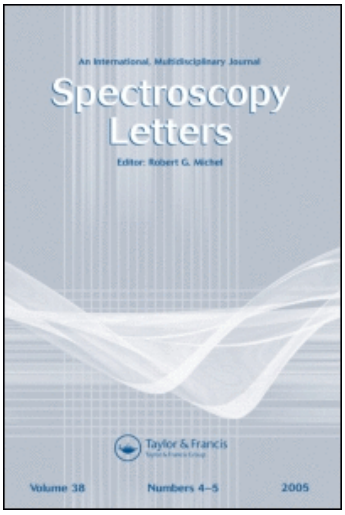

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Ultraviolet Fluorescence of Cyanidin and Malvidin Glycosides in Aqueous Environment

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Ultraviolet Fluorescence of Cyanidin and Malvidin Glycosides in Aqueous Environment

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Abstract: The fluorescence of cyanidin 3-glucoside (Cy 3-glc), cyanidin 3,5-diglucoside (Cy 3,5-diglc) and malvidin 3,5-diglucoside (Mv 3,5-diglc) in binary solvents (water–methanol, 1:1, v/v) and in water at pH 4–5.5 has been studied. The absorption spectra, steady-state fluorescence spectra, and fluorescence excitation spectra have been measured. Fluorescence excitation was in the UV range at the absorption maxima of cyanidin and malvidin glycosides ($\lambda_{exc} = 220$ nm) and at the characteristic absorption band of about $\lambda_{exc} = 280$ nm. In the aqueous environment, Cy 3-glc exhibits short-wavelength fluorescence F_{SH} at $\lambda^{fl}_{max} = 299$ nm which was most effectively excited at 220 nm. Similar short-wavelength fluorescence F_{SH} was observed for Cy 3,5-diglc ($\lambda^{fl}_{max} = 308$ nm) and Mv 3,5-diglc ($\lambda^{fl}_{max} = 293$ nm) in a binary solvent system.

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We postulate that the observed short-wavelength fluorescence F_{SH} is related to the hemiacetal forms of the analyzed anthocyanin glycosides.

Keywords: Cyanidin glycoside malvidin glycosides, UV fluorescence, UV-Vis absorption spectra

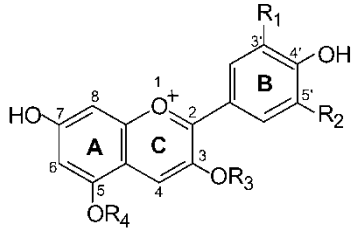
INTRODUCTION

Cyanidin, malvidin, delphinidin, and pelargonidin are aglycones of a group of the most common anthocyanins. Structures of various anthocyanins are presented in the Scheme 1.

Cyanidin and malvidin similar to other anthocyanins easily undergo glycosylation, methylation, and acylation.[1–5] Anthocyanins play an important biological role as antioxidants and free-radical scavengers.[2–4,6–10] In aqueous solutions, anthocyanins exist in various structural forms, depending on pH of the medium, among them flavylium cation (AH^+), quinonoidal base (A), two hemiacetal (B) and two chalcone isomers (C) (Scheme 2.[11,12]) In acidic media ($pH \leq 0.2$), anthocyanins exist as the flavylium ion only (AH^+). In alkaline solutions, the quinonoidal base may be present as ion A^-.[13,14]

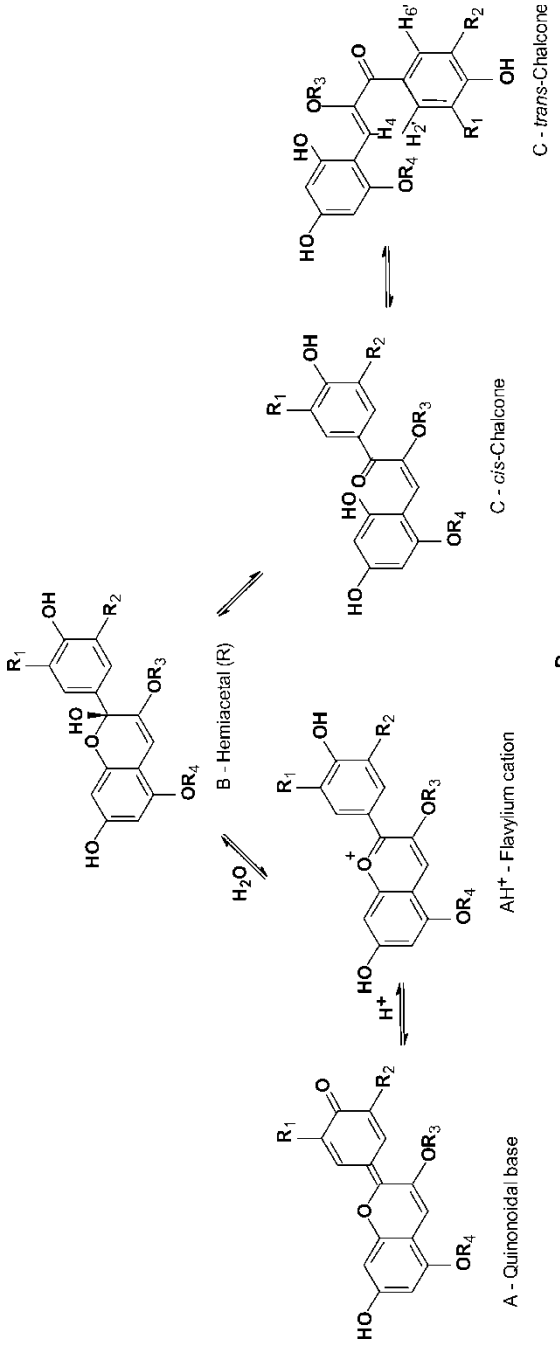
As a result of pH changes, particular forms of anthocyanins undergo reversible changes. Studies conducted using ^1H NMR techniques confirmed the occurrence of two hemiacetal forms (isomer R and S) of anthocyanins.[11,12,15,16] Figueiredo and Lima[13,17] found that malvidin 3,5-diglucoside exists as both *cis*-chalcone and *trans*-chalcone isomers as well as ionized *cis*-chalcone in the excited state.

