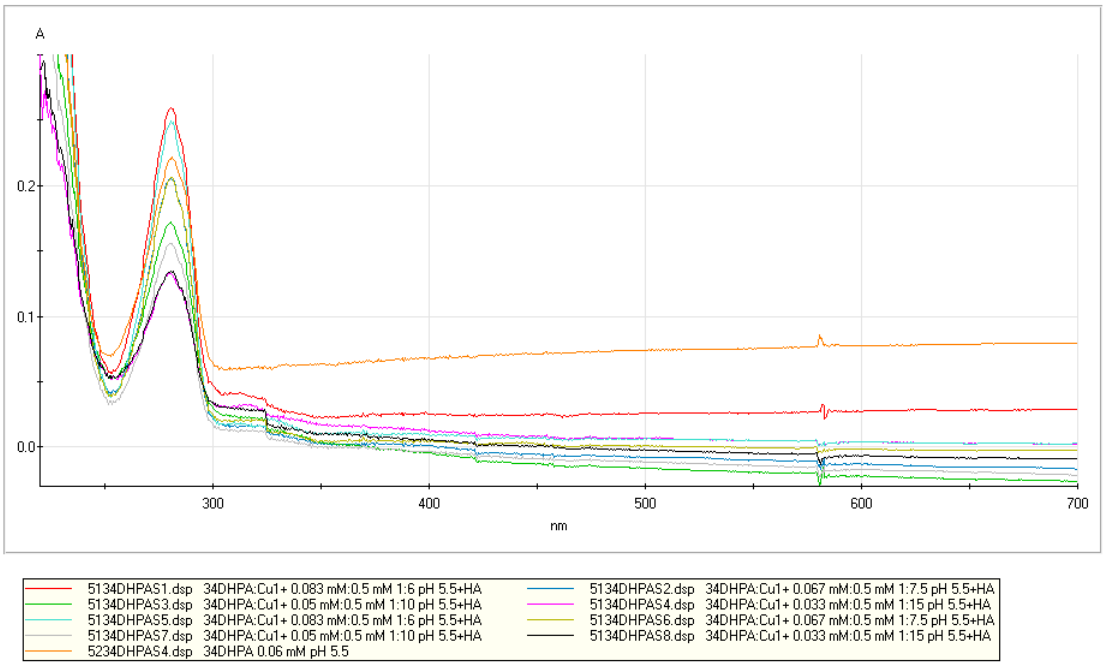

Figure 28: Absorption spectra of DOPAC and its complex with Cu^{+} at pH 5.5. Interestingly, some slight modifications of spectra were observed.

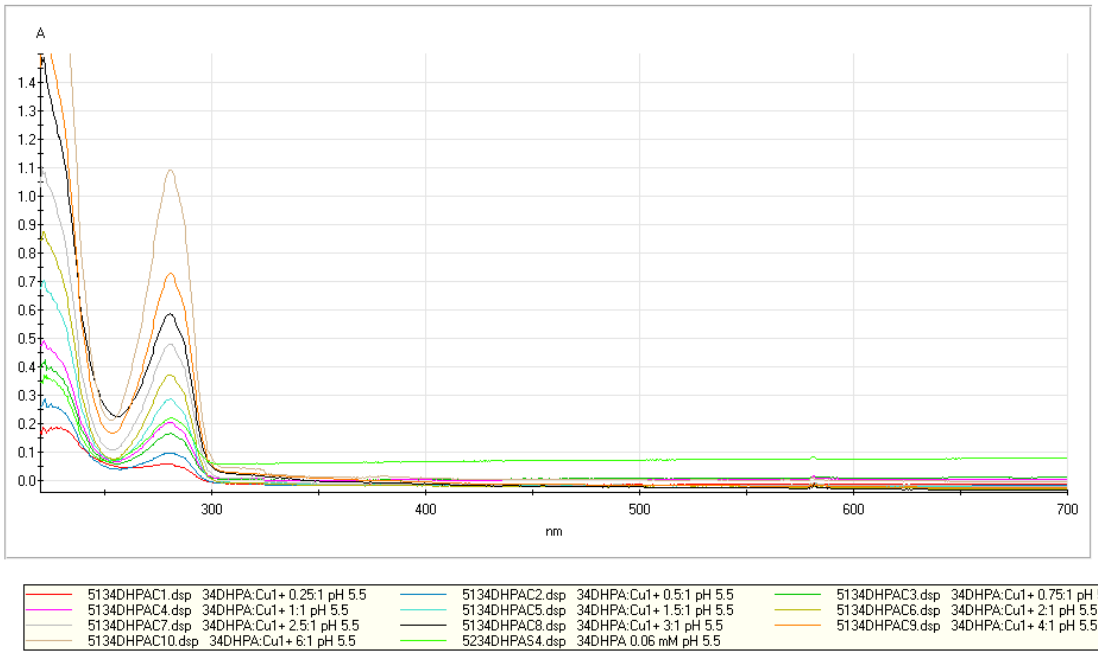

Figure 29: Complementary approach spectra of DOPAC compared to DOPAC with Cu^{+} at pH 5.5. No clear modification of spectra was observed.

Interestingly, there was a slight modification of spectra of DOPAC after addition of Cu^{+} at pH 5.5 (Fig. 28). However, both Job's (data not shown) and complementary approach (Fig. 29) were not able to demonstrate significant complex formation, suggesting low affinity of DOPAC toward copper at this condition. This result was expected, as we know that neither iron nor copper was chelated at 4.5 and 5.5 pH conditions. Contrarily, chelation was observed at pH 6.8 (Fig. 30).

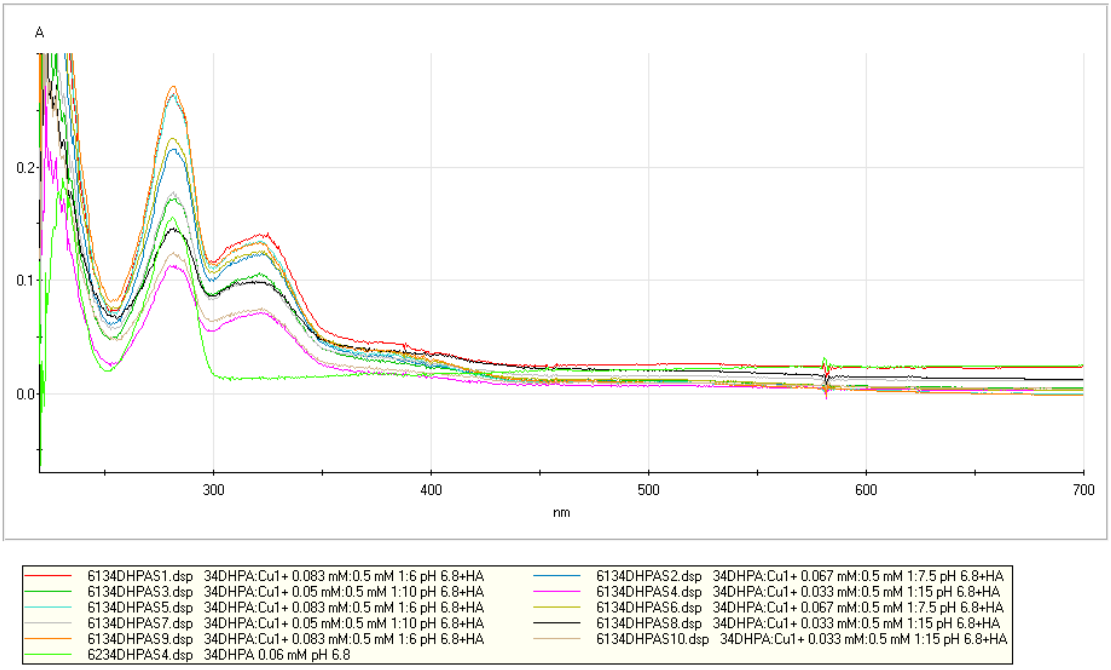

Figure 30: Absorption spectra of DOPAC and its complex with Cu^{+} at pH 6.8. In this case, very clearly, the complex was formed – compare the pure DOPAC solution (light green line) in the range from 300 to 350 nm to other samples (DOPAC + Cu).

