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Original Scientific Paper

Nitroxide Mediated Degradation of Anthocyanidins

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Abstract. The degradation of the six anthocyanidins (pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin) mediated by the nitroxides: 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo), 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol) and 4-methoxy-2,2,6,6-tetramethylpiperidine1-oxyl (4-CH_3O-Tempo) at 25 ºC in aqueous acid solution was investigated spectrophotometrically and by EPR and HPLC measurements. The reaction kinetics were followed under pseudo-first order conditions using a large excess of nitroxide reactants. The spontaneous degradation of anthocyanidins under these conditions is several orders of magnitude slower, and it did not influence the measurements. However, it was found that the reaction rate increases with the age of acidified nitroxide solutions, reaching a maximum after 24 hours. This result indicates that in every case the oxoammonium cation, generated by disproportionation of nitroxyl radicals, is somewhat more reactive toward anthocyanidins than the nitroxyl itself. The products were identified by HPLC as ring substituted benzoic acids. The relative reactivities of the six anthocyanidins and the accelerating influence of the *p*-substituent of nitroxides on the reaction is discussed.

Keywords: anthocyanidins; kinetics; structure activity; 2,2,6,6 tetramethylpiperidine -1-oxyl =Tempo; 4-hydroxy-Tempo, 4-methoxy-Tempo

INTRODUCTION

Polyphenols are the most abundant diet antioxidants. They are widespread in fruits, vegetables and processed foods and beverages like juices and wines and may play a useful role in reducing disease risk. Plant polyphenols are multifunctional and can act as reducing agents, hydrogen atom donors, antioxidants, and singlet oxygen quenchers. Most can also form stable radical species, and some can react with metal ions.

Anthocyanin polyphenols are generally accepted as the largest and most important group of water-soluble pigments in nature.[1–5] Their color is a function of the number and position of hydroxyl groups in the molecule. Anthocyanin intake by humans is associated with reduced risk of several degenerative diseases such as atherosclerosis, cardiovascular disease, cancer and diabetes.[6,7] Owing to their ability to scavenge free radicals, anthocyanins can also serve as potential chemopreventive substances. A great number of studies have been carried out on the potential benefits of anthocyanins to human health.

Anthocyanins consist of an aglycone-anthocyanidin with a glycone-sugar mostly substituted in the C-ring.[8] Around 90 % of all anthocyanins are based on only six anthocyanidins: pelargonidin (**1**), cyanidin (**2**), delphinidin (**3**), peonidin (**4**), petunidin (**5**), and malvidin (**6**), Chart 1.

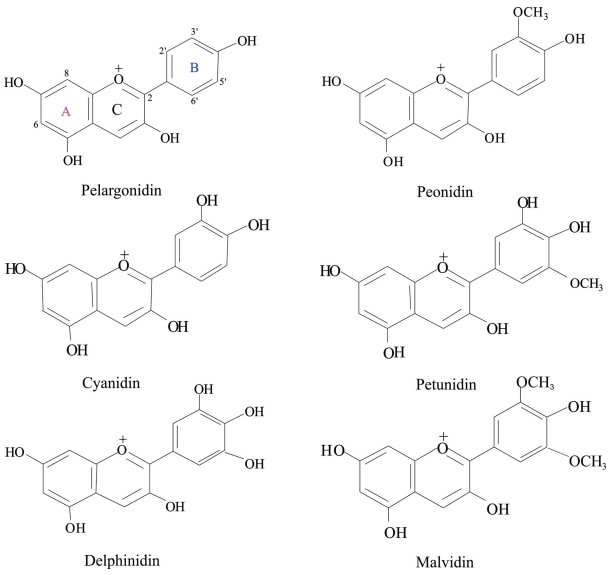
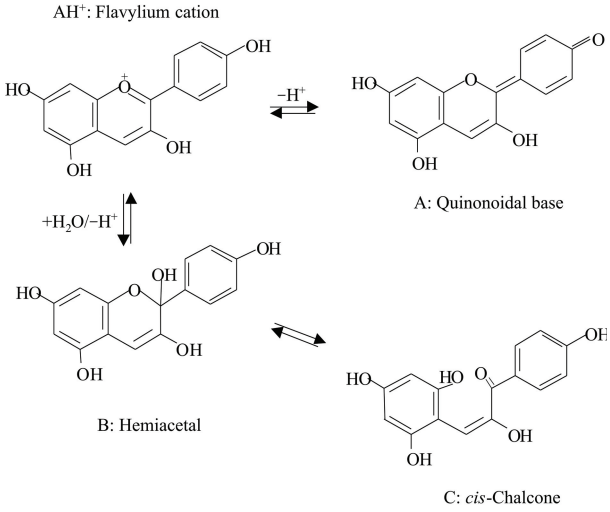


