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ABSORPTION SPECTROSCOPY STUDY OF ACID-BASE AND METAL-BINDING PROPERTIES OF FLAVANONES

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We have used absorption spectroscopy to study the acid-base and metal-binding properties of two structurally similar flavanones: taxifolin and naringenin. We have determined the acid dissociation constants for taxifolin (pK_{a1} = 7.10 ± 0.05, pK_{a2} = 8.60 ± 0.09, pK_{a3} = 8.59 ± 0.19, pK_{a4} = 11.82 ± 0.36) and naringenin (pK_{a1} = 7.05 ± 0.05, pK_{a2} = 8.85 ± 0.09, pK_{a3} = 12.01 ± 0.38). The appearance of new absorption bands in the visible wavelength region let us determine the stoichiometric composition of the iron (II) complexes of the flavanones. We show that at pH 5, in solution there is a mixture of complexes between taxifolin and iron (II) ions in stoichiometric ratio 2:1 and 1:2, while at pH 7.4 and pH 9, we detect a 1:1 taxifolin:Fe(II) complex. We established that at these pH values, naringenin forms a 2:1 complex with iron (II) ions. We propose structures for the complexes formed. Comprehensive study of the acid-base properties and the metal-binding capability of the two structurally similar flavanones let us determine the structure-properties relation and the conditions under which antioxidant activity of the polyphenols appears, via chelation of variable-valence metal ions.

Keywords: *flavanones, taxifolin, naringenin, absorption spectroscopy, acid dissociation constants, metal-chelating properties.*

Introduction. Flavonoids are polyphenol compounds which are widely found in nature and are ingested by humans and animal with food. Recently flavonoids have been considered as efficient antioxidants, capable of not only "trapping"active oxygen species (AOS) but also preventing their formation by binding transition metal ions such as iron and copper ions, which leads to inhibition of the Fenton reaction [1–4]. Data are available indicating that under certain conditions, flavonoids and their complexes with transition metal ions are capable of enhancing oxidative stress, thus having a prooxidant effect [5–7]. Many papers are devoted to study of the properties of these compounds in different model systems [1–9], but nevertheless there are not so many studies of the physical and chemical properties of structurally similar flavonoids [10–13]. In fact, such an approach may be quite effective both for development of new antioxidant agents and for studying the mechanisms of action for these compounds. In order to study the antioxidant mechanism of action of polyphenols in the presence of variable-valence metal ions, we need a clear idea about the structural features of the complexes formed in aqueous solutions, which is not possible without knowing the conditions for deprotonation of individual hydroxy groups of the polyphenols. In particular, it has been shown [11] that in most cases, flavonoids have an antioxidant effect via binding transition metal ions under deprotonation conditions for at least one of the hydroxy groups. Furthermore, the antioxidant action of polyphenols via "trapping" active oxygen species is also connected with dissociation of the proton and loss of the electron by the hydroxy groups in these compounds [12, 13]. Thus we need to determine the acid-base properties of the flavonoids in order to understand the mechanism for their antioxidant effect.

In this paper, we focus our attention on spectral studies of two structurally similar flavanones: taxifolin and naringenin. These compounds are found in plant products [14–17] and also are considered as precursors of biologically active compounds [18–20]. Taxifolin and naringenin are dihydroflavanones, in the structure of which the 2,3-double bond is missing and as a result there is no conjugation between the catechol/phenol and benzoyl moieties of the molecule. Probably due to this structural feature the authors of [8], when studying the interaction of flavonoids with iron and copper ions, under

