

REVIEW

Investigation of metal–flavonoid chelates and the determination of flavonoids *via* metal–flavonoid complexing reactions*

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Abstract: Flavonoids constitute a large group of polyphenolic phytochemicals with antioxidant properties which are overwhelmingly exerted through direct free radical scavenging. Flavonoids also exhibit antioxidant properties through chelating with transition metals, primarily Fe(II), Fe(III) and Cu(II), which participate in reactions generating free radicals. Metal–flavonoid chelates are considerably more potent free radical scavengers than the parent flavonoids and play a prominent role in protecting from oxidative stress. To unravel the origin of their potent biological action extensive physico–chemical studies were undertaken to reveal the chemical structure, chelation sites, assess the impact of the metal/ligand ratio on the structure of the complexes and the capacity of flavonoids to bind metal ions. In spite of such extensive efforts, data on the composition, structure and complex-formation properties are incomplete and sometimes even contradictory. The aim of this paper is to give a personal account on the development of the field through a retrospective evaluation of our own research which covers approximately 40 complexes of flavonoids from different flavonoids subclasses (rutin, quercetin, 3-hydroxyflavone, morin and hesperidin) with several metal ions or groups and suggest directions for future research. Special emphasis will be given to the site of the central ion, the composition of the complexes, the role of pH in complex formation, the stability of metal–flavonoid complexes and their potential application for analytical purposes.

Keywords: flavonoids, chelates, free radical scavengers, stability constants, quantitative analysis.

FLAVONOIDS

The Hungarian Nobel laureate Albert Szent-Györgyi discovered flavonoid compounds in 1936.¹ Using evidence from his own experiments, he hypothesized that a new vitamin – vitamin P works synergistically with vitamin C in citrus extracts to strengthen capillaries.² Although the description of flavonoids as vitamins was eventually found to be inaccurate, research into their beneficial potentials continued and has increased dramatically over the past two decades.^{3–6}

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Flavonoids (*flavus* – yellow), or bioflavonoids, are a ubiquitous group of polyphenolic substances which are present in most plants, concentrated in the seeds, fruit skin or peel, bark and flowers.³ More than 4000 different flavonoids have been identified to date, making them the largest group of plant chemicals. Many fruits and vegetables, especially buckwheat, apple and onion, are some of these sources. Beverages prepared from plant extracts (beer, tea, wine, fruit juice) are the principal source of dietary flavonoid intake.^{7–16}

Health benefits of flavonoids

Scientific studies conducted in the last few years generated a growing interest in the potentially important role of flavonoids in maintaining human health. A considerable number of plant medicines contain flavonoids, which have been reported by many authors as having anti-bacterial, anti-inflammatory, anti-allergic, anti-mutagenic, anti-viral, anti-neoplastic, anti-thrombotic, and vasodilatory actions.^{17–31} Overwhelmingly, the pharmacological effects are related to the anti-oxidant activity of flavonoids, arising through their ability to scavenge free radicals. When generated in excess, free radicals can damage biomolecules, and are therefore implicated in the etiology of several diseases and ageing.³² Radical scavenging by flavonoids occurs *via* electron donation from the free hydroxyls on the flavonoid nucleus with the formation of a less reactive flavonoid aroxyl radical, which is stabilized by resonance and therefore plays only a moderate role in the propagation of radical-induced damage in biological systems. The anti-oxidant activity of flavonoids correlates well with their physiological function *in vivo*, because oxidative stress is known to participate in the initial process of atherosclerosis leading to coronary heart disease and other patho-physiological events. A number of studies have revealed that flavonoids act as anti-oxidants by scavenging reactive oxygen species.^{33–42}

Specifically, flavonoids reduce the risk of stroke and heart disease (the so-called French paradox, the lack of a positive correlation between a high intake of saturated fat and the occurrence of coronary heart disease is related at least partly to the consumption of red wine,⁴³ which is rich in flavonoids), protect against age-related vision disorders, relieve hay fever, sinusitis, asthma symptoms, alleviate inflammatory skin conditions, reduce inflammation in joints and muscles, common to rheumatoid arthritis, minimize menopausal hot flashes, shrink hemorrhoids, reduce varicose veins and battle viral infections.^{17–31,44}

A considerable number of pharmaceutical preparations containing flavonoids as active substance are commercially available today. For example, *Ginkgo biloba* leaf extract,⁴⁵ used in the treatment of symptoms in the early stages of Alzheimer's disease, vascular dementia and memory impairment,⁴⁶ is the most widely sold phytomedicine in Europe. Some of the commercial pharmaceutical preparations which include the flavonoid rutin are widely used for curing veins diseases. Quercetin, the most biologically active and common dietary flavonoid, is generally used as a dietary supplement.

Structure and spectral characteristics of flavonoids

The carbon atoms in flavonoid molecules are assembled in two aromatic rings, commonly denoted as A and B, which are connected by a three-carbon “bridge”: C₆–C₃–C₆, thus forming a diphenyl-propane structure with the central unit being a benzo- γ -pyrone (chromone). Multiple hydroxyl groups, sugar, oxygen, or methyl groups are attached to this core structure. Depending on the oxidation state of the heterocyclic ring, flavonoids are classified as flavones, flavanols, flavanones, flavonols, flavanones or isoflavones (Fig. 1).³

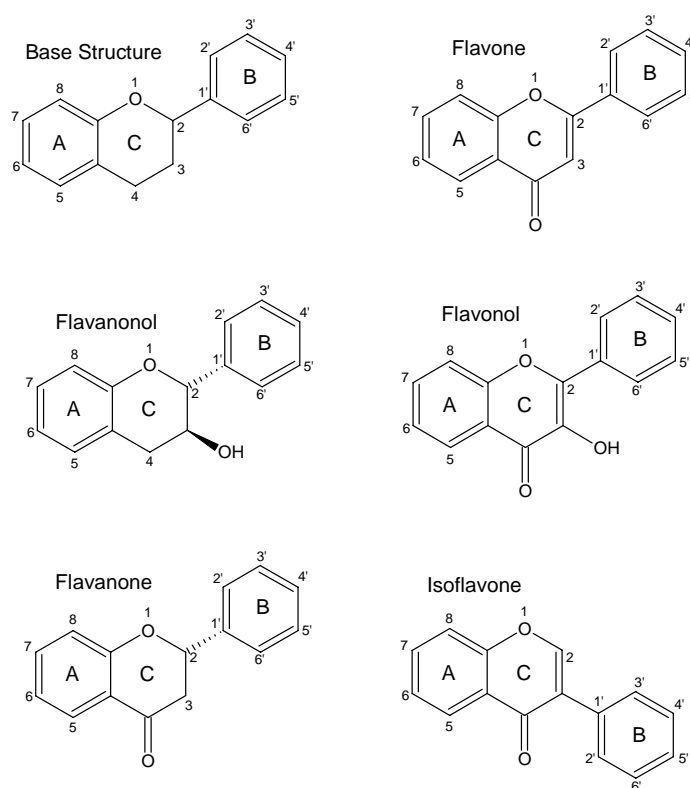


Fig. 1. The flavonoid nucleus consists of benzo- γ -pyrone (ring A and ring C) and benzene (ring B). The major classes of flavonoids are flavone, flavanonol, flavonol, flavanone and isoflavone.

Being pigments responsible for the color of leaves, herbs and petals, flavonoids strongly absorb ultraviolet (UV) radiation. Therefore UV-Vis spectroscopy remains the main tool for structural analysis of flavonoids. Typically, two major absorption maxima are observed in the UV-Vis spectrum of flavonoids. The absorption maximum observed in the range 240–285 nm is referred to as band II and the one between 300–400 nm as band I. In general terms, absorption band II may be considered as originating from $\pi \rightarrow \pi^*$ transitions in the A ring, a ben-

zene system, whereas the absorption band I is attributed to transitions in the B ring, a cinnamoyl system. The position of band I in flavones is between 304–350 nm, while flavonols absorb in the range 352–385 nm. Highly oxygenated flavones and flavonols tend to absorb at longer wavelengths than those with fewer oxygen substituents. Methylation or glycosylation of the hydroxyl groups on the flavonoid nucleus usually results in hypsochromic shifts, predominantly of band I. The book by Marby⁵ provides a detailed catalogue of UV spectra of 175 flavonoids.