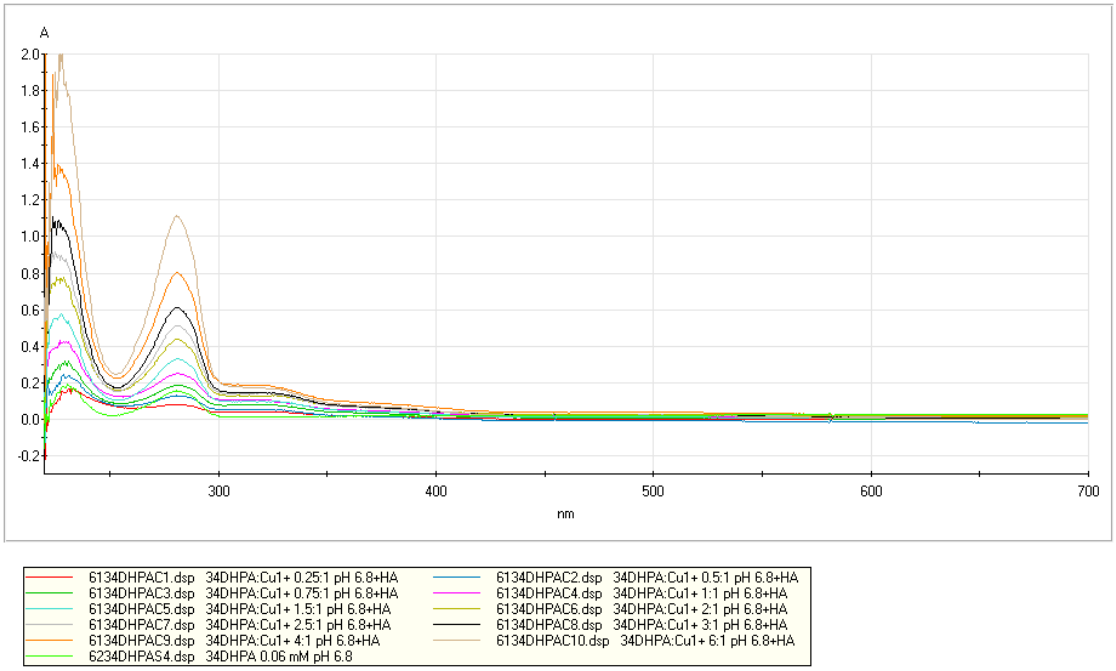

Figure 31: Complementary approach spectra of DOPAC compared to DOPAC with Cu^{+} at pH 6.8.

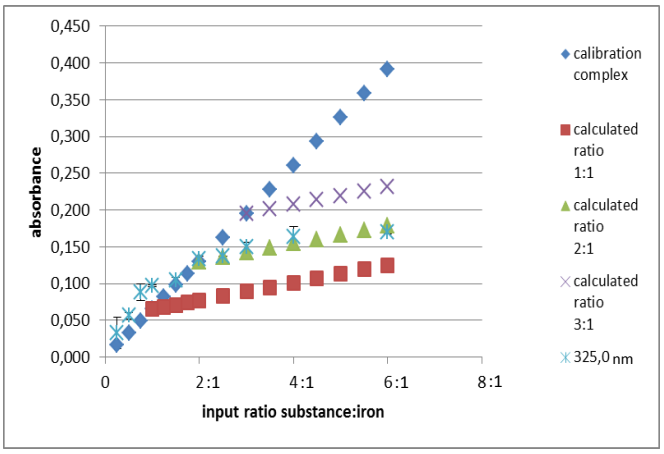

Figure 32: Results of mathematical analysis with method V, Cu^{+} pH 6.8 (2 repetitions of measurements).

While testing the potential formation of complexes of DOPAC and Cu^{+} at pH 6.8, we detected very low absorbance values, which may be the reason for 2 methods giving different results. According to Job's, it is hard to say if there is any chelation ratio visible (data not showed), however a 1:1 ratio can be speculated. In method V, the results of chelation ratio are unambiguously 2:1 (Fig. 31, 32). Similarly, at pH 7.5, cuprous ions formed a complex with DOPAC (Fig. 33), complementary approach suggested complex formation with 2:1 or 3:1 stoichiometry (Fig. 34, 35).

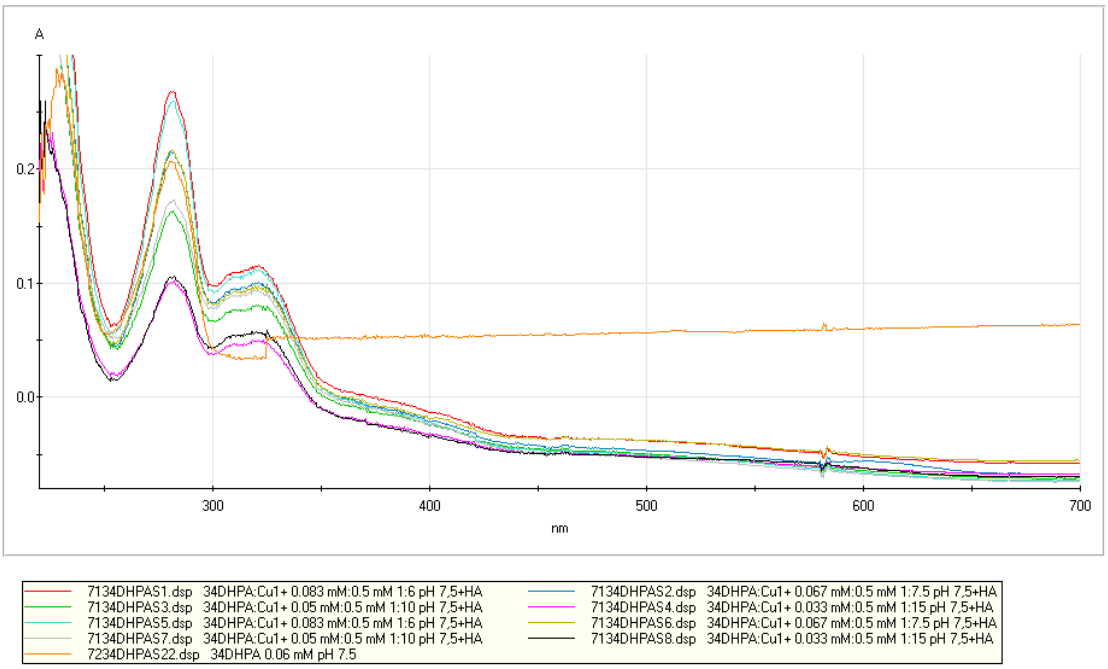

Figure 33: Absorption spectra of DOPAC and its complex with Cu^{+} at pH 7.5. Although no such clear spectrum, as in the case of pH 6.8, was measured, the complex formation again is undisputable.

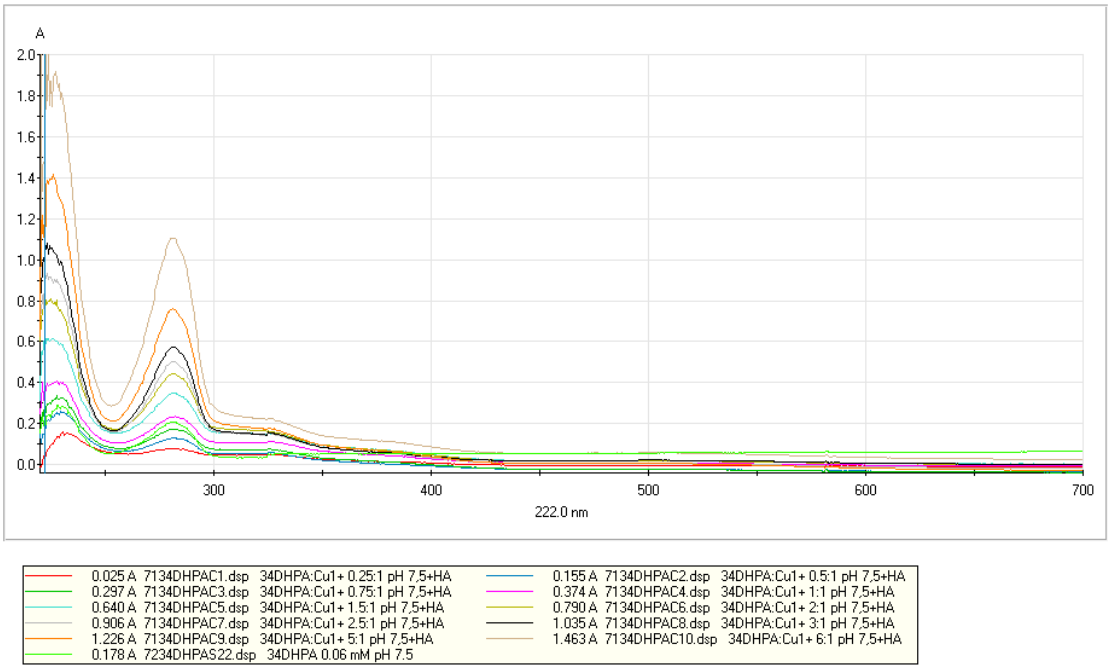

Figure 34: Complementary approach spectra of DOPAC compared to DOPAC with Cu^{+} at pH 7.5.

