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Delphinidin–Aluminum(III) Complexes in Aqueous and Non-Aqueous Media: Spectroscopic Characterization and Theoretical Study

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Summary. The study of delphinidin complexation with trivalent aluminum in acidic aqueous buffered (*pH* 3.0 and 3.8) and methanolic solutions was performed utilizing electronic absorption spectroscopy and quantum chemical calculations. In its structure delphinidin possesses several chelating sites in competition towards aluminum(III). Molar ratio plots denoted the formation of only one aluminum(III):delphinidin complex of stoichiometry of 1:1 in both investigated media. Semiempirical calculations, performed at the restricted *HF* AM1 level, enabled the determination of the structural features of free delphinidin and structural modifications caused by chelation of aluminum(III). Considering the pigment molecular structure and the results of the theoretical calculations it is possible to equally implicate C3′–C4′ and C4′–C5′ hydroxyl groups as those with the predominant chelating power.

Keywords. Delphinidin; Aluminum; Complex formation; Absorption spectra; Stoichiometry; Stability constants; Semiempirical calculations.

Introduction

Flavonoids are aromatic secondary plant metabolites ubiquitously found in plants [1, 2]. These molecules are receiving renewed attention of many researchers during the last decade because of their remarkable array of biological and physiological effects [3–5], complexity of the biosynthesis and metabolism, possible industrial applications, and constantly rising commercial interest and relevance in the processes involved in nutrition, flavours, and aromas [6, 7]. Flavonoids play multiple roles in the ecology of plants. To some extent flavonoid action in plants may be related to primary metabolism. Due to their brilliant colors, coming primarily from anthocyanins, they act as attractors for pollinating insects. They also act as catalysts in the light phase of the photosynthesis and as regulators of iron channels associated with phosphorylation. The numerous investigations provided some circumstantial evidence that flavonoids are involved in the UV-protection of plants and protection of plants against microbial invasion [4, 5, 8].

The basic flavonoid structure consists of 15 carbon atoms arranged in three rings, two benzene rings joined with the γ-pyrone ring (C_6H_5(A)–C_3–C_6H_5(B)). A group of flavonoids is differentiated in several classes according to the degrees of oxidation and unsaturation of the heterocyclic C ring.

Anthocyanins are the most intensively colored group of flavonoid class [1]. Structurally they are hydroxylated and methoxylated derivates of flavylium, 2-phenyl-1-benzopyrilium, salts. They are vacuolar pigments synthesized exclusively by organisms of the plant kingdom and have been observed to occur in all tissues of higher plants, providing color in leaves, stems, flowers, and fruits [8]. The stability of the structure of these molecules *in vivo*, conditioning the stability of their color, is influenced by the

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number and the nature of the substituents, *pH* value of the medium, soil composition, and climate conditions. Their stability is also partially improved by glycosylation and acylation but also very dependent upon possible copigmentation, self-association, and metal complexation reactions [9–14].

Besides being very sensitive and powerful color stabilization mechanism developed in higher plants *in vivo* [1, 2] metal complex formation is also one of the mechanisms which enable accumulation of metals in peripheral tissues reducing the possibility of their migration to eco systems suppressing metal toxicity [15–17]. At the same time metal complexation is a very efficient mechanism of protecting plants from pathogens and plant eaters. The antioxidative activity of flavonoids, besides the direct free radical scavenging, includes as well metals chelation reactions [18]. This molecular property is also very often used for colorimetric purposes in the detection of metal traces in solutions.

The objective of this paper is *in vitro* investigation of the delphinidin–aluminum(III) complex formation in two different media, methanol and buffered aqueous solutions. Delphinidin (Fig. 1) is the most common anthocyanidin molecule in blue flowers [4, 5]. Delphinidin is the most effective in production of blueness which is associated with its availability as oxygen donating ligand in metal chelation reaction [4, 5]. In this paper delphinidin is used as a model compound concerning the fact that it does not exist in nature in its aglycon form. Aluminum is a trivalent cation found in its ionic form in most kinds of animal and plant tissues and in natural waters everywhere [15–17, 19]. It is the third most prevalent element and the most abundant metal in the earth's crust. Aluminum toxicity is among the most widespread problems of ion toxicity stress in plants. Aluminum occurs in most soils, but its availability to plants is highly *pH* dependent. Although there is some evidence to suggest that aluminum availability increases in strongly alkaline soils, most aluminum toxicity is reported in strongly acidic soils [15–17].