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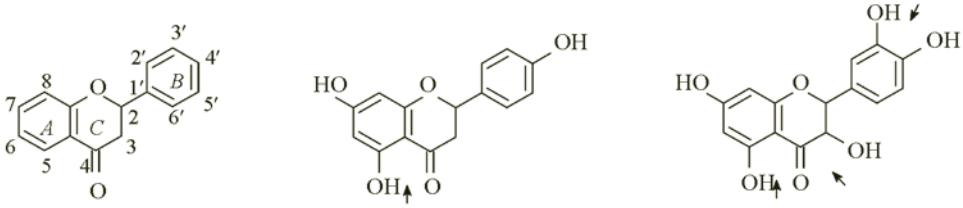
the experimental conditions did not observe appreciable changes in the absorption spectra of taxifolin and naringenin that are characteristic for other compounds containing a 2,3 double bond. Furthermore, naringenin is a structural analog of taxifolin, in the *B* ring of which there is no OH group (in the 3′ position). Consequently, for this compound there is no site for chelation of metal ions on the *B* ring. Thus study of the chelating properties of the indicated compounds will allow us to determine the structure-property relation and the conditions for the appearance of antioxidant activity via binding of mixed-valence metal ions. The aim of this work was to use optical spectroscopy to study the acid-base and metal-binding properties of the structurally similar flavanones taxifolin and naringenin.

Materials and Methods. We used taxifolin (Flamena NPF, Russia), sodium chloride, iron (II) sulfate, sodium acetate trihydrate, acetic acid, potassium chloride, boric acid, sodium phosphate monobasic ($NaH_2PO_4{\cdot}2H_2O$), sodium phosphate dibasic ($Na_2HPO_4{\cdot}12H_2O$), sodium hydroxide (Reakhim, Russia). Buffer solutions with pH 5.0 (sodium acetate/acetic acid), pH 7.4 (sodium phosphate monobasic/dibasic), pH 9.0 (boric acid/potassium chloride/sodium hydroxide) were prepared in distilled water, additionally purified on a Milli-Q apparatus.

The acid dissociation constants of the flavanones were determined using absorption spectroscopy. The solutions of the flavanones (25 μM), brought to pH 4.0 by hydrochloric acid, were titrated against an NaOH solution until pH 12.6 was reached. All the solutions had an ionic strength corresponding to a 0.10 M NaCl solution. The absorption spectra were recorded in the range 200–400 nm. The acid dissociation constants were calculated in Microsoft Excel, using data from at least three independent experiments, each of which included more than 15 spectra. The acid dissociation constants are given as the average value ± the standard error of the mean.

In connection with the low solubility of Fe(II) hydroxide, which is formed in solution as a result of hydrolysis of iron salts, we determined the metal-binding properties of the flavanones at pH 5 both in an acetate buffer solution and in a 0.1 M NaCl solution. The flavanone/metal stoichiometric ratio in the complex was determined by the isomolar series method (the Ostromyslensky–Job method) [21, 22]. The study was conducted by mixing isomolar solutions of the components in the following flavanone/Fe(II) ratios: 0.1, 0.25, 0.5, 1, 1.5, 2, 4, 9. In order to assess the metal-binding properties of the flavanones in the neutral and basic pH region, we prepared stock solutions of the flavanone (0.1 M) and also of iron sulfate plus the flavanone (in a 1:2 flavanone/metal ratio for naringenin and 1:6.5 for taxifolin) in DMSO. Then the buffered (phosphate (pH 7.4) or borate (pH 9)) solution of 0.5 mM flavanone was titrated against a solution containing iron (II) sulfate plus the flavanone in DMSO. The flavanone/Fe(II) ratio in solution was varied from 10.5:1 to 1:1 for naringenin and from 5:1 to 0.5:1 for taxifolin. We were unable to increase the fraction of iron in the system due to precipitation of its hydrolysis products. The change in the absorption spectra was recorded in the visible wavelength range.

Results and Discussion. *Acid-base properties of the flavanones*. Taxifolin and naringenin are dihydroflavanones with the distinguishing feature that there is no 2,3 double bond, and as a result there is no conjugation of electron density between the catechol/phenol and benzoyl moieties of the molecule:



(the arrows indicate possible sites for binding of metal ions by the flavanones).

