**DETERMINING STABILITY CONSTANTS OF
NARINGENIN (4', 5, 7-TRIHYDROXY FLAVANONE)
COMPLEXES WITH ALUMINIUM (III) AND IRON (II)
BY POTENTIOMETRIC AND
SPECTROPHOTOMETRIC METHODS**

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ABSTRACT

Naringenin and its metal complexes are used extensively in chemistry, biology and dye industry. In this study, complexes of naringenin with aluminium and iron are examined by spectrophotometric and potentiometric methods. The stability constants of these complexes were determined potentiometrically by using Calvin-Bjerrum and Irving Rossotti methods. The evaluated protonation constants of the naringenin were found: $\log K_1 = 10.19$, $\log K_2 = 8.31$, and $\log K_3 = 7.06$ at the 25°C. Logarithms of the stability constants were found: for Aluminium complex : $\log K_1 = 15.39$, $\log K_2 = 7.12$, and $\log K_3 = 6.47$ and for Iron complex : $\log K_1 = 10.11$, $\log K_2 = 6.40$, and $\log K_3 = 6.11$ at the 25°C. The components of metal-naringenin complexes are given 1:3 (metal: naringenin ratio) both Al(III) and Fe(II) from the results.

Key words: naringenin, iron, aluminium, stability constant, potentiometry, spectrophotometry

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INTRODUCTION

Flavonoids comprise a large group of naturally existing polyphenolic compounds containing over 4000 different chemical structures /1/ widely distributed throughout the plant kingdom. These are found in fruits, vegetables, nuts and seeds as well as most types of tea and red wine /2/. These compounds are recognized by the presence of a C₆-C₃-C₆ basic ring skeleton. Flavonoids differ from one another in the orientation of the substituents (hydroxyl and/or methyl etc.), the degree of unsaturation, the type of sugar moiety attached and the position of the benzenoid substitution /3/. Basic flavan nucleus;

The major classes are flavones, isoflavones, flavans, anthocyanins, proanthocyanidins, flavanones, chalcones, and aurones /4/.

Diverse biochemical properties of flavonoids have provoked interest in biology, biochemistry, food chemistry, dye industry, medicinal chemistry, and cosmetics. These compounds have exhibited a broad range of biological and pharmacological activities such as antioxidant, anti-allergic, antibacterial, anti-inflammatory, antimutagenic, anticancer, and anti-atherogenic effects /5, 6/.

They usually contain one or more aromatic hydroxyl groups and this moiety is responsible for the antioxidant activity of the flavonoid. Due to their chemical composition, the flavonoid aglycons span the whole range from fully water- to fully lipid-soluble, allowing them to perform their antioxidative mode of action both in water solutions and in biological membranes /1/.

Flavonoids are known to exhibit various biological effects such as inhibiting platelet aggregation, scavenging free radicals, preventing cell proliferation and reducing levels of low-density lipoproteins in plasma. In addition, these compounds are reported to modulate vascular tone. Several

epidemiological studies revealed the inverse association between flavonoid intake and reduction in occurrence of cardiovascular diseases, such as myocardial infarction /3,7/.

Flavonoids are the most important pharmacologically active constituents in propolis. Flavonoids are thought to account for much of the biologic activity in propolis. Among the most potent microbicidal compounds in propolis are flavonone pinocembrin (5,7-dihydroxyflavanone) and its 3-OH analogue flavonol galangin (3,5,7-trihydroxyflavon) /8/.

Flavonoids are main individual chemical components of natural dye extracts, and have been used as natural dyes since ancient times. Flavonoids in natural dyes in extracts from wool and silk fibres from archeological textiles are determined. The examined objects originate from 4th to 12th Century Egypt and belong to the collection of Early Christian Art of the National Museum in Warsaw /9/. Natural dyes are reported as potent antimicrobial agents owing to the presence of a large amount of tannins. Several other sources of plant dyes are reported to exhibit antibacterial and antifungal activity /10,11/.