Investigations on the equilibrium of anthocyanin forms in aqueous solutions at various pH show that the minimum concentration of the forms



	R_1	R_2	R_3	R_4
CYANIDIN	OH	H	H	H
CYANIN	OH	H	Glucose	H
MALVIDIN	OCH_3	OCH_3	H	H
MALVIN	OCH_3	OCH_3	Glucose	Glucose
DELPHINIDIN	OH	OH	H	H
PELARGONIDIN	H	H	H	H

Scheme 1. Structures of selected anthocyanins.



Scheme 2. See Scheme 1 for explanation of abbreviations.

that absorb in the UV-V is region (AH^+, A) can be observed at pH 5.[14,18] Studies on the concentration of structural forms B, C, and A of malvidin 3,5-diglucoside indicate that in weakly acidic solutions (pH > 3.5), the dominant form is hemiacetal B.[13,17,19] Anthocyanins can form dimers and oligomers in water environment. The self-association occur at high concentrations ($>10^{-3}$ mol dm^{-3}).[20]

Some anthocyanins show measurable fluorescence, but information on this topic in the literature is scarce. Previously published monographs describe fluorescence of various forms of malvidin 3,5-diglucoside (Mv 3,5-diglc) and cyanidin 3-glucoside (Cy 3-glc) in aqueous environment at various pH. In particular, it was found that chalcones fluoresce in the spectral range 420–450 nm ($\lambda_{exc} = 320–340$ nm), and the fluorescence of Mv 3,5-diglc centered about 370 nm is assigned to the hemiacetal form. The flavylium ions (AH^+) of both compounds show weak fluorescence, which at $\lambda_{exc} = 520$ nm is characterized by the following parameters: Mv 3,5-diglc, $\lambda^{fl}_{max} = 620$ nm, fluorescence quantum yield ($\Phi_{fl} = 4.1 \times 10^{-3}$; Cy 3-glc, $\lambda^{fl}_{max} = 570$ nm, $\Phi_{fl} = 10^{-4}$) while the quinonoidal base A as ion A^- fluoresces in the range 600–665 nm.[13,17,20] Our previous studies of red cabbage extracts, containing mainly cyanidin derivatives, show that the colorless compounds present in these extracts fluoresce, and that this emission is pH dependent.[21,22]

The fluorescence of anthocyanins has usually been investigated using excitation in the visible region and near UV, that is, at $\lambda_{exc} >$ 270 nm.[13,17,20,21] Low fluorescent quantum yield, especially of colored forms of anthocyanins, is one of the reasons why it is rarely applied in analytics. Nonetheless, during spectroscopic analyses of chokeberry extracts (at pH 5) in the UV region, fluorescence of relatively high intensity was recorded with excitation at 220 nm. The dominant chemical species in these extracts was Cy 3-glc.[23] Therefore, in the current paper, particular attention was paid to the short-wavelength excitation range of anthocyanins, where the absorption band of the highest absorption coefficient is found, particularly for Cy 3-glc.

The aim of the study was to determine the fluorescence of cyanidin 3-glucoside, cyanidin 3,5-diglucoside, and malvidin 3,5-diglucoside, excited in the UV range (220–230 nm and ~280 nm). The studies were conducted in water and a binary solvent (water–methanol at pH 4–5.5). These compounds are widely used as eluents and solvents of anthocyanin pigments.[24,25]

MATERIALS AND METHODS

Chemicals

Cyanidin 3-glucoside (Cy 3-glc) and cyanidin 3,5-diglucoside (Cy 3,5-diglc) as chloride salt were obtained from The Department of Fruit and Vegetable Technology, Agricultural University of Wrocław (Poland). Their purities was measured by high-performance liquid chromatography (HPLC), NMR, and MS.[26] Kuromanin chloride (Cy 3-glc) and malvin chloride (Mv 3,5-

diglc) were purchased from Extrasynthese (France), and L-tyrosine (98% TLC) was from Sigma-Aldrich (Germany). Methanol (spectral grade) was from Fluka (Germany), and all other analytical grade chemicals were obtained from Merck (Germany) and POCH (Poland).

Sample Preparation

Cy 3-glc, Cy 3,5-diglc, and Mv 3,5-diglc were dissolved in two kinds of solvents: water and binary solvent (water–methanol, 1 : 1, v/v). Stock solutions (1.5×10^{-4} to 3.1×10^{-4} mol dm^{-3}) were left to equilibrate in the dark at 2–6°C for about 24 hr and subsequently diluted to final concentrations of 1.4×10^{-5} to 3.6×10^{-5} mol dm^{-3}. The required pH was achieved by addition of hydrochloric acid or sodium hydroxide. The final pH of the Cy 3-glc, Cy 3,5-diglc, and Mv 3,5-diglc solutions ranged from 4 to 5.5. Prior to measurements, the samples were left in a water bath to equilibrate for 2 hr at 25°C, and after this period absorption and fluorescence spectra of the samples were stable for at least 24 hr. L-Tyrosine was dissolved in water, 0.1 mol dm^{-3} phosphate buffer pH 7.[27,28] Ten solutions were prepared at concentrations from 1.5 10^{-5} mol dm^{-3} to 4.3 10^{-4} mol dm^{-3}.