The aim of the present paper is to investigate the possibility of delphinidin to enter a complexation reaction with aluminum in model solutions, to determine the stoichiometric composition, stability of the complexes, and structural modifications caused by the chelation of aluminum(III).

Results and Discussion

At *pH* 3.0 and 3.8 the electronic spectra of free delphinidin exhibit, in the visible range of the spectrum, absorption bands of cationic transformation form positioned at $\lambda_{\max(pH\ 3.0)} = 524\,\text{nm}$ and $\lambda_{\max(pH\ 3.8)} = 527\,\text{nm}$ (Figs. 2 and 3). Chosen *pH* values are close to an average, physiological *pH* value of the plant tissue [20, 21] so the obtained results could give better insight into the processes of biological relevance *in vivo*. The *pH* interval in which the reaction was monitored was limited due to some reasons. The *pH* values below *pH* 3.0 were not investigated because they are not characteristic to natural media. On the other hand, complexation reaction could not be monitored at higher *pH* values because of the strong hydrolytic properties of aluminum ion which, at the concentrations used in the experiment, precipitated due to the formation of various types of aluminum hydroxo and acetate complexes [22].

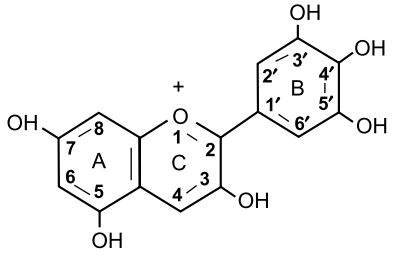

Fig. 1. Structural formula of delphinidin

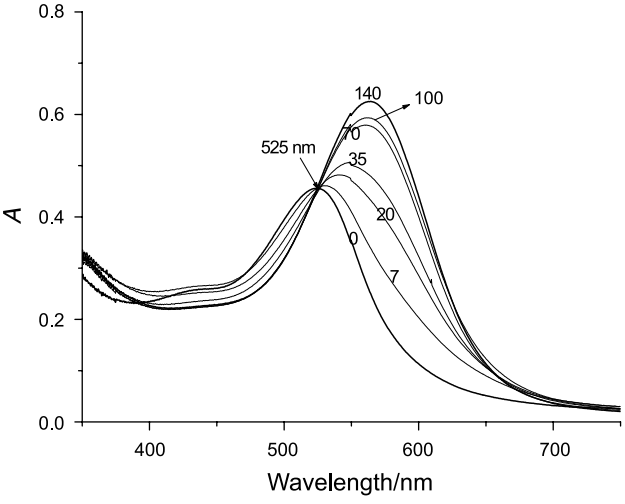

Fig. 2. Electronic spectra of equilibrated solutions of delphinidin ($5 \times 10^{-5}\,\text{mol} \cdot \text{dm}^{-3}$) in *pH* 3.0 buffer at different aluminum–delphinidin mole ratios (indicated on spectra)

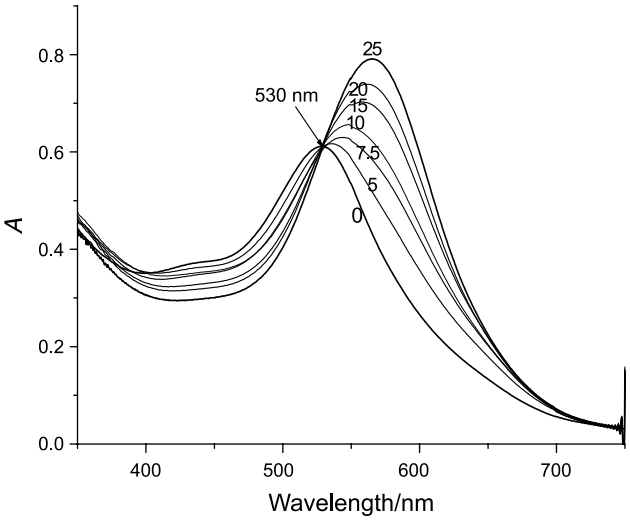

Fig. 3. Electronic spectra of equilibrated solutions of delphinidin ($5 \times 10^{-5}\,\mathrm{mol \cdot dm^{-3}}$) in *pH* 3.8 buffer at different aluminum–delphinidin mole ratios (indicated on spectra)