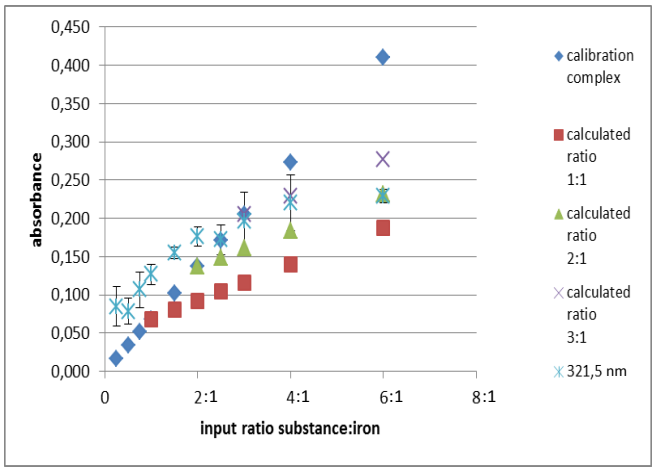

Figure 35: Results of method V for Cu^{+} at pH 7.5 have shown a 2:1 or 3:1 chelation ratio (3 repetitions of measurements).

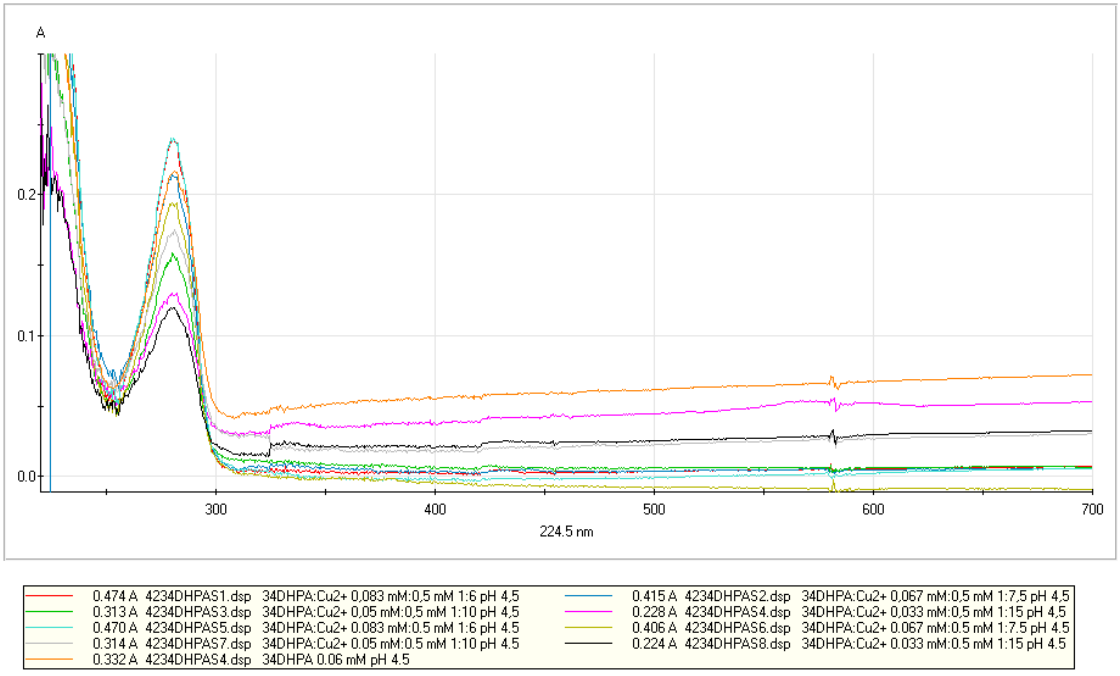

Figure 36: Absorption spectra of DOPAC and its mixture with Cu^{2+} at pH 4.5. Apparently no complex is formed after addition of cupric ions.

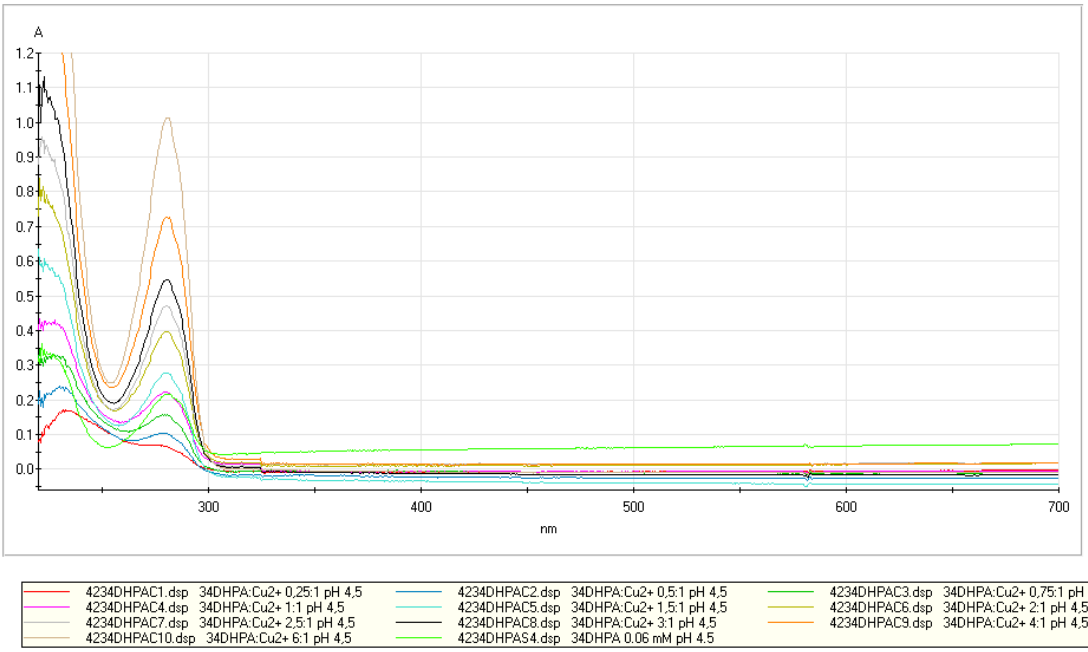

Figure 37: Complementary approach spectra of DOPAC compared to DOPAC with Cu^{2+} at pH 4.5.