Spectroscopic Methods

The absorption spectra were measured using a spectrophotometer Cary 300 (Varian, Australia).A quartz cuvette with a 1-cm optical path was used for recording the absorption spectra. Steady-state fluorescence measurements (emission and excitation spectra) were performed using a Perkin-Elmer LS50B luminescence spectrometer with the slit widths 6 nm or 8 nm for the excitation and emission. The fluorescence was measured using front face excitation, with emission measured at 90° angle to the exciting beam. A quartz cuvette with an 0.2-cm optical path was used for recording the fluorescence spectra. Such conditions of measurement substantially limited the phenomenon of reabsorption, which was in all cases within the experimental error limits and therefore neglected in subsequent analyses. The fluorescence spectra were corrected for photomultiplier spectral output and whenever necessary by subtracting blank solvent emission. The fluorescence excitation spectra were automatically corrected for spectral distribution of the light source. All experiments were carried out at room temperature and repeated two or three times.

Estimation of Fluorescence Quantum Yield of Cy 3-glc in Binary Solvent

To estimate F_{SH} fluorescence quantum yield η_{SH} of Cy 3-glc, the L-tyrosine as quantum yield standard was used. The fluorescence spectra of these compounds are similar in the sense of λ_{max} and half width. The fluorescence

of tyrosine solution was recorded at the same instrumental setup as F_{SH} fluorescence of Cy 3-glc. For estimation of quantum yield, identical absorbance of Cy 3-glc and tyrosine were applied and quantum yield calculated according to Lakowicz.[28]

Gauss Deconvolution of the Absorption Spectra

The deconvolution of absorption spectra (Gaussian curves) was done with the use of PeakFit v.4.01 software by SPSS Inc. The spectra were preprocessed prior to the deconvolution. The wavelength in nanometers was converted into wavenumbers in cm^{-1} and, if necessary, the baseline parallel to abscissa axis was subtracted. Curves were then calculated using Savitsky-Golay 1% smoothing and 'Gauss Amp' function without the 'Vary Width' option and without the 'Allow Negative' option. The remaining parameters were used as default options.

RESULTS

Absorption and Fluorescence of Cyanidin 3-Glucoside in Aqueous Environment

The absorption and fluorescence properties of Cy 3-glc in binary solvent and water at pH 5 have been examined. The absorption spectra in the UV-Vis region are presented in Fig. 1, curves 1 and 2. Comparison of these spectra

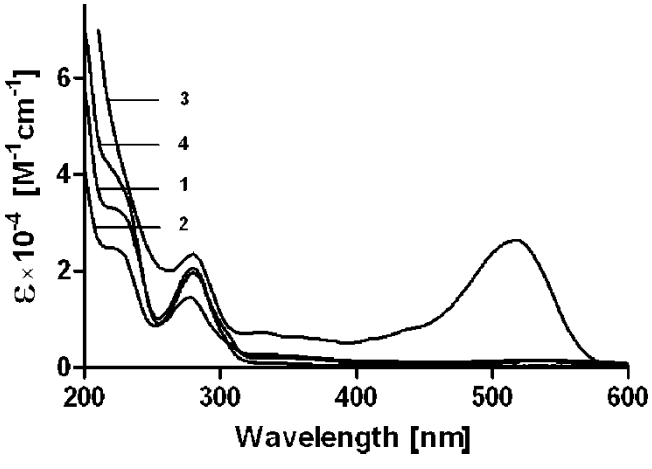


Figure 1. Absorption spectra of Cy 3-glc in binary solvent (water–methanol, 1:1, v/v) at (1) pH 5 and (3) pH 0.2, and (2) in water at pH 5; (4) absorption spectrum of Cy 3,5-diglc in binary solvent at pH 5.

with the absorption spectrum of the flavylium ion (Fig. 1, curve 3, pH 0.2) shows that in binary solvent and water at pH 5, mainly Cy 3-glc colorless forms exist. Their absorption spectrum in the UV region differs from that of the flavylium ion especially at 220–230 nm (the range used to excite the fluorescence, see below). These results agree with the literature data reported for anthocyanins.[2,13,14,17,29]

The fluorescence of Cy 3-glc in the binary solvent at pH 5 was excited in the range of 210–290 nm, with the most effective excitation at $\lambda_{exc} = 220$ to 230 nm and $\lambda_{exc} = 280$ to 290 nm. It was found that Cy 3-glc shows short-wavelength fluorescence F_{SH} with the maximum at $\lambda^{fl}_{max} = 299$ nm (Fig. 2a, curves 1 and 2) and long-wavelength fluorescence of lower intensity with maximum at about 356 nm (Fig. 2a, curve 3). The fluorescence excitation spectra, monitored at 320 nm, show maxima at 275 nm and 220 nm, the latter of much higher intensity (Fig. 2a, curve 4). The excitation maximum of the long-wavelength fluorescence ($\lambda^{fl}_{max} = 356$ nm) is observed at about $\lambda_{exc} = 282$ nm (Fig. 2a, curve 5), but the most selective excitation of this band is achieved at $\lambda_{exc} = 290$ nm, so this wavelength was used for further study.