METAL–FLAVONOID CHELATES

Due to their specific chemical structure, flavonoids easily chelate metal ions and create complex compounds.

Biological activity of metal–flavonoids complexes: chelates as free radical scavengers

In addition to direct free radical scavenging, flavonoids exert anti-oxidant activity through interactions with the reduced form of transition metals, primarily Fe(II), Fe(III) and Cu(I), which participate in reactions generating free radicals.⁴⁷ Flavonoids may sequester metal ions by chelating and preventing metal-mediated generation of free radicals and, accordingly, may protect the potential biological targets from oxidative stress. Thus, the overall anti-oxidant action of flavonoids appears to be a combination of a direct reaction with free radicals and chelating the metal ions responsible for the production of reactive oxygen species.

It was confirmed in numerous studies that flavonoids function as anti-oxidants mainly by chelating metal ions.^{48–60} Moreover, experimental data have shown that the chelates are considerably more effective free radical scavengers than the free flavonoids. Kostyuk *et al.*⁵⁶ found that complexes of rutin, dihydroquercetin or epicatechin with Fe(II), Fe(III), Cu(II) or Zn(II) are more effective radical scavengers than the free flavonoids, due to the acquisition of additional superoxide dismutating centers. These complexes show elevated efficiency in protecting red blood cells against asbestos-induced oxidative injury *in vitro*. According to the same authors, the Cu–rutin complex was found to be the most effective anti-oxidant against asbestos-induced lipid peroxidation in pulmonary tissue *in vivo*.⁵⁶ Moridani *et al.*⁵⁷ found that the Fe(III) complexes of flavonoids were much more effective than the free flavonoids in protecting isolated rat hepatocytes against hypoxia–reoxygenation injury. By using the 1,1-diphenyl-2-picrylhydrazyl radical scavenging method, de Souza and de Giovani⁵⁸ found antioxidant activities of the quercetin, rutin, galangin, and catechin complexes more effective than free flavonoids. Afanas'ev *et al.*⁵⁹ found that Fe(II)– and Cu(II)–rutin complexes were more efficient free radical scavengers *in vitro* and *ex vivo*. These complexes decreased the production of oxygen radicals by xanthine oxidase, rat liver microsomes and the rat peritoneal macrophages, as well as the generation of oxygen radicals by bronchioalveolar cell from bleomycin-treated rats by 2–30 times compared to the parent rutin.⁵⁹ The anti-oxidative activities of morin and its

Pd(II)- and Pt(II)-complexes were also investigated.⁶⁰ The anti-oxidative effects (scavenging superoxide radicals) of the complexes were greater than that of morin itself, while the Pt(II)-complex exhibited stronger scavenging efficacy than the Pd(II)-complex. Both the Pd(II)- and Pt(II)-complexes showed an inhibitory effect on lipid peroxides which was greater than that of free morin.⁶⁰ Due to anti-oxidative mechanisms, morin complexes with La(III), Gd(III) and Lu(III) ions against three bacterial strains, *i.e.*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* showed inhibitory action.⁶¹ Rare earth metal(III) complexes with quercetin can bind to DNA thereby changing its transcription and repressing the growth of tumor cells.⁶²

Complexes of flavonoids play an important role in limiting metal bioavailability and suppressing metal toxicity. For example, aluminum has been implicated in neurological and bone disorder. The complexation of Al(III) by quercetin reduces aluminum overload in the diet.⁶³ By forming complexes, flavonoids appear to be a suitable antidote for heavy metal poisoning *in vivo*.⁶⁴ Quercetin, as an active biological ligand, might be an appropriate Mo(VI) chelator in the case of molybdenum deficiency caused by irradiation, since the use of molybdenum salts is undesirable because of their high toxicity.⁶⁵

Physico-chemical features of metal-flavonoid complexes

Understanding the mechanisms of chelation of flavonoids by metal ions permits a better understanding of their complex anti-oxidant properties. Therefore, many metal-flavonoid complexes have been synthesized and characterized in the past several years.⁶⁶⁻⁷⁴ Elemental and thermal analyses, conductivity and cyclic voltammetry, as well as IR, Raman, ¹H-NMR, ¹³C-NMR, UV-Vis and fluorescence spectroscopy have been used to assess relevant interactions of flavonoids and metal ions, the chelation sites, the dependence of the complex structure on the metal/ligand ratio, the capability of flavonoids in binding metal ions, *etc.* However, data on the composition, structure and complex-formation features are sometimes incomplete and contradictory. The outcome largely depends on the experimental condition and the type of assay employed.

Metal-flavonoid complex compounds are the subject of our longtime research during which their properties, composition, complex formation features, stability constants, as well as analytical appraisal were investigated.⁷⁵⁻¹¹¹ Starting in the early 1980's until now, approximately 40 complexes of flavonoids from different flavonoids subclasses (rutin, quercetin, 3-hydroxyflavone, morin and hesperidin, Fig. 2) with a number of metal ions or metal groups have been investigated. Considering the importance of metal chelation for the understanding of the anti-oxidant behavior of flavonoids, our aim is to give a personal account on the development of the field through a retrospective of our own research. In addition, analytical methods suitable for routine analysis involving the spectrophotometric determination of flavonoids *via* complexing reactions are also reported.

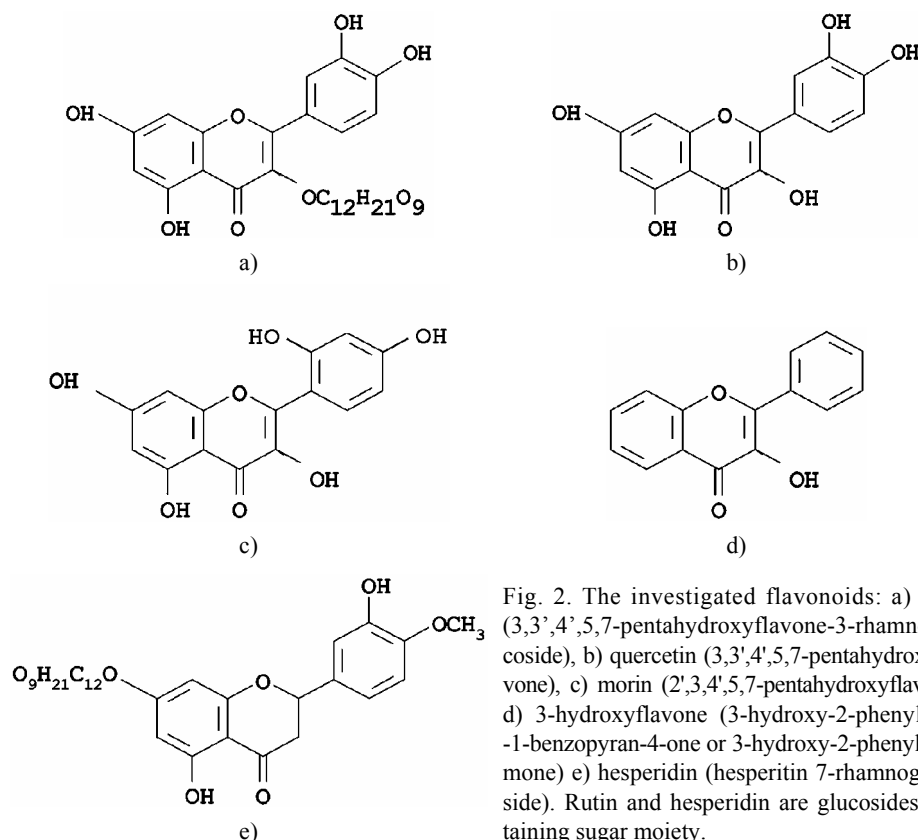


Fig. 2. The investigated flavonoids: a) rutin (3,3',4',5,7-pentahydroxyflavone-3-rhamnoglucoside), b) quercetin (3,3',4',5,7-pentahydroxyflavone), c) morin (2',3,4',5,7-pentahydroxyflavone), d) 3-hydroxyflavone (3-hydroxy-2-phenyl-4*H*-1-benzopyran-4-one or 3-hydroxy-2-phenylchromone) e) hesperidin (hesperitin 7-rhamnoglucoside). Rutin and hesperidin are glucosides containing sugar moiety.