Chart 1. Structures of anthocyanidins studied in this work: pelargonidin (1), cyanidin (2), delphinidin (3), peonidin (4), petunidin (5) and malvidin (6).

Most anthocyanins are unstable toward light, heat, presence of oxygen, acidity and basicity. The stability of anthocyanins can be enhanced through intramolecular or intermolecular copigmentation with other compounds. Anthocyanins interact with other flavonoids, polyphenols, amino acids and related compounds including the anthocyanins themselves. This association is the main mechanism of stabilisation of color in plants.[9,10]



Scheme 1. Chemical forms of antocyanidins as a function of pH.

The electron-deficient flavylium nucleus is unstable and decomposes in acidic and neutral aqueous solutions. As shown in Scheme 1, the mechanisms proposed for this process generally assume the existence of flavylium cation AH^+ at sufficiently acidic pH, quinonoidal base A, formed by deprotonation of the flavylium cation (pH = 2–4). After addition of a molecule of water and deprotonation, the flavylium cation is converted to hemiacetal B (pH = 5), which is transformed to *cis*-chalcone (pH = 6). The *trans*-chalcone is result of the isomerization of the *cis*-chalcone. The chalcone form is characterised by the opening of the pyrylium ring at C2 whereby the planarity of the species is destroyed.[11–15]

In strongly acidic solutions, the dominant species is the flavylium cation AH^+. Because of its positive charge, this species is susceptible to nucleophilic attack, principally at C2. The mechanisms of the various reaction paths for flavylium ions depend on the solvent and acidity of the medium.

According to current theories, the bimolecular reactions between oxygen-centered radicals and phenols take place by hydrogen atom abstraction or electron transfer or proton transfer.[16,17] The first of these processes does not involve charge separation and can be characterized as homolytic scission of the phenolic O–H bond, which is most likely to occur in non-polar solvents.

The present study focused on the effect of cyclic nitroxyl radicals (Tempo, Tempol and 4-CH_3O-Tempo, Chart 2) on the degradation of six anthocyanidins (1–6, Chart 1) in 0.10 mol dm^{-3} aqueous $HClO_4$.

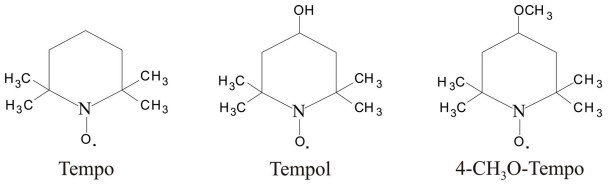
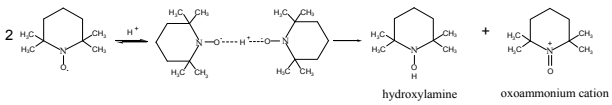


Chart 2. Nitroxyl radicals used in this work.

Nitroxyl radicals have been reported to protect effectively against oxidative stress and to act as potential new therapeutic agents[18,19] as well as mediators in some organic reactions.[20,21]

We carried out experimental and theoretical investigations of flavonoids; the radical formation,[22] kinetics,[23] and gas phase reactions with metal ions.[24] Results have convinced us that combined theoretical (quantum chemical calculation), analytical (HPLC, EPR, UV-Vis, mass spectrometry) and a kinetic (with appropriate model reactants) approach is needed to understand the complicated transformations of flavonoids that occur while they perform their beneficial activity. With the choice of the relatively stable nitroxyl radicals to initiate the transformation by changing their structure slightly we expect to better understand and elucidate its mechanisms in polar media.