In the investigated buffers aluminum bonded moderately to oxygen donating delphinidin requiring larger mole ratios of the components, especially in the *pH* 3.0 buffer (over 100), for a measurable effect of the reaction. The addition of aluminum to delphinidin solutions resulted in important spectral modifications with the apparition of new bands. The new bands, bathochromically shifted up to 44 nm ($\lambda_{\max(pH\ 3.0)} = 568\,\mathrm{nm}$, Fig. 2 and $\lambda_{\max(pH\ 3.8)} = 558\,\mathrm{nm}$, Fig. 3), could be attributed to the new species, metal complexes formed. The clear isosbestic points, which appear in both buffer solutions ($\lambda^{\mathrm{is}}_{pH\ 3.0} = 525\,\mathrm{nm}$, Fig. 2 and $\lambda^{\mathrm{is}}_{pH\ 3.8} = 530\,\mathrm{nm}$, Fig. 3), indicate fairly simple flavylium cation-aluminum equilibrium that involves two species, free and complexed ligand molecule.

By the positions of the new bands and from the well known complex structural equilibrium [23], which is generally established between colored (flavylium cation and anhydrobase) and colorless (pseudobase and chalcone) transformation forms, it is most likely possible to conclude that these bands correspond to the anhydrobase transformation forms of delphinidin molecule. Knowing the fact that these transformation forms are normally characteristic to higher *pH* values [23], neutral, weekly alkaline or alkaline, but not to acidic ones, it is quite possible to presume the ability of the small and hard, charged, aluminum(III) ion to deprotonate flavylium chromophore even in acidic medium and make the molecule adopt some of its anhydrobase forms.

Although methanol is not characteristic to the natural environment complexation in methanol was also investigated because it is well known as a good complexing medium so the obtained results could be compared. Addition of aluminum to methanolic delphinidin solution resulted as well in spectral modification, the apparition of a new band bathochromically shifted approximately for $\Delta\lambda = 40\,\mathrm{nm}$. The absorption spectra also cross at an isosbestic point at $\lambda^{\mathrm{is}} = 560\,\mathrm{nm}$, which also indicates the presence of only one complex species in the system.

The complex formation can proceed by proton detachment from the ligand molecule, and can be presented by Eq. (1) [24]:

$$m\mathrm{Al}^{3+} + n\mathrm{H}_xL \leftrightarrows \mathrm{Al}_m(\mathrm{H}_yL)_n + (x-y)\mathrm{H}^+ \qquad (1)$$

where m is the number of metal ions and n the number of ligand molecules.

The corresponding stability constant value is:

$$\gamma = \frac{[\mathrm{Al}_m(\mathrm{H}_yL)_n][\mathrm{H}^+]^{(x-y)}}{[\mathrm{Al}^{3+}]^m[\mathrm{H}_xL]^n} \qquad (2)$$

when the *pH* value is constant Eq. (2) is transformed into Eq. (3) giving rise to stability constant β:

$$\beta = \frac{[\gamma]}{[\mathrm{H}^+]^{(x-y)}} = \frac{[\mathrm{Al}_m(\mathrm{H}_yL)_n]}{[\mathrm{Al}^{3+}]^m[\mathrm{H}_xL]^n} \qquad (3)$$

Stability constant value β, presented by Eq. (3), is the relative one and in the case when the complexation reaction proceeds without proton detachment (when $x = y$) it equals $\beta = \gamma$. By the molar ratio method [24] all the parameters present in Eq. (3) can be correlated with the spectroscopically obtained results. Doing so Eq. (3) is transformed into Eq. (4):

$$\log \frac{A_x}{(A_0 - A_x)^n} = \log c_{\mathrm{Al}^{3+}} \log \beta \qquad (4)$$

where (A_x) and $(A_0 - A_x)$ represent absorbance values that correspond to equilibrium concentrations of the complex and ligand. A_0 is the absorbance value of the horizontal part of the curve which presents the dependence of the complex absorbance values *vs.* aluminum(III) concentration. Figure 4 presents such dependence in buffer solution *pH* 3.8. The horizontal part of the curve, reached in each medium at certain aluminum concentrations, correspond to the completely complexed delphinidin molecule. For certain number of ligand molecules and metal ions bonded in the complex structure, n and m