UV-Vis spectra

Metal-flavonoid chelates are usually colored. In the presence of metal ions, a bathochromic shift is typically observed in the absorption spectra of flavonoids. The reaction with AlCl_3 , described for the first time in 1962,³ is actually the earliest presented complexing reaction of flavonoids with aluminum as the central ion and flavone as the ligand. Already then it was observed that complex formation causes a bathochromic shift in both absorption bands, I and II, and that the shift is reversed by increasing the acidity of the medium. A similar behavior is observed for all flavonoid subclasses possessing 5-hydroxy-4-keto, 3-hydroxy-4-keto and/or *o*-dihydroxy groups, suggesting that these moieties are important for chelation. This red shift is caused by the increased conjugative effect when the complexes are formed to give a new ring (Fig. 3).

The site of the central ion

In each flavonoid molecule, there are three domains that can likely interact with metal ions, *i.e.*, the 3',4'-dihydroxy group located on the B ring, the 3-hydroxy or 5-hydroxy and the 4-carbonyl groups in the C ring. Generally, the chela-

ting properties of flavonoids toward metal ions have been attributed to the presence of the 3- or 5-hydroxypyran-4-one, rather than the *ortho*-hydroxyl groups in the B ring.¹¹² Our own IR spectroscopic results the Pd(II)-quercetin¹⁰² (Fig. 4) and UO₂(II)-rutin¹⁰¹ (Fig. 5) complexes also confirm that the benzoyl moiety is the major site for metal chelation. There are, however, studies proposing the catechol moiety as the major site for metal chelating.^{54,66} The results of Bodini *et al.*¹¹³ indicate that coordination to the catechol group of quercetin is the strongest for iron, even in acidic media. Cornard and Merlin⁷⁰ assert the opposite, that in acidic media the *ortho*-dihydroxyl groups of quercetin are never involved in complexation with Al(III). The same authors found two binding sites in the Al(III)-quercetin complex; the first one involved in complex formation is the 3-hydroxychromone and the second one is the *ortho*-hydroxy groups, which are strongly dependent on the medium and pH. Depending on the experimental conditions, as well as with an excess of the metal ion, Torreggiani *et al.*⁷² also found that two chelating processes occurred consecutively, implicating two binding sites in the Cu(II)-quercetin complex. Based on ¹H-NMR, DTA curves and fluorescence spectroscopy, Tang *et al.*⁶⁰ proposed the site of metal ion in chelation given in Fig. 6.

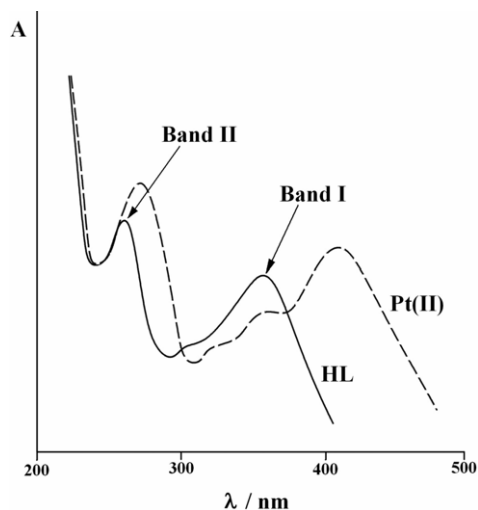


Fig. 3. The red shift of band I and band II in morin (HL) after reaction with Pt(II).⁶⁰

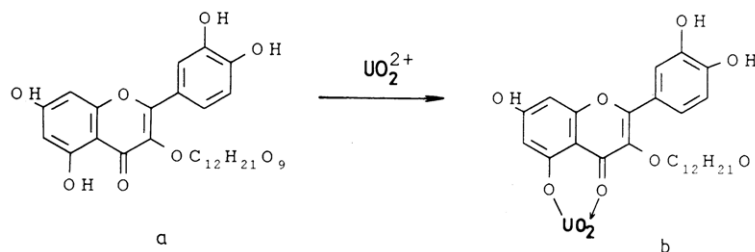


Fig. 4. Formation of uranyl-rutin complex in a 1:1 stoichiometric ratio: a) rutin, b) complex. Favored metal site is 5-hydroxypyran-4-one, rather than the *ortho*-hydroxyl groups in the B ring.¹⁰¹

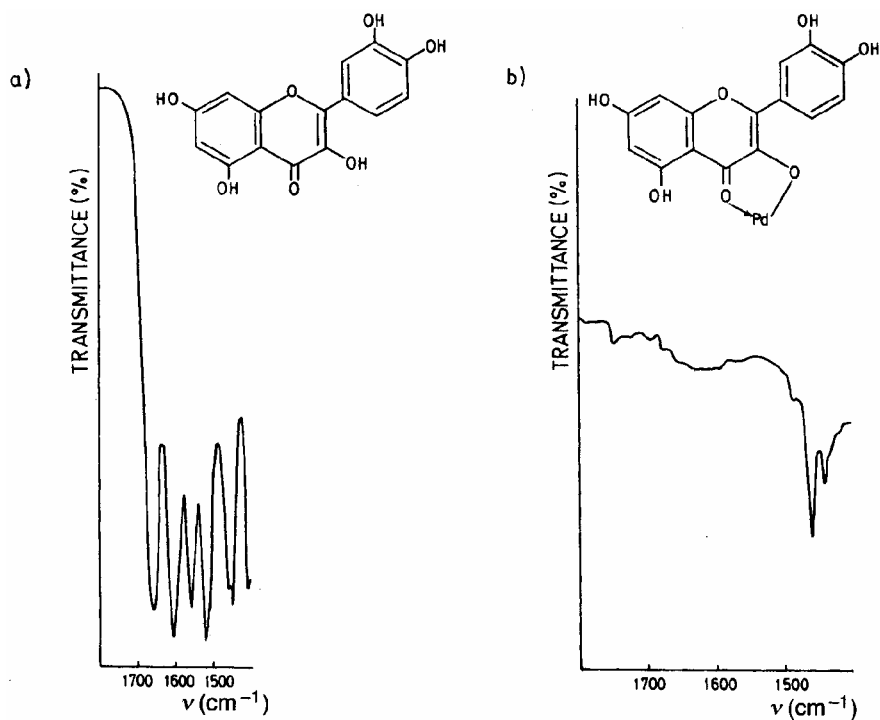


Fig. 5. Composition of the Pd(II)–quercetin complex. Favored metal site is 3-hydroxypyran-4-one, as confirmed by IR spectroscopy.¹⁰²

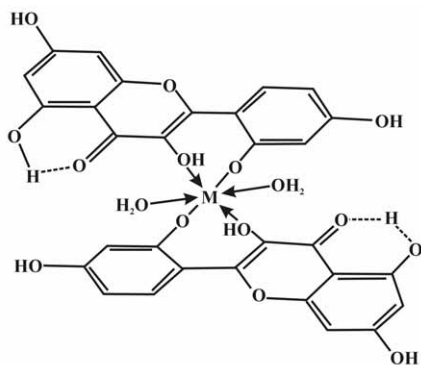


Fig. 6. Probable structure of M(II)–morin complexes $M = \text{Pt(II)}, \text{Pd(II)}$ and Zn(II) . The structure is suggested due to the fixation of ring B caused by the effect of coordination after the complex was formed.⁶⁰

Composition of the complexes

Based on our research, only mononuclear complexes (with one central ion) were formed under the investigated experimental conditions (water–alcoholic solution, neutral or acidic media, maximum concentration of $0.0025 \text{ mol dm}^{-3}$ for rutin, quercetin, morin and 3-hydroxyflavone and $0.0015 \text{ mol dm}^{-3}$ for hesperidin). The maximum number of flavonoids in a complex never exceeded two. Though complexes comprising a greater number of flavonoids as ligands are

sterically unfavorable, Zhou and co-workers⁶² found by fluorescence spectroscopy 1:3 complexes of quercetin with eight rare-earth metal ions as the central ions.