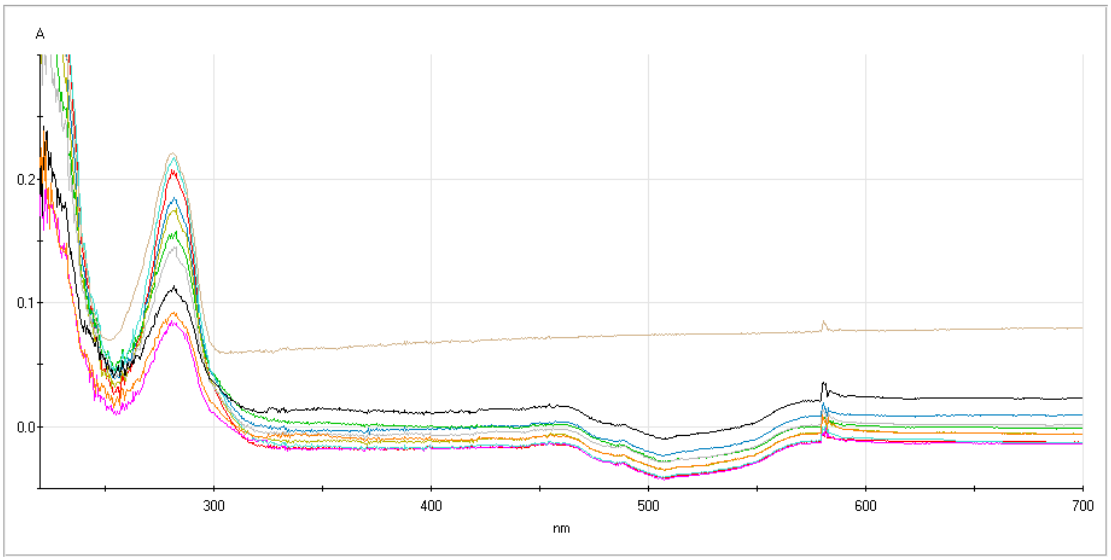

Figure 38: Absorption spectra of DOPAC and its mixture with Cu^{2+} at pH 5.5. Again, apparently no complex of DOPAC with cupric ions is formed.

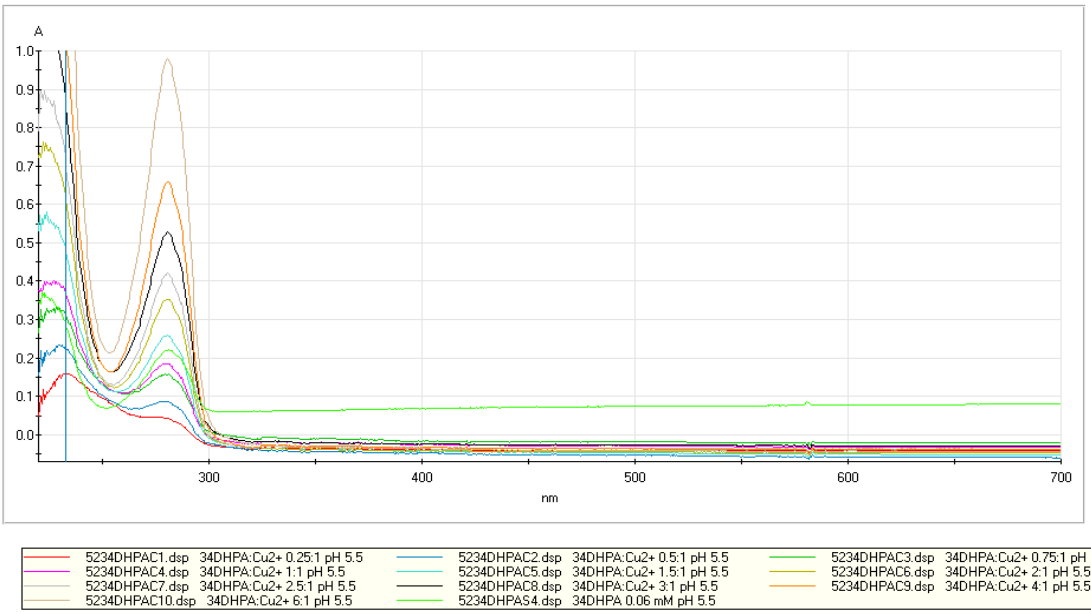

Figure 39: Complementary approach spectra of DOPAC compared to DOPAC with Cu^{2+} at pH 5.5.

As well as iron, copper also doesn't form complexes with DOPAC in pH 4.5 and 5.5 (Fig. 36- 39).

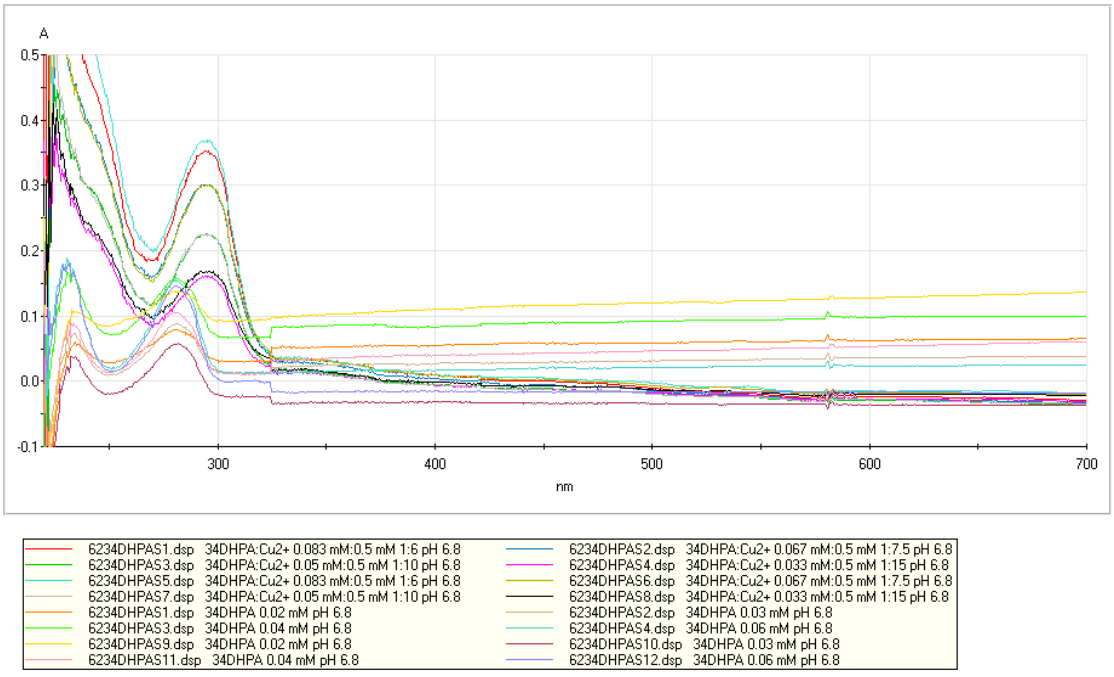

Figure 40: Absorption spectra of DOPAC and its complex with Cu^{2+} at pH 6.8. A complex with cupric ions is formed at this pH.

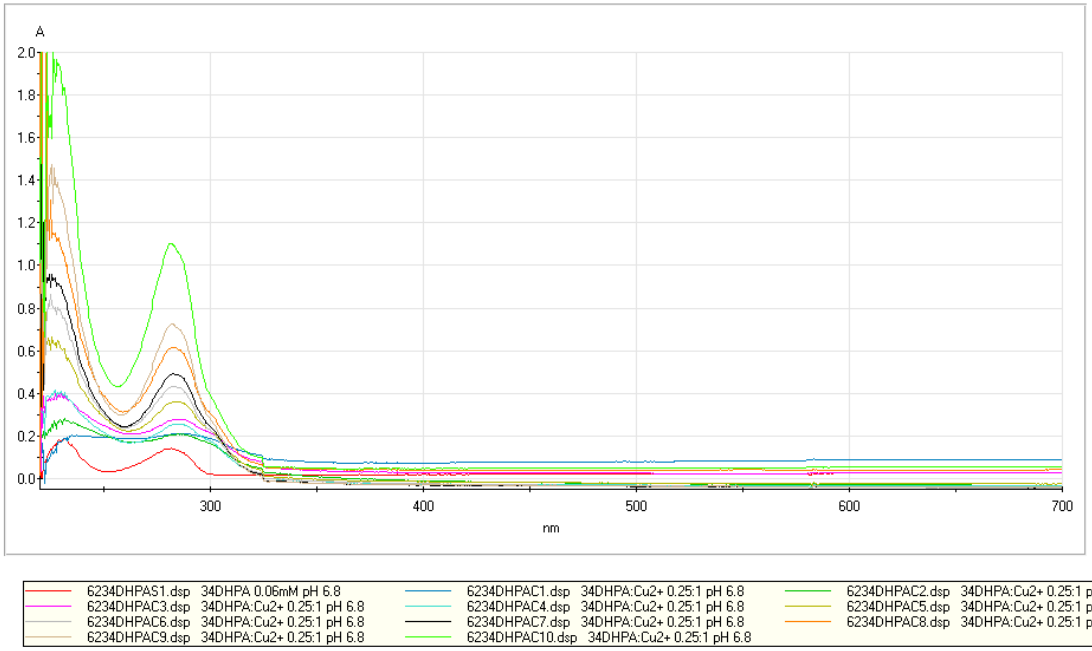

Figure 41: Complementary approach spectra of DOPAC compared to DOPAC with Cu^{2+} at pH 6.8.