One of the main groups of flavonoids that occur as glycosides are flavanones. Flavanones were exclusively analyzed in citrus fruits (oranges and grapefruits) since these compounds are concentrated in this plant family and are not likely to be found in others. Naringenin was related to a large number of beneficial effects such as cancer chemopreventive agents, mutagenic inhibitor, genotoxicity inhibitor, as an agent to lower plasma cholesterol with hypocholesterolemic properties, an antioxidant agent and has blood lipid-lowering activity. One likely explanation for these effects is that dietary plants contain substances able to control basic cellular processes such as the endogenous defense against oxidative stress e.g. by means of the regulation of the glutation levels and cytochromes P450 /12/.

More recently, the protective effect of naringenin against ethanol-induced gastric lesions in rats is reported. Epidemiological studies indicate a protective relationship between the consumption of citrus fruits or juices and the risk of ischaemic stroke and lung cancer. Most flavanones are found as glycosides (bound to sugar). The antioxidant activity of naringenin could also be ascribed to its phenolic acid metabolites in rats namely *p*-hydroxyphenylpropionic acid, *p*-coumaric acid and *p*-hydroxybenzoic acid. In conclusion, naringenin, a natural occurring flavonoid, attenuates the nephrotoxicity of cisplatin in rats /2/.

Naringenin are found the most in Everlostings or strawflowers (varieties of *Helichrysum*). Naringenin, a glycon of naringin, was found to inhibit the formation of aortic atherosclerotic lesions in rabbits fed a high cholesterol diet. In addition, previous studies showed that naringenin and its derivative exhibited hypocholesterolemic activities by reducing cholesterol biosynthesis and cholesterol esterification in high cholesterol-fed rats /5,13/.

It is resistant for washing and abrasion with lightfastness, so it is used as a natural yellow dye extract in dye industry /14/.

Chemical structure of naringenin (4', 5, 7-trihydroxy flavanone)

The protonation constants of naringenin and stability constants of naringenin-metal complexes are determined. The stability constants of complexes have been calculated potentiometrically using Calvin-Bjerrum and Irving-Rossotti methods /15/.

Because of the insolubility of naringenin in water, dioxane-water mixture (1:1.v/v) was used in all workings. Behaviour of H^+ ion in the water-dioxane (1/1:v/v) mixture is the same as aqueous solutions /16,17,18,19/.

EXPERIMENTAL

Instrumentation

For spectrophotometric studies Shimadzu-UV-1600PC UV-VIS double-beam spectrophotometer was used. For potentiometric studies, Radiometer TIM800 Titration Manager, ABU 901 Autoburette and HI 1131B Combination pH electrode were used. Computer calculations were performed on the pH-metric data.

Chemicals and Reagents

HClO₄ (%70-72, 1,67 g/mL), NaClO₄, 1,4-dioxane, Al(NO₃)₃.9H₂O were obtained from Merck, Fe(NH₄)₂(SO₄)₂. 6H₂O was purchased from Carlo Erba. Naringenin (4', 5, 7-trihydroxy flavanone) was provided from Carl Roth GmbH. All reagents were of highest commercial quality. A solution of 0,01 N NaOH was prepared from "Titrisol Merck". The concentration of Fe(II) and Al(III) ions in the solution was typically kept at 4.10⁻³ molL⁻¹ and determined accurately by titrating it with standart ethylenediamine tetraacetic acid (EDTA). A solution of 0.1 N EDTA was prepared from "Titrisol Merck". The purity and the concentrations of the prepared solution were checked by potentiometric titration method. The ionic strength of the reaction medium was kept constant at 25°C (I=0.0045) using NaClO₄ solution.

METHODS

Potentiometric Studies

The determination of Protonation and Stability Constants

In order to determine the protonation constants the solutions including HClO₄ and ligand (naringenin) + HClO₄ were titrated potentiometrically using 0.010 molL⁻¹ NaOH (Fig.1). Average $\bar{n}_A$ values were calculated from the titration curves.