The stoichiometric composition of flavonoid complexes is typically determined by the following methods: the method of continual variation of equimolar and non-equimolar solutions, the molar-ratio method, the Bent-French and Nach methods.^{114,115} According to longtime experience, the most reliable results were obtained by the method of continual variation of equimolar solutions, while the method of continual variation of non-equimolar solutions is not recommended for metal-flavonoids complexes because of low reproducibility. It was also found that the Bent-French method is not sufficiently accurate for complexes with a 1:2 stoichiometric ratio, but is acceptable for complexes with a 1:1 composition. According to recent literature, these methods were not widely utilized by other authors (only the molar ratio method and method of continual variation were used by Cornard and co-authors),⁶⁹⁻⁷¹ but we find them still suitable taking into account their simplicity and inexpensiveness. The compositions of the investigated flavonoid complexes, as well as the methods used, are presented in Table I.

TABLE I. Composition of the metal-flavonoid complexes. MR: Mole ratio method, B-F: Bent-French method

Flavonoid	Method	Metal ion	Metal/flavonoid	pH	Ref.	
Rutin	Job, MR	Cu(II)	1:2	6.1	78	
	MR, B-F	Zn(II)	1:1	6.2	79	
	MR, Nach, Job	Pb(II)	1:2	4.5	80	
	MR, Nach, Job	Ni(II)	1:2	6.2	82	
	MR, Nach	Co(II)	1:1	5.0	83	
	B-F, Job, MR	MoO ₄ ²⁻	1:1	6.3	91	
	MR	WO ₄ ²⁻	1:2	7.0	92	
	Job, MR	Eu(III)	1:2	5.0	98	
	Job, MR	UO ₂ (II)	1:1	6.8	101	
	Job	Pd(II)	1:2	8.2	103	
	Job, MR	TiO(C ₂ O ₄) ₂ ²⁻	1:2	6.4	107	
	Quercetin	MR, Nach	Ni(II)	1:1	5.0	81
		MR, Nach	Co(II)	1:1	5.0	84
Job, MR		Pd(II)	1:1	6.2	102	
Job, MR		TiO(C ₂ O ₄) ₂ ²⁻	1:2	6.4	109, 111	
Morin	Job, MR	Cu(II)	1:2	5.8	76	
	Job, MR, Nach	Zn(II)	1:2	5.5	77	
	Job	WO ₄ ²⁻	1:2	5.2	97	
	Job	Pd(II)	1:1	5.5	106	
	Job, MR	TiO(C ₂ O ₄) ₂ ²⁻	1:2	4.3	110	
	MR, B-F	Ba(II)	1:1	4.2	85	

TABLE I. Continued

Flavonoid	Method	Metal ion	Metal/flavonoid	pH	Ref.
3-Hydroxyflavone	Job	Zn(II)	1:1	5.8	75
	Job, MR, Nach	Pb(II)	1:1	6.1	93
	Job, MR	Ni(II)	1:1	6.1	95
	Job	Co(II)	1:1	6.2	86
	Job, MR	MoO ₄ ²⁻	1:2	6.3	90
	Job	WO ₄ ²⁻	1:2	8.6	94
	Job	Eu(III)	1:2	5.7	96
	Job, MR, B-F	UO ₂ (II)	1:1	3.5	88
	Job, MR	TiO(C ₂ O ₄) ₂ ²⁻	1:2	5.0	108
	Job, MR	Mn(II)	1:1	6.3	87
Hesperidin	Job, MR	Cu(II)	1:2	5.7	105
	Job, MR	UO ₂ (II)	1:2	3.7	104
	Job, MR	Al(III)	1:1	3.7	100
	Job, MR	Zr(IV)	1:1	3.6	99

The role of pH in complex formation

Flavonoids are weak polybasic acids that tend to protonate.¹¹⁴ Therefore, pH has a considerable impact on complex formation. According to our results, complexes with the highest coordination number are typically formed in slightly acidic or neutral pH, rarely in basic media. The optimal pH for complex formation, although strongly dependent on the features of the metal ion, is around pH 6. Complex formation at pH values lower than 3.0 is difficult because the flavonoids are predominantly present in their undissociated form. Although high pH values favor deprotonation of flavonoids and, consequently, more complex species, at higher pH values metal ions are often involved in side reaction (hydrolysis) and hydroxo-complexes are formed.⁷⁵⁻¹¹¹

Complexation with flavonoids as unidentate or bidentate ligands leads to the formation of complexes that contain protons in addition to the metal ion and ligand (so-called protonated complexes) which tend to dissociate at higher pH values. Thus, the bathochromic shift that can be observed in the absorption spectra of metal-flavonoid complexes at higher pH values can be attributed to the dissociation of the protonated complexes, rather than the formation of complexes with different stoichiometric compositions.¹⁰²⁻¹⁰⁹ The absorption spectra of the titanyl-oxalato-quercetin complex at different pH values are presented in Fig. 7. The majority of the investigated metal-flavonoid complexes follow this pattern.¹⁰²⁻¹⁰⁹

Stability of complexes

Several methods for spectrophotometric determination of the stability constants of metal-ligand complexes have been described, such as the Sommer,

Bent–French and Nach method, the method of continual variation of equimolar solutions, the molar-ratio method and the Bjerrum method.^{114–115} We found the Bjerrum method to be the most appropriate for metal–flavonoid complexes, having satisfactory reproducibility and accuracy. The method was modified and simplified by Malešev and the adopted method (denoted with Bjerrum* in Table II), described in detail in the literature,^{91,94,100,102} was extensively used in our research. The stability constant of approximately twenty metal–flavonoid complexes were calculated using this method, Table II (note that only one value for the stability constant is presented although two or more methods were applied in the study, but gave approximately equal results).

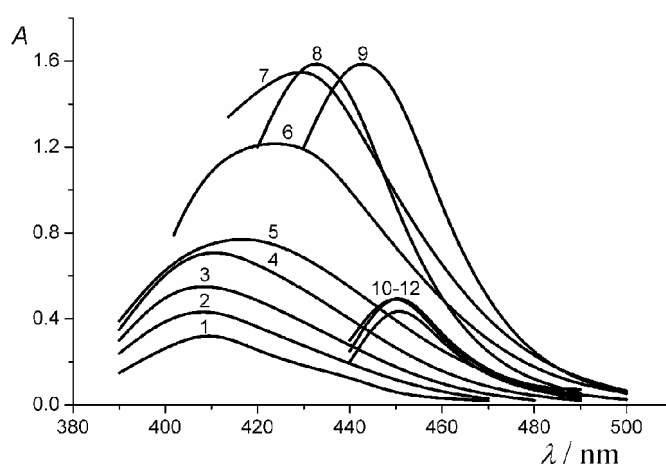


Fig. 7. Absorption spectra of the tityloxalato–quercetin complex obtained at different pH values; curves 1–12: pH: 3.6 (1), 4.3 (2), 5.0 (3), 5.6 (4), 6.8 (6), 7.2 (7), 8.2 (8) 9.2 (9), 10.0 (10), 12.2 (11), 10.6 (12). The bathochromic shift is due to dissociation of the complex.¹⁰⁹

