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ORIGINAL PAPER

Photophysical Properties of Some Flavones Probes in Homogeneous Media

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Abstract Photophysical properties of five hydroxyflavones (HF) (some typical models of flavonols), (3 - HF, 6 - HF, 7-HF, 3, 6 - diHF and 3, 7 - diHF) were studied in homogeneous media by means of UV–vis and steady-state and time resolved fluorescence spectroscopies. Their absorption and fluorescence characteristics based on the flavonols structure are presented and discussed. It was found that the fluorescence of the flavonols depends on the nature of the solvent and on their molecular structure, especially on the position and the number of the -OH groups of the substituted phenyl ring. Attention is paid to the number of the -OH groups that influence the excited-state intramolecular proton transfer (ESIPT) process. The fluorescence quantum yield and the lifetime of the flavonols in heterogeneous media have been also determined. The results are discussed with relevance to the flavonols as sensitive fluorescence probe and to their microenvironments in the systems of biological interest and especially in a typical protein environment.

Keywords Flavones · Fluorescence spectroscopy · ESIPT · Heterogeneous media

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Introduction

Flavones are related compounds of the flavonoid group and studies concerning them are largely focused on two important aspects: flavones as biologically active natural compounds and as fluorescent probes. The first aspect concerns their therapeutic properties: antioxidants, antiradicals, angioprotectives, making them agents against cancers, tumors, allergies, inflammation, dietary agents especially of high potency and low systemic toxicity [1–5]. The second interesting aspect of flavonoids is related to their fluorescence emission behavior. Because of their molecular systems that exhibit intramolecular excited state proton transfer and fluorescence behavior, the flavonoids are useful models for mechanistic studies regarding the photophysical aspects [6–9].

The fluorescence properties of flavones are sensitive to the surrounding medium (polarity, hydrogen bonding effects, pH, Temperature) [8–10] Studies on the electronic excited – relaxation processes including excited-state proton transfer (ESPT) of a prototype model flavonols, such as 3-hydroxiflavone (3-HF) and 7-hydroxiflavone (7- HF), have been studied [6, 9, 11, 12]. In these lines, applications of the dual emission parameters of flavonols as exquisitely sensitive environmental probes have been reported [13–15]. It was found that by comparison with 3-HF and its derivatives, where the ESPT is intrinsic (proceeding across an internal H-bond of the molecule) and barrier free [16], the ESPT in 7-HF, where proton donor and acceptor sites are not located adjacent to each other, is solvent assisted and consequently strongly depends on the nature of the solvent medium. Here, time resolved fluorescence spectroscopy and transient absorption measurements showed that the ESPT of 7-HF in methanol solution involves the formation of two types of phototautomers in the excited state as well as in the ground state [17].

According to Frolov et al. [18], the luminescence of 7-HF in alcohol glass at 77 K depends on the excitation wavelength. It was also shown that for an increase of the

temperature, to 298 K, the blue fluorescence (λ_{em}~450 nm) shifted to longer wavelengths leading to a green fluorescence (λ_{em}~537 nm). The behavior was found due to selective excitation of the same ground state species, namely the conjugate anion of 7-HF (7-HFA), its fluorescence being strongly modulated by solvent relaxation owing to a large change in dipole moments between the ground and excited states of 7HFA [19–21]. Studies on the excited-state intramolecular proton transfer in 3-HF isolated in solid argon, have been reported by Dick and Ernsting [22]. It was observed that upon electronic excitation, the molecules undergo rapid intramolecular proton transfer and moreover, no fluorescence from the excited state of the normal form of the molecule has been detected [22]. An apparent tautomer fluorescence rise time of 2.7 ps and a rate constant greater than 10^{12} s^{-1}, for excited-state intramolecular proton transfer in 3-HF, were found [22]. Concerning the temperature dependence, it was reported that the dual – fluorescence quantum efficiency is originated in a varying degree of complexation with hydrogen-bonding impurities. A violet fluorescence attributed to the normal form of the 3-HF, is evidenced [6].