Nitroxyl radicals are stable in aqueous solutions except under strongly acidic conditions. Those that do show some stability at pH = 1 are probably not protonated at that acidity.[25] As excellent hydrogen bond acceptors[26,27] nitroxyls form hydrogen-bonded dimers in acidic solutions followed by disproportionation to hydroxylamine and oxoammonium cations, Eq. (1). Aged solutions of Tempo thus contain both the oxidizing and reducing species, both of which can be involved in the reactions with added substrates.



(1)

EXPERIMENTAL SECTION

Materials and methods

Anthocyanidins were purchased from Karl Roth (pelargonidin chloride = 3,4',5,7,-tetrahydroxyflavylium chlo-

ride, cyanidin chloride = 3,3',4',5,7-pentahydroxyflaylium chloride), ChromoDex (malvidin chloride = 3,4',5,7-tetrahydroxy-3',5'-dimethoxyflavylium chloride, petunidin chloride = 3,3',4',5,7-pentahydroxy-5'-methoxyflavylium chloride) and Extrasynthese (delphinidin chloride = 3,3',4',5,5',7-hexahydroxyflavylium chloride, peonidin chloride = 3,4',5,7-tetrahydroxy-3'-methoxyflavylium chloride) and were used without purification. The nitroxide radicals 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol) were purchased from Fluka, and 4-methoxy-2,2,6,6-tetramethylpiperidine1-oxyl (4-CH_3O-Tempo) from Lancaster Synthesis. Tempo was recrystallized from methanol. Perchloric acid (analytical grade, Merck) was used as received. Doubly destilled water was additionally purified by passage through a Milly-Q water purification system.

Kinetic investigation of the reactions of anthocyanidins with nitroxide was done spectrophotometrically with HP Agilent 8453 diode array spectrophotometer and a Durrum D-110 stopped-flow instrument.

Stock solutions of each anthocyanidin (0.30−9.85 × 10^{-5} mol dm^{-3}) were prepared in 0.10 mol dm^{-3} perchloric acid. The reactions were initiated by addition of the nitroxide solution, either fresh or aged, in 0.10 mol dm^{-3} aqueous perchloric acid. The kinetics were followed at an absorption maximum of the flavylium ions: 505 nm (pelargonidin), 515 nm (cyanidin, peonidin) and 520 nm (delphinidin, petunidin and malvidin). The data were collected under pseudo-first order conditions using a large excess of nitroxide over the flavylium ions. All of the kinetics experiments were carried out at 25 °C in 0.10 mol dm^{-3} aqueous perchloric acid.

The EPR spectra were monitored with an X-band Varian E-109 spectrometer. Data were collected using the software supplied by the manufacturer.[28]

Reaction products were analysed by Knauer HPLC System wih Diode Array Detector K-2800 and a Kromasil C18 column (5μ, 100A).

RESULTS AND DISCUSSION

Solutions of nitroxyl radicals in 0.10 mol dm^{-3} HCl or 0.10 mol dm^{-3} $HClO_4$ decayed slowly over a 24-hour period as shown by changes in the absorption and EPR spectra (Figure 1). The remaining experiments in this work utilized 0.10 mol dm^{-3} $HClO_4$ as solvent.

The disproportionation of nitroxyl radicals in acidic solution involves the oxidation of the radical the protonated counterpart of which yields hydroxylamine and oxoammonium cation, Eq. (1). The reaction is reversible and nitroxyl radicals are regenerated upon neutralization of H^+, as evidenced by EPR measurements.[29]