In general, the majority of the investigated complexes can be characterized as moderately ($5 < \log\beta < 10$) or highly ($\log\beta > 10$) stable. It is interesting to note that in the highly stable complexes, the central ion is typically an anion, *i.e.*, tityloxalate $\text{TiO}(\text{C}_2\text{O}_4)_2^{2-}$ and WO_4^{2-} in complexes with rutin, morin and 3-hydroxyflavone and MoO_4^{2-} in the complex with 3-hydroxyflavone, rather than a cation. This is in discordance with the crystal field theory, which describes bonding in transition metal complexes and states that the interactions between ligand and metal are partly electrostatic. However, the molecular orbital theory allows overlapping of atomic orbitals with the same symmetry between the central ion and the ligands. Thus, bonding in metal–flavonoid complexes probably occurs by electron transfer from the d orbital of the metal ion to the π^* orbital of the flavonoid.¹¹⁵

For several investigated rutin complexes, the values of the thermodynamic parameters for complex formation were determined and they imply that the reaction between a flavonoid and a metal ion occurs spontaneously at room temperature.¹¹¹

TABLE II. Stability constants of metal–flavonoid complexes. Bjerrum* denotes Bjerrum's modified method

Flavonoid	Method	Metal ion	Stability constant $\log\beta$	pH
Rutin	Sommer, Nach	Cu(II)	10.76	6.1
	Nach, B–F	Zn(II)	4.68	6.2
	Sommer, Nach	Pb(II)	13.81	4.5
	MR, Nach	Ni(II)	8.95	6.2
	Nach, MR	Co(II)	6.04	5.0
	B–F, Bjerrum*	MoO ₄ ²⁻	8.01	4.0
	Bjerrum*	WO ₄ ²⁻	13.44	4.0
	Bjerrum*	Eu(III)	10.59	5.0
	Bjerrum*	UO ₂ (II)	6.57	4.0
	Bjerrum*	Pd(II)	10.15	8.0
Bjerrum*	TiO(C ₂ O ₄) ₂ ²⁻	10.80	6.5	
Quercetin	MR	Ni(II)	5.57	5.0
	Sommer	Co(II)	4.87	5.0
	Bjerrum*	Pd(II)	6.05	5.0
	Bjerrum*	TiO(C ₂ O ₄) ₂ ²⁻	11.84	6.5
Morin	Sommer	Cu(II)	4.94	5.8
	Sommer	Zn(II)	6.74	5.5
	Bjerrum*	WO ₄ ²⁻	11.6	3.0
	Bjerrum*	Pd(II)	4.55	4.0
	Bjerrum*	TiO(C ₂ O ₄) ₂ ²⁻	7.35	8.0
	MR, B–F	Ba(II)	4.55	4.2
3-Hydroxyflavone	Sommer	Zn(II)	8.51	5.8
	Nach	Pb(II)	7.74	6.1
	Nach	Ni(II)	7.63	6.1
	B–F, Nach	Co(II)	10.87	6.2
	Bjerrum*	MoO ₄ ²⁻	15.13	6.3
	Bjerrum*	WO ₄ ²⁻	16.45	4.0
	Bjerrum*	Eu(III)	13.47	6.0
	B–F, Bjerrum*	UO ₂ (II)	8.68	4.0
	Bjerrum*	TiO(C ₂ O ₄) ₂ ²⁻	16.65	5.0
	B–F, Bjerrum*	Mn(II)	5.43	6.0
	B–F, Bjerrum*	Cd(II)	5.90	6.2
Hesperidin	Bjerrum*	Cu(II)	5.78	7.0
	Bjerrum*	UO ₂ (II)	7.00	6.0
	Bjerrum*	Al(III)	4.54	5.0

Analytical appraisal

Due to their significant health benefits, sensitive analytical methods are required for the quantitative determination of flavonoids in crude plant materials/extracts, plant-based beverages and pharmaceutical preparations. Until now, a number of analytical techniques have been described for the quantification of flavonoids. Liquid chromatography is currently the most commonly applied method

for routine determination of flavonoids. Reversed phase RP-HPLC approaches established in the late 1980s¹¹⁶ aim at the separation, identification and quantization of flavonoids in crude plant materials/extracts and plant-based beverages.^{117–126}

Given that metal-flavonoid chelates are usually colored and absorb at a different wavelength, the complexing reactions of flavonoids with metal ions can be optimized and utilized for the quantitative determination by indirect spectrophotometric methods. By measuring the absorbance of the newly formed colored complex, the amount of the complex-forming constituent (metal ion or ligand flavonoid) can be quantified. Flavonoid-complexing reactions are particularly suitable for analytical purpose because the solutions of the complexes show excellent characteristics for detection by spectrophotometric techniques (intensive color, clear, translucent and rather stable in time).^{75–111} The intensity and the hue of the color of the complexes depend strongly on the chemical properties of the flavonoid, in particular the number and the position of the hydroxyl groups in the flavonoid molecules, as well as the properties of the metal ion. The length of the conjugated π -bond system which includes the reactive groups affects considerably the absorption intensity and hence sensitivity.

Several official methods for flavonoid determination recommend the employment of complexing reactions. For example, the Romanian Pharmacopoeia X established the usage of AlCl_3 as a colorimetric reagent for the quantitative analysis of flavonoids in *Cynarae folium*,¹²⁷ and a general color test for identification of flavonoids in extracts obtained from stems, leaves and flowers prescribes the reaction with 5 % FeCl_3 .¹²⁸ During the late 1960s and early 70s, interactions of flavonoids with metal ions were investigated for analytical purposes.^{129–145} In these articles, chelating reactions of flavones and flavonols were predominantly described, emphasizing their potential for analytical determination of metal ions in the atmosphere, natural water, biological materials and alloys. The following detection limits for some of the metal ions were achieved: 5.5 ppm for Zr(IV), 2.8 ppm for Mo(VI), 3.7 ppm for W(IV) and 0.5 ppm for Fe(III) based on complexing reaction with rutin.¹⁴⁵ To improve the sensitivity and selectivity of metal determination, multi-ligand complexes incorporating a metal, flavone and some other ligands (antipyrine, ClO_4^- , SO_4^{2-}) were synthesized.¹⁴⁵ Being suitable for metal determination, complexing reactions of flavonoids have been extensively investigated during last ten years.^{146–153} For this purpose, quercetin and morin were the most widely used flavonoids for the determination of Al(III), Cr(III), W(IV), Zn(II), Ti(IV), Fe(III) and Mo(VI).

Determination of flavonoids via colored complexing reactions

Numerous complexing reactions have been optimized and adjusted for spectrophotometric determination of the parent flavonoid.^{88–109} Alcohol-water mixtures were used as the solvent. The composition of the solvent was optimized to achieve complete dissolution of both components, and ranged typically between

50 to 80 % ethanol (methanol for hesperidin complexes). The ionic strength and pH were adjusted to a constant value. Parameters, such as linearity, interval (range), specificity, estimated limit of detection (*LOD*), estimated limit of quantization (*LOQ*), recovery (*R*, %), precision or relative standard deviation (*RSD*, %) were established.

Generally, the Beer law was obeyed in the range from 10^{-6} to 10^{-3} mol dm⁻³ for the considered flavonoid, with excellent linearity. Among a variety of selected metal salts, potassium titanyl oxalate is particularly suitable for analytical purposes.¹⁰⁹ For the determination of rutin *via* the titanyl oxalate complex, the best detection limit *LOD* = 0.67 µg ml⁻¹ was obtained.¹⁰⁷

Complexing reactions were also optimized in an attempt to develop a simple, rapid and inexpensive method for the routine determination of flavonoids in commercially available pharmaceutical preparations (Table III). Using the proposed method, it was possible to eliminate the matrix interference since the flavonoid molecules exclusively form the complex. A good selectivity (ability to accurately measure an analyte in the presence of other substances that may be present in the sample matrix) with respect to other constituents in the sample matrix (tablet excipients and diluents, other active principles) was achieved by the proposed assays. The low values of the obtained *RSD* (less than 3 %) and recovery lying in the stated range (Ph EUR 97), indicate good application of the method for flavonoid determination in oral dosage forms.