For the calculation, the following equation are given below:

$$\bar{n}_A = y + \frac{(V_1 - V_2)(N + C)}{(V_0 + V_1)C_L} \quad (a)$$

The protonation constant of the naringenin and the stability constant of naringenin-metal complexes which were used as ligand were determined in Table 1.

The stability constant of the binary complexes were determined potentiometrically using the Irwing-Rossotti method /15/. Therefore the mixture which the metal ions were titrated with standard 0.010 molL⁻¹ NaOH solution potentiometrically and the titration curves were plotted (Fig.1). $\bar{n}_I$ values were calculated using $\bar{n}_A$ values and the equation given below; pL

values were calculated using $\bar{n}_L$ values to calculate stability constants. The following equation was used to calculate $\bar{n}_L$ values:

$$\bar{n}_L = \frac{(V_3 - V_2) [N + C + C_L (y - \bar{n}_A)]}{(V_0 + V_2) \cdot \bar{n}_A \cdot C_M} \quad (b)$$

Where:

V_0 = Initial volume (mL)	: 25.00 mL
N = Molarity of the base (NaOH)	: 0.01000 molL ⁻¹
C_M = Metal concentration	: 0.00040 molL ⁻¹
C_L = Naringenin(L) concentration	: 0.00040 molL ⁻¹
C = HClO ₄ concentration	: 0.00254 molL ⁻¹
y = Number of protons given for naringenin	: 1

V_1, V_2, V_3 = NaOH volumes of V_1, V_2 and V_3 were read from the titration curves which contain HClO₄, ligand + HClO₄ and HClO₄ + Ligand + Metal ions.

$\bar{n}_A = \bar{n}_A$ values which correspond to different pH values were calculated by using the volumes of V_1 and V_2 and were plotted in function of pH, i.e. $\bar{n}_A = f(\text{pH})$.

The following equation was used to calculate pL values:

$$\text{pL} = \log [] \quad \frac{1 + \beta_1[\text{H}^+] + \beta_2[\text{H}^+]^2 + \dots + \beta_n[\text{H}^+]^n}{C_L - \bar{n}_L \cdot C_M} \quad (c)$$

$\bar{n}_L$ = The values were calculated using $\bar{n}_A$ values and the equation (b)

pL = pL values were calculated using β values (β values were calculated as follows).

The relation $\bar{n}_L = f(\text{pL})$ was plotted using $\bar{n}_L$ and pL values which were calculated for each metal ion. The stability constants were determined from these graphs (Fig. 3, 6 and Table 1).

The formation constant (β) were determined from stability constant metal-ligand complexes/20/.

For naringenin-Al(III) complex

$$(\log K_1 = 15.39) \quad \beta_1 = 2,45.10^{11}$$

$$\begin{aligned}(\log K_2 = 7.12) \quad \beta_2 &= 3,23.10^{18} \\(\log K_3 = 6.47) \quad \beta_3 &= 2,34.10^{36}\end{aligned}$$

For naringenin-Fe(II) complex

$$\begin{aligned}(\log K_1 = 10.11) \quad \beta_1 &= 1,29.10^{10} \\(\log K_2 = 6.40) \quad \beta_2 &= 3,24.10^{16} \\(\log K_3 = 6.11) \quad \beta_3 &= 2,55.10^{27}\end{aligned}$$

The following equation was used to calculate the conditional formation constant ($\log K$) for pH 0 to 14:

$$\log K = \log K_{1,2,3}^M \cdot \alpha_M \cdot [(\log K_1^L \cdot \log K_2^L \cdot \log K_3^L) / [(H^+)^3 + K_1^L \cdot (H^+)^2 + K_1^L \cdot K_2^L \cdot (H^+) + K_1^L \cdot K_2^L \cdot K_3^L]] \quad (d)$$

$$\begin{aligned}\log K_{1,2,3}^L &= \text{Stability constants of ligand(naringenin)} \\ \log K_{1,2,3}^M &= \text{Formation constant of metal – ligand complex} \\ H^+ &= \text{The values at various pH 0 to 14}\end{aligned}$$

α_M = The fraction of α_M of the metal ion in the uncomplexed form metal may be calculated/20/.