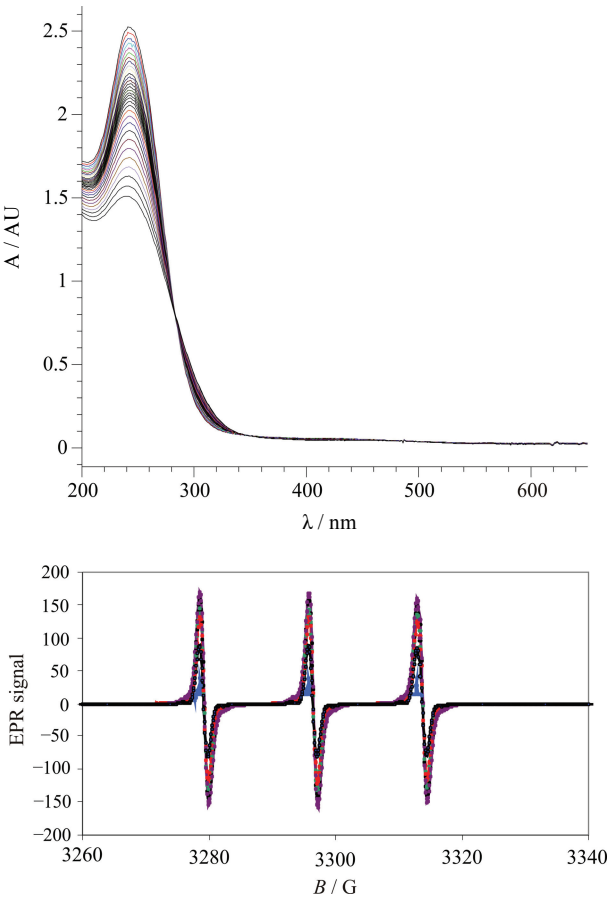


Figure 1. Time dependence decrease of the absorption and EPR spectra of 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo) in 0.10 mol dm^{-3} perchloric acid. Total time: 24 h.

For comparison, a structurally related nonradical species, pyridine-N-oxide, proved stable in acidic aqueous solution and had no effect on the degradation of anthocyanidins.

The reaction between pelargonidin in 0.10 mol dm^{-3} $HClO_4$ and an aqueous solution of Tempo was slow, but not close to that of spontaneous degradation of pelargonidin (*i.e.*, $k_{obs} \approx 1.6 \times 10^{-5}$ s^{-1}). These results suggest that the reaction observed was the acid catalyzed disproportionation of nitroxyl. Kinetics experiments were done with both freshly prepared solutions of the radicals (strong EPR signal) and solutions that had lost most of their paramagnetism. Kinetics of the reactions of flavylium cations with a large excess of nitroxyl radicals were studied as a function of concentration. Spectral scans for the pelargonidin reaction with aged solution of Tempo are shown in Figure 2 along with the kinetic trace at 505 nm. Standard treatment of the exponential kinetic traces yielded first order rate constants k_{obs} which were independent of the concentraton of the limiting reagent, and increased linearly with the initial

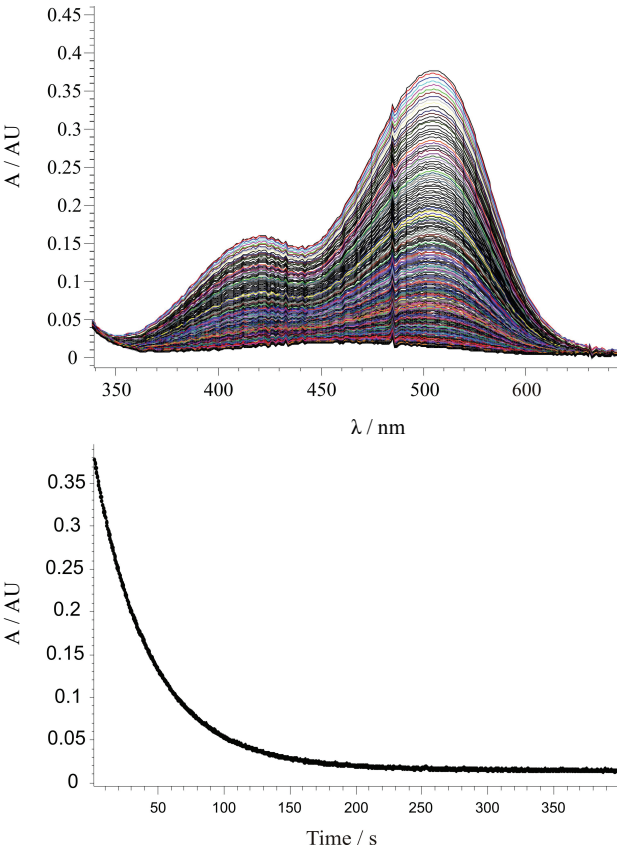


Figure 2. Spectral change at 505 nm and kinetic trace for the reaction between pelargonidin (2.1×10^{-5} mol dm^{-3}) and 2,2,6,6-tetramethylpiperidine-1-oxyl (1.23×10^{-3} mol dm^{-3}) in 0.10 mol dm^{-3} $HClO_4$ (aged solution) at 25.0 °C. (Total time 400 s).