The fluorescence spectra of Cy 3-glc in water, recorded at the same conditions as above, are presented in Fig. 2b. Two maxima at 300 nm and ~356 nm were observed (Fig. 2b, curve 1 or 2 and curve 3), but fluorescence intensity of Cy 3-glc in water is about twofold lower than that measured in the binary solvent (see Figs. 2a and 2b, curves 1). The fluorescence excitation spectrum of Cy 3-glc in water at pH 5 for $\lambda_{em} = 300$ nm indicates that the most effective excitation of short-wavelength fluorescence F_{SH} is achieved at $\lambda_{exc} = 220$ nm, and less intensive at $\lambda_{exc} = 272$ nm (Fig. 2b, curve 4). The long-wavelength fluorescence (~356 nm) was excited at about $\lambda_{exc} = 282$ nm (Fig. 2b, curve 5). Similar fluorescence patterns were observed in the binary solvent at pH 5 (see above, Fig. 2a). We conclude that Cy 3-glc generates similar fluorescence species in water at pH 5 as those in the binary solvent at the same pH.

The short-wavelength fluorescence quantum yield η_{SH} of Cy 3-glc was estimated, and $\eta_{SH} = 0.0032$ was obtained. The estimated quantum yield of Cy 3-glc can be treated as apparent fluorescence quantum yield, because in our experiment it was impossible to eliminate both the effect of inner filter and the quenching by another structural form of Cy 3-glc that coexists in solution.

When Cy 3-glc solutions were excited at $\lambda_{exc} = 330$ nm (i.e., within the absorption band characteristic of the chalcone forms of anthocyanins,[13,17,20,30]) only traces of fluorescence characteristic to these forms (~450 nm) were observed.

Absorption and Fluorescence of Cyanidin 3,5-Diglucoside in Binary Solvent

The absorption spectrum of Cy 3,5-diglc in the binary solvent at pH 5 is shown in Fig. 1, curve 4. Figure 2c shows the fluorescence spectrum (curve 1) and the

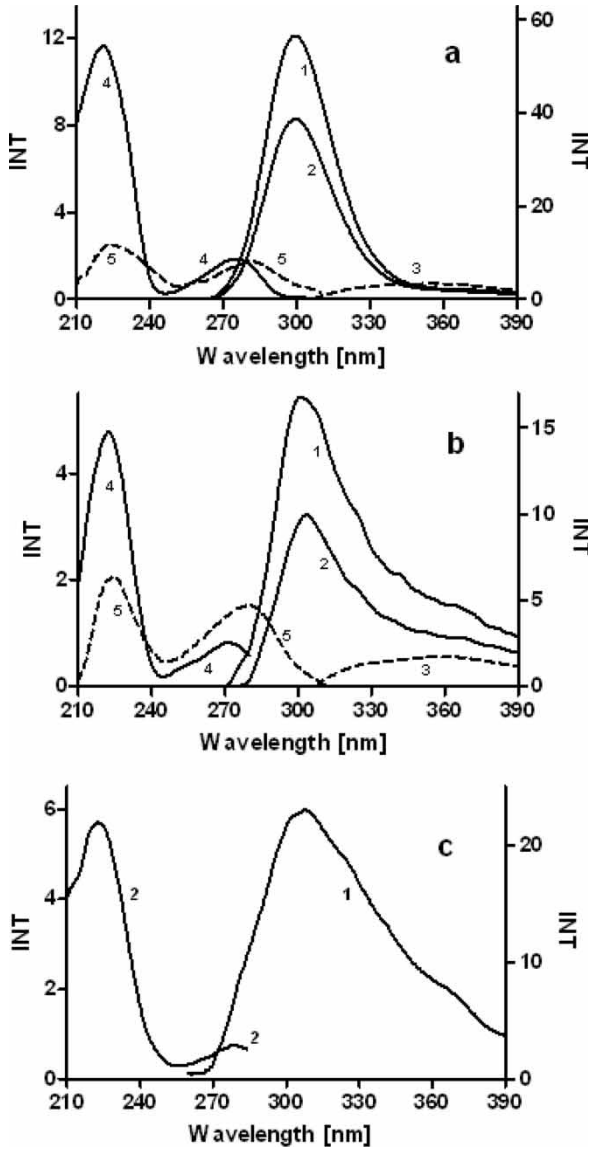


Figure 2. Fluorescence spectra (right axis) and fluorescence excitation spectra (left axis) of Cy 3-glc and Cy 3,5-diglc. (a) Cy 3-glc in binary solvent (water–methanol, 1:1, v/v, pH 5); fluorescence spectra: (1) $\lambda_{exc} = 220$ nm, (2) $\lambda_{exc} = 230$ nm, (3) $\lambda_{exc} = 290$ nm; fluorescence excitation spectra: (4) $\lambda_{em} = 320$ nm, (5) $\lambda_{em} = 360$ nm. (b) Cy 3-glc in water, pH 5; fluorescence spectra: (1) $\lambda_{exc} = 220$ nm, (2) $\lambda_{exc} = 230$ nm, (3) $\lambda_{exc} = 285$ nm; fluorescence excitation spectra: (4) $\lambda_{em} = 300$ nm, (5) $\lambda_{em} = 360$ nm. (c) Cy 3,5-diglc in binary solvent, pH 5; fluorescence spectrum: (1) $\lambda_{exc} = 220$ nm; fluorescence excitation spectrum: (2) $\lambda_{em} = 300$ nm. INT, fluorescence intensity (a.u.).

fluorescence excitation spectrum (monitored at 300 nm, curve 2) of the Cy 3,5-diglc. This compound is also characterized by a short-wavelength fluorescence F_{SH}, centred at 308 nm (Fig. 2c, curve 1). The fluorescence excitation spectrum shows two maxima, at 220 nm and around 280 nm, the former more intense (Fig. 2c, curve 2). This suggests that the S_1^* state, which corresponds with the absorption band of 280 nm, is the lowest energy excited state from which short-wavelength fluorescence F_{SH} occurs.

