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Study of Acid-Base Properties of Morin by Tristimulus Colorimetry

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Abstract—Acid-base properties of morin in aqueous solutions have been studied by means of chemical tristimulus colorimetry, and the ionization constants have been determined. The p*K* values have been assigned to the corresponding functional groups, and their dissociation scheme has been suggested. Diagram of distribution of ionic and molecular forms of morin at pH 1–13 has been constructed. Spectral parameters of equilibrium acid-base forms of morin have been determined.

Keywords: morin, chemical tristimulus colorimetry, ionization constant, distribution diagram

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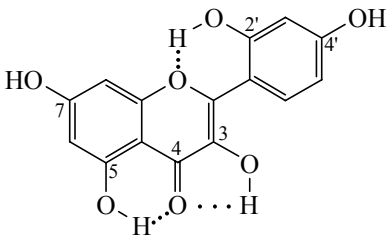
Wide range of pharmacological activity of flavornoids has determined the emerging interest to this numerous class of natural phytogenous compounds [1, 2]. These polyphenol compounds have been recognized as strong antioxidants exhibiting P-vitamin activity [3, 4]. The behavior of flavonoids in pharmacological studies can be understood in view of the data on the ionization constants (p*K*) of their functional groups [5–7]. The p*K* values are important parameters underlying the observed adsorption, distribution, and metabolism (ADME) phenomena [8].

In this work we studied one of quercetin isomers, morin (3,5,7,2',4'-pentahydroxyflavone), behaving as a weak polyprotic acid in aqueous solutions (H_5R).



Depending on the environment acidity morin can exist in seven acid-base forms: protonated (H_6R^+), molecular (H_5R), and deprotonated (H_4R^-, H_3R^{2-}, H_2R^{3-}, HR^{4-}, and R^{5-}) ones. Acid-base properties of morin have been measured in a number of papers by diverse procedures [9–18]. However, the contradictory p*K* values have been reported in these contributions complicating the selection of reliable data for further analysis. Moreover, the information on acid-base properties of each of morin functional groups (Table 1) and the order of their dissociation has been absent. The acidic character of the OH group in position 7 has been marked in [10, 12], whereas other studies [14, 15] have mentioned the primary deprotonation of hydroxy groups of the resorcin fragment.

Analysis of the data given in Table 1 has led to conclusion that the classical physicochemical methods are not suitable for determination of p*K* values of each of the functional groups of morin. Discrepancy in the reported values can be due to several reasons. The study of protolytic properties of flavonoids containing functional groups of similar acidity should account for the special features of the compounds under consideration: low solubility in water and alcohols, easy oxidation with air oxygen, and the closeness of the spectral parameters of the equilibrium ionic and molecular forms. The flavonoids contain functional groups capable of dissociation as well as isolated electronegative atoms involved in intramolecular and intermolecular interactions. These interactions are accompanied with electron density redistribution in the molecules. This usually changes the quantitative parameters of ion-molecular equilibria and significantly affects the order of the functional groups dissociation (i.e., the scheme of the polyfunctional compound dissociation as a function of pH and the environment nature). Morin and quercetin molecules contain pairs of bifurcating hydrogen bonds $C^5OH\cdots OC^4$ and

Table 1. Available reference data on ionization constants of morin

Method	pK_1	pK_2	pK_3	pK_4	pK_5	pK_6
Potentiometry [10]	–	4.99	–	8.29	10.33	–
Electrophoresis [12]	–	5.06	–	8.64	10.62	–
Spectrophotometry [13]	2.30	6.65	–	7.86	9.58	12.26
[16]	–	5.09	–	8.46	10.17	14.24
QSPR [12, 14]	–	6.93	–	8.32	9.98	11.98

$C^3OH\cdots OC^4$ [19]. However, additional hydrogen bond between the 2'-OH group of the resorcin fragment and the heterocyclic oxygen ($C^{2'}OH\cdots O$) appears in the case of morin, owing to the change in the position of OH group in the benzene ring from 3' (quercetin) to 2' (morin). As a result, morin molecule contains several pseudo aromatic cycles involved into a common conjugated system; this should likely affect the order of dissociation of the functional groups.