In contrast to naringenin, in the structure of taxifolin there are two additional hydroxyl groups, one of which is located in the 3′ position of the *B* ring and the other is located in the 3 position of the *C* ring. The presence in taxifolin of a group which is not part of an aromatic system (3 position of the *C* ring) allows us to say that it will not contribute to the change in the absorption spectrum, and consequently we can obtain pK_a values only for four hydroxy groups of taxifolin. Thus analysis of the change in the absorption spectra of the flavanones as the pH of the solutions varies and also analysis of the structural features of these compounds allows us to assign the acid dissociation constants obtained to specific hydroxyl groups in the structure of the flavanones. During photometric titration, the absorption of the solutions of both flavanones changes at 232, 242, 269, 287, and 323 nm (Fig. 1). Furthermore, for taxifolin we observed a change in the absorption spectrum near

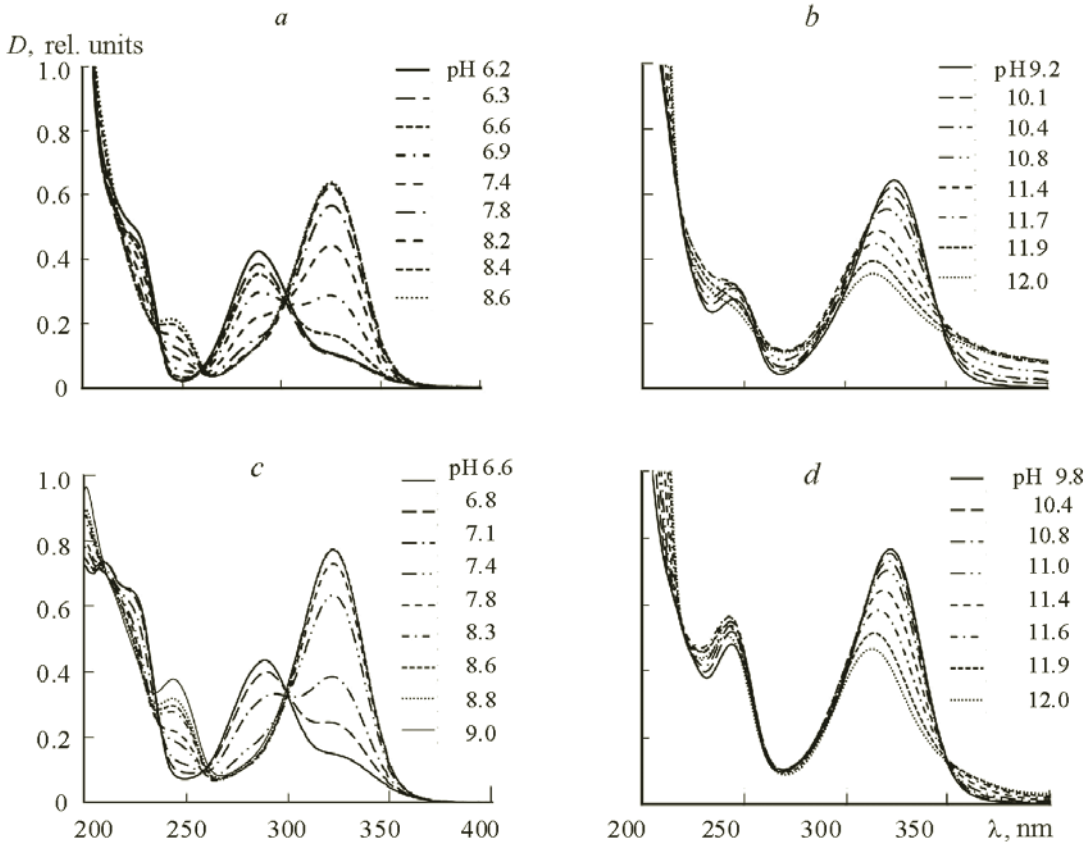


Fig. 1. Change in the absorption spectra of taxifolin (a, b) and naringenin (c, d) when titrated against an NaOH solution.