Sample Preparation

The following solutions were prepared:

- 1) 0.001 molL⁻¹ HClO₄ and 0.01 molL⁻¹ NaClO₄ are dissolved in 25.0 mL dioxane-H₂O mixture and mixed with 25.0 mL dioxane-H₂O mixture.
- 2) 0.001 molL⁻¹ HClO₄ , 0.01 molL⁻¹ NaClO₄ and 0.004 molL⁻¹ naringenin are dissolved in 25.0 mL dioxane-H₂O mixture and mixed 25.0 mL dioxane-H₂O mixture.
- 3) 0.001 molL⁻¹ HClO₄ , 0.01 molL⁻¹ NaClO₄ and 0.004 molL⁻¹ naringenin are dissolved in 25.0 mL dioxane-H₂O. Then it is mixed with 0.006 mol L⁻¹ Al(III) in 25mL dioxane-H₂O mixture.
- 4) 0.001molL⁻¹ HClO₄ , 0.01 molL⁻¹ NaClO₄ and 0.004 molL⁻¹ naringenin are dissolved in 25.0 mL dioxane-H₂O mixture. Then it is mixed with 0.004 molL⁻¹ Fe(II) in 25mL dioxane-H₂O mixture.

Each mixture was separately titrated potentiometrically with 0.010 mol L⁻¹ NaOH (Fig. 1). The separation among (HClO₄), (HClO₄ + L) and (HClO₄ +L+Metal) plots in all potentiometric titration curves showed the formation

of a metal-ligand complex.

Fig. 1: Potentiometric titration curves.

For the titration of $\text{HClO}_4 + \text{NaClO}_4$ with mixture of $\text{HClO}_4 + \text{NaClO}_4 +$ naringenin, $\bar{n}_A = f(\text{pH})$ dependence was prepared. ($\bar{n}_A$ was calculated according to equation (a)). Then these protonation constants were found: $\log K_1 = 10.19$, $\log K_2 = 8.31$, and $\log K_3 = 7.06$ at 25°C (Fig. 2).

To obtain the $\bar{n}_L = f(\text{pL})$ dependence for the potentiometric titrations with $\text{HClO}_4 + \text{NaClO}_4 +$ naringenin + $[\text{Al(III)}]$ or $[\text{Fe(II)}]$ mixtures, equation (b) and equation (c) were applied. (Figs. 3, 6).

By using calculated formation constants, the relative abundances of different species of metal-ligand complexes were found and were plotted as a function of pH (Fig. 4, 7).

Furthermore, by using equation (d), the conditional formation constants were calculated and were plotted as a function of pH (Fig. 5, 8).

Fig. 2. $\bar{nA} = f(\text{pH})$ curves for naringenin.

Fig. 3: $\bar{nL} = f(\text{pL})$ curves for the Al (III)-naringenin complex.

Fig. 4: Relative abundance of ionic and molecular species calculated as a function of pH for Al(III) – naringenin complex.

Fig. 5: Conditional formation constant as a function of pH for Al(III)-naringenin complex.

Fig. 6: $nL = f(pL)$ curves for the Fe(II)-naringenin complex.

Fig. 7: Relative abundance of ionic and molecular species calculated as a function of pH for Fe(II) – naringenin complex.

Fig. 8: Conditional formation constant as a function of pH for Fe(II)-naringenin complex.

Spectrophotometric Studies

In spectrophotometric measurements, $\text{HClO}_4 + \text{NaClO}_4 + \text{dioxane} + \text{H}_2\text{O}$ mixture is used as reference, because the same composition is used for the potentiometric titration. Then, naringenin, aluminium(III) and iron(II) solutions are added separately to the reference mixture and absorption spectra are recorded for each (Figs. 9, 10). A similar procedure for metal ions was performed separately in the absence of naringenin (Figs. 9 and 10).