Due to their high sensitivity to solvent properties, connected with excited state intramolecular proton transfer, studies on flavonols as metal – ion chelators, have been performed [23]. It was found that in the excited state chelating magnesium complexes are more stable than in the ground state, and they are produced both from normal and from phototautomer forms of flavonols [23]. Very recently, synthesis of 3-HF fluorescent probes and study of their fluorescence properties as well as studies of the solvent effects on the electronic absorption spectra of flavones and 7-HF in neat and binary solvent mixture, have been performed [24, 25]. It was found that the basic fluorescence properties of 3HFs are maintained in all probes in terms of a strong blue shift in the maximum fluorescence emission, the fluorescence quantum yield being 100 fold greater in organic solvents in comparison with the systems in aqueous Hepes buffer, pH 7.4 [24]. Also, it was shown that both specific hydrogen bond donor ability and non-specific dipolar interactions of the solvents play an important role in absorption maxima of flavones in pure solvents [25].

In this paper the photophysical properties of five hydroxyflavones (HF), (some typical models of flavonols), (3 - HF, 6 - HF, 7-HF, 3, 6 - diHF and 3, 7 - diHF) were studied in homogeneous media by means of UV–vis and steady-state and time resolved fluorescence spectroscopies. Their absorption and fluorescence characteristics based on the flavonols's structure are presented and discussed. The fluorescence of the flavonols depends on the nature of the solvent and the position in which the – OH groups substitute the phenyl group. This last aspect is discussed with relevance to its influence on the process of the excited-state intramolecular proton transfer (ESIPT) process. The fluorescence quantum yield and the lifetime of the flavonols in heterogeneous media have been also determined. The results are discussed with relevance to the flavonols as sensitive fluorescence probes and also to their microenvironments in the systems of biological interest and especially in a typical protein environment.

Experimetal Section

Materials

3-Hydroxyflavone (3-HF) and 7-HF were purchased from Sigma. 6-HF, 98 % with 3, 6-diHF, 98 % and 3, 7-diHF hydrate, 97 % were purchased from Aldrich. All these HFs were used without further purification. The structure of the studied flavones is shown in Scheme 1. The stock solutions were prepared in Methanol. Aliquotes from stock solutions were dried at room temperature and then diluted with various solvents, Acetonitrile (ACN), Ethanol (EtOH) and Dioxan, to a final working concentration in the range of $4.8–6\times10^{-5}$ M. Methanol of spectrophotometric grade was purchased from Sigma. EtOH was purchased from Merck, ACN and Dioxan were purchased from Uvasil.

Methods and Apparatus

The absorption measurements were recorded using a Perkin Elmer, Lambda 35, UV–vis Spectrometer at a scan rate of 480 nm/min and a spectral resolution of 1 nm.

The fluorescence emission and excitation spectra were recorded with a Jasco FP-6500 Spectrofluorometer, using 3 nm bandpasses for the excitation and the emission monochromators, the detector response of 1 s, data pitch of 1 nm, the scanning speed of 100 nm/min. The excitation wavelength was 365 nm.