211 nm, which is not characteristic for naringenin. Most likely, these changes are connected with the process of protonation/deprotonation of the hydroxyl group of taxifolin located in the 3′ position of the *B* ring and missing in naringenin. The change in the absorption intensity at the selected wavelengths in the pH range 4.0–12.5 allowed us to determine the acid dissociation rate constants for taxifolin ($pK_{a1} = 7.10 \pm 0.05$, $pK_{a2} = 8.60 \pm 0.09$, $pK_{a3} = 8.59 \pm 0.19$, $pK_{a4} = 11.82 \pm 0.36$) and naringenin ($pK_{a1} = 7.05 \pm 0.05$, $pK_{a2} = 8.85 \pm 0.09$, $pK_{a3} = 12.01 \pm 0.38$). Based on the structural features of the indicated compounds, we can hypothesize that the presence of a carbonyl group on the *C* ring has a negative mesomeric effect on redistribution of electron density in the *A* ring, leading to an increase in the dissociation constant for the hydroxyl group in the 7 position (*para* position) of the *A* ring. Most likely the lowest values of pK_{a1} obtained for taxifolin and naringenin correspond to specifically this group. The acid dissociation constant $pK_{a2} = 8.60 \pm 0.09$ for taxifolin corresponds to the hydroxyl group in the 3′ position of the *B* ring (missing in the naringenin structure), while $pK_{a3} = 8.59 \pm 0.19$ for taxifolin and $pK_{a2} = 8.85 \pm 0.09$ for naringenin correspond to hydroxyl groups at the 4′ position of the *B* ring.

The values of $pK_{a4} = 11.82 \pm 0.36$ for taxifolin and $pK_{a3} = 12.01 \pm 0.38$ for naringenin correspond to the dissociation constant for the OH group in the 5 position. Probably the high value of pK_a for this group is connected with formation of a hydrogen bond between the proton of the hydroxy group in the 5 position and the carbonyl group in the 4 position. Thus the hydroxy group in the 5 position has a smaller dissociation constant than the hydroxy group of an analogous compound not having a carbonyl group in the *ortho* position, evidence for which comes from literature data for the structurally similar phenol compounds in [23]. The still undetermined dissociation constant for the OH group of taxifolin in the 3 position of the *C* ring has an even higher pK_a than the groups mentioned above. This hypothesis is connected with the fact that the indicated group is not part of an aromatic system, and its dissociation constant will be close to the values for aliphatic alcohols. Thus based on the results obtained, the structural features, and literature data for structurally similar compounds, we can arrive at the conclusion that the dissociation constants for the hydroxyl groups of taxifolin and naringenin increase in the series: 7-OH > 4′-OH ≈ 3′-OH > 5-OH > 3-OH.

