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Tautomerism of flavonol glucosides: relevance to plant UV protection and flower colour

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Abstract

The fluorescence *emission* spectra of the naturally occurring flavonoids, quercetin-7-glucoside, a 3′,4′-dihydroxyflavonol, and kaempferol-7-glucoside, a 4′-hydroxyflavonol, have been determined as a function of concentration in aqueous solutions. These spectra indicate that the extent of keto–enol phototautomerism in both flavonoids is greatest at high concentrations: a situation which favours molecules aggregation/dimerization. Such behaviour is consistent with phototautomerism being facilitated by a concerted, intermolecular transfer of protons between the partners in the flavonoid dimer. This excited state tautomerism dissipates absorbed energy harmlessly and as such provides a possible mechanism by which these molecules may function in the protection of plants from damaging UV radiation. The fluorescence *excitation* spectra of both kaempferol and quercetin-7-glucosides at high concentrations in aqueous solutions indicate the presence of significant amounts of the enolic tautomeric form in the ground-state. At lower concentrations only the kaempferol glucoside spectrum shows this. When kaempferol- and quercetin-7-glucosides were deposited on a cellulosic support, their reflectance and fluorescence excitation spectra could be resolved into contributions from keto and enol ground-state chromophores. The absorption of the enolic tautomer is at longer wavelengths (ca. 450 nm) than that of the keto tautomer (ca. 370 nm) and as it extends into the blue spectral region, would account for the yellow appearance of these flavonols in aggregation on cellulose and in concentrated solution in petal vacuoles.

Keywords: Flavonoids; Tautomerism; UV protection; Flower colour

1. Introduction

Flavonoids are a group of polyphenolics, which are present in virtually all land-based plants. They display strong absorption in the UV spectral region and the flavonols, that is, 3-hydroxyflavones, exhibit fluorescence with excitation maxima about 350–370 nm. In many solvents and molecular environments two emission bands are apparent with maxima at 400–440 nm and 510–540 nm [1–9]. These fluorescences correspond to the radiative relaxations from the first excited singlet states (S_1^*) of the keto form of the molecules (type structure I) and from the enolic benzopyrilium tautomer (type structure IIe), respectively [2–9], as depicted in Fig. 1. This fluorescence behaviour has been investigated widely and provides a valuable means of examining the photophysics associated with tautomerism in the excited states of these molecules. Excitation of flavonols facilitates excited state intramolecular proton transfer (ESIPT) from the 3-hydroxy group to the neighbouring carbonyl group [1–9]. ESIPT is very sensitive to competition and interference from intermolecular proton transfer in hydrogen-bonding solvents/molecular environments. In the case of the simplest and most extensively studied flavonol, namely, 3-hydroxyflavone, the presence of small amounts of water in hydrocarbon solvents causes a substantial reduction in tautomerism which is apparent as a diminution of the fluorescence yield of the long-wavelength band [2–9].

In addition to the flavonols, other aromatic molecules possessing a basic carbonyl group and neighbouring OH or NH group exhibit intramolecular proton transfer and tautomerism in their excited states [10–12]. In molecules such as 7-hydroxyquinoline and N-phenylbenzamide this process is concentration-dependent and it has been concluded that a concerted biprotic transfer between the constituent monomeric partners of a dimer occurs in concentrated solutions [10].

It has been suggested that protection against UVB-induced damage may be an important function of flavonols in plants [13], a role which is supported by the finding that