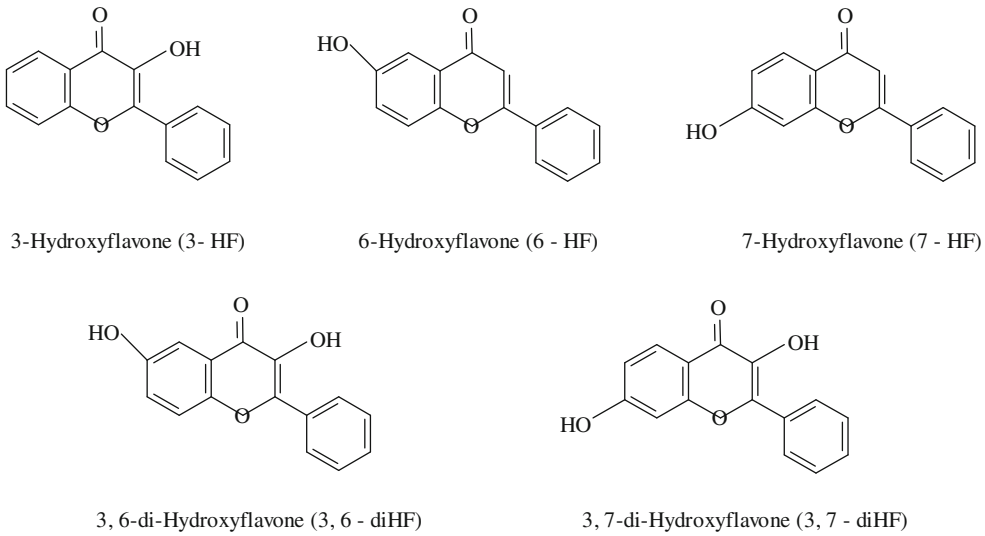
The fluorescence quantum yield was determined by comparison to dilute quinine bisulfate solution in 0.1 N H_2SO_4 with 0.55 absolute quantum yield [26], using the following relationship:

$$\phi_x = (F_x A_{ref} \phi_{ref}) \Big/ F_{ref} A_x$$

where F is the area under the fluorescence emission curve, for quinine bisulfate (F_{ref}) and for the studied compound (F_x) over the wavelength region 420 – 650 nm, A_{ref} is the absorbance for quinine bisulfate at 346 nm.

A least – squares iterative curve fitting of the fluorescence excitation spectra was performed with Gaussian bands using the Peak Fit Analysis Program (Sea Solve,

Scheme 1 Molecular structure of the investigated flavones



MA, USA). The position of each band was chosen from the second derivative spectrum. The shift of the position was allowed for fitting, leading to a lower error. The quality of the data fit was judged using statistical parameters: the correlation coefficient, r^2; the standard error, *SdErr* and the coefficient of statistical significance, *Fstat*.

The fluorescence lifetime decays were recorded in a time-correlated single photon counting FLS920 system from Edinburgh Instruments, with laser excitation at 375.6 nm, a lifetime scale of 50 ns and 1024 channels. The data were fitted with a multi-exponential decay (reconvolution) and the accuracy of the fit was checked on grounds of χ^2, which was around 1. Intensity-averaged lifetimes were calculated according to the equation [27]:

$$\langle\tau\rangle = \frac{\sum_i \alpha_i \cdot \tau_i^2}{\sum_i \alpha_i \cdot \tau_i} \quad (1)$$

where α_i is the preexponential factor and τ_i the lifetime of the *i*th component. The fractional intensity of the *i*th component is defined as:

$$f_i = \frac{\alpha_i \cdot \tau_i}{\sum_i \alpha_i \cdot \tau_i} \quad (2)$$

while the fractional amplitude, interpreted as the relative population of the respective state, is:

$$a_i = \frac{\alpha_i}{\sum_i \alpha_i} \quad (3)$$

The TRES (time resolved emission spectrum) spectra were obtained by slicing the lifetime decays recorded as above at several wavelengths from 400 to 650 nm, with a step of 2 nm.

Results and Discussion

UV–vis Absorption Measurements

Figure 1 presents the electronic absorption spectra of 3-HF and 7-HF in dioxan. Two absorbance bands are evidenced. For the bare 3-HF, the band I can be found in the range of 356 to 425 nm, while band II around 340 nm. In direct comparison, 7-HF was found to present only one structured absorption band, centered at 336 nm. According to Sancho et al., 2011, 7-HF presents a very limited solubility for non-polar solvents [25].