Let us point to the drawbacks of the conventional physicochemical methods as far as study of flavonoids are concerned. The study of acid-base properties of organic compound by means of potentiometry and spectrophotometry basically requires the purity of the tested compound and its stability over the whole probed pH range; on top of that, the functional groups should significantly differ in the acidity. Furthermore, the spectrophotometric determination of pK requires the detectable difference in the spectral properties of the molecular-ionic forms; and potentiometric study is possible only in the cases of the compounds soluble in aqueous or aqueous-alcoholic medium. Capillary electrophoresis method allows the study of acid-base properties of contaminated or unstable compounds, but does not allow determination of pK of the functional groups close in acidity.

We regard the tristimulus colorimetry method as a complementary approach to study of acid-base properties of such flavonoids as morin. This method consists in calculating the color coordinates of the equilibrium ionic and molecular forms basing on their electron absorbtion spectra. Application of this method allows discrimination of the spectrally similar forms of the compound, thus gaining additional information on the dissociation process [20–22]. We have earlier applied the tristimulus colorimetry method to study of acid-base properties of quercetin in aqueous solutions [23]. In particular, we have managed to determine the pK values of each of its functional groups.

Processing of the spectrophotometry data collected by measuring the spectra under nitrogen atmosphere gave the plot of the chromaticity SCD function (Specific Color Discrimination) of the morin solution as a function of the medium acidity (Fig. 1).

The presence of six maxima in the plot evidenced the existence of seven ionic-molecular forms of the dye in the dynamic equilibrium, depending on the medium pH. The pH values corresponding to each of the maxima were equal to the pK values of the morin functional groups in the solution (Table 2). It should be noted that the products of oxidative decomposition of morin (phloroglucinol and hydroxybenzene acids) did not interfere with the colorimetric determination of pK of the functional groups, since they exhibited the light absorption in the UV range, and the chromaticity functions were calculated from the spectral data over the visible range (380–780 nm).

As seen from the data in Table 2, we failed to determine the pK values of the hydroxy groups in positions 3 and 2' (close in the acidity) as well as the protonation constant of the carbonyl oxygen atom by means of spectrophotometry. The QSPR (Quantitative Structure–Property Relationship) approach allowed estimation of the pK values of three of morin functional groups; they were close to the values determined by means of colorimetry and spectrophotometry. Comparative analysis of experimental and computational data given in Table 2 demonstrated the acceptable reproducibility and narrow confidence interval of the results of the colorimetric method. The data processing using the Student criterion [24] confirmed that the mean values of the morin functional groups pK as determined via the colorimetric method were not significantly different from those obtained by spectrophotometry and QSPR data. Hence, the colorimetric method was free of the systematic errors and produced correct results. Comparison of the pK values of the corresponding functional groups of morin and quercetin

revealed the effect of the substituent position on the molecule acid-base properties. In particular, the formation of the hydrogen bonds system in morin molecule enhanced the basicity of the carbonyl oxygen atom, whereas the incorporation of the 3-OH and 5-OH groups of morin in the pseudo aromatic highly stable cycle owing to the formation of the $C^{2'}OH\cdots O$ hydrogen bonds noticeably weakened their acidic properties as compared to quercetin [23]. The enhancement of the acidity of hydroxy groups in the benzene ring of morin was probably due to the redistribution of the electron density owing the formation of stable system of hydrogen bonds.

Basing on the set of the colorimetric and spectrophotometry data as well as quantum-chemical simulation results, we suggested the following scheme of ion-molecular equilibria in the morin solutions over wide pH range.