concentration of nitroxyl radicals (Figure 3), according to the rate law in Eq. (2). The second-order rate constants k_{II} were obtained from the slopes of the lines in Figure 3.

$$\frac{d[\text{Anthocyanidin}]}{dt} = k_{II}[\text{Anthocyanidin}][\text{Nitroxide}] \quad (1)$$

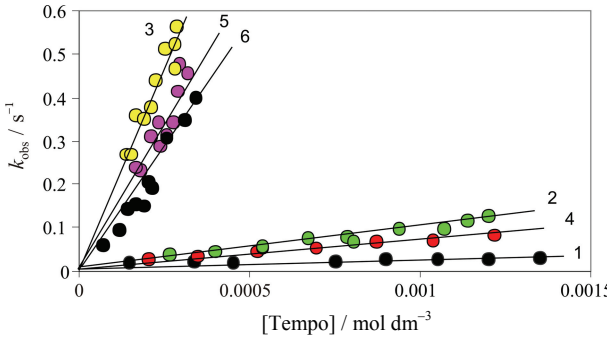


Figure 3. Plot of k_{obs} *vs.* the concentrations of 2,2,6,6-tetramethylpiperidine-1-oxyl (aged solution) for the reaction with pelargonidin (1), cyanidin (2), delphinidin (3), peonidin (4), petunidin (5) and malvidin (6).

Table 1. Kinetic data for the reaction of pelargonidin (2.9×10^{-5} mol dm^{-3}) with a solution of Tempo aged by increased amounts of time (25 °C, 0.1 mol dm^{-3} $HClO_4$)

t / h	10^3[Tempo] / mol dm^{-3}	$10^2 k_{obs}$ /s^{-1}
0	1.22	1.62
0.30	1.22	1.69
0.7	1.22	1.77
1.5	1.22	1.92
3	1.22	2.01
24.15	1.22	2.22
25.5	1.22	2.22
27.3	1.22	2.28
49.66	1.22	2.24
	1.28*	2.48
	1.28*	2.48

* With a new solution of Tempo aged for five days.

In general, reaction rates initially increased with the age of Tempo solutions, and leveled off at about 24 hours after solution preparation. The measured constants, k_{obs}, for the reaction of pelargonidin with freshly prepared and aged solutions of Tempo are shown in Table 1.

Because the rate constant does not change significantly with time, and for some of our reductants it even increases, even though no nitroxyl remains after several days; thus, another species must be responsible for the reaction with aged solutions. The most likely candidate is the oxoammonium cation, itself a powerful oxidant[30,31] and known to be formed by disproportionation of Tempo in acidic solutions.[29]

To confirm this hypothesis, the oxoammonium cation was generated independently from Tempo and Ce(IV). The reaction of the cation with pelargonidin was then examined under the same conditions utilized in the study of the Tempo-pelargonidin reaction (Table 2). It was found that the cation indeed reacted with pelargonidin. Moreover, the rate constant for the cation-pelargonidin reaction is similar to that for the Tempo-pelargonidin reaction, as required by our hypothesis.