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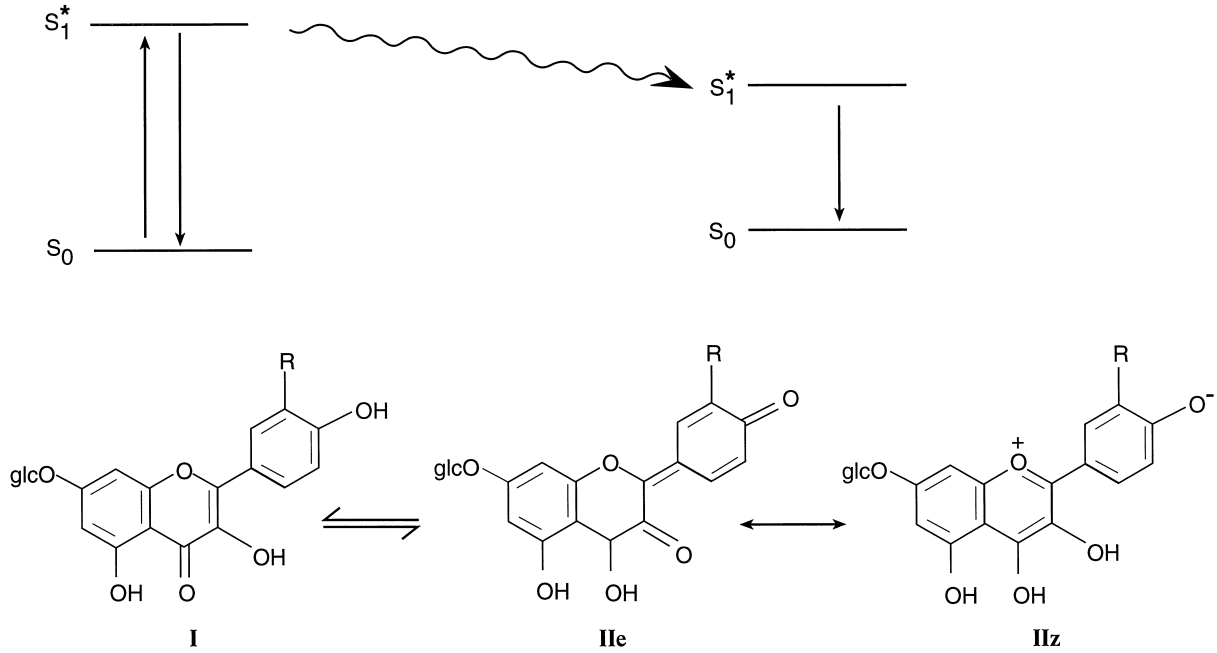

Fig. 1. Proposed scheme for photo-induced tautomerism of flavonol-7-O-glucosides together with relative energy levels of their ground and first excited electronic states. I-keto tautomer (R=H, kaempferol-7-O-glucoside; R=OH, quercetin-7-O-glucoside), IIe=enolic tautomer, IIz=zwitterionic form of enolic tautomer.

their biosynthesis in UV-tolerant plants is commonly stimulated by exposure of these plants to UVB radiation [1,9]. UV protection of plants by flavonols may be achieved in a number of ways including radiation screening/absorption and free radical or singlet oxygen scavenging [14,15]. Although flavonols are effective UVB absorbers, they must also have the capacity to dissipate absorbed UV radiation energy harmlessly in some way. Indeed, UVB absorbance by itself cannot account for the different protective effectivenesses observed for similar UV-absorbing flavonols such as kaempferol and quercetin, and apigenin and luteolin [16]. The photoinduced tautomerism which has been observed for a number of flavonoids could afford the energy dissipation mechanism required of a protective UV screening molecule.

It is also possible that the ground-state of the enolic tautomer is present in flavonol dimers/aggregates formed by intermolecular proton transfer, analogous to that described for 7-hydroxyquinoline in concentrated solutions [11]. It is apparent from Fig. 1 that the ground-state (S_0^*) of an enolic tautomer would absorb lower energy radiation (red-shifted) than the ground-state of the predominant keto form. This would have implications for the colour of the flavonol. A number of examples have been reported in which intense yellow flower colouration is produced by flavonols which are essentially colourless in solution [17,19]. So far no explanation has been advanced for this phenomenon.

To date the best photophysically characterised flavonoid system, that is, 3-hydroxyflavone (flavonol) in non-aqueous environments [1–9], are not found in nature. Most of the flavonoids in plants are present in the cell vacuole as water-soluble glycosides and sometimes at very high concentrations [17–19]. The photophysical and spectroscopic features of two naturally occurring flavonol glycosides which are relevant to UV protection and flower colour are examined in the work described in this report. Specifically, the concentration dependencies of keto–enol tautomerism of quercetin-7-glucoside (Q7G) and kaempferol-7-glucoside (K7G) in aqueous and alcoholic solutions have been determined by measuring the fluorescence associated with the keto and enol forms of these flavonols.