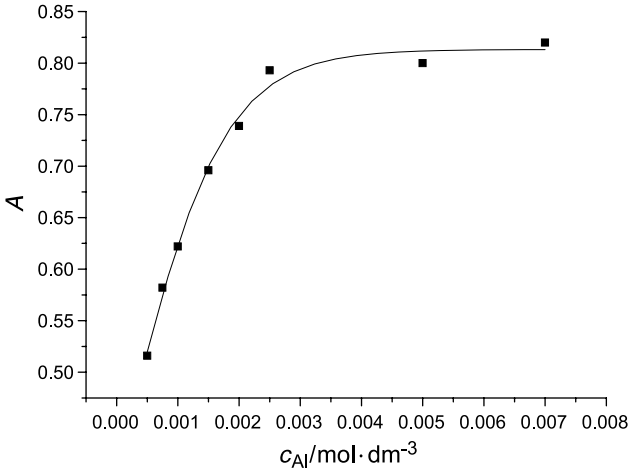

Fig. 4. The changes of the absorbance values of the complex *vs.* aluminum(III) concentration, *pH* 3.8

values, respectively, Eq. (4) gives a linear dependence of $\log(A_x/(A_0 - A_x)^n)$ *vs.* $\log c_{Al^{3+}}$ with an intercept which equals $\log \beta$. Figures 5a and 5b

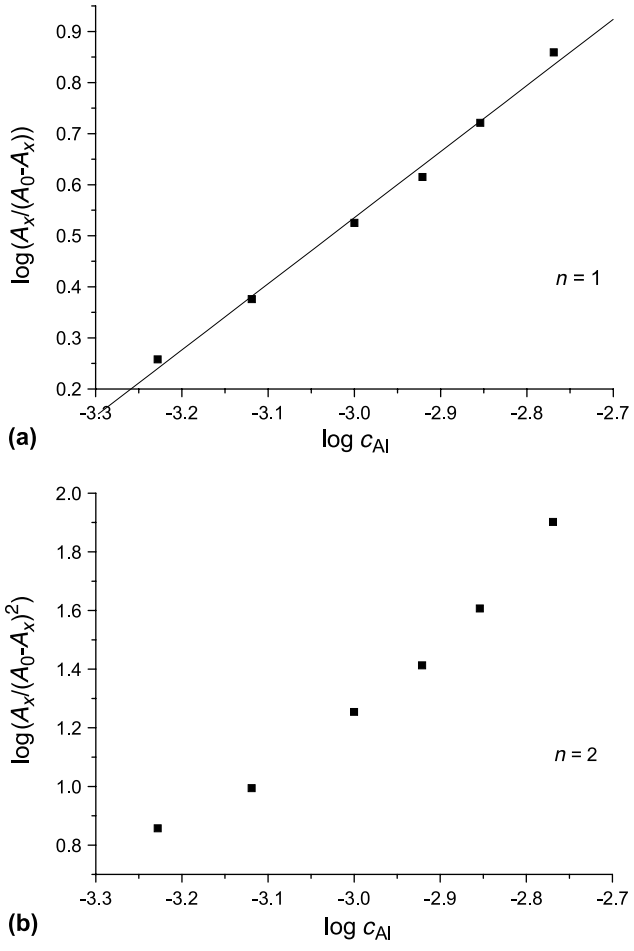

Fig. 5. Relative absorbance values, $\log(A_x/(A_0 - A_x)^n)$, *vs.* aluminum concentration: a) $n = 1$ b) $n = 2$, (*pH* 3.8)

Table 1. Stability constant values and stoichiometric ratios of the components for the delphinidin–aluminum(III) complex formation

Complexing medium	$\log \beta$	n
pH 3.0	2.75	1
pH 3.8	4.25	1
methanol	4.45	1

present molar ratio plots for the complexation in buffered solution *pH* 3.8, taking $n = 1$ and 2 and $m = 1$ in both cases. From Fig. 5a it is evident that only the $n = 1$ value gives a linear dependence indicating 1:1 stochiometry for the complex formed at *pH* 3.8. The same stoichiometry is also obtained for the solution buffered at *pH* 3.0 and complex formed in methanol. The total dominance of the 1:1 species in both solvents could be expected since the complex formation was observed only at a considerable excess of aluminum(III). Relative stability constant values are listed in Table 1. From Eq. (3), which can also be presented as $\log \beta = \log \gamma + 2pH$, it is evident that the complex is more stable at *pH* 3.8 $(\log \beta = 4.25)$ than at *pH* 3.0 $(\log \beta = 2.75)$. These findings go along with what is generally observed in the very large majority of the cases, concerning the fact that the calculated constant values are the relative ones, and that our results have not been corrected for the ligand dissociation. The formation of the more stable complex in the *pH* 3.8 buffer could also support the suggestion that aluminum availability increases as *pH* increases [17]. The fact that the difference between two stability constant values, at *pH* 3.0 and 3.8, $(\Delta \log \beta = 1.5)$ is almost twice the difference in *pH* values $(\Delta pH = 0.8)$ could indicate that the complex formation mechanism, most probably, proceeds with the detachment of two H^+ ions. The stability constant γ, which could be calculated from Eq. (3) $(\log \beta = \log \gamma + 2pH)$, is independent of the *pH* value of the medium. It is found to be $\log \gamma = (-3.30 \pm 0.05)$.