According to Scheme 1, the increase in the solution pH primarily resulted in proton elimination from the protonated carbonyl group (H_6A^+) to form neutral morin molecule (H_5A). The QSPR analysis suggested that the 3-OH group (in the *ortho* position with respect to the electron-acceptor carbonyl group) should be the next to be deprotonated with the increasing solution pH. Indeed, the hydroxy groups of quercetin in positions 3 and 5 were fairly acidic [23]. However, the system of strong hydrogen bonds in morin structure impeded their dissociation; the quantum-chemical simulation suggested that the dissociation of the 7-OH group occurred to form the H_4A^- anion, coinciding with the data in [10, 12]. Ionization of the 7-OH group induced the mesomeric effect reflected in the electron density transfer onto the resorcin fragment, thus impeding dissociation of the hydroxy groups in positions 2' and 4' and affecting the strength of hydrogen bonding between the 5-OH group and the carbonyl oxygen. As a result, hydroxy groups in position 5 became more acidic than those in positions 3, 2', and 4'. The suggested dissociation of the 5-OH

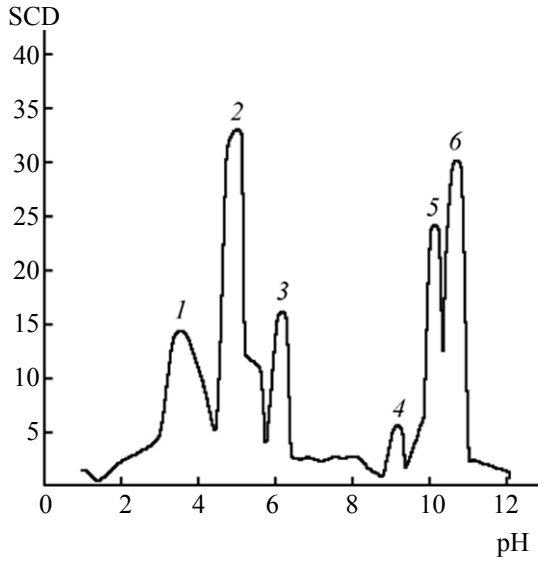


Fig. 1. Chromaticity function SCD of morin solution as function of pH under nitrogen atmosphere. (*1*) $pK_{C=OH^+}$, (*2*) $pK_{7\text{-OH}}$, (*3*) $pK_{5\text{-OH}}$, (*4*) $pK_{3\text{-OH}}$, (*5*) $pK_{2'\text{-OH}}$, and (*6*) $pK_{4'\text{-OH}}$.