Absorption and Fluorescence of Malvidin 3,5-Diglucoside in Aqueous Environment

Mv 3,5-diglc in water and binary solvent was examined at pH 4.2. The absorption spectra in UV are shown in Fig. 3, curves 1 and 2, respectively (cf. Refs.[2,11,13,17,30]). The absorption spectrum of Cy 3-glc (curve 3) is given for comparison. It is evident that these spectra differ profoundly within the ranges 220 to 230 nm and 300 to 400 nm, the latter characteristic to the chalcone forms.[17] Lower values of a molar extinction coefficient around 330 nm observed for Mv 3,5-diglc in binary solvent, in comparison with water, indicate lower concentration of the chalcone forms in the former solvent (Fig. 3, curves 1 and 2).

The fluorescence spectra of Mv 3,5-diglc were measured at several excitation wavelengths in the UV region, with $\lambda_{exc} = 330$ nm and $\lambda_{exc} = 220$ to 230 nm being the most effective. These spectra are shown in Figs. 4a and 4b, respectively. In water, the fluorescence of Mv 3,5-diglc centred at 495 nm was observed for the excitation at $\lambda_{exc} = 330$ nm (Fig. 4a, curve 1).

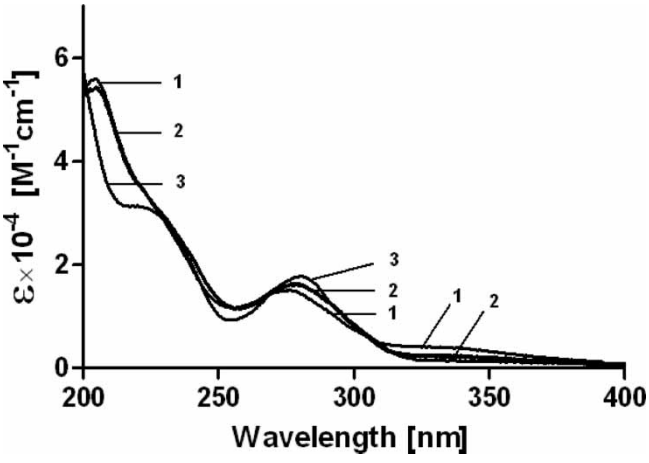


Figure 3. Absorption spectra of Mv 3,5-diglc (1) in water at pH 4.2, (2) in binary solvent (water–methanol, 1:1, v/v, pH 4.2); Cy 3-glc in binary solvent (3) at pH 5.2.

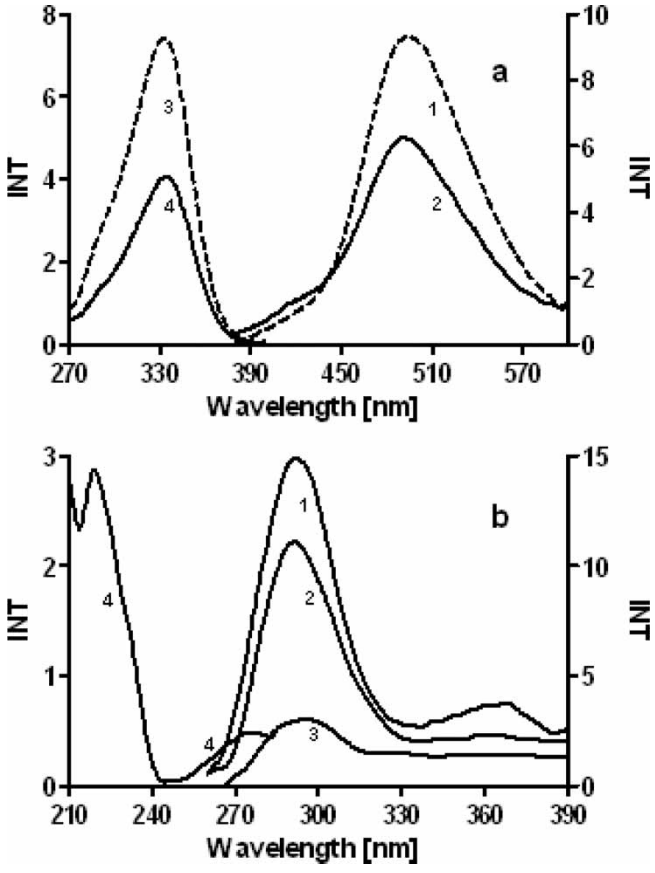


Figure 4. Fluorescence spectra (right axis) and fluorescence excitation spectra (left axis) of Mv 3,5-diglc in binary solvent (water–methanol, 1:1, v/v) and water, pH 4.2. (a) Fluorescence spectra ($\lambda_{exc} = 330$ nm): (1) in water and (2) in binary solvent; fluorescence excitation spectra ($\lambda_{em} = 490$ nm): (3) in water and (4) in binary solvent. (b) Fluorescence spectra in binary solvent: (1) $\lambda_{exc} = 220$ nm and (2) $\lambda_{exc} = 230$ nm; in water (3) $\lambda_{exc} = 220$ nm; (4) fluorescence excitation spectrum ($\lambda_{em} = 300$ nm) in binary solvent. INT, fluorescence intensity (a.u.).