2. Experimental details

Samples of synthetic kaempferol- and quercetin-7-O-glucosides were provided by Prof. H. Geiger, Department of Botany, University of Saarland, Saarbrucken, Germany.

Single distilled water was deionised by passage through a Milli-Q ion exchange and filter system. The pH of this water was 6.0–7.0. Solutions of Q7G and K7G, covering a range of concentrations up to 5×10^{-4} mol dm^{-3}, were prepared in HPLC-grade methanol. Saturated solutions of the flavonols were prepared in water by sonication at room temperature followed by centrifugation to remove any undissolved solute remaining in suspension. The concentrations of these saturated solutions were 3.0×10^{-5} mol dm^{-3} and 6×10^{-5} mol dm^{-3} for Q7G and K7G, respectively. A series of aqueous solutions of lower concentrations was prepared by dilution. Absorption spectra were determined using a Hewlett Packard 8451A diodearray spectrophotometer and fluorescence excitation and emission spectra were recorded on a Hitachi 3010 spectrofluorimeter. The relative

fluorescence yields of the various flavonoid fluorophores were determined by measurement of the area under the bands of the fluorescence spectra. The spectrofluorimeter was also used to determine the reflectance spectra of the flavonols which were deposited from their methanol solutions on Whatman 3MM chromatography paper (which had been prewashed with methanol). The paper coated with flavonol was thoroughly dried by gentle heating.

3. Results

3.1. Fluorescence emission in solution

In methanolic solutions, the fluorescence emission spectra of Q7G and K7G excited at 370 nm are shown in Fig. 2. These spectra exhibit two broad bands with maxima at about 450 and 530 nm. The shorter wavelength band of K7G can be resolved into two components with maxima at 425 and 470 nm. The fluorescence yields of the longer wavelength emissions are 1.6 and 2.4 times greater than that at shorter wavelengths for Q7G and K7G, respectively, and are independent of concentration. The fluorescent excited states are produced as a result of excitation of a single form of the ground-state which has an absorption maximum at 370 nm for Q7G and at 365 nm for K7G.

In an aqueous solution, the fluorescence emission spectra of Q7G and K7G at different concentrations are shown in Fig. 3((a) and (b)), respectively. As was the case in methanolic solutions, two emission bands are apparent for the flavonols in water. The broad, shorter wavelength bands have maxima at ~405 and ~440 nm for Q7G and K7G, respectively, and the longer wavelength bands at 510 nm. In water the relative yields of fluorescence associated with the two major emission bands depend on flavonol concentration. There is an increased yield of the long wavelength emission band relative to that of the shorter wavelength band at higher concentrations. This concentration dependence of relative fluorescence yields is summarised in Table 1.

Table 1
The concentration dependence of the relative fluorescence yields of the keto forms, F_k and enol forms, F_E, of Q7G and K7G

Flavonol	Concentration (mol dm^{-3})	F_K/F_E
Q7G	3×10^{-5}	1.0
	1.5×10^{-5}	1.0
	5×10^{-6}	1.4
	2×10^{-6}	1.7
	1×10^{-6}	3.5
K7G	6×10^{-5}	0.9
	3×10^{-5}	0.9
	3×10^{-6}	1.0
	1.5×10^{-6}	1.0
	1.0×10^{-6}	3.6

3.2. Fluorescence excitation in solution

In aqueous solutions at lower concentrations, that is, $<10^{-5}$ mol dm^{-3}, Q7G exhibits a single fluorescence excitation band for emission at both 450 and 520 nm. This excitation band has a maximum at 360 nm which corresponds to the absorption maximum of this flavonol. However, at higher concentrations, near saturation, a second excitation band is apparent for emission at 520–530 nm