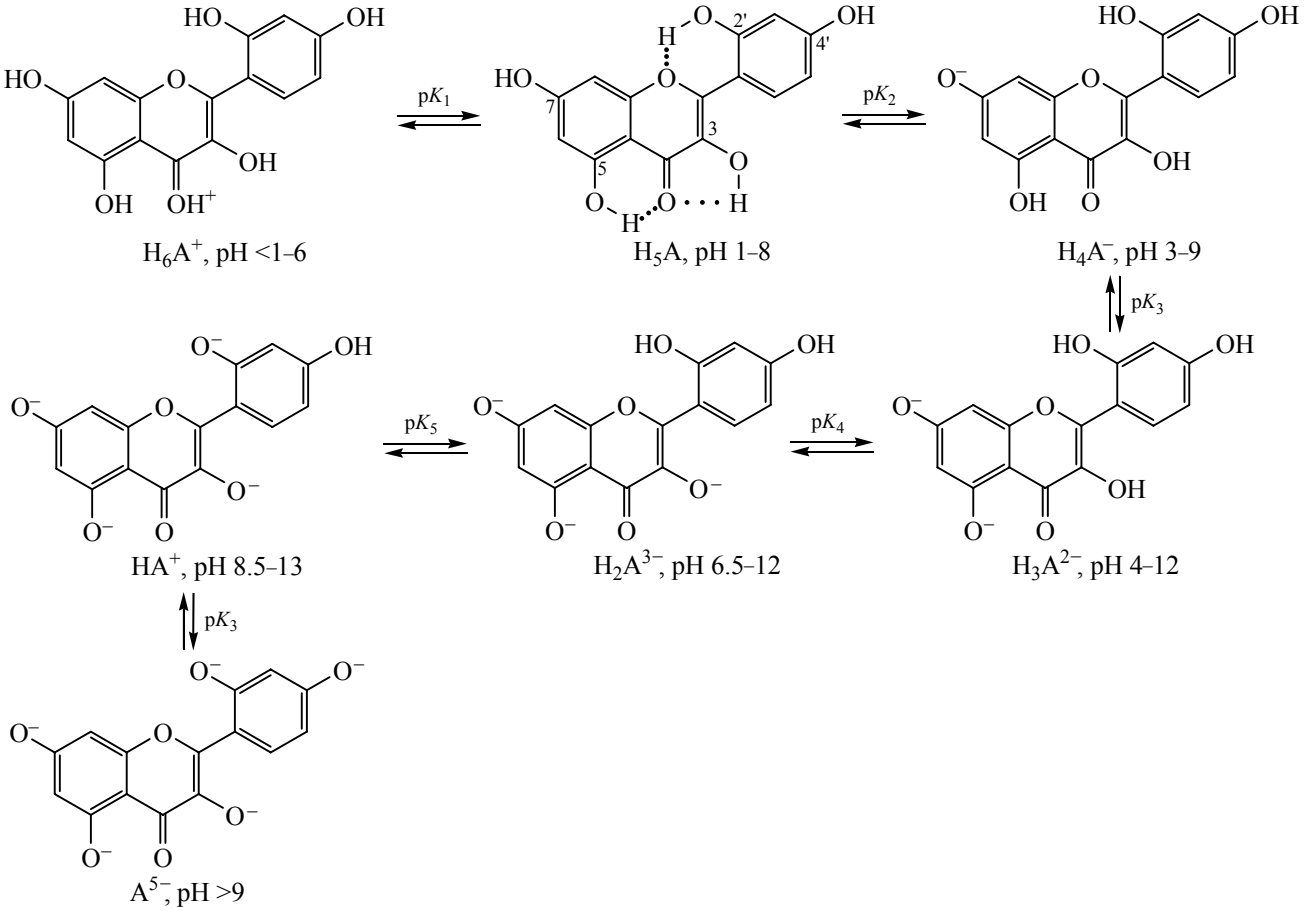
group to form the H_3A^{2-} anion further hampered dissociation of hydroxy groups of the resorcin fragment. In view of that, dissociation of the 3-OH group was the most probable, yielding the H_2A^{3-} form. Further stepwise dissociation of the hydroxy groups in positions 2' and 4' gave the HA^{4-} and A^{5-} forms, respectively, as followed from the quantum-chemical simulation results. The listed forms of morin co-existed in the solution in the dynamic equilibrium, and the fraction of each form as a function of pH could be plotted in the distribution diagram (Fig. 2).

The data given in Fig. 2 suggested that five or six acid-base forms of morin could simultaneously exist in the aqueous solution over the narrow range of pH 8–10, thus seriously complicating determination of their pK using optical and electrochemical methods. Indeed, the reliable information on pK of the hydroxy groups in positions 3 and 2' as well as 2' and 4', close in acidity, has not been found in the reference literature.

Table 2. Ionization constants of morin (n = 3, P = 0.95)

Method	$pK_{C=OH^+}$	$pK_{7\text{-OH}}$	$pK_{5\text{-OH}}$	$pK_{3\text{-OH}}$	$pK_{2'\text{-OH}}$	$pK_{4'\text{-OH}}$
Chemical tristimulus colorimetry	3.5±0.2	5.0±0.1	6.2±0.1	9.2±0.2	10.1±0.1	10.7±0.1
Spectrophotometry	–	5.0±0.1	6.6±0.2	–	–	10.6±0.2
QSPR	–	5.5	–	9.3	–	10.7

Scheme 1.