TABLE III. Spectrophotometric determination of rutin and quercetin in oral dosage forms, and hesperidin in orange juices *via* the complexing reaction

Metal ion	Rutin		Quercetin		Hesperidin in orange juice			Ref.
	Recovery %	<i>RSD</i> %	Recovery %	<i>RSD</i> %	Trade name	Found mg l ⁻¹	<i>SD</i>	
Al(III)	–	–	–	–	“Happy day”	268	168	100
					”Bravo”	6.79	7.88	
Pd(II)	102.5	0.57	–	–	–	–	–	103
UO ₂ (II)	97.9	0.75	–	–	–	–	–	101
TiO(C ₂ O ₄) ₂ ²⁻	97.3	0.87	104.5	2.80	–	–	–	107, 111

However, the selectivity with respect to other compounds which are structurally/chemically related to flavonoids is low in complex matrices, such as *Ginkgo biloba* preparations. However, in mixtures of flavonoids and other relevant components containing one component in a relatively large excess, the same procedure can be applied. For example, the content of hesperidin in orange juice is significantly higher than other flavonoids. Thus, using the complexing reaction with Al(III), it was possible to determine hesperidin in orange juice.¹⁰⁰ Since the amount of hesperidin differs significantly in different species, even in one and the same plant material, depending strongly on the region of growth and the sea-

son, the content of hesperidin is usually not declared by producers, but the concentration obtained in the investigated brands of orange juice with this particular reaction, corresponds to those found by other authors.¹⁵⁴

CONCLUSIONS

The overall anti-oxidant action of flavonoids is achieved through synergy between a direct reaction with free radicals and the chelation of metal ions which are responsible for the production of reactive oxygen species. To unravel the origin of their potent biological action, many metal-flavonoid complexes have been synthesized and characterized. Our research in metal-flavonoid complexes covers approximately 40 complexes of five flavonoids (rutin, quercetin, 3-hydroxyflavone, morin and hesperidin) with a number of metal ions or metal groups. Only mononuclear complexes were formed under the employed experimental conditions. The 3- or 5-hydroxy group and the 4-carbonyl group in the C ring are main metal complexing domains which interact with metal ions. The majority of the investigated complexes are moderate ($5 < \log\beta < 10$) or highly stable ($\log\beta > 10$) complexes. Some complexing reactions were utilized to develop simple methods for routine determination of rutin, quercetin or hesperidin in commercially available pharmaceutical preparations or beverages.

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ИЗВОД

ИСПИТИВАЊЕ МЕТАЛ-ФЛАВОНОИД ХЕЛАТА И ОДРЕЂИВАЊЕ ФЛАВОНОИДА ПРЕКО КОМПЛЕКСИРАЈУЋЕ РЕАКЦИЈЕ МЕТАЛ-ФЛАВОНОИД

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Флавоноиди представљају велику групу полифенолних једињења биљног порекла, који имају антиоксидантна својства због директног «хватања» слободних радикала. Антиоксидантно дејство флавоноиди такође остварују стварањем хелата са јонима прелазних метала, првенствено са јонима Fe(II), Fe(III) и Cu(II), који сами учествују у реакцији стварања слободних радикала. Хелати метал-флавоноид су много ефикаснији «хватачи» слободних радикала од матичних флавоноида и могу заштитити потенцијалне мете у организму од оксидативног стреса. Да би се разјаснио механизам биолошког деловања комплекса метал-флавоноид, испитиване су физичко-хемијске карактеристике комплекса: место везивања јона метала, зависност структуре комплекса од односа метал/лиганд, афинитет флавоноида за везивање металног јона, итд. Међутим, подаци о саставу, структури и особинама комплекса су некомплетни и понекад контрадикторни. Стога је намера овог рада да кроз ретроспективу наших резултата дамо лични допринос у испитивању метал-флавоноид комплекса. У приказаном ревијалном раду сакупљени су сви наши резултати о приближно 40 комплекса образованих између пет флавоноида (рутин, кверцетин, 3-хидроксифлавоон, морин и хесперидин) и већег броја металних јона или металних група. Истакнути су подаци о месту везивања централног јона у комплексу, стехиометријском односу метал-лиганд, утицају рН на форми-

рање комплекса и константама стабилности комплекса. Такође, у овом раду приказали смо и примену метал–флавоноид комплекса у аналитичке сврхе.

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REFERENCES

1. S. Rusznyak, A. Szent-Györgyi, *Nature* **138** (1936) 798
2. A. Szent-Györgyi, *Physiol. Chem.* **225** (1938) 126
3. E. Middleton, C. Kandaswami, in: *The Flavonoids: Advances in Research Since 1986*, J. B. Harborne, Ed., Chapman & Hall, London, 1994
4. L. Jurd, in: *The Chemistry of Flavonoid Compounds*, T. A. Geissman, Ed., Pergamon Press, New York, 1962
5. T. J. Mabry, K. R. Markham, M. B. Tomas, *The Systematic Identification of Flavonoids*, Springer-Verlag, Berlin, 1970
6. J. B. Harborne, H. Baxter, *The Handbook of Natural Flavonoids*, Wiley, Chichester, 1999
7. M. G. L. Hertog, P. C. H. Hollman, M. B. Katan, D. Kromhout, *Nutr. Cancer* **20** (1993) 21
8. M. G. L. Hertog, P. C. H. Hollman, M. B. Katan, *J. Agric. Food. Chem.* **40** (1992) 2379
9. N. C. Cook, S. Samman, *J. Nutr. Biochem.* **7** (1996) 66
10. J. Kuhnhan, *World Rev. Nutr. Diet.* **24** (1976) 24
11. J. A. Ross, C. M. Kasum, *Annu. Rev. Nutr.* **22** (2002) 19
12. A. R. Ness, J. W. Powles, *Int. J. Epidemiol.* **26** (1997) 1
13. A. L. Klatsky, G. D. Friedman, M. A. Armstrong, *Am. J. Epidemiol.* **132** (1990) 479
14. H. D. Sesso, J. M. Gaziano, J. E. Buring, C. H. Hennekens, *Am. J. Epidemiol.* **149** (1999) 162
15. C. A. Brown, C. Bolton-Smith, M. Woodward, H. Tunstall-Pedoe, *J. Epidemiol. Com. Health* **47** (1993) 171
16. D. A. Balentine, S. A. Wiseman, L. C. M. Bouwens, *Food Sci. Nutr.* **37** (1997) 693
17. M. L. Neuhouser, *Nutr. Cancer* **50** (2004) 1
18. N. Schmiedebergs, *Arch. Pharmacol.* **370** (2004) 290
19. M. Katan, *Am. J. Clin. Nutr.* **65** (1997) 1542
20. R. R. Huxley, H. A. Neil, *Eur. J. Clin. Nutr.* **57** (2003) 904
21. M. G. L. Hertog, E. J. M. Feskens, P. C. H. Hollman, M. B. Katan, D. Kromhout, *Lancet* **342** (1993) 1007
22. W. Ren, Z. Qiao, H. Wang, L. Zhu, L. Zhang, *Med. Res. Rev.* **23** (2003) 519
23. P. Knekt, R. Järvinen, A. Reunanen, J. Maatela, *Br. Med. J.* **312** (1996) 478
24. E. B. Rimm, M. B. Katan, A. Ascherio, M. J. Stampfer, W. C. Willett, *Ann. Intern. Med.* **125** (1996) 384
25. M. G. L. Hertog, E. J. M. Feskens, D. Kromhout, *Lancet* **349** (1997) 699
26. L. Yochum, H. Kushi, K. Meyer, A. R. Folsom, *Am. J. Epidemiol.* **149** (1999) 943
27. B. Starvic, *Clin. Biochem.* **27** (1994) 245
28. M. G. L. Hertog, P. C. H. Holmann, *Eur. J. Clin. Nutr.* **50** (1996) 63
29. C. Manach, F. Regeat, O. Texier, G. Agullo, C. Demigne, C. Remesy, *Nutr. Res.* **16** (1996) 517
30. N. C. Cook, S. Sammn, *J. Nutr. Biochem.* **7** (1996) 66
31. V. Kuntić, I. Filipović, D. Malešev, S. Jelić, *Yugoslav. Med. Biochem.* **18** (1999) 167
32. B. N. Ames, M. K. Shigenaga, T. M. Hagen, *Proc. Natl. Acad. Sci.* **90** (1993) 7915
33. D. Amic, D. Davidovic-Amic, D. Beslo, N. Trinajstic, *Croat. Chem. Acta* **76** (2003) 55
34. M. M. Silva, M. R. Santos, G. Caroco, R. Rocha, G. Justino, L. Mira, *Free Rad. Res.* **36** (2002) 1219
35. C. A. Rice-Evans, N. J. Miller, G. Paganga, *Free Rad. Biol. Med.* **20** (1996) 933
36. N. Salah, N. J. Miller, G. Paganga, L. Tijburg, G. P. Bolwell, C. Rice-Evans, *Arch. Biochem. Biophys.* **322** (1995) 399