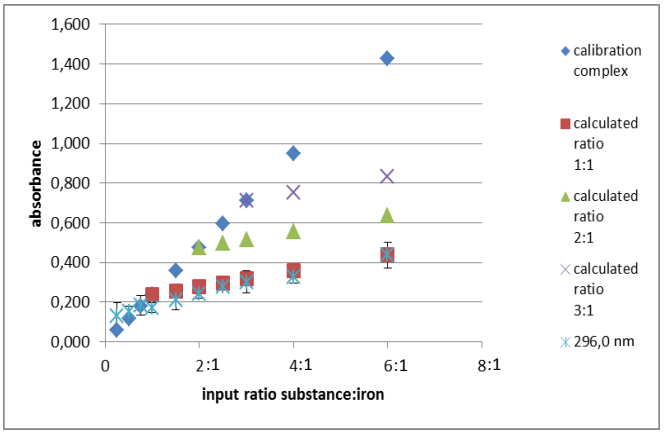

Figure 42: Results of mathematical analysis with method V, Cu^{2+} pH 6.8 (2 repetitions of measurements).

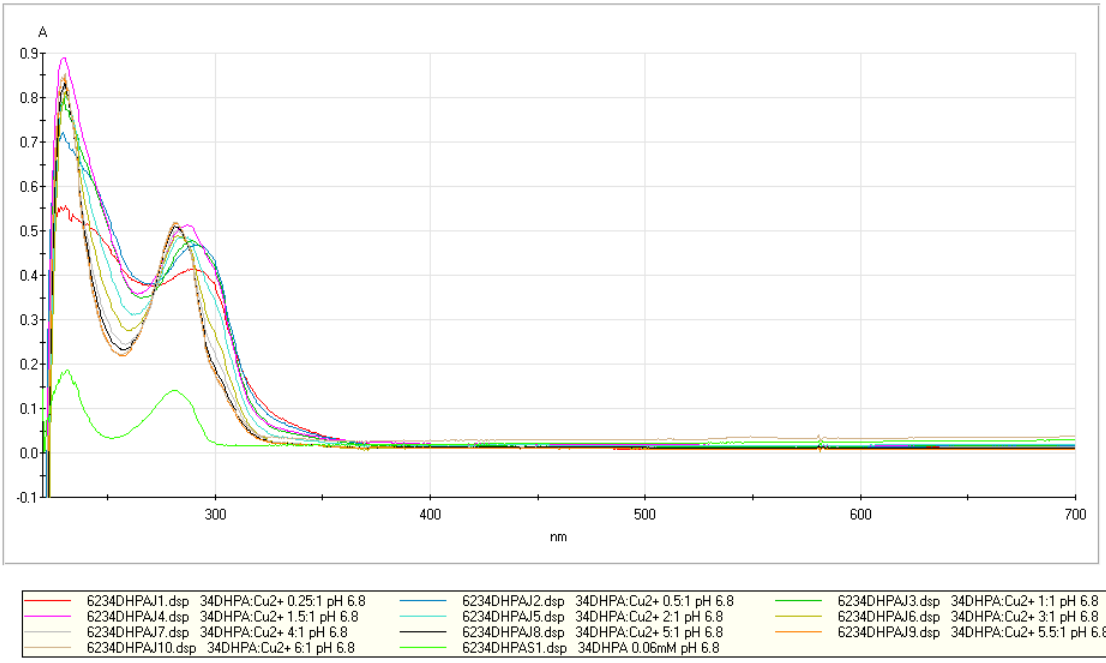

Figure 43: Spectra from Job's approach, Cu^{2+} pH 6.8.

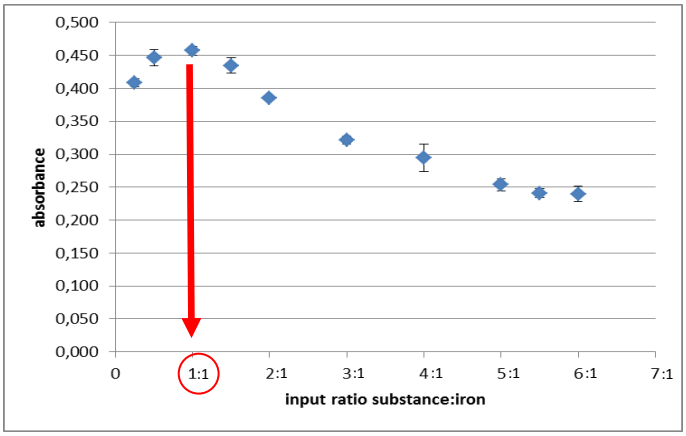

Figure 44: Results of Job's approach, Cu^{2+} pH 6.8 (2 repetitions of measurements).

At pH 6.8, the chelation of cupric ions by DOPAC is clearly suggested in Fig. 40. Complementary approach (Fig. 41) as well its mathematical depiction (Fig. 42) implicated the stoichiometric ratio 1:1. Interstingly, Job´s method did not give clear results in previous conditions, but here, the results (Fig. 43 and 44) are very clear. We can conclude that there is a 1:1 chelation ratio of DOPAC and Cu^{2+} at pH 6.8.

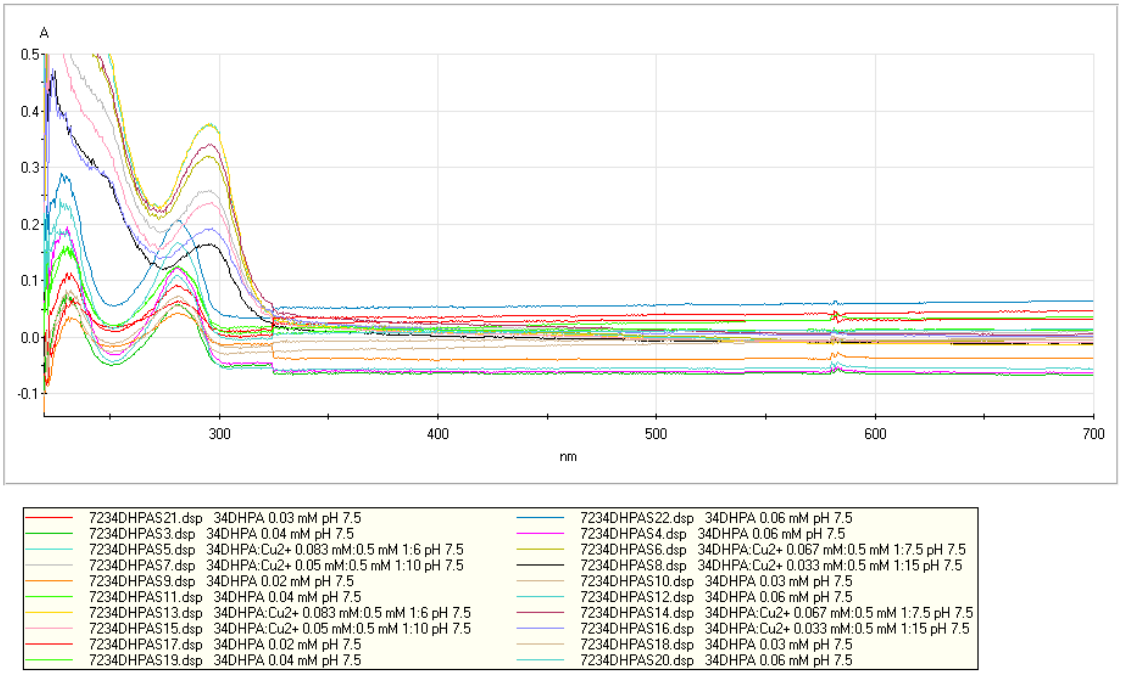

Figure 45: Absorption spectra of DOPAC and its complex with Cu^{2+} at pH 7.5. Again, the addition of cupric ions resulted in complex formation with DOPAC.