Table 2. Rate constants for the reaction of anthocyanidins with 24 hours aged Tempo prior to the experiment in 0.1 mol dm^{-3} $HClO_4$ (Tempo = 2,2,6,6-tetramethylpiperidine-1-oxyl)

ANTHOCYANIDINS	k_{II} / mol^{-1} dm^3 s^{-1} (Tempo-aged)
PELARGONIDIN (1)	21.7
PEONIDIN (4)	54.6
CYANIDIN (2)	91.1
MALVIDIN (6)	1469
PETUNIDIN (5)	1491
DELPHINIDIN (3)	1853

The observed rate constant was 38 ± 4 mol^{-1} dm^3 s^{-1}. The value derived from aged solutions of Tempo (where only 50 % of the initial nitroxyl radical was converted to the cation) was 40 ± 4 mol^{-1} dm^3 s^{-1}. All these results confirm that the species responsible for the reaction of aged solutions of Tempo is indeed the cation. It is surprising that the radical and the cation react at such similar rates. The greater reduction potential of the cation and the radical nature of nitroxyl almost certainly require a change in mechanism. Similar kinetics may be a coincidence, but it is more likely that other factors, such as steric crowding at the reaction site, play a major role. This may be the reason for reduced reactivity of Tempo in the present study. Oxoammonium cations, which presumably react by electron transfer, should be affected less because electron transfer does not require such a close approach as hydrogen atom transfer does. Also, to rule out the possibility that some unreacted Ce(IV), used to generate the cation, was not responsible for the observations in the cation-pelargonidin reaction, the oxidation of pelargonidin with Ce(IV) was examined directly and found to be very fast, thus ruling out an interference by Ce(IV).

The degradation of anthocyanidins occurs by oxidative cleavage of the pyrylium ring. The products of the reaction of the pelargonidin and Tempo were analyzed by HPLC assay. One product of the degradation of pelargonidin was identified as 4-hydroxybenzoic acid and another signal appearing from the rest of parent molecules. There is no evidence to formation of the chalcone or α-diketone, however, this form is rather unstable and its formation is favored at high pH and also at higher temperatures.[12] The thermal and photochemical degradation of anthocyanidins led to production of the OH-substituted benzoic acid and 2,4,6-trihydroxybenzaldhyde. Photochemical reaction goes through the direct photochemical conversion of the flavylium cation to the product. During the thermal degradation pyrylium ring opens to give chalcone followed by its cleavage to give products.[32,33]

Table 3. Rate constants for the reaction of aged solutions of substituted 2,2,6,6-tetramethylpiperidine-1-oxyl with anthocyanidins at 25 ºC and 0.1 mol dm^{-3} $HClO_4$

ANTHOCYANIDINS	k_{II} / mol^{-1} dm^3 s^{-1} 4-OCH_3-Tempo	k_{II} / mol^{-1} dm^3 s^{-1} 4-OH-Tempo
PELARGONIDIN (1)	92.6	147.4
PEONIDIN (4)	575.5	871.6
CYANIDIN (2)	1200.6	1258.1
MALVIDIN (6)	1138.9	1088.1
PETUNIDIN (5)	1162.8	1670.0
DELPHINIDIN (3)	1672.5	1809.5

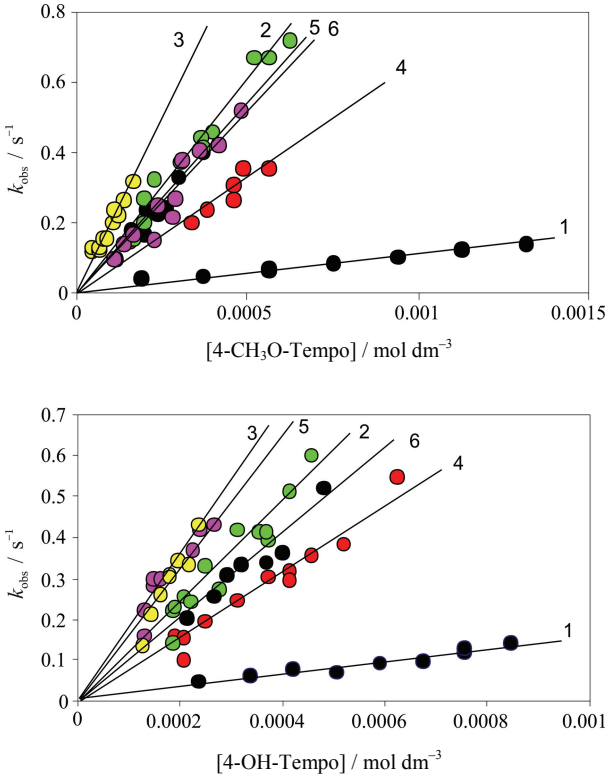


Figure 4. Plot of k_{obs} *vs*. the concentrations of *p*-substituted 2,2,6,6-tetramethylpiperidine-1-oxyl for the reaction with pelargonidin (1), cyanidin (2), delphinidin (3), peonidin (4), petunidin (5) and malvidin (6).