37. I.B.Afanas'ev, E.A.Ostrachovitch, N.E.Abramova, L.G.Korkina, *Biochem. Pharmacol.* **50** (1995) 627
38. W. Bors, M. Saran, *Free Rad. Res. Commun.* **2** (1987) 289
39. L. Mira, M. Silva, C. F. Manso, *Biochem. Pharmacol.* **48** (1994) 753
40. G. Cao, E. Sofic, R. L. Prior, *Free Rad. Biol. Med.* **22** (1997) 749
41. S. R. Husain, J. Cillard, P. Cillard, *Phytochemistry* **26** (1987) 9
42. K. Ioku, T. Tsushida, Y. Takei, N. Nakatani, J. Terao, *Biochim. Biophys. Acta* **1234** (1995) 99
43. S. Renaud, M. deLorgeli, *Lancet* **339** (1992)1523
44. W. Bors, W. Heller, C. Michel, in: *Flavonoids in Health and Disease*, C. A. Rice-Evans, L. Packer, Eds., Marcel Dekker, New York, 1998, p. 111
45. *German Pharmacopoeia*, Deutscher Apotheker Verlag, Stuttgart, 2000
46. T. A. van Beek, *J. Chromatogr. A* **967** (2002) 25
47. H. J. H. Fenton, *J. Chem. Soc.* **65** (1894) 889
48. H. E. Hajji, E. Nkhili, V. Tomao, O. Danglas, *Free Rad. Res.* **40** (2006) 303
49. S. van Acker, G. P. van Balen, B. van der Berg, W. J. F. van der Vijgh, *Biochem. Pharmacol.* **56** (1998) 935
50. I. B. Afanas'ev, A. I. Dorozhko, A. V. Brodskii, V. A. Kostyuk, A. I. Potapovitch, *Biochem. Pharmacol.* **38** (1989) 1763
51. J. E. Brown, H. Khodr, R. C. Hider, C. A. Rice-Evans, *Biochem. J.* **330** (1998) 1173
52. M. Fiorani, R. De Sancitis, R. De Bellis, M. Dacha, *Free Rad. Biol. Med.* **32** (2002) 64
53. S. Fleschin, K. R. Jennings, *Free Rad. Res.* **36** (2002) 1199
54. L. Mira, M. T. Fernandez, M. Santos, R. Rocha, M. H. Florenico, K. R. Jennings, *Free Rad. Res.* **36** (2002) 1199
55. M. T. Fernandez, M. L. Mira, M. H. Florenco, K. R. Jennings, *J. Inorg. Biochem.* **92** (2002) 105
56. V. A. Kostyuk, A. I. Potapovich, E. N. Vladykovskaya, L. G. Korkina, I. B. Afanas'ev, *Arch. Biochem. Biophys.* **385** (2001) 129
57. M. Y. Moridani, J. Pourahmad, H. Bui, A. Siraki, P. J. O'Brien, *Free Rad. Biol. Med.* **34** (2003) 243
58. R. F. De Souza, W. F. De Giovanni, *Redox. Rep.* **9** (2004) 97
59. I. B. Anafas'ev, E. A. Ostrakhovitch, E. V. Mikhail, G. A. Ibragimova, L. G. Korkina, *Biochem. Pharmacol.* **61** (2001) 677
60. H. Tang, X. Wang, S. Yang, L. Wang, *Rare Metals* **23** (2004) 38
61. M. Kopacz, E. Woznicka, J. Gruszecka, *Acta Pol. Pharm.* **62** (2005) 65
62. J. Zhou, L. Wang, J. Wang, N. Tang, *J. Inorg. Biochem.* **83** (2001) 41
63. Z. Deng, C. Coudray, L. Gouzoux, A. Mazur, Y. Rayssiguier, D. Pepin, *Biol. Trace Elem. Res.* **76** (2000) 245
64. S. X. Wang, F. J. Zhang, Q. P. Feng, Y. L. Li, *J. Inorg. Biochem.* **46** (1992) 251
65. R. Kh. Yuldashev, Kh. M. Makhkamov, Kh. T. Sharipov, Kh. U. Aliev, *Chem. Nat. Comp.* **35** (1999) 420
66. P. Viswanathan, V. Sriram, *J. Agric. Food. Chem.* **48** (2000) 2802
67. A. C. Boudet, J. P. Cornard, J. C. Merlin, *Spectrochim. Acta* **56** (2000) 829
68. J. P. Cornard, J. C. Merlin, *J. Mol. Struct.* **569** (2001) 129
69. J. P. Cornard, A. C. Boudet, J. C. Merlin, *Spectrochim. Acta* **57** (2001) 591
70. J. P. Cornard, J. C. Merlin, *J. Inorg. Biochem.* **92** (2002) 19
71. R. F. De Souza, E. M. Sussuchi, W. F. De Giovanni, *Synth. Reac. Inorg. Met.-Org. Chem.* **33** (2003) 1125
72. A. Torreggiani, M. Tamba, A. Trincherro, S. Bonora, *J. Mol. Struct.* **744-747** (2005) 759
73. M. D. Engelman, E. R. Hutchenson, I. F. Cheng, *J. Agric. Food Chem.* **53** (2005) 2953