Figure 2 shows the electronic absorption spectra of 3, 6 - diHF and 3, 7-diHF in ethanol (Fig. 2a) and in dioxan (Fig. 2b). As it can be observed, in the 350 to 425 nm range, the shape of the spectra are almost the same, a 5 nm

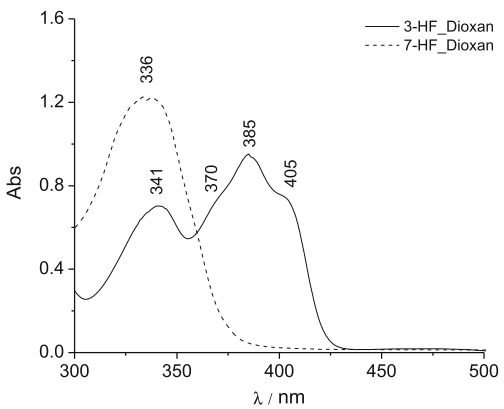
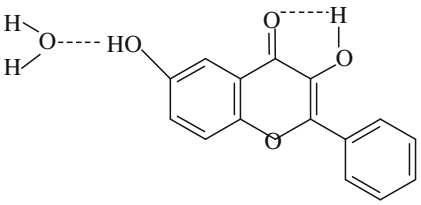
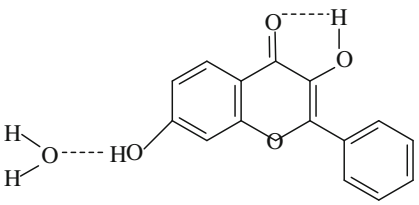


Fig. 1 UV–vis absorption spectra of 3 - HF and 7 - HF in Dioxan

hipsochromic-shift is evidenced for 3, 7-diHF. It is interesting to notice the appearance of a new absorption band in the 425–525 nm range, 458 nm for 3, 7- diHF and 470 nm, for 3, 6-diHF. The behavior may be attributed to the formation of H-bonded complex with the water traces from the used polar protic solvent, ethanol. The intermolecular H-bonds between HFs and the polar protic solvent, together with the stabilization of the emission species as well as with the reducing of the energy of the excited HFs, may be presented as following:





In direct comparison, Fig. 2b presents the electronic absorption spectra of 3, 6-diHF and 3, 7-diHF in a non-polar solvent, dioxan. Here, no significant absorption bands in the 425–525 nm range, have been detected.

Comparing the electronic absorption bands, Fig. 2, the influence of the following factors: the nature of the solvent, the number and the position of the side phenyl rings, of the –OH group (3,6 and 3,7) on the shape and on the wavelength of the absorption is considered. In these lines, Table 1 summarizes the maximum absorption wavelength and the molar extinction coefficient of all investigated HFs in various solvents.

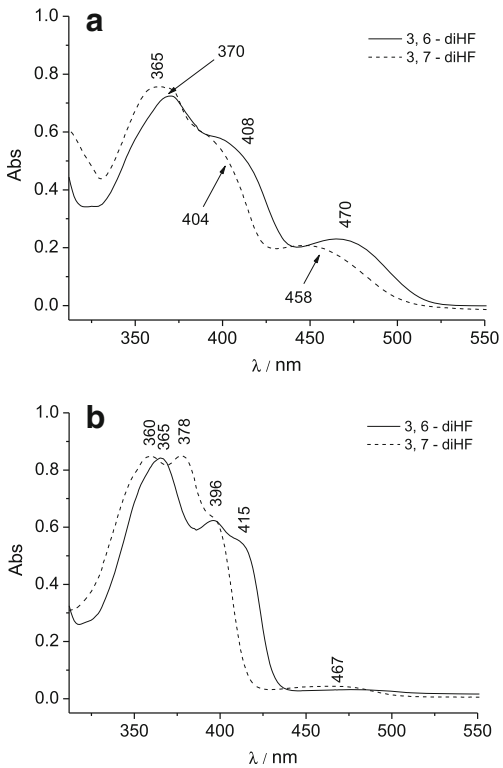


Fig. 2 UV–vis absorption spectra of 4.8×10^{-5} M of the 3, 6 - diHF and 3, 7 - diHF in ethanol (**a**) and in dioxan (**b**)

Steady - State Fluorescence Measurements

The reaction of proton phototransfer characteristic of flavonols with a dual fluorescence behavior, consisting of normal (blue emission) and intramolecular ESPT tautomer (green emission) bands is well known [6, 9, 11, 18].