This fluorescence was attributed to the chalcone form ionized in excited state C^{*-}. In the case of Mv 3,5-diglc dissolved in the binary solvent, somewhat weaker fluorescence with a maximum at about 492 nm was recorded together with a shoulder at about 420 nm, attributed to chalcone forms C^{*-} and C, respectively (Fig. 4a, curve 2). The fluorescence excitation spectra measured for the emission wavelength of 490 nm in both media (Fig. 4a, curves 3 and 4) confirms the presence of malvidin chalcone forms, as the active absorption band of these forms lies within the spectrum range close to 330 nm.[11,13,15,17]

Additionally, in binary solvents of Mv 3,5-diglc, where the proportion of chalcone forms responsible for the absorption and fluorescence is lower (see above), a short-wavelength fluorescence F_{SH} was detected, centered at 293 nm (at an excitation of $\lambda_{exc} = 220$ and 230 nm) with a shoulder at about 368 nm (Fig. 4b, curve 1 and 2). In water, this F_{SH} fluorescence is significantly less intense, but still detectable (Fig. 4b, curve 3). The excitation spectrum of F_{SH} fluorescence (Fig. 4b, curve 4) shows maximum intensity at $\lambda_{exc} = 220$ nm, with another, less effective, excitation band at about 280 nm. The latter indicate location of the $S_1^* \leftarrow S_0$ electronic transition of the form responsible for the F_{SH} fluorescence.

Attempts at Separating the Active Absorption in the Absorption Spectrum of the Anthocyanins

Comparing the absorption spectra of the analyzed anthocyanins and their excitation spectra indicates that only part of the absorption spectra corresponds with active absorption. Attempts have been made to isolate the bands of active absorption which excites F_{SH} fluorescence in Cy 3-glc, Cy 3,5-diglc, and Mv 3,5-diglc in binary solvent.

To achieve this goal, absorption spectra of Cy 3-glc, Cy 3,5-diglc, and Mv 3,5-diglc, were analyzed by Gauss deconvolution within the range of 50,000 to 38,461 cm^{-1} (200 to 260 nm). The distribution in which one of the Gauss peaks corresponded with active absorption (i.e. centred at about 220 nm) was assumed to be the most likely (see fluorescence excitation spectra, Fig. 2a, 2c, and 4b). Figures 5a and 5b show the Gaussian distribution of the absorption spectra of Cy 3-glc and Mv 3,5-diglc, respectively, in binary solvent. The distribution of the absorption spectrum of Cy 3,5-diglc is similar in character to that of Cy 3-glc. Gauss bands no. 3 in Figs. 5a and 5b, which occur in excitation range centered at 220 nm, can be treated as the active bands for F_{SH} fluorescence of Cy 3-glc and Mv 3,5-diglc.

A comparison of the obtained distributions with the corresponding excitation spectra (e.g., Fig. 5a and Fig. 2a, curve 4) indicates that within the spectral range of the applied excitation (220–230 nm) of F_{SH} fluorescence, the active absorption band is accompanied by two or three bands of non-active absorption. The amount of absorbed energy at $\lambda_{exc} = 220$ nm was calculated ($E_a = 1$ to 10^{-A_a}, where A_a is active absorbance within the range of the absorption band 3) and expressed as percent of the total amount of energy E_{tot} absorbed at that wavelength (E_a/E_{tot}). This value equals 90.5% for Cy 3-glc and 89.4% for Cy 3,5-diglc and much less for Mv 3,5-diglc (only 61.0%). This means that the structural forms of anthocyanins that do not emit F_{SH} fluorescence absorb part of the energy and create the so-called inner filter effect. This effect is particularly pronounced when Mv 3,5-diglc solutions are excited at 220 nm.

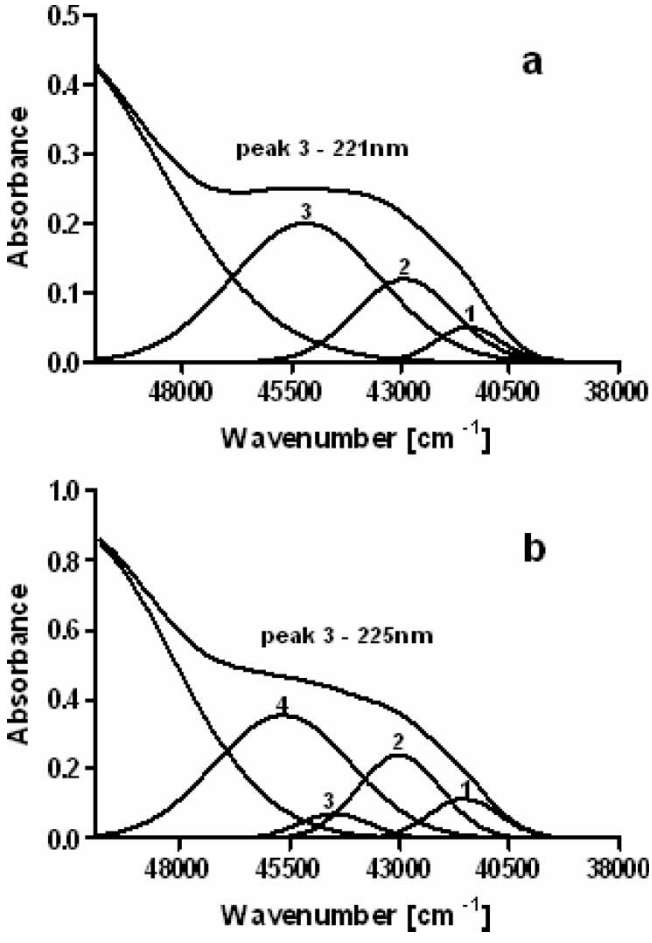


Figure 5. The Gaussian deconvolution of absorption spectra in binary solvent: Cy 3-glc (a); Mv 3,5-diglc (b). The Gaussian peak 3 is the band of the active absorption for F_{SH} fluorescence.