74. R. F. De Souza, W. F. De Giovanni, *Spectrochim. Acta* **61** (2005) 1985
75. Z. Radović, D. Malešev, *Arh. Farm.* **4** (1983) 147
76. Z. Radović, D. Malešev, *Arh. Farm.* **5** (1983) 221
77. D. Malešev, Z. Radović, *Bull. Soc. Chim.* **48** (1983) 745
78. Z. Radović, D. Malešev, *Pharmazie* **39** (1984) 870
79. D. Malešev, Z. Radović, *Pharmazie* **40** (1985) 133
80. Z. Radović, D. Malešev, *Mikrochim. Acta* **2** (1985) 247
81. Z. Radović, D. Malešev, *Arch. Pharm.* **320** (1987) 188
82. Z. Radović, D. Malešev, *Acta Polon. Pharm.* **XLIV** 5 (1987) 433
83. D. Malešev, Z. Radović, *Pharmazie* **42** (1987) 57
84. D. Malešev, Z. Radović, *Pharmazie* **42** (1987) 59
85. Z. Radović, D. Malešev, *Pharmazie* **43** (1988) 135
86. D. Malešev, Z. Radović, *Pharmazie* **44** (1989) 496
87. D. Malešev, Z. Radović, M. Jelikić-Stankov, *Pharmazie* **44** (1989) 863
88. D. Malešev, Z. Radović, M. Jelikić-Stankov, *Monatsh. Chem.* **121** (1990) 455
89. D. Malešev, Z. Radović, M. Jelikić-Stankov, *J. Serb. Chem. Soc.* **55** (1990) 607
90. D. Malešev, Z. Radović, M. Jelikić-Stankov, *Monatsh. Chem.* **122** (1991) 429
91. D. Malešev, Z. Radović, M. Jelikić-Stankov, M. Bogavac, *Anal. Lett.* **24** (1991) 1159
92. D. Malešev, Z. Radović, M. Jelikić-Stankov, *Pharmazie* **46** (1991) 534
93. D. Malešev, Z. Radović, M. Jelikić-Stankov, *Arh. Farm.* **46** (1991) 223
94. D. Malešev, Z. Radović, M. Jelikić-Stankov, *Spectrosc. Lett.* **25** (1992) 1089
95. Z. Radović, D. Malešev, *Arh. Farm.* **42** (1992) 27
96. D. Malešev, Z. Radović, M. Jelikić-Stankov, *Arh. Farm.* **42** (1992) 163
97. D. Malešev, Z. Radović, M. Jelikić-Stankov, *J. Serb. Chem. Soc.* **58** (1993) 557
98. D. Malešev, Z. Radović, M. Jelikić-Stankov, *Spectrosc. Lett.* **26** (1993) 1985
99. Z. Radović, D. Malešev, M. Jelikić-Stankov, *Pharmazie* **51** (1996) 8
100. D. Malešev, Z. Radović, V. Kuntić, M. Kosanić, *Anal. Lett.* **30** (1997) 917
101. V. S. Kuntić, D. L. Malešev, Z. V. Radović, M. M. Kosanić, U. B. Mioč, V. B. Vukojević, *J. Agric. Food Chem.* **46** (1998) 5139
102. V. Kuntić, S. Blagojević, D. Malešev, Z. Radović, M. Bogavac, *Monatsh. Chem.* **129** (1998) 41
103. V. Kuntić, M. Kosanić, D. Malešev, Z. Radović, *Pharmazie* **53** (1998) 724
104. V. Kuntić, M. Kosanić, D. Malešev, Z. Radović, U. Mioč, *J. Serb. Chem. Soc.* **63** (1998) 565
105. V. Kuntić, S. Blagojević, D. Malešev, Z. Radović, *Pharmazie* **54** (1999) 548
106. S. Blagojević, D. Malešev, Z. Radović, *Arh. Farm.* **5-6** (1999) 287
107. V. Kuntić, D. Malešev, Z. Radović, V. Vukojević, *Monatsh. Chem.* **131** (2000) 769
108. M. Aleksić, S. Blagojević, D. Malešev, Z. Radović, *J. Serb. Chem. Soc.* **65** (2000) 631
109. N. Pejić, V. Kuntić, D. Malešev, *Pharmazie* **57** (2002) 216
110. S. Blagojević, M. Aleksić, D. Malešev, Z. Radović, *Arh. Farm.* **5** (2003) 17
111. V. Kuntić, N. Pejić, S. Mičić, V. Vukojević, Z. Vujić, D. Malešev, *J. Serb. Chem. Soc.* **70** (2005) 753
112. P. G. Pietta, *J. Nat. Prod.* **63** (2000) 1035
113. M. E. Bodini, G. Copia, R. Tapia, F. Leighton, L. Herrera, *Polyhedron* **18** (1999) 2233
114. J. Inczedy, *Analytical applications of complex equilibria*, Ellis Horwood Ltd., New York, 1976
115. M. Obradović, D. Veselinović, P. Đurđević, *Fizičko-hemijske metode ispitivanja ravnoteža u kompleksirajućim sredinama*, Filozofski fakultet Niš, Fakultet za fizičku hemiju, Beograd, 1996
116. D. J. Daigle, E. J. Conkerton, *J. Liq. Chromatogr.* **11** (1988) 309
117. J. P. V. Leite, L. Rastrelli, G. Romussi, A. B. Oliveira, J. H. Y. Vilegas, C. Pizza, *J. Agric. Food Chem.* **49** (2001) 3796

118. W. K. Li, J. F. Fitzloff, *J. Chromatogr., B: Biomed. Appl.* **765** (2001) 99
119. C. Queija, M. A. Queiros, L. M. Rodrigues, *J. Chem. Educ.* **78** (2001) 236
120. C. L. Moreira, S. R. De Paiva, J. L. M. Da Costa, M. R. Figueiredo, *J. High. Resol. Chromatogr.* **22** (1999) 527
121. A. Crozier, E. Jensen, M. E. J. Lean, M. S. McDonald, *J. Chromatogr., A* **761**(1997) 315
122. U. Justesen, K. Pia, L. Torben, *J. Chrom. A* **799** (1997) 101
123. P. L. Menghinello, F. Cucchiarini, D. Palma, M. Agostini, V. Dacha, *J. Liq. Chromatogr. Relat. Technol.* **22** (1999) 3007
124. L. Bramati, F. Aquilano, P. Pietta, *J. Agric. Food. Chem.* **51** (2003) 7472
125. A. B. Teris, *J. Chromatogr., A* **967** (2002) 21
126. A. Hasler, O. Sticher, B. Meier, *J. Chromatogr., A* **605** (1992) 41
127. *XXX Romanian Pharmacopoeia X*, Ed. Medicală, București, 1993
128. M. Stanciu-Rotaru, M. Mititelu, M. Crasnaru, D. Balaban, *Roum. Biotech. Lett.* **8** (2003) 1093
129. V. A. Bandyukova, A. L. Shinkarenko, *Zh. Analit. Khim.* **21** (1966) 232
130. F. D. Drawert, W. Heimann, A. Ziegler, *Anal. Chem.* **217** (1966) 22
131. N. A. Tykavkina, K. I. Lapteva, N. G. Devyatko, *Zh. Analit. Khim.* **24** (1969) 777
132. A. B. Blank, I. I. Mirenskaya, *Zh. Analit. Khim.* **27** (1972) 1883
133. A. Korcuc, K. Lesz, *Chem. Anal.* **17** (1972) 855
134. E. P. Shkrobot, *Zh. Analit. Khim.* **17** (1962) 186
135. N. L. Olenovich, L. I. Kovalchuk, *Zh. Analit. Khim.* **28** (1973) 2162
136. A. B. Blank, I. I. Mirenskaya, L. M. Satanovsii, *Zh. Analit. Khim.* **30** (1975) 1116
137. A. B. Blank, I. I. Mirenskaya, L. P. Eksperiandova, *Zh. Analit. Khim.* **28** (1973) 1331
138. A. T. Pilipenko, T. U. Kukibaev, A. I. Volkova, T. E. Getman, *Ukr. Khim. Zh.* **39** (1973) 819
139. A. T. Pilipenko, T. U. Kukibaev, A. I. Volkova, *Zh. Analit. Khim.* **28** (1973) 510
140. A. P. Golovina, A. P. Tsintsevich, A. F. Karpovich, E. U. Kovalenko, N. B. Zorov, *Zh. Analit. Khim.* **28** (1973) 815
141. A. T. Pilipenko, A. I. Zhebentayev, A. I. Volkova, *Ukr. Khim. Zh.* **38** (1972) 363
142. M. Katyal, *Talanta* **15** (1968) 95
143. A. Korcuc, *Wiadom. Chem.* **23** (1969) 345
144. E. M. Nevskaya, V. A. Nazarenko, *Zh. Analit. Khim.* **27** (1972) 1699
145. M. Katyal, S. Prakash, *Talanta* **24** (1977) 367
146. E. A. Saad, L. H. Khalil, M. T. M. Zaki, A. A. Abu El-Ella, *Mikrochim. Acta* **140** (2002) 87
147. A. M. Jamaluddin, H. Jamail, *Talanta* **42** (1995) 1135
148. A. Saffavi, M. Mirzace, H. Abdollahi, *Anal. Lett.* **36** (2003) 699
149. M. Z. Gonzalez, M. E. Diaz Garcia, A. Sanz-Medel, *Talanta* **36** (1989) 919
150. A. Y. El-Sayed, E. A. Saad, B. M. M. Ibrahime, M. T. M. Zaki, *Mikrochim. Acta* **135** (2000) 19
151. N. Pourreza, K. Alizadeh, *J. Anal. Chem* **53** (1998) 520
152. A. Y. El-Sayed, M. M. H. Khalil, *Talanta* **43** (1996) 583
153. M. Tarek, M. Zaki, A. K. Abdel-Kader, M. M. Abdalla, *Fresenius J. Anal. Chem* **339** (1991) 197
154. D. Obendorf, E. Reichart, *Electroanalysis* **7** (1995) 1075.