Figure 3a shows the fluorescence emission spectra of 3-HF in non-polar, polar-protic and polar-aprotic solvents, for an excitation wavelength of 365 nm. Typical dual fluorescence emission behavior of normal band (N^*) at 470 nm and intramolecular ESPT tautomer (T^*) bands around 530 nm, have been evidenced. As we can observe, the intensity ratio of tautomer : normal fluorescence depends on the solvent polarity. In the polar aprotic solvent, such as ACN, no fluorescence of N* form of 3-HF is evidenced and the fluorescence of T^* form is stronger, centered at 527 nm. In the non-polar solvent, dioxan for instance, the fluorescence of the N* appears at 470 nm while for T^* form, it decreases and is 4 nm red-shifted, at 531 nm respectively. In protic solvent, both fluorescence emissions of 3-HF forms, the N^* as well as the T^*, strongly decrease and are red - shifted. Also, changes in the fluorescence of T^* on the exited state, as a function of solvent polarity are observed in the fluorescence excitation spectra, Fig. 3b.

Figure 4a presents the fluorescence emission spectra of 6 and 7-HF in the polar protic solvent, ethanol at an excitation wavelength of 365 nm. Very weak fluorescence emission for both probes with major changes in the intensity ratio of the two emission bands, N^* and T^*, were detected. The feature is

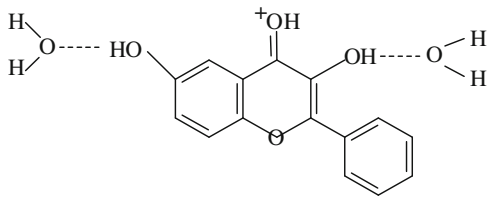
Table 1 Absorption (λ_{Abs} and ε), Emission (λ_{em} and Fluorescence Quantum Yield (ϕ_f)) and Stokes Shift ($\Delta\nu$) of the flavonols in different solvents. λ_{ex}=365 nm

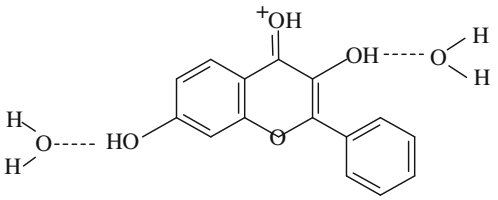
Probe	Solvent	λ_{Abs} (nm)	ε (L M^{-1} cm^{-1})	λ_{em} (nm)	$\Delta\nu$ (cm^{-1})	ϕ_f
3-HF *(6× 10^{-5}M)*	ACN	341; *383*; 402 sh[a]	16333	527	7148	0.099
	EtOH	341; *388*, 475 bb[a]	15000	464; 533	7106	0.052
	Dioxan	341; *385*; 405 sh;	15833	470; 531	7099	0.106
6-HF *(6× 10^{-5}M)*	ACN	300; *334*; 376 sh	12166	–	–	–
	EtOH	*304*; 342; 394 sh	20000	456; 522 sh	10939	0.0073
	Dioxan	*304*; 326 sh; 340sh; 371	22333	–	–	–
7-HF *(5.3× 10^{-5}M)*	ACN	336	21200	–	–	–
	EtOH	302; *345*; 437 sh	22139	474 sh; 529	10005	0.0087
	Dioxan	336	22701	–	–	–
3, 6-diHF *(4.8× 10^{-5}M)*	ACN	*364*; 394 sh; 411 sh; 483 bb	17500	465; 527	8482	0.183
	EtOH	*370*; 408; 470	15000	473; 530 sh	6019	0.095
	Dioxan	*365*; 396; 415	17500	532	8561	0.207
3, 7-diHF *(4.8× 10^{-5}M)*	ACN	*358*; 375; 396 sh; 468 bb	16666	466; 530	9028	0.039
	EtOH	*365*; 404 sh; 458	15833	473; 531	8597	0.080
	Dioxan	*360*; 378; 396 sh; 467 bb	17708	467; 535	9176	0.053