The reactivity order Pelargonidin < Peonidin < Cyanidin < Petunidin < Malvinidin < Delphinidin demonstrates the overwhelming importance of steric bulk at B-ring in the reaction with Tempo (Table 2). These six common anthocyanidins differ in the positions of the hydroxy and methoxy groups in their B-rings.[34] Delphinidin with three hydroxy group on B-ring is most reactive followed by cyanidin, with two and pelargonidin with a single hydroxy group in the B-ring. Methylation of one of two hydroxy groups of cyanidin (peonidin) reduces significantly the reactivity while the methylation of one or two of the three hydroxy groups in delphinidin (petunidin or malvidin, respectively) causes only slightly and nearly same reactivity reduction. Our experimental data show that the number of OH substituent in the B-ring determinates the reactivity of anthocyanidins.

We also examined how the degradation rates of six anthocyanidins (**1–6**) depend on the nitroxide structure. Kinetics with the other two nitroxides were measured with solutions prepared on the previous day.

The reactivity for two other reactants, Tempol and 4-CH_3O-Tempo in the reaction with anthocyanidins is listed in Table 3 and the corresponding plots of observed rate constants *vs*. the concentrations in Figure 4.

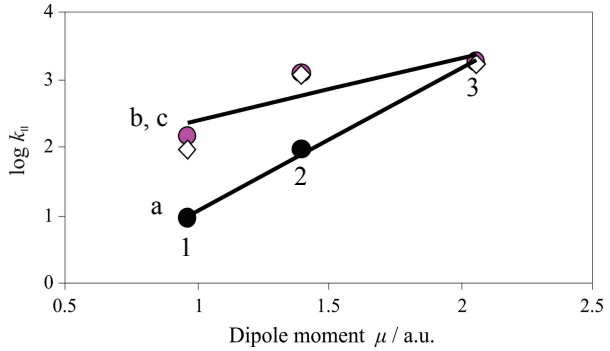


Figure 5. Plot of log k_{II} *vs.* calculated dipole moment μ for the reaction 2,2,6,6-tetramethylpiperidine-1-oxyl (a) and *p*-substituted 2,2,6,6-tetramethylpiperidine-1-oxyl (b,c) with pelargonidin (1), cyanidin (2) and delphinidin (3).

An investigation of the kinetics of the structural transformation of anthocyanidins in acidic medium in the reaction with *p*-substituted 2,2,6,6-tetramethylpiperidine-1-oxyl reactants shows that increase of the number of hydroxyl groups in flavylium nucleus also increases the rate constants. Pelargonidin with one OH group substituted in the B-ring is the least reactive species. In the present series of nitroxyl reactants, cyanidin with an *o*-di-OH substitution in the B-ring reacts twice as rapidly as peonidin that has a 3'-methoxy and 4'-hydroxy substitution and approximately ten times faster then pelargonidin. Cyanidin is more susceptible to degradation in the reaction with *p*-substituted 2,2,6,6-tetramethylpiperidine-1-oxyl reactants than in the reaction with the unsubstituted reactant.

However, as we compare the structure and the rate constants for the delphinidin, petunidin and malvidin, all with 3',4',5'-substitution in the B-ring, we observe that delphinidin with 3',4',5'-tri-OH substitution is the most reactive anthocyanidin. The petunidin and malvidin with one and two methoxy group substituted in the B-ring react slowly but effect of the number of methoxy groups is negligible for the reaction with 4-CH_3O-Tempo, whereas with 4-OH-Tempo malvidin is reacting slower than the other two aglycones with 3',4',5'-substitution. This differences in sensitivity toward *p*-substituted nitroxides is a result of geometric influence and electronic effects of the substituents. Only pelargonidin, cyanidin and peonidin show remarkable difference in reactivities with the three nitroxide reactants, whereas the reactivities of delphinidin toward all nitroxides were similiar. The trend in rate among the number of OH substituents in the B-ring is in the same direction as the dipole moment in each species[35] (Figure 5).