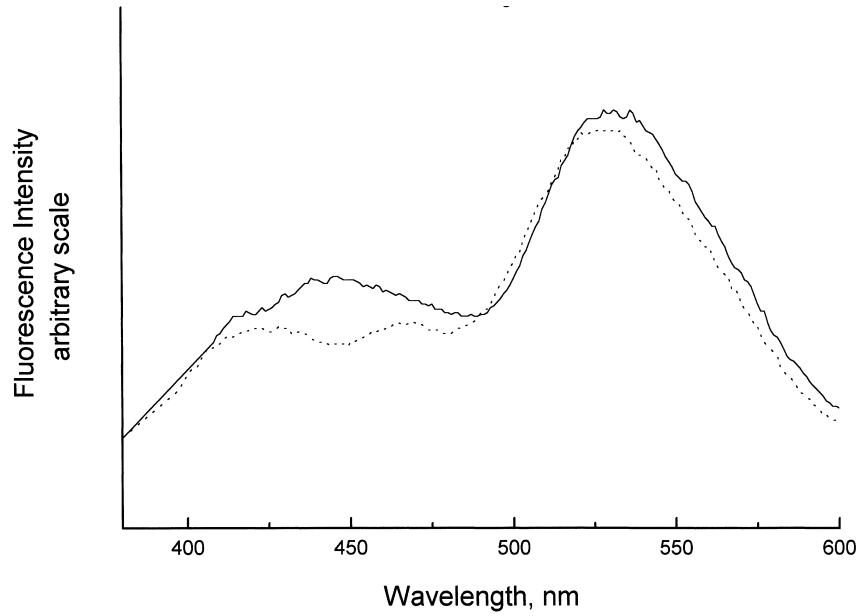

Fig. 2. Fluorescence emission spectra of the flavonols in methanol. ——— Q7G, and ..... K7G. The units of the fluorescence intensity are arbitrary.

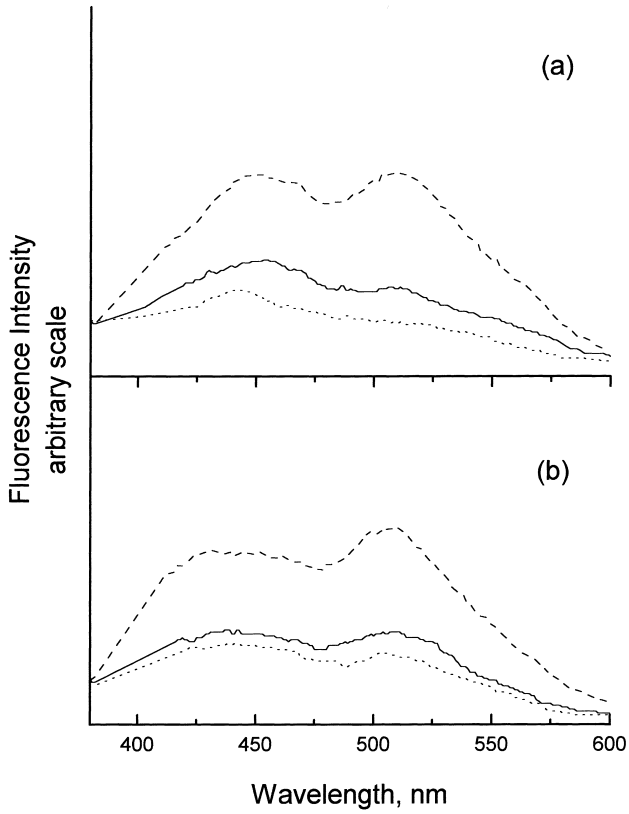

Fig. 3. Fluorescence emission spectra of the flavonols in an aqueous solution as a function of concentration (a) Q7G, - - - 3×10^{-5} mol dm^{-3}; —— 5×10^{-6} mol dm^{-3}; ..... 1×10^{-6} mol dm^{-3} (b) K7G; - - - 4×10^{-5} mol dm^{-3}; —— 4×10^{-6} mol dm^{-3}; ..... 2×10^{-6} mol dm^{-3}.