DISCUSSION

Cyanidin and malvidin glycosides, similar to other anthocyanins, exist in aqueous environment in various structural forms, depending on pH. Spectral investigations (NMR and electronic absorption) revealed coexistence of flavylium cation (AH^+), quinonoidal base (A), two hemiacetal (B), and two chalcone isomers (C). Equilibrium between the foregoing structures is highly pH-dependent, and, except for cis–trans chalcone isomerizations, all transformations are fully reversible with reaction half-time of several minutes or less.[11,12,15–17,30]

At pH < 2, anthocyanins exist predominaetly as flavylium cations, characterized by the extended π-electronic system, and, consequently, electronic absorption spectra in the visible region. Upon elevation of pH to 4–6, the flavylium cation undergoes two processes (see Scheme 2):

- fast deprotonation of AH^+, leading to two tautomeric quinoidal bases, and/or,
- hydration via the nucleophilic attack, leading in effect to two optical isomers R and S of the hemiacetal structure (B); the latter can subsequently undergo ring-opening, forming *cis*-chalcone (C_{cis}) with restituted large π-electron system (see Scheme 2), with possibility of further (usually slow) isomerisation to the *trans*-chalcone (C_{trans}) structure.

Kinetic and thermodynamic investigations of anthocyanins in aqueous environment, utilizing ^{1}H NMR revealed the existence of dynamic equilibrium between hemiacetals and chalcone structures. At weakly acidic pH (4–6), this equilibrium is usually shifted toward the hemiacetal forms but somewhat differs for various anthocyanins (in particular, it is sensitive to substituents in the B ring), and for various solvents.[11–19,30]

In our investigations, anthocyanin stock solutions (concentration 10^{-4} mol dm^{-3}) were dissolved to final concentrations of $\sim 10^{-5}$ mol dm^{-3}, and pH adjusted accordingly. The new equilibrium dependent on the above factor was established.[16,19,30] The low concentration of dyes indicate that anthocyanin exists as monomers in these solutions.

Short-Wavelength Fluorescence of Cyanidin Glycosides

The cyanidin glycosides are virtually regarded as nonfluorescent or exhibit very faint fluorescence, at least in neutral and weakly acidic aqueous media, and malvidins emit weakly in the visible region.[11,12,14–17,20] To our knowledge, there is no report in the literature describing the short-wavelength fluorescence F_{SH} in cyanidin and malvidin glycoside solutions. This emission is preliminarily characterized in the current work.

The results of emission measurements of Cy 3-glc in binary solvents at pH 5 (Fig. 2a) show that the short-wavelength fluorescence F_{SH} with relatively high intensity is characterized by $\lambda^{fl}_{max} = 299$ nm, that is, $\tilde{\nu}^{fl}_{max} = 33{,}445$ cm^{-1}, half width $\Delta\tilde{\nu}_{1/2\,max}^{fl} = 3500$ cm^{-1}, and estimated fluorescence quantum yield $\eta_{SH} = 0.0032$. In water at pH 5, Cy 3-glc generates similar fluorescence species as in the binary solvent (Fig. 2b). F_{SH} fluorescence Cy 3-glc, observed in aqueous media, is most effectively excited at 220 nm, where the molar extinction coefficient of the compound is high (Figs. 1 and 2). Other effective excitation range is near 280 nm, characteristic of absorption band of the flavonoids with no π-electron coupling between the two rings systems (e.g., hemiacetal forms of anthocyanins.[11]) In aqueous media

is also observed faint long-wavelength fluorescence of Cy 3-glc at $\lambda_{max}^{fl} = 356$ nm.

Attempts have been made to assign the observed emission to particular structural forms of Cy 3-glc by comparing the results of our investigations with available spectroscopic data from the literature. The fluorescence excitation spectrum, monitored at 320 nm (Fig. 2a, curve 4), shows that the long-wavelength excitation maximum for F_{SH} fluorescence, corresponding with the maximum of active absorption ($S_0 \rightarrow S_1$ transition), is centered at $\sim$275 nm. This is a range of the absorption band characteristic of the hemiacetal forms of anthocyanins[11–13,15–17,19,29] and is clearly distinct from the long-wavelength absorption bands of the chalcones ($\sim$330 nm), thus excluding the latter structures as putative sources of F_{SH} fluorescence. It is therefore justified to postulate that fluorescence F_{SH} ($\lambda_{max} = 299$ nm), excited at either 275 nm or 220 nm should be assigned to the hemiacetal isomers of Cy 3-glc. If chalcones and/or other forms of anthocyanins absorb at about 220 and 280 nm, it is nonactive absorption for F_{SH} fluorescence.

The short-wavelength fluorescence F_{SH} can be also observed in Cy 3,5-diglc (Fig. 2c), with maximum emission shifted to 308 nm and excitation maxima at 220 and 280 nm. We interpret this emission the same as above.

This interpretation is also confirmed by observation that malvidin glycosides, in addition to the fluorescence of the chalcone form ($\lambda_{max}^{fl} = 495$ nm), are also characterized by F_{SH} fluorescence (Figs. 4a and 4b). The short-wavelength fluorescence F_{SH} of Mv 3,5-diglc in binary solvent (Fig. 4b), with its maximum shifted hypsochromically to 293 nm, has much lower intensity in comparison with the Cy 3,5-diglc or Cy 3-glc when measured under similar conditions. In water this F_{SH} fluorescence is hard to observe for Mv 3,5-diglc. This observation supports our interpretation of F_{SH} fluorescence as originating from the hemiacetals. The short-wavelength fluorescence F_{SH} spectra of malvidin and cyanidin glycosides, measured in binary solvent under similar conditions, are displayed in Fig. 6.