[a] *sh* is shoulder and *bb* is broad band

attributed to intramolecular H-bond species that may influence the intensity ratio of tautomer: normal fluorescence (I_{N^*}/I_{T^*}) and also the excited - state proton – transfer formation. As it can be seen, for 6-HF no fluorescence of T^* was detected and the I_{N^*} which appears at 456 nm is very weak. On the contrary, for 7-HF, no fluorescence of N^* form was detected and the I_{T^*} that appears at 529 nm, is not significant. For these probes, larger Stokes Shifts were determined ($\Delta\nu\sim10000$ cm^{-1}) and the fluorescence quantum yields, ϕ=0.0073 for 6-HF and ϕ=0.0087 for 7-HF, are 10-fold lower than the fluorescence quantum yield of 3-HF, ϕ=0.052, Table 2.

Comparatively, Fig. 4b shows the fluorescence emission spectra of 3, 6 -diHF and 3, 7-diHF in ethanol using the same excitation wavelength, 365 nm. We can observe that for both probes, the fluorescence emission of the N^* form appears at 473 nm and of the T^* form appears at 531 nm. In this case only the intensity ratio of the tautomer : normal fluorescence changes. The possibilities of tautomer formation for 3, 6 - diHF and 3, 7 - diHF, are the following:





This is why we can expect differences in the T^* emission band and the N^* band of the 3, 6 - diHF and 3, 7 - diHF by comparsison to those of the bare 3-HF.

In polar protic solvent, the fluorescence quantum yield of 3, 6 -diHF (ϕ=0.095) is higher than that of 3-HF (ϕ=0.052) and 3, 7-diHF (ϕ=0.052), Table 2.

Differences between the fluorescence excitation spectra of 3, 6-diHF and 3, 7-diHF in ethanol at an emission wavelength of 530 nm have been evidenced. The shapes of the excitation profiles (the results are not shown) are different by comparison to their absorption spectra. The feature is due to a difference in the behavior of the 3, 6 - diHF and 3, 7 - diHF on the excited state. A slight variation in the Stokes Shift values ($\Delta\nu$=6019, for 3, 6 - diHF and 8597 cm^{-1}, 3, 7 - diHF A), Table 1, induces a change in the geometry of their excited state by comparison to that of the ground state. In addition, the explanation concerning the differences observed between the 3, 6- and 3, 7-diHF substituted phenyl ring are due to a higher S_0–T_2 gap, increasing thus the rate of the overall non-radiative intersystem crossing processes. Also, the nature of

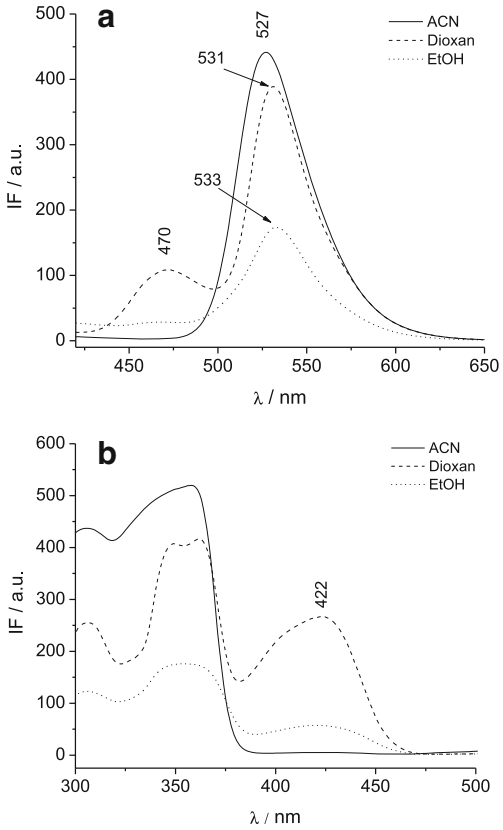


Fig. 3 Fluorescence emission (**a**) and excitation (**b**) spectra of 3-HF in different solvents; λ_{ex}=365 nm; λ_{em}=530 nm

the first excited singlet state is perturbed by these positions of the -OH groups of the phenyl ring.