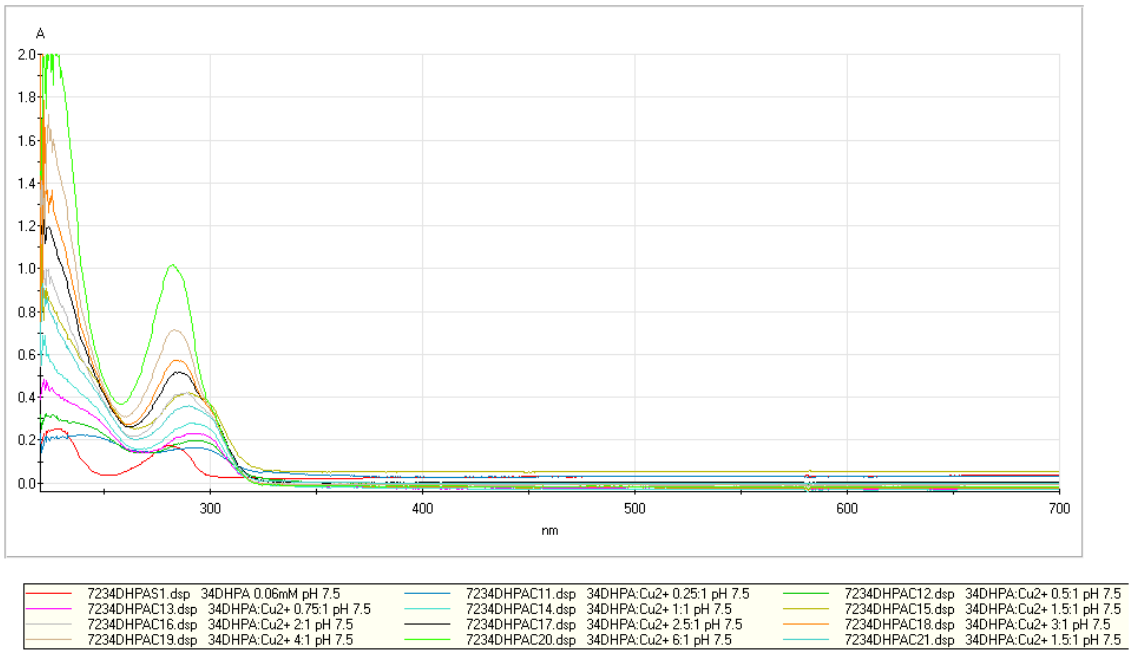

Figure 46: Complementary approach spectra of DOPAC compared to DOPAC with Cu^{2+} at pH 7.5.

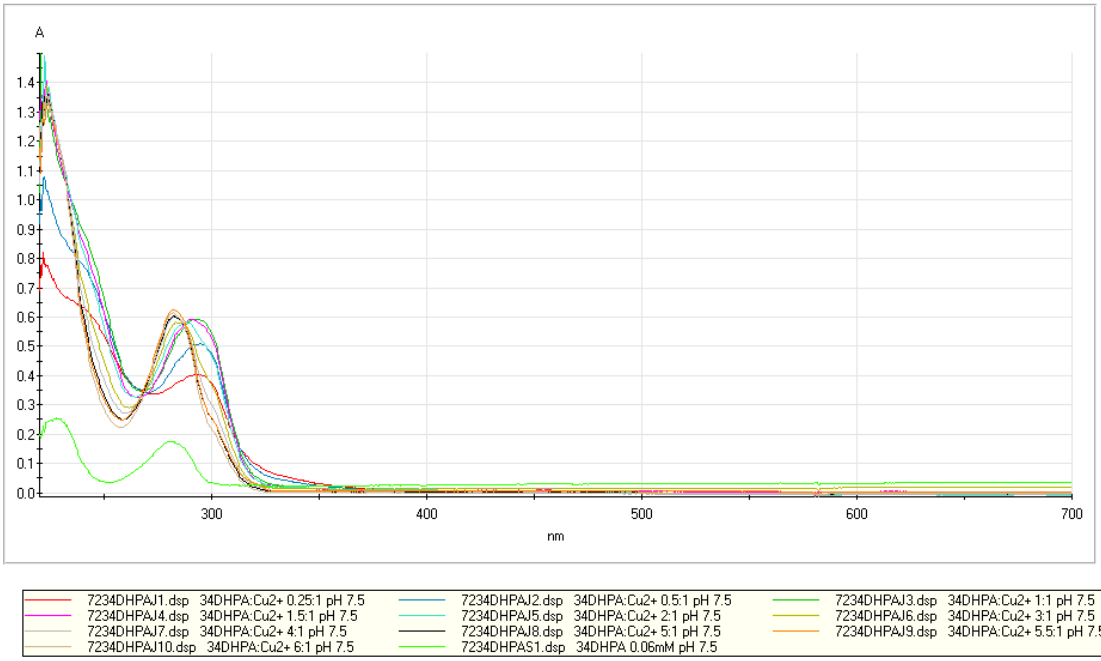

Figure 47: Results of Job's approach, Cu^{2+} pH 7.5.

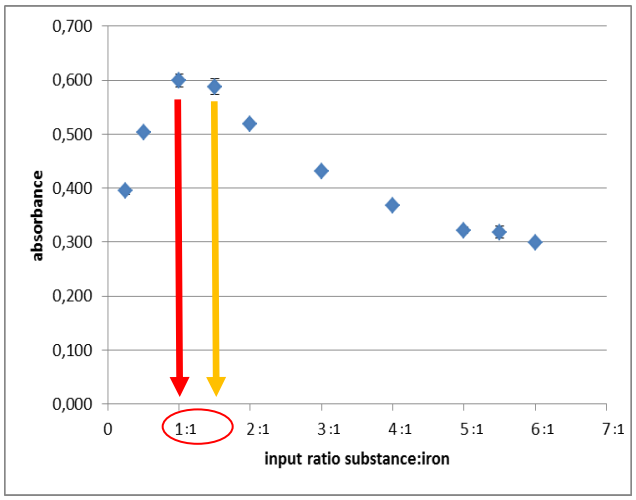

Figure 48: Results of Job's approach, Cu^{2+} pH 7.5 (2 repetitions of measurements). The ratio is not clear; it might be 1:1 or potentially 3:2 (1.5:1).

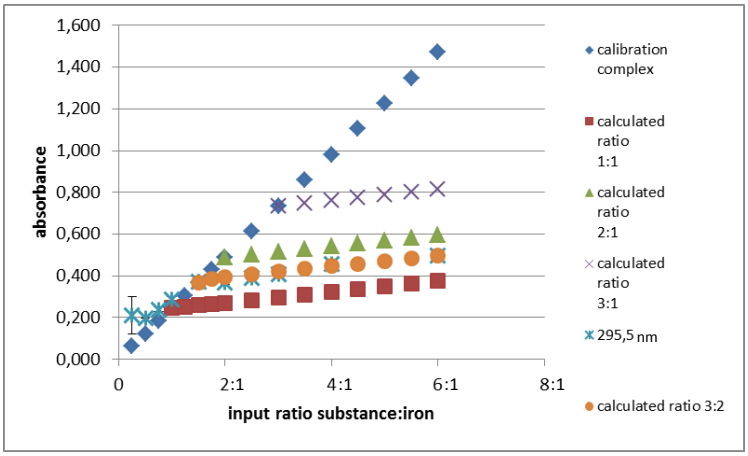

Figure 49: Results of mathematical analysis with method V, Cu^{2+} pH 7.5. Similarly to results from Job´s plot, the ratio seems to be 3:2 (2 repetitions of measurements).

Again, at pH 7.5 the chelation of cupric ions by DOPAC does take place (Fig. 45). Complementary method is depicted in Fig. 46 and its mathematical transformation in Fig. 47. Job´s method (Fig. 48, 49) suggested ratio 3:2.