Nonetheless, it is reasonable to expect at this point that the short-wavelength and relatively intense fluorescence F_{SH} centered at $\sim$300 nm may have analytical applications as an indicator of hemiacetal-type structures of anthocyanins in solutions.

The Effect of Binary Solvent on the Short-Wavelength Fluorescence of Anthocyanin Glycosides

Qualitative observations indicate that short-wavelength fluorescence F_{SH} of cyanidin and malvidin glycosides is more intense in water–methanol solvents in comparison with water solutions of the same pH. This may result either from the equilibrium shift in favor of the hemiacetal forms or from the increase of the intrinsic fluorescence yield.

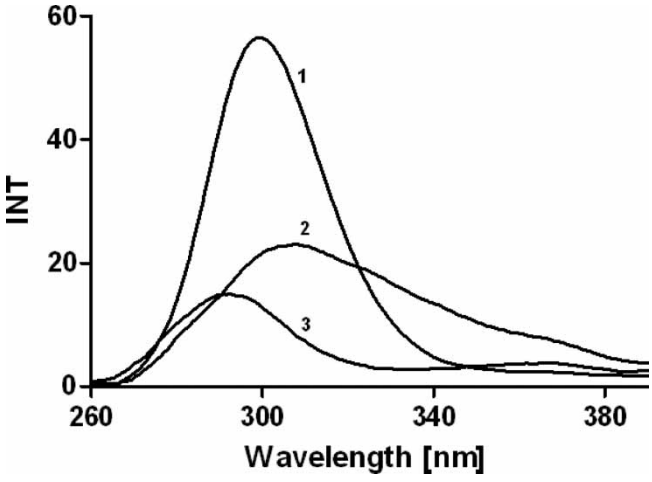


Figure 6. Short-wavelength fluorescence spectra (F_{SH}) of Cy 3-glc, Cy 3,5-diglc, and Mv 3,5-diglc in binary solvent pH 4.2–5, $\lambda_{exc} = 220$ nm: (1) Cy 3-glc, (2) Cy 3,5-diglc, and (3) Mv 3,5-diglc. INT, fluorescence intensity (a.u.).

It is known that flavonoids in methanol solvents can interact with the solvent via hydrogen bonding.[31–33] In binary solvent (water–methanol, 1:1, v/v), where water and methanol molecules have a similar dipole moment, 1.85D and 1.70D respectively, the solvation shell of Cy 3-glc contains both water and methanol molecules.[34] It is suggested that specific interactions via the hydrogen bonding of methanol to the anthocyanin rings in solvation shell can stabilize the F_{SH} fluorescing hemiacetal forms of Cy 3-glc, Cy 3,5-diglc, and Mv 3,5-diglc in applied solutions in this study.

We have performed quantitative analysis of the UV absorption spectra of the investigated anthocyanin glycosides in binary solvent in an attempt to identify the active absorption bands responsible for the short-wavelength fluorescence F_{SH}. For the active absorption band, the absorbance at 220 nm has been calculated to constitute $\sim$90% of total absorbance for cyanidin glycosides but only 61% for Mv 3,5-diglc (Figs. 5a and 5b, bands 3). These calculations are fully in line with the previous conclusions and indicate that the non-active absorption of chalcone forms may act as an internal filter, thus further lowering the intensity of F_{SH} fluorescence. It indicates that at higher concentrations of the anthocyanins, such as encountered *in vivo*, this effect can be more intensive. This may explain why the short-wavelength fluorescence was not detected in previous works.

It must also be taken into account that the excitation energy of the hemiacetal forms can undergo radiationless transfer to other (e.g., chalcone) forms of anthocyanins, causing dynamic quenching of the short-wavelength fluorescence F_{SH}. Therefore, the intensity of F_{SH} fluorescence of cyanidin and malvidin glycosides depends not only on the fluorescence quantum yield

and the concentration of the fluorescing form but also on the concentrations of other structural forms present in the solution. These forms may play the role of both an internal filter and an energy acceptor in dynamic quenching.

CONCLUSIONS

The results of the experiments presented in this paper show that Cy 3-glc in binary solvent (water–methanol, 1:1, v/v) and in water at pH 5 exhibits short-wavelength fluorescence F_{SH} with $\lambda^{fl}_{max} = 299$ nm. The estimation of F_{SH} fluorescence quantum yield η_{SH} gave the value of 0.0032. The excitation of this fluorescence was most effective at $\lambda_{exc} = 220$ nm. F_{SH} fluorescence can also be observed in binary solvent of Cy 3,5-diglc ($\lambda^{fl}_{max} = 308$ nm) and Mv 3,5-diglc, the latter with $\lambda^{fl}_{max} = 293$ nm. It seems that the observed F_{SH} fluorescence may be attributed to the hemiacetal forms of the analyzed anthocyanins.

The intensity of the observed F_{SH} fluorescence of Mv 3,5-diglc ($\lambda^{fl}_{max} = 293$ nm) correlates negatively with the intensity of fluorescence of chalcone forms of Mv 3,5-diglc ($\lambda^{fl}_{max} = 496$ nm), at least in the solutions investigated in this study. This implies that conversion of hemiacetal forms into chalcones of Mv 3,5-diglc in aqueous media may compete with the creation of the F_{SH} fluorescing form of Mv 3,5-diglc. The presence of methanol in the solution stabilizes forms responsible for the F_{SH} fluorescence of Cy 3-glc, Cy 3,5-diglc, and Mv 3,5-diglc.

The intensity of F_{SH} fluorescence of cyanidin and malvidin glycosides in aqueous environment depends not only on the fluorescence quantum yield and the concentration of the fluorescing form but also on the concentrations of other structural forms present in solution. These forms may act as of both internal filters and energy acceptors in dynamic quenching.

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