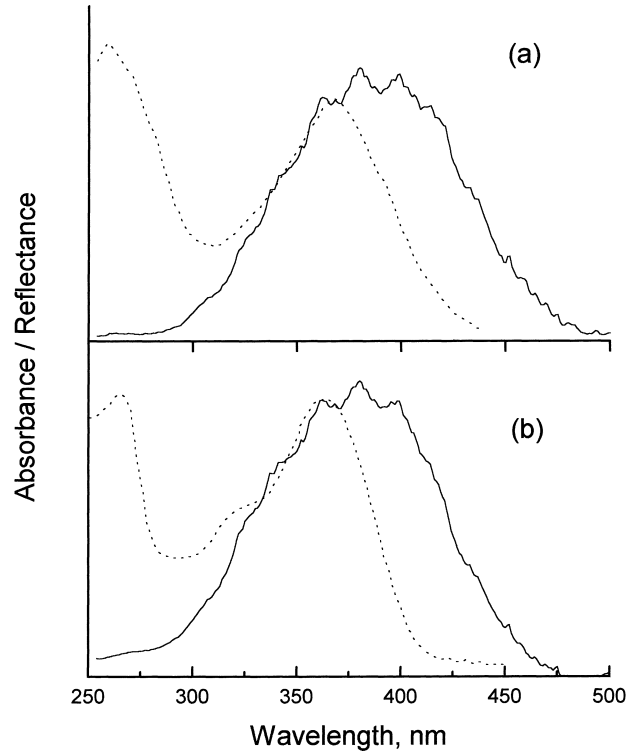

Fig. 4. (a) Fluorescence excitation spectrum of Q7G deposited on dry cellulose, —— compared with the absorption spectrum in aqueous solutions, ... (b) Fluorescence excitation spectrum of K7G deposited on dry cellulose, —— compared with the absorption spectrum in aqueous solution, ...

with a maximum at 450 nm. This contrasts with the fluorescence excitation spectrum of K7G which exhibits two excitation bands for emission at 520–530 nm at all of the concentrations examined; one with a maximum at 450 nm and the other at the absorption maximum of K7G in aqueous solution, that is, 360 nm.

3.3. Fluorescence and reflectance spectra on cellulose

The fluorescence emission spectra of Q7G and K7G bound to a dry solid cellulose substrate are similar to those observed for these compounds in concentrated aqueous solution with two bands having maxima at 460 nm and 530 nm for Q7G and at 450 and 530 nm for K7G. The excitation spectra for emission at 530 nm also exhibit two bands with maxima at 350 and 450 nm, and 360 and 440 nm, for Q7G and K7G, respectively.

The reflectance spectra of Q7G and K7G on cellulose are shown in Fig. 4((a) and (b)), respectively. The bands are significantly broadened and the maxima are red-shifted relative to the absorption spectral maxima of the corresponding flavonol in solution. This is consistent with the presence of an additional overlapping, long wavelength chromophore associated with the flavonols when they are bound to cellulose.

4. Discussion

4.1. Fluorescence emission in solution

The phenomenon of dual fluorescence has been the subject of many previously published accounts of the photophysical behaviour of flavonol aglycones dissolved in hydrocarbon and other non-aqueous solvents [1–9]. It has been demonstrated that the shorter wavelength fluorescence originates from the first excited singlet state of the unmodified keto form of the flavonol and the Stokes-shifted, longer wavelength emission is from an enolic/benzopyrilium, tautomeric form of the molecule formed by intramolecular proton transfer from the 3-hydroxy group to the basic carbonyl group in the excited state. This process is facile in non-hydrogen-bonding environments where competition from intermolecular hydrogen bonding with the carbonyl and/or 3-hydroxy groups of other molecules is absent or minor [2–5,20,21].

For the first time, similar dual fluorescence is reported for solutions of the naturally occurring 7-glucosides of quercetin and kaempferol in alcohol and water. As in the case for aglycones in non-aqueous solvents, the appearance of two fluorescence bands in alcohol can be attributed to emissions from the excited singlet states of the keto and enolic

tautomers of the flavonols. In aqueous solutions, the shorter wavelength emissions of the flavonols are mirror images of their longest wavelength absorption bands and, as was the case with these molecules in other solvents, this fluorescence is attributed to emission from the excited singlet state of the keto tautomer [1–5].