4.3. Summary of Obtained Data and Discussion

During the mathematical analysis of the data, it became clear that some methods provided acceptable results, in particular method V, and also method I or VI (data not shown) and Job´s method in a few cases. The reason seems to be in accordance with previous reports from our laboratory (32) and likely lies in small bathochromic shifts in measured spectra after addition of transient metals, e.g. only 3.5 nm at pH 6.8 in case of ferrous ions (Tab. X and XI). The summary of obtained results is demonstrated in Tab. XII for iron and Tab. XIII for copper.

Table X: Changes in absorption maxima of pure substance and absorption maxima of complexes with Fe^{2+} and Fe^{3+}.

pH	λ_{max} DOPAC	λ_{max} DOPAC + Fe^{2+}	λ_{max} DOPAC	λ_{max} DOPAC + Fe^{3+}
4,5	281.5	281.5	281.0	288.0
5,5	281.0	282.0	281.0	290.0
6,8	280.5	284.0	280.5	281.0
7,5	281.5	289.5	281.0	282.0

As we see from the absorption maxima differences between the λ_{max} of pure substance and λ_{max} of the complexes, it is clear that the differences are negligible at pH 4.5 and 5.5 but can be observed in the case of Fe^{2+} at pH 6.8 and 7.5.

Table XI: Changes in absorption maxima of pure substance and absorption maxima of complexes with Cu^{+} and Cu^{2+}.

pH	λ_{max} DOPAC	λ_{max} DOPAC + Cu^{+}	λ_{max} DOPAC	λ_{max} DOPAC + Cu^{2+}
4,5	281.5	281.0	281.5	280.5
5,5	281.0	324.5	281.0	281.5
6,8	280.5	325.0	280.5	296.0
7,5	281.5	321.5	281.5	295.5

In the case of copper, it is clearly seen that the λ_{max} vary more. There are clear differences at pH 5.5, 6.8 and 7.5 when comparing DOPAC and DOPAC with Cu^{+} from which the λ_{max} is rising. Therefore, we can say that at these conditions complexes are being formed and affect the final measured λ_{max}. Also, when testing the substance with the addition of Cu^{2+} at pH 6.8 and 7.5 the formation of complexes occurs and shows as the difference in λ_{max}.

Table XII: Summarized results for stoichiometric ratio of DOPAC with iron, according to the complementary and Job's approach.

Method/ pH	**Fe^{2+} 4.5**	**Fe^{2+} 5.5**	**Fe^{2+} 6.8**	**Fe^{2+} 7.5**	**Fe^{3+} 4.5**	**Fe^{3+} 5.5**	**Fe^{3+} 6.8**	**Fe^{3+} 7.5**
Method I	N	N	?	?	N	N	N	N*
Method V			2:1 changing to 3:1	1:1				
Method VI			2:1 changing to 3:1	?				
Job's approach			?	?				

N – no complex formation, ? –the method was not able to determine the stoichiometry, * - no or very low chelation. Other methods are not shown because they did not give acceptable results.

Table XIII: Summarized results for stoichiometric ratio of DOPAC with copper, according to the complementary and Job's approach.

Method/ pH	Cu^{+} 4.5	Cu^{+} 5.5	Cu^{+} 6.8	Cu^{+} 7.5	Cu^{2+} 4.5	Cu^{2+} 5.5	Cu^{2+} 6.8	Cu^{2+} 7.5
Method I	N	N*	2:1	?	N	N	?	?
Method V			2:1	2:1 or 3:1			1:1	3:2
Method VI			?	?			?	?
Job's approach			?	?			1:1	1:1 or 3:2

N – no complex formation, * – no or very low chelation, ? – the method was not able to determine the stoichiometry. Other methods are not shown because they did not give acceptable results.

As DOPAC has iron/copper binding moiety (the catechol group), it is expected that formation of complexes occurs. However, the stoichiometric ratios of iron/copper DOPAC complexes differ in relation to different pH conditions and the presented metal. Different pathophysiological relevant pH conditions, ranging from low to higher pH, are important for many reasons. Low pH conditions are of importance because pathological acidosis may occur in inflammatory conditions including acute myocardial infarction and tumors (33, 34). By varying the pH, we also mimic the physiological differences in various body compartments (e.g. stomach, lysosomes). The most acidic pH used in this experiment was 4.5, which can be found in lysosomes – organelles important for iron trafficking. The pH conditions of 5.5 and 6.8 were used to mimic severe or moderate ischemia, where iron in particular is known to participate in tissue damage. The differences in iron and copper chelation can be explained by different dissociation of protons of hydroxyl groups in different pH conditions. It is vital that the metal binding chelator is present in the deprotonated form, which is in this case the form of catecholate. As confirmed by the results, the catechol ring has chelating properties at physiological conditions, but has low chelating activity at a lower pH (33). Therefore, a low pH is unfavorable for formation of complexes. Moreover, that can be confirmed for DOPAC, as 3,4-dihydroxyl group has a predicted pKa of 6.7 and is therefore sensitive to deprotonation at physiological pH conditions (34). Metal ions such as Fe^{2+} and Fe^{3+} prefer octahedral geometry so they are able to coordinate up to three catecholate groups. Therefore, it might be expected that polyphenols with a catechol group would bind in a 3:1 fashion (Fig. 50). However, it is not

so, as the complexes formed are pH dependent and exhibit variable coordination modes (5). As seen in the results, the ratios can range from 3:2, 2:1, 1:1 in different conditions. The same is true for Cu^{2+}.

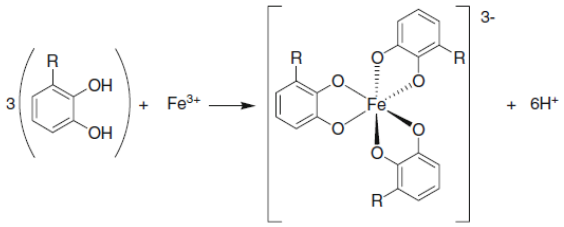

Figure 50: Predicted octahedral coordination geometry of iron-polyphenol complexes (5).

Radical scavenging antioxidant activity of hydroxyflavones can also be quantified using a so called TEAC value. This assay was used in a study of Lemanska et al. (34), where experimental data for deprotonation and antioxidant activities was determined and compared to computer calculated parameters for OH-deprotonation, electron donation and hydrogen atom donation as well. The results of the study showed significant the pH dependent influences on the antioxidation action of hydroxyflavones and also revealed a change in the mechanism of antioxidant action upon hydroxyl moiety deprotonation. The TEAC value for 3,4-dihydroxyflavone was found to be increasing with the pH (34). This is also visible in the results given above. Deprotonation generally enhances the antioxidation action of the hydroxyflavones and the ionization potential becomes lower upon deprotonation, so it can be concluded that electron donation is the dominant mechanism of antioxidant action after deprotonation (34).

In the case of Fe^{3+}, polyphenols can reduce the iron to Fe^{2+} upon binding of a catecholate ligand. The substance is oxidized to a semiquinone during the process and is protonated at a low pH, therefore forming a neutral ligand. Once the semiquinone form is generated, it can reduce another equivalent of Fe^{3+}, simultaneously oxidizing the semiquinone to quinone. To investigate such Fe^{3+} reduction, the study needs to be performed at a very low pH. However, as mentioned before, DOPAC doesn't chelate at low pH conditions, with data showing that it reduces Fe^{3+}. At a higher pH, the formation of complexes (two or three polyphenol ligands coordinated to a single metal ion) with iron inhibiting these Fe^{3+} reduction reactions which therefore may occur much more slowly at around pH 7 (5).

Quite a surprising result is that DOPAC seems to chelate only ferrous but not ferric ions. In general, the results of this study show, that in comparison to iron, copper seems to be a slightly better chelating target at least in the case of this agent. Other studies came to identical conclusions. In the study of Perron and Brumaghin (2009), stability constants for Cu^{2+} catecholate complexes were found to be greater than for Fe^{2+}. It was also noted that in contrast with other metal ions found in biological systems, Cu^{2+} has a positive reduction potential in aqueous solutions. It is facilitating the reduction of Cu^{2+} to Cu^{+} and promoting Cu^{2+} binding to electron rich ligands, such as oxygen atoms. The tendency to copper reduction and the tendency of polyphenols to oxidize, results in the formation of complexes, especially in the presence of ROS (5).