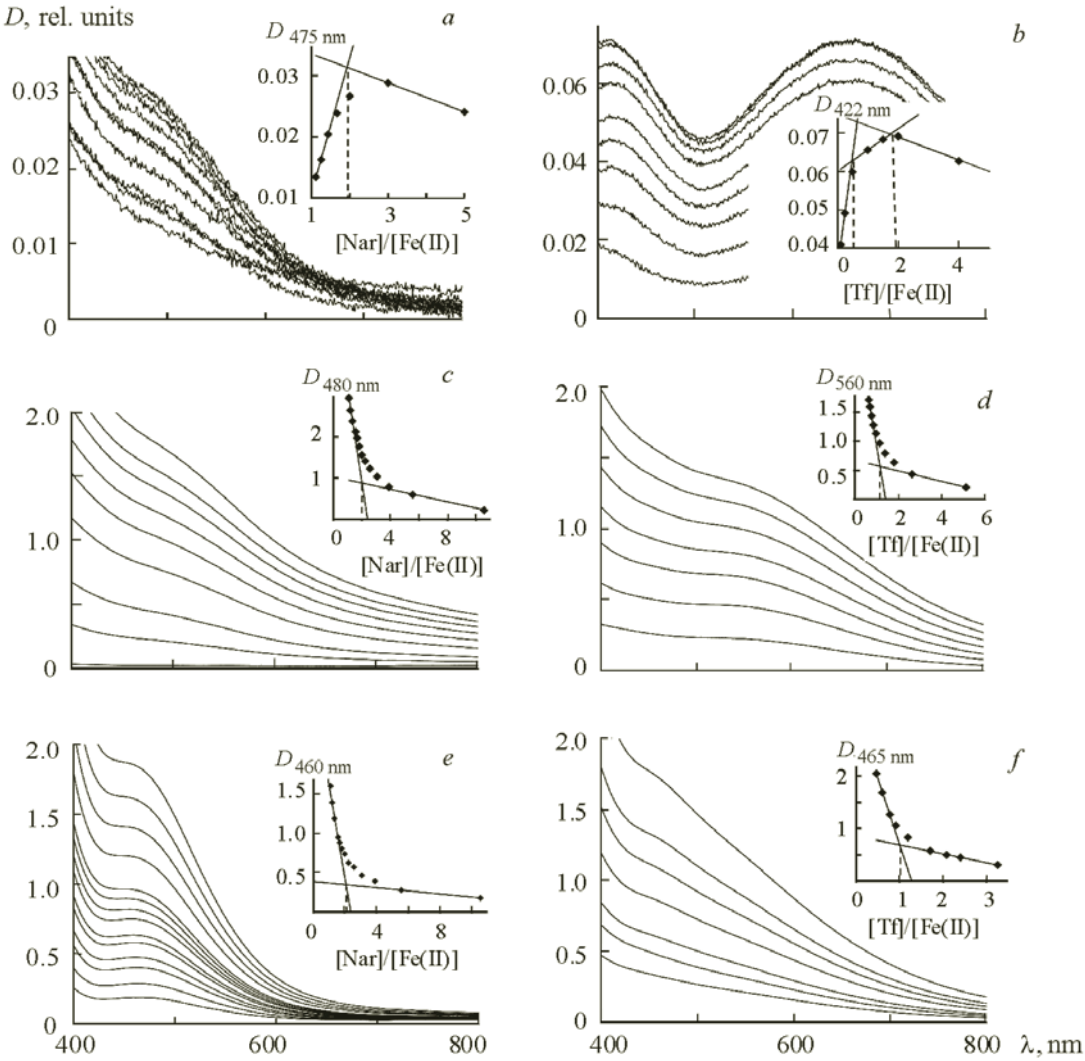


Fig. 2. Changes in the absorption spectra of the complexes of naringenin (Nar) (a, b, e) and taxifolin (Tf) (b, d, f) with Fe^{2+} ions at pH 5 (a, b), pH 7.4 (c, d), and pH 9 (e, f) as a function of the flavanone/Fe^{2+} mole ratio. Insets: absorption intensity at the corresponding wavelengths vs. flavanone/Fe(II) mole ratio.

Metal-chelating properties of flavanones. Today, the antioxidant effect of flavonoids is apparent in their ability to not only trap active oxygen species but also to bind transition metal ions. In this paper, we focus our attention on the ability of flavanones to chelate Fe(II) ions, which as we know can catalyze generation of active oxygen species and lead to formation of highly reactive hydroxyl radicals via the Fenton reaction. Based on the structural features, the acid-base properties of the flavonoids, and literature data, we can identify several possible sites for chelation of metal ions by the flavonoids (see above, indicated by the arrows): 1) at the carbonyl group in the 4 position of the *C* ring and the 5-OH group; 2) at the carbonyl and 3-OH groups; 3) at the 3′ and 4′ hydroxy groups of the *B* ring.

Under acid pH conditions, it has been established that depending on the flavanone/Fe(II) mole ratio, we observe changes in absorption in the visible region of the spectrum: new absorption bands appear with maxima located near 475 nm for naringenin, 422 nm and 665 nm for taxifolin (Fig. 2a and b). The appearance of new absorption bands that are not characteristic for free flavonoids suggests formation of complexes in solution. A change in the flavanone/metal mole ratio leads to a change in the concentration of the complex formed in solution, which allows us to determine the stoichiometric ratio of the components in the complex. The presence of two absorption bands for a solution containing an iron (II) salt plus taxifolin is characteristic only for low pH values, which is probably connected with formation of a mixture of complexes