A feature of the fluorescence exhibited by aqueous solutions of Q7G,and to a lesser extent K7G, is the dependence on concentration (see Table 1 and Fig. 3). The yield of the longer wavelength fluorescence, relative to the emission from the keto-excited singlet, is increased at higher concentrations. Like Q7G and K7G, 7-hydroxyquinoline in water displays two fluorescence bands that have relative fluorescence yields dependent on concentration [11]. The fluorescence bands are attributed to emission from the keto and enol tautomeric forms of this molecule. The keto-to-enol tautomerism is facilitated by a concerted, excited-state proton transfer between the component partners of dimers present in solutions at higher concentrations. Similarly, in aqueous solutions of Q7G and K7G at higher concentrations where dimerization is favoured, the concentration dependence of the relative fluorescence yields of the two bands is consistent with phototautomerism via dual, proton transfer within dimers to give an excited state of the enol tautomer.

The assignment of emission with a maximum at 510 nm to the excited singlet state of an enol dimer is also supported by the observation of a second, excitation band at ~450 nm for this emission from concentrated solutions as discussed below.

Previous work has shown that tautomer formation by intramolecular hydrogen transfer is less probable in solvents/molecular environments where the intramolecular process is subject to interference from hydrogen bonding with the solvent [2,20,21]. This effect, together with deprotonation of the excited states of these flavonols in aqueous solutions, accounts for the lower levels of the enolic tautomer relative to the keto form present in water compared with that in methanol.

The shorter wavelength fluorescence band of K7G in undried methanol can be resolved into two bands with maxima at 425 and 470 nm. The shorter wavelength fluorescence bands of both K7G and Q7G in aqueous solutions are very broad suggesting that there are also overlapping contributions to the spectra from more than one species. The longer wavelength emission in this band can be described in terms of an increased charge transfer character of those keto-excited states within the total population which experience greater solvation/partial detachment of the 3-hydroxyl proton. Such members of the excited state ensemble will be stabilised by coulombic, solute–solvent interactions in polar solvents.

4.2. Fluorescence excitation in solution

In methanol, the excitation spectra of fluorescence originating from the excited states of the keto and the enolic tautomer forms, exhibit a single band which matches the flavonoid ground-state absorption spectrum. This is the behaviour which has been reported previously for flavonol aglycones in non-aqueous solutions [1–9]. In these systems any keto-to-enol tautomerism is via the first excited singlet state and no evidence has been presented previously for direct excitation of a ground-state enolic tautomer. However, in aqueous solutions of K7G at all concentrations, in addition to the keto excitation band with a maximum at 360 nm, there is a second band with a maximum at ~450 nm for the emission at wavelengths >529 nm. This indicates that the ground-state of the enolic tautomer of K7G is present in water. By contrast, dilute solutions of Q7G display only a single fluorescence excitation band, corresponding to the ground-state keto tautomer. However, in a near-saturated aqueous solution, a second excitation band associated with the enolic tautomer is observed.

The different relative amounts of enolic, ground-state tautomers of K7G and Q7G present in aqueous solutions may be rationalised as follows. Kaempferol is a 4′-hydroxylated flavonol and quercetin is a 3′,4′-dihydroxylated flavonol with structures as in Fig. 1. The most acidic hydroxyl group in the 7-glucosides of these molecules is that which is sited at C-4′ [22–24]. In the ^{13}C NMR spectra of 4′-hydroxy and 3′,4′-dihydroxyflavonoids [25], resonances associated with the C-4′ carbon atom move to a higher field on the introduction of the 3′-hydroxyl group. An increase the electron density on the C-4′ atom is thus indicated which would decrease the acidity of the 4′-OH group. Thus, the formation of the conjugate base anion by deprotonation of the 4′-OH group in aqueous environments will be favoured for kaempferol compared with quercetin. Electronic rearrangement and protonation of the carbonyl group accompanying the formation of the conjugate base would produce the ground-state, enolic tautomer evident in the fluorescence excitation spectrum of K7G in water.

Although no ground-state of the enolic tautomer is evident in the fluorescence excitation spectra of dilute aqueous solutions of Q7G, this species is apparent at higher concentrations in near-saturated solutions. This type of concentration dependence for ground-state tautomer formation in aqueous solutions has been reported previously for 7-hydroxyquinoline. Such behaviour was shown to be the result of dimerization of this molecule at high concentrations with dual hydrogen bond formation which facilitates proton transfer between the keto and N–H groups of both monomeric partners of the dimer. At high concentrations of Q7G in water, a situation which is favourable for molecular association/dimerization, the excitation spectra betray the presence of the ground-state of the enolic tautomer with an excitation maximum at 450 nm.