For further and more extensive analysis of DOPAC and its chelation of iron and copper, other methods like Raman or mass spectrometry could be used. A research of similar flavonol substances to DOPAC could give more relevant data about the importance of structure in the case of chelation of copper/iron. However, it is of great importance to realize that the results obtained in vitro cannot be simply extrapolated to the conditions in vivo, but still may be taken into account as a demonstration of substance behavior under physiologically relevant conditions (33).

5. CONCLUSION

As phenolic compounds represent an important part of the human diet and are known to have free radical scavenging activity, they are being researched for positive health effects, mainly in the therapy of cardiovascular, neurodegenerative diseases and cancer.

Since structure-activity relationships consistently rank quercetin as one of the most powerful antioxidants in the class of flavonoids capable of chelation, DOPAC could be assumed to also have some potential as a chelator agent. However, the results of this research have shown that it might not be so, mostly because of pathological conditions which occur at low pHs, such as acute myocardial infarction and tumors, where a powerful chelator active in acidic condition is needed, which DOPAC is not. H_2QpyQ /2, 6-bis[4(1-phenyl-3-methylpyrazol-5-one)carbonyl]pyridine, which is an iron chelator proven to have increased affinity with decreasing pH conditions (35), is of particular interest.

In conclusion, the results have shown that DOPAC doesn't chelate at low pH conditions, which confirms our first hypothesis. As it is visible from the results, we can also confirm the ability of DOPAC to chelate with Fe^{2+} in the 1:1 ratio at pH 7.5. However, no or very low chelation was detected in the case of Fe^{3+}. While studying copper chelation, the formation of complexes was confirmed in the case of Cu^{+} in the 2:1 ratio at pH 6.8, while in Cu^{2+}, chelation occurred at pH 6.8 (1:1 ratio) as well as 7.5 (2:1 ratio). Therefore, we cannot confirm the second hypothesis as chelation was expected in both ions at pH 6.8 and 7.5. Furthermore, additional research is needed for studying iron and copper binding stability, as well as for proper in vivo and in vitro antioxidant activity of phenolic compounds. It is to be kept in mind, that an ideal chelator would possess a high selectivity for iron and/or copper and would have a minimum impact on chelation of other biologically essential metal ions.

6. LITERATURE

1. Andrews N: Molecular control of iron metabolism. Best Practice and Research Clinical Haematology 2005; 18: 2: 159-169.
2. Liu Z, Hider R: Design of iron chelators with therapeutic application. Coordination Chemistry Reviews 2002; 232: 151-171.
3. Horwitz L, Rosenthal E: Iron mediated cardiovascular injury. Vascular Medicine 1999; 4: 93-99.
4. Papanikolaou G, Pantopoulos K: Iron metabolism and toxicity. Toxicology and Applied Pharmacology 2005; 202: 199-211.
5. Perron N, Brumaghim J: A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. Cell Biochemistry and Biophysics 2009; 53; 75-100.
6. Perron N, Hodges J, Jenkins M, Brumaghim J: Predicting how polyphenol antioxidants prevent DNA damage by binding to iron. Inorganic Chemistry 2008; 47: 6153-6161.
7. Arrendondo M, Nunez M: Iron and copper metabolism. Molecular Aspects of Medicine 2005; 26: 313-327.
8. Ferenci P: Pathophysiology and clinical features of Wilson disease. Metabolic Brain Disease 2004; 19: 229-239.
9. Skjorringe T, Moller L, Moos T: Impairment of interrelated iron- and copper homeostatic mechanisms in brain contributes to the pathogenesis of neurodegenerative disorders. Frontiers in pharmacology 2012; 3: 1-14.
10. Cooper G, et al.: Regeneration of the heart in diabetes by selective copper chelation. Diabetes 2004; 53: 2501-2508.
11. Scalbert A, et al.: Absorption and metabolism of polyphenols in the gut and impact on health. Biomedicine and Pharmacotherapy 2002; 56: 276-282.
12. Bhooshan Pandey K, Ibrahim Rizvi S: Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Medicine and Cellular Longevity 2009; 2: 5: 270-278.
13. Morton L, et al.: Chemistry and biological effects of dietary phenolic compounds: relevance to cardiovascular disease. Clinical and Experimental Pharmacology and Physiology 2000; 27: 152-159.

14. Arts I, Hollman P: Polyphenols and disease risk in epidemiologic studies. American Journal of Clinical Nutrition 2005; 81: 317S-25S.
15. Duthie G, Duthie S, Kyle J: Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. Nutrition Research Reviews 2000; 13: 79-106.
16. Heim K, Tagliaferro A, Bobilya D: Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. Journal of Nutritional Biochemistry 2002; 13: 572-584.
17. Croft K: The chemistry and biological effects of flavonoids and phenolic acids. Annals of the New York Academy of Science 1998; 854: 435-442.
18. Carrasco-Pozo C, et al.: 3, 4-dihydroxyphenylacetic acid, a microbiota-derived metabolite of quercetin, protects against pancreatic β-cells dysfunction induced by high cholesterol. Experimental Cell Research 2015; http://dx.doi.org/10.1016/j. yexcr.2015.03.021
19. Lopes Jesus A, et al.: Conformational preferences of 3, 4-dihydroxyphenylacetic acid (DOPAC). Spectrochemica Acta Part A: Molecular and bimolecular spectroscopy 2015; 140: 54-64.
20. Okabe N, Kyoyama H: 3, 4-Dihydroxyphenylacetic acid. Acta Crystallographica Section E 2001; 57: 715-716.
21. Eldrup E: Significance and origin of DOPA, DOPAC, and dopamine- sulphate in plasma, tissue and cerebrospinal fluid. Danish medical bulletin 2004; 15: 34-62.
22. Goldstein DS, et al.: Decreased vesicular storage and aldehyde dehydrogenase activity in multiple system atrophy. Parkinsonism and Related Disorders 2015; http://dx.doi.org/10.1016/j.parkreldis.2015.03.006
23. Khokhar S, Owusu Apenten R: Iron binding characteristics of phenolic compounds: some tentative structure-activity relations. Food Chemistry 2003; 81: 133-140.
24. Andjeloveć M, et al.: Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. Food Chemistry 2006; 98: 23-31.
25. Sroka Z, Cidowski W: Hydrogen peroxide scavenging, antioxidant and anti- radical activity of some phenolic compounds. Food and Chemical Toxicology 2003; 41: 753-758.
26. Mladenka P, et al.: Cardiovascular effects of flavonoids are not caused by direct antioxidant activity. Free Radical Biology and Medicine 2010; 49: 963-975.

27. Horwitz L, Rosenthal E: Iron-mediated cardiovascular injury. Vascular Medicine 1999; 4: 93-99.
28. Crisponi G, Remelli M: Iron chelating agents for the treatment of iron overload. Coordination Chemistry Reviews 2008; 252: 1225-1240.
29. Havsteen B: The biochemistry and medical significance of the flavonoids. Pharmacology and Therapeutics 2002; 96: 67-202.
30. Nijveldt R, et al.: Flavonoids: a review of probable mechanisms of action and potential applications. The American Journal of Clinical Nutrition 2001; 74: 418-425.
31. Stookey LL: Ferrozine- A new spectrophotometric reagent for iron. Analytical chemistry 1970; 42: 779- 781.
32. Filipsky T, et al.: Mathematical calculations of iron complex stoichiometry by direct UV-Vis spectrophotometry. Bioorganic Chemistry 2013; 49: 1-8.
33. Mladenka P, et al.: In vitro analysis of iron chelating activity of flavonoids. Journal of Inorganic Biochemistry 2011; 105: 693-701.
34. Lemanska K, et al.: The influence of pH on antioxidant properties and the mechanism of antioxidant action of hydroxyflavones. Free radical biology and medicine 2001; 31: 869-881.
35. Filipsky T., et al.: In vitro characteristics of 1-phenyl-3-methyl-4-acylpyrazol-5-ones iron chelators. Biochimie 2012; 94: 1: 125-131.