The higher reactivity can be explained by the structural fragility of anthocyanidin molecules with three substituents in the B-ring, thus making them more prone to C-ring opening. The para substitution of 2,2,6,6-tetramethylpiperidine-1-oxyl reactant was shown to have little effect on the gas phase electronic structure of radicals and also on their reactivity in electron transfer reaction.[36] The different effect of Tempo and its *p*-substituted analogs on anthocyanidins reactivities could be result of the different basicity of nitroxyl group in aqueous acidic media. Nitroxyls with electron releasing groups show a lower stability to the acid catalyzed disproportionation and higher reactivity. The data indicated Tempol as the most reactive species with nearly all anthocyanidins.

Observation from numerous other studies shows that the reactivity of flavylium ions depends on the B ring structure. Two assays for the antioxidant activity, the oxygen radical capacities (ORAC),[37] and the Trolox equivalent antioxidant activity (TEAC/mmol dm^{-3})[2] showed that the most reactive anthocyanidins were delphinidin and cyanidin with three and two OH substituents in the B-ring, respectively.

The scavenging activity of the series of the anthocyanins toward the superoxide radical was in the following order: delphinidin > petunidin > malvidin ~ cyanidin > peonidin > pelargonidin and the reactivity order in the reaction with $ONOO^-$ was: delphinidin > cyanidin ~ petunidin > malvidin > peonidin > pelargonidin. It was concluded that the scavenging activity was determined primarily by the aglycone structure and not by the nature of the sugar moiety.[38] All these results as well as those obtained in the present study suggest that the reactivity of anthocyanidins is the result of electron distribution within the molecule making the polarity of the aglycones the most important factor in their activity. As a result, delphinidin is a highly reactive species. All this also confirms the nature and extent of B-ring substitution to be responsible for anthocyanidin reactivity.

CONCLUSION

This work presents results of the nitroxide mediated degradation of a series of anthocyanidins in acidic medium. The overall results suggest that the stability of anthocyanidins is greatly influenced by B-ring substituents. Nitroxyl radical undergoes a disproportionation reaction in acidic solution. The resulting oxoammonium cation is somewhat more reactive toward anthocyanidins than the nitroxyl itself. This suggests that anthocyanidins in biological systems provide protection not only against harmful radicals, but also against other oxidants. The effect of *p*-substituted analogs of Tempo on anthocyanidin degradations is a result of different basicities of the nitroxyl group in acidic solution.

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SAŽETAK

Raspad antocijanidina potpomognut nitroksidom

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Istraživana je brzina raspada šest antocijanidina (pelargonidin, cijanidin, delfinidin, peonidin, petunidin i malvidin) potpomognuta nitroksidima: 2,2,6,6-tetrametilpiperidin-1-oksil (Tempo), 4-hidroksi-2,2,6,6-tetrametilpiperidin-1-oksil (Tempol) i 4-metoksi-2,2,6,6-tetrametilpiperidin-1-oksil (4-CH_3O-Tempo) u kiseloj vodenoj otopini kod 25 ºC. Reakcije s nitroksidima praćene su spektrofotometrijski i EPR-om a produkti su analizirani HPLC-om. Kinetike su vođene u uvjetima pseudo-prvog reda s nitroksidom u velikom višku. Spontani raspad antocijanidina je u ispitivanim uvjetima vrlo polagan tako da nije imao utjecaja na istraživanu reakciju. Zapaženo je da se reakcija ubrzava stajanjem kisele otopine nitroksida, a brzina se ustali nakon 24 sata. To ukazuje da je oksoamonijum kation koji nastaje disproporcioniranjem nitroksi radikala reaktivniji od njega. Supstituirana benzojeva kiselina identificirana je kao produkt raspada. Razmatrane su relativne reaktivnosti antocijanidina i utjecaj na brzinu reakcije *p*-supstituenta na nitroksidima.