In order to better describe the individual equilibrium forms of morin, we applied the iteration algorithms and multiple regression analysis to extract their pure absorption spectra. The spectral parameters were calculated utilizing the determined pK values of the functional groups and the additive properties of the solution absorbance. The spectrophotometry data were processed using SpectroCalc-H_5A software [25]. The so determined molar absorptivities (ε_λ) are given in Table 3, and the corresponding spectra are presented in Fig. 3.

As seen in Fig. 3 (curves *1* and *2*), deprotonation of the carbonyl oxygen atom of morin at pH < 1–6 was accompanied by the significant red shift of the absorption band, by 35 nm (Table 3). In turn, the stepwise dissociation of hydroxyl groups in positions 7, 5, 3, 2', and 4' resulted in the slight red shift of the absorption band ($\Delta\lambda \approx 5$–20 nm) and the hyperchromic effect (Fig. 3). The coexisting pairs of the ionic morin forms H_4A^-, H_3A^{2-} and H_2A^{3-}, HA^{4-} were spectrally identical (Table 3), and the available chemometric methods did not allow extraction of their pure spectra, therefore, Fig. 3 shows the spectra of the H_4A^-+H_3A^{2-} and H_2A^{3-}+ HA^{4-} mixtures (curves *3* and *4*). That complicated determination of pK values of hydroxy groups in positions 5 and 2' via the conventional spectrophotometry methods [17, 18] but could be partially overcome using the multiwavelength spectrophotometry [13, 14] and the iteration algorithm used in this work as implemented in Spectro Calc-H_5A software [25] (Table 2).

The data shown in Fig. 3 and Table 3 confirmed that several equilibrium forms of morin were only slightly different in the spectral properties. On top of that, several functional groups of morin exhibited close pK values (Table 2). However, those complications as well as easy oxidation of morin in the solution did not prevent study and quantitative description of its acid-base tristimulus colorimetry by means of the colorimetry method.

In conclusion, we demonstrated that using the tristimulus colorimetry function of the analytical signal

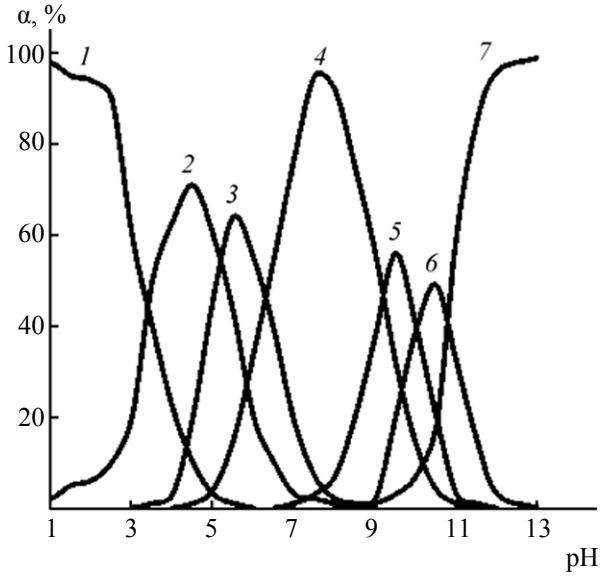


Fig. 2. Distribution diagram of ionic and molecular forms of morin in the solution. (*1*) H_6A^+, (*2*) H_5A, (*3*) H_4A^-, (*4*) H_3A^{2-}, (*5*) H_2A^{3-}, (*6*) HA^{4-}, and (*7*) A^{5-}.