Delphinidin possesses three hydroxyl groups in the B ring (Fig. 1) which are in competition towards aluminum. The strong affinity of octahedrally hexacoordinated aluminum(III) to this ligating function is known and can result in formation of highly stable, bidentate 1:1, 1:2, and 1:3, complexes [22]. The obtained 1:1 stoichiometry implicates the formation of chelate structure complex $[DpAl]^{2+}$, with possible participation of C-4′–C-5′ and C-3′–C-4′ hydroxyl groups as chelating sites. This assumption

is consistent with the literature data concerning complexation of some synthetic and natural anthocyanidin molecules [25]. The calculated constant values also agree with the results on the deprotonation constant values of the six anthocyanin flavylium transformation forms in water ($pK' = 3.50$–4.85) [26].

Somewhat bigger stability constant value in methanol ($\log\beta = 4.45$, Table 1) can be attributed to the change of the relative permittivity value of the solvent. Comparing to water the solvation process in methanol is much less pronounced so the complexation is probably driven by stronger electrostatic forces. Another explanation could also be that the deprotonation of the hydroxyl group is easier in methanol than in acidic medium. The stability constant values in methanol are also rather consistent with the data [27–31] referring to the complexation of aluminum(III) with differently substituted flavones, molecules structurally very similar to anthocyanidins.

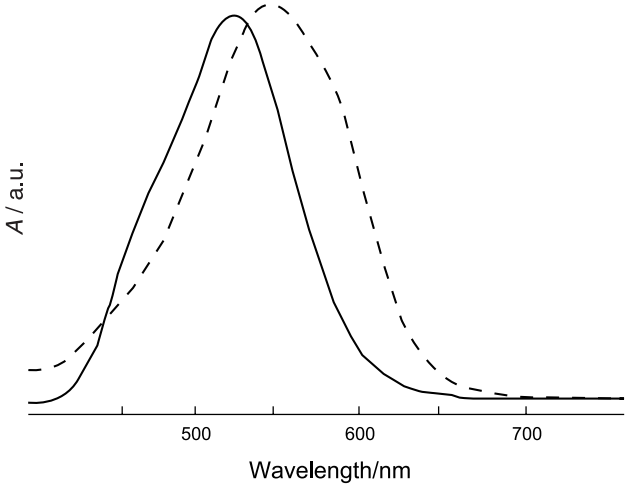

Fig. 6. Experimentally (– – – –) and theoretically (——) obtained electronic spectra of delphinidin–aluminum(III) complex formed in methanol

The energy minimization with semiempirical AM1 method shows that delphinidin is more stable in *gauche* conformation, with $\omega(C1–C2–C'1–C'6) = 28.9°$. The optimized geometry of delphinidin is presented in Fig. 6. In DFT study of anthocyanidins [32] it was found out that this torsion angle was out of plain by 3.8° for delphinidin. It is intriguing that the same torsion angle for a very similar molecule of petunidin was found to equal 28.1° [32].

There are two possible pathways for complexation of delphinidin with aluminum(III) in the investigated media. The complexation in both solvents occurs with the loss of the protons of the hydroxyl groups in the B ring and the breaking of the hydrogen bonds. The complex model obtained by the AM1 calculations indicates that one molecule of delphinidin bonds to aluminum *via* two hydroxyl groups of delphinidin. Figure 6 presents experimentally and theoretically obtained spectra of the 1:1 complex formed in methanol. The fact that the agreement between theoretical and experimental wavelengths is rather good can validate the chosen semiempirical method. The solvent effects are not taken into account in the calculations. Figure 7 presents two, the most stable conformations (I and II) of the complex formed in methanol, which are realized with very similar energies, 31.8 and 24.7 kJ/mol, indicating the possible participation of C-4′–C-5′ and C-3′–C-4′ hydroxyl groups as chelating sites. A conformational search is performed for both possible products by means of the AM1 method. In addition, two molecules of methanol are coordinated to aluminum *via* oxygen atoms.