4.3. Fluorescence and reflectance spectra on cellulose

In this molecular environment both K7G and Q7G exhibit emission bands with maxima about 520 nm which are

characteristic of the excited singlet states of the enolic tautomers. This behaviour is likely to be a consequence of the presence of flavonol–flavonol molecular aggregates. There is also a contribution to fluorescence emission from the excited singlet state of the keto form of the flavonol which is stabilized in an environment such as this, where ESIPT is subject to significant interference from intermolecular hydrogen bonding with the cellulosic substrate.

The fluorescence excitation spectra indicate that the K7G and Q7G excited states of the keto tautomers are only produced by excitation of the keto forms of the ground-states. However, the enolic tautomer emissions are excited from the ground-states of both the keto and enolic forms of the flavonols. The significant quantities of enolic tautomer ground-states present in the dry cellulose environment can be explained in terms of a dual, concerted proton transfer between the carbonyl and 3-OH groups of the monomer partners of flavonol dimer/aggregates. These are present in this system because of the high number density of flavonol molecules deposited on, or bound to solid cellulose.

Absorption by these species extends the decreased reflectance (i.e. absorption) well into the blue region of the visible spectrum and thereby accounts, at least in part, for the yellow appearance of these flavonols on solid cellulose.

5. Conclusions

Previous work on the photophysics of flavonol aglycones has demonstrated that ESIPT can be an important non-radiative step in the relaxation of excited states of these molecules, particularly in aprotic or non-hydrogen-bonding solvents [1–9]. In this process, the excited state of an enolic tautomer relaxes to the keto form of the ground-state and as such, no net photo-induced, chemical change occurs. Flavonol glucosides occur naturally in plants, and since they have a high absorbance in the biologically damaging, UVB spectral region, and are relatively photochemically stable and unreactive, they are ideally suited to screening/protecting vital plant components from UV damage [26]. However, photophysical studies to date have dealt with flavonol systems which are not physiologically relevant. In the present work it has been shown that ESIPT also occurs in conditions which more closely approximate those encountered in the cell vacuole, that is, concentrated, aqueous solutions of the flavonol glucosides. Flavonols could therefore fulfil their putative UV protective role in plants through harmlessly dissipating absorbed energy by ESIPT. Analyses of the flavonoid compositions of a number of UV-tolerant plants have shown an increase of B-ring dihydroxylated flavonoids (e.g. quercetin) relative to the monohydroxylated equivalents (e.g. kaempferol) as a result of exposure of the plant to UVB radiation [27,28]. This suggests that quercetin is a more effective UV-protective flavonoid than kaempferol even though the UVB extinction coefficients of both are approximately equivalent. The greater effectiveness observed for Q7G relative to K7G [29] can be rationalised by the results of the current research in the following way. The ESIPT mechanism for the non-reactive dissipation of energy involves initial absorption of UV energy to produce an excited singlet state followed by rapid relaxation to an unreactive tautomeric form of the excited state and subsequent return to the original ground-state. It has been shown in this work that K7G differs from Q7G in that a significant amount of the ground-state enol tautomeric form of K7G is present in aqueous solutions. This tautomer absorbs in the blue rather than in the UVB spectral region. Thus, with K7G there is correspondingly less of the UV absorbing keto form present and therefore, a diminished capacity to screen and protect from UVB radiation.

The blue-absorbing, ground-state of the enolic tautomers of both Q7G and K7G formed as a result of flavonol–flavonol association are present both in saturated aqueous solutions and when they are bound to a cellulosic substrate. One consequence of this is to extend the reduced reflectance (i.e. absorption) of Q7G and K7G in these molecular environments well into the blue spectral region, to produce a yellow colouration. This finding is likely to have relevance in flower colouration in which largely colourless flavonol glycosides are known to produce intense yellow colouration. Observed flavonol glycoside levels within the petal vacuoles in the published examples are very high [17,19] and thus molecular association and consequent ground-state enol formation, may well account for the yellow colouration in such circumstances.

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