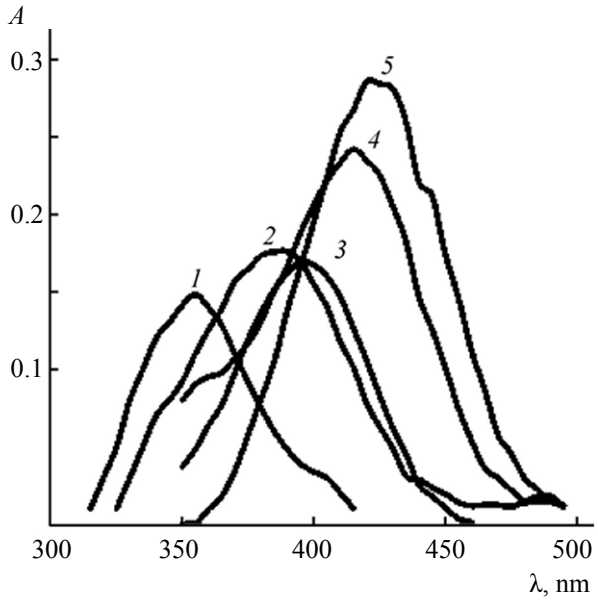


Fig. 3. Absorbance spectra of equilibrium acid-base forms of morin at pH (*1*) 1, (*2*) 4.5, (*3*) 7, (*4*) 10, and (*5*) 12.

extended the possibility of the study of the acid-base properties of dyes and gave the comprehensive description of the protolytic equilibria in their solutions over wide pH range.

EXPERIMENTAL

The chemicals used in this work were of analytically pure grade. The stock solution of morin ($c = 1 \times 10^{-3}$ mol/L) was prepared by dissolution of a weighed amount of the dye in 50 wt % ethanol just before use. The required acidity level was maintained by addition of sodium hydroxide or sulfuric acid solution.

Electron absorption spectra were recorded using SF-56 spectrophotometer (LOMO-Spektr) in the cells with the optical pathlength of 1 cm, over 380–780 nm. The medium acidity was monitored using an I-130 ionometer equipped with an ESL-63-07 glass electrode and an EVL-1M3 silver chloride reference electrode, calibrated using standard buffer solutions.

The spectra for analysis were recorded using the solutions of 1 mL of the stock morin solutions of the required pH, diluted to 50 mL in a volumetric flask; the pH was varied between 1 and 13 with the step of 1 unit. In order to prevent morin oxidation with air oxygen, the solutions were flushed with nitrogen (about 5 mL/min). When the spectral changes were not clear, the step of pH change was decreased to 0.1 unit in order to enhance the p*K* values differentiation. The ionic strength was maintained constant at 0.05 mol/L by addition of the calculated amount of KCl. Taking into account the dilution, concentration of ethanol in the studied solution did not exceed 10 wt %, and its effect on the p*K* values could be neglected [26]. The following chromaticity functions were used: X, Y, and Z are the color coordinates in the CIEXYZ space; L, A, and B are the color coordinates in the CIELAB space; S, the color saturation; and SCD, specific color discrimination. Their calculation method is described in detail in [23].

Table 3. Spectral parameters of equilibrium forms of morin

Form	H_6A^+	H_5A	H_4A^-	H_3A^{2-}	H_2A^{3-}	HA^{4-}	A^{5-}
pH	1.0	4.5	6.0	7.5	9.5	10.5	13.0
λ_{max}, nm	355	390	395		415		420
ε_λ	1480	1750	1690		2410		2860

Spectrophotometry under the same conditions was used as the alternative analysis method. The p*K* values were determined by processing the electron absorption spectra using SpectroCalc-H_5A software. The computation algorithm was based on the iteration method and multiple linear regression analysis via the least-squares method; the technique has been proved to be applicable even in the case of significant overlap of the absorption spectra of the individual forms [25].

Geometry of the ionic and molecular forms of morin was optimized using the MM+ molecular mechanics method, and the electron density distribution was simulated using the CNDO, MNDO, AM1, and PM3 semiempirical methods implemented in HyperChem Pro software. The p*K* values of morin were theoretically predicted from the corresponding forms structure with the optimized geometry using Marvin 5.9.1 and ACDLabs Professional 6.0 software packages. The p*K* values computation method was based on the QSPR approach.

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