The reaction of the complexation induces some important structural changes of the delphinidin mol-

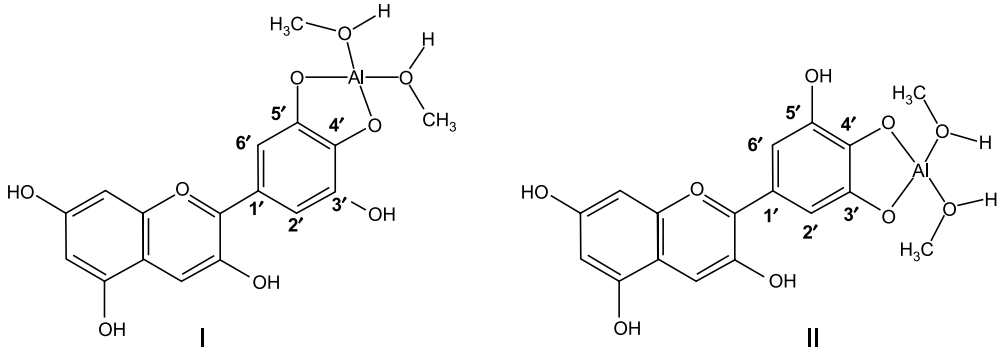

Fig. 7. Two conformers for the complex of delphinidin with aluminum in methanol

Table 2. Bond lengths (Å) calculated by the AM1 method for free delphinidin and delphinidin–aluminum complex in methanol

Bond	*Dp*	Complex
O1–C2	1.368	1.369
C2–C3	1.405	1.404
C3–C4	1.403	1.406
C4–C10	1.400	1.394
C5–C6	1.386	1.387
C5–C10	1.437	1.439
C6–C7	1.417	1.415
C7–C8	1.407	1.413
C8–C9	1.394	1.389
C9–C10	1.424	1.429
C9–O1	1.380	1.380
C2–C1′	1.448	1.456
C1′–C2′	1.408	1.409
C2′–C3′	1.393	1.392
C3′–C4′	1.414	1.423
C4′–C5′	1.413	1.401
C5′–C6′	1.400	1.405
C6′–C1	1.404	1.405
C3–O2	1.376	1.375
C5–O3	1.360	1.358
C7–O4	1.355	1.351
C3′–O5	1.375	1.374
C4′–O6	1.365	1.371
C5′–O7	1.369	1.367
H(O6)O5	2.271	
H(O7)O6	2.279	2.431
O6–Al		1.754
O5–Al		1.757

Table 3. Bond angles (°) calculated by the AM1 method for free delphinidin and delphinidin–aluminum complex in methanol

Bond angle	*Dp*	Complex
O1–C2–C3	120.7	120.5
C2–C3–C4	120.7	120.6
C3–C4–C10	118.5	118.9
C4–C10–C5	123.8	123.9
C5–C6–C7	119.4	119.7
C6–C7–C8	121.7	121.6
C7–C8–C9	117.7	117.6
C8–C9–C10	123.1	123.3
C10–C5–C6	121.2	121.0
C9–O1–C2	119.6	120.1
C3–C2–C1′	126.6	127.7
C2–C1′–C2′	119.8	119.9
C1′–C2′C3′	119.3	117.8
C2′–C3′–C4′	120.9	121.4
C3′–C4′–C5′	119.2	120.1
C4′–C5′–C6′	120.1	118.8
C5′–C6′–C1′	118.8	120.5
C6′–C1′–C2′	120.6	121.4
C2–C3–O4	122.1	117.2
O3–C5–C6	123.6	123.9
O4–C7–C8	122.7	115.6
O5–C3′–C4′	115.2	116.2
O6–C4′–C5′	116.9	122.6
O7–C5′–C6′	117.0	117.3
O6AlO5		98.4

Table 4. Main dihedral angles (°) calculated by the AM1 method for free delphinidin and delphinidin–aluminum complex in methanol