in solution. Plotting the change in the absorption intensity at the given wavelengths vs. the flavanone/Fe(II) mole ratio allowed us to determine the stoichiometric ratio of the complexes. The results obtained suggest that under these conditions, in solution there is a mixture of complexes between taxifolin and iron (II) ions in 2:1 and 1:2 stoichiometric ratios, while for naringenin the naringenin/Fe(II) ratio found is 2:1. Based on the structural features of the studied compounds and the acid-base properties, we can conclude that in contrast to taxifolin, in naringenin there is only one most likely Fe(II) binding site: at the carbonyl and the 5-OH group. Probably in the 2:1 naringenin/Fe(II) complex, two naringenin molecules are coordinated around the iron ion with participation of these groups. We do not rule out the possibility that such coordination of the molecules is observed in the case of a 2:1 taxifolin/Fe(II) complex. Furthermore, for taxifolin, besides the binding site mentioned above, chelation of metal ions by the catechol group (*B* ring) is likely, and accordingly we can hypothesize that in the 1:2 taxifolin/Fe(II) complex, the iron ions are bound by the carbonyl and 5-OH groups and the 3′, 4′ hydroxy groups of the *B* ring. We note that for flavonoids whose structure includes a 2,3 double bond, in the literature a third possible binding site for metal ions is considered: at the carbonyl and 3-OH groups [1, 24]. Nevertheless, the absence of the indicated bond in the studied compounds, and also the experimental results on the acid dissociation constants, the metal-reducing capability (not shown), and the literature data [1, 25, 26] for structurally similar compounds allow us to suggest that the most likely region for interaction between metal ions and taxifolin is the catechol group (the *B* ring).

A change in the conditions for the medium (pH, ionic strength, polarity of the solvent, etc.) can lead to a change in the ratio of the complexes present in solution, their structure, and also their spectral characteristics. Along with the 2:1 and 1:2 taxifolin/Fe(II) complexes, taxifolin can form a 1:1 complex, which was observed in aqueous solutions at pH 7.4 and pH 9. At pH 7.4, new absorption bands appear with maxima at 475 nm for naringenin and 560 nm for taxifolin (Fig. 2c and d). As mentioned, the taxifolin/Fe(II) stoichiometric ratio obtained is 1:1, while for the naringenin/Fe(II) complex this ratio is 2:1 (Fig. 2c and d, inset). Under alkaline conditions, complexes are also formed: absorption bands appear near 460 nm and 465 nm for naringenin and taxifolin (Fig. 2e and f). The flavanone/Fe(II) stoichiometric ratios are 1:1 and 2:1 respectively for taxifolin and naringenin (Fig. 2e and f, inset).

When the concentration of the components in the system increases in acetate buffer (pH 5) and especially phosphate buffer (pH 7.4), a precipitate is formed which is not present in borate buffe (pH 9) at the same flavanone and Fe(II) concentrations. The data obtained suggest that under acid and neutral pH conditions, we observe precipitation of the flavanone/Fe(II) complexes formed, and at pH 7.4 minimal solubility of the complexes in aqueous solutions occurs.

Thus the studied flavanones are capable of chelating iron (II) ions in aqueous solutions and can form complexes of different compositions under acid, neutral, and alkaline pH conditions. The ability of taxifolin and naringenin to chelate transition metal ions and to thus inhibit the Fenton reaction may play a key role under oxidative stress conditions. However, the prooxidant effect of polyphenols observed under certain conditions also may be the result of interaction between the indicated compounds and transition metal ions (Fe^{3+}, Cu^{2+}), and specifically their metal-reducing capability [6–8]. After binding of Fe^{3+} ions followed by their reduction down to Fe^{2+}, the latter may participate in the Fenton reaction. The exhibition of prooxidant or antioxidant properties by flavonoids will depend on the stability constants of the complexes they form, and also on the conditions in the surrounding medium.

Conclusion. We have used optical spectroscopy to study the acid-base and metal-binding properties of two structural analogs: taxifolin and naringenin, which allowed us to identify the conditions for the formation of polyphenol/metal complexes and their structural features.

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