Fig. 9: UV-vis spectra of Al(III) ions, naringenin and Al(III)-naringenin complex in 1:3 dioxane+H₂O+HClO₄+NaClO₄ (50 μ M at pH 8.50).

Fig. 10: UV-vis spectra of Fe(II) ions, naringenin and Fe(II)-naringenin complex in 1:3 dioxane+H₂O+HClO₄+NaClO₄ (50 μ M at pH 8.50).

RESULTS AND DISCUSSION

Some of the metal salts, i.e. the aluminium(III) and iron(II) salts, which were used as mordant in traditional dyeing process with natural dyes, consist of flavonoids. In this study, the protonation constants of naringenin were determined potentiometrically by the Irwing-Rosotti method. The conditional formation constants were calculated and these constants were found to be in agreement with the formation constants of binary systems. In this calculation, pK values of ligand and the formation constants of complexes that they formed with metals were used as data. The formation constants of complexes were found from the formation curves $\bar{n}L=f(pL)$ which were drawn for aluminium(III) and iron(II) complexes. The conditional formation constants equal the “ β values” of the complex. The formation constants of metal-naringenin complexes found in this study are in agreement with the calculated conditional formation constants of β . The conditional formation constants, namely the stability constants of complexes, can also be calculated. The behaviour of aluminium(III) and iron(II) ions has been examined in this work.

Fig. 11: Proposed structure of naringenin-metal complexes: (M = Al(III) or Fe(II) ions).

From the experiment results, we can conclude that naringenin-metal complexes 3:1 (naringenin:metal ratio) both Al(III) and Fe(II). Since coordination number for both iron and aluminium is 6, that complex contains 3 water molecules /18,19/. The proposed structure of naringenin-metal complexes is given in Figure 11. The protonation constant of the naringenin and the stability constant of naringenin-metal complexes were determined in Table 1.

Table 1
The stability constants for Al(III)-naringenin and
Fe(II)-naringenin complexes

	LogK ₁	LogK ₂	LogK ₃
Naringenin	10,19	8,31	7,06
Al(III)-Naringenin	15,39	7,12	6,47
Fe(II)-Naringenin	10,11	6,40	6,11

The conditional formation constant for Al(III)-naringenin complex was found as the maximum value 16,94 at the range of pH= 8-9 (Fig. 5). This value is in accordance with the relative abundance (Fig. 4). The conditional formation constant for Fe(II)-naringenin complex was found as the maximum value 7,41 at the range of pH = 7-11,5 (Fig. 8). This value is in accordance with the relative abundance (Fig. 7). The complex formation shows a maximum in a narrow pH range and by decreasing with the increase of pH. (Fig 5,8).

Table 2
Maximum absorption wavelengths.

Naringenin	290,50
Al(III)	303,00
Al(III)-Naringenin	327,00
Fe(II)	310,50
Fe(II)-Naringenin	326,50

According to the spectra in Figure 9, Al(III) ions, naringenin and Al(III)-naringenin complex exhibit maxima at the wavelength of 303.00, 290.50, and

327.00 nm respectively (Table II). At 290.50 nm and 303.00 nm absorption of Al(III) ions and naringenin decrease, while absorption of the Al(III)-naringenin is increased at 327.00 nm. After the reaction was completed, no absorption signal originating either from Al(III) or naringenin was observed. Similar conclusions could be drawn from the spectrum of Fe(II)-naringenin complex. Although Fe(II) ions absorb at 310.50 nm, the iron(II)-naringenin complex absorb at 326.50 nm (Table II). Therefore, with the progress of the complexation, absorption signals of naringenin and Fe(II) ions decreased, while absorption of Fe(II)-naringenin complex was enhanced (Fig. 10). After the reaction was completed, one could observe only the latter.

The results presented are in good agreement with the previous ones for naringenin (258 nm; 373 nm) obtained by Markham /21/.

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