Dihedral angle	*Dp*	Complex
O1–C2–C1′–C6′	28.9	26.2
C2′–C3′–O5–H	5.5	
C3′–C4′–O6–H	1.4	
C4′–C5′–O7–H	0	2.7
C2′–C3′–O5–Al		179.6
C3′–C4′–O6–Al		0.1

ecule. All important structural parameters for free delphinidin and the most stable delphinidin complex formed in methanol, bond lengths, bond angles and torsional angles, are presented in Tables 2, 3, and 4. Due to the changes in the B ring the bond length between B and C rings (C2–C1′) is longer than in free delphinidin, whereas, H(O7)-O6 hydrogen bond is much weaker than corresponding bond in free delphinidin. The consequence of bonding aluminum to delphinidin *via* O5 and O6 oxygen atoms is that C3′–C4′ bond becomes longer, whereas C4′–C5′ becomes shorter.

All valence angles of the complex are almost identical to the corresponding valence angles of free delphinidin. The presence of aluminum causes small geometrical deformations of the B ring of the complex with an increase of the O6–C4′–C5′ and O5–C3′–C4′ bond angles. The torsion angle between B and C rings becomes close to the value in free delphinidin. The new valence angle O6–Al–O5 of 98.4° is close to the values obtained for other similar aluminum complexes. Corresponding new torsion angles C2′–C3′–O5–Al and C3′–C4′–O6–Al indicate that aluminum is coplanar with the B ring in the complex.

Experimental

Materials

Delphinidin chloride (Pfaltz and Bauer, USA), $AlCl_3 \times 6H_2O$ (Fluka, Switzerland), sodium chloride (p.a., Merck, USA),

acetic acid (Merck, USA), methanol (Uvasol. Merck, USA), sodium hydroxide (Merck, USA) and hydrochloric acid (Merck, USA). The purity of delphinidin was checked chromatographically [33] and the aluminum chloride hexahydrate was used as received.

Solutions

Acetate buffered solutions of *pH* 3.0 and *pH* 3.8, of constant ionic strength, adjusted by sodium chloride ($5 \times 10^{-1}\,\mathrm{mol \cdot dm^{-3}}$), were used. The solutions were obtained by mixing acetic acid ($5 \times 10^{-2}\,\mathrm{mol \cdot dm^{-3}}$) and sodium hydroxide ($1.5\,\mathrm{mol \cdot dm^{-3}}$). Stock solution of delphinidin ($1 \times 10^{-3}\,\mathrm{mol \cdot dm^{-3}}$), prepared in methanol and 0.1% hydrochloric acid, was left to equilibrate in the dark for 1 h. This solution was diluted to the concentration of $5 \times 10^{-5}\,\mathrm{mol \cdot dm^{-3}}$ by addition of the buffers and methanol. The stock solutions of aluminum chloride hexahydrate ($1 \times 10^{-2}\,\mathrm{mol \cdot dm^{-3}}$), prepared in corresponding buffers and in methanol, were diluted to fit different metal:pigment mole ratios. All the solutions were equilibrated for half an hour before each spectroscopic measurement.

Electronic Spectra

The electronic spectra were recorded on a *Cintra GB-10* UV-Vis spectrophotometer. Quartz cuvettes of 10 mm optical path length were used. Each spectroscopic measurement was repeated three times.

pH Measurements

An Iskra MA 5730 (Kranj, Slovenia) *pH* meter with a Sentek (Essex, England) combined electrode was used for the *pH* measurements.

Theoretical Methods

The stability constant values and the composition of the complexes formed were obtained by the molar ratio method [24].

Semiempirical Calculations

Semiempirical treatment of the structure of delphinidin and its complexes was performed using the AM1 (Austin Model 1) method [34] reported as the most appropriate to determine the structure of the flavonoid compounds and to reproduce experimental data, particularly electronic or vibrational spectra [35, 36]. All the calculations are performed with the program package Spartan 02 [37]. The geometry optimization performed at the restricted *HF* AM1 level [34] provided the opportunity to describe the electronic spectra of both free and complexed delphinidin. The theoretical harmonic vibrational frequencies are obtained from the analytical second derivatives to verify if the equilibrium structures are true minima. Conformation search is performed to determine stable conformers of delphinidin molecule. Optimal structure is used for further investigations. The geometries of the free and complexed molecules are fully optimized, without restriction. The electronic excitations are calculated by the INDO1/S [38] method with the geometries obtained through the AM1 optimization by using the ZINDO program [39–41].

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