The fluorescence emission spectra of 3, 6 –diHF and 3, 7-diHF in EtOH and in ACN in direct comparison with the bare 3 –HF, are presented in Fig. 4c and d, respectively. Changes in intensity ratio of tautomer: normal fluorescence (I_{T*}/I_{N*}) are well evidenced, especially in EtOH, with a batochromic shift of the N^* and a slight blue-shift of the T^* forms, Fig. 4c. In ACN, the major T^* forms are observed with no significant shifts in the wavelength emission.

In direct comparison, the fluorescence emission spectra of 3, 6-diHF (Fig. 4e) and of 3,7-diHF (Fig. 4f) in polar-aprotic (ACN), polar protic (EtOH) and non-polar (Dioxan) solvents, are presented.

It can be noticed, Fig. 4e, that for 3, 6-diHF in Dioxan, the fluorescence emission of the T^* form is 5 nm batochromic shifted (λ_{em} =532 nm) and the I_{T*} is more intense. In EtOH, the N^* form appears to be more intense than the T^* form. From Fig. 4f, both the N^* and T^* forms are well structured with no significant sifts in the wavelength emission. Here, only the fluorescence intensity ratio, I_{T*}/I_{N*}, changes as a function of solvent polarity; The I_{T*}/I_{N*} increases in the following order: EtOH > ACN > Dioxan. We assume that for 3, 6-diHF, a fast intramolecular ESPT takes place by comparison with 3, 7- diHF. This fact is attributed to weaker intermolecular H- bonding between 3, 6-diHF and the solvent molecule in the excited state. For 3, 7-diHF, Fig. 4f, the supposition is that the acidity of the –OH group from the position 7 increases upon excitation, fact that leads to a stronger intermolecular H-bond with the solvent molecule in the excited state by comparison to the ground state. Subsequently, in this case the intramolecular ESPT is slower than in the case of 3, 6-diHF.

Overall, discrepancies in the data about the behavior regarding proton transfer provide evidence of the presence of different tautomer configurations, especially of the 3, 7-diHF. Moreover, the proton transfer appears to be promoted by the solvent polarity as well as by the position and the number of the -OH groups of the phenyl ring.

Time - Resolved Fluorescence Measurements

To corroborate the results found in steady-state fluorescence analysis, fluorescence lifetime studies of HFs in several solvents at an emission wavelength of 530 nm, were performed. The decay parameters, average fluorescence lifetimes and χ^2 values are listed in Table 2. The short component (< 1 ns) is predominant at 530 nm, so it belongs to the ESPT state, while the long one (~ 3 ns), which increases in fraction at 480 nm (as seen from the decay in Fig. 5a), is attributed to the normal excited state. The ESPT state is less fluorescent than the normal one, as the fractional intensity f_1 is always lower than the relative population a_1. The data demonstrate that in the fluorescence of 3-HF the emission from the tautomer form is higher than the emission from the normal one in all the solvents used. 3, 6-diHF predominantly emits from its T^* state in non-protic solvents, but N^* emission dominates in EtOH, probably due to intermolecular solute-solvent H-bond formation. 3, 7-diHF behaves entirely different, as its emission corresponds mainly to the N^* form, no matter of the polarity or type of solvent. A more striking picture of the dynamics of the two excited states and the shape of the spectrum on a nanosecond timescale is given by the TRES spectra, as can be seen in Fig. 5b and c for 3-HF. In ACN, the T^* band is the most intense on virtually the whole timescale. The deconvolution of the spectrum shows that initially I_{N*}/I_{T*}=0.16. The situation is different in EtOH. Although the N^*/T^* intensity ratio seems to be more than unity, as the T^* band appears as a shoulder, the deconvolution of the spectrum reveals that in fact T emission predominates initially (I_{N*}/I_{T*}=0.89). As this component has a shorter lifetime, it rapidly decreases and N^* emission prevails. Another observation is that the T^* band has a larger full width at half maximum than the N^* one, e.g. 90 nm vs. 40 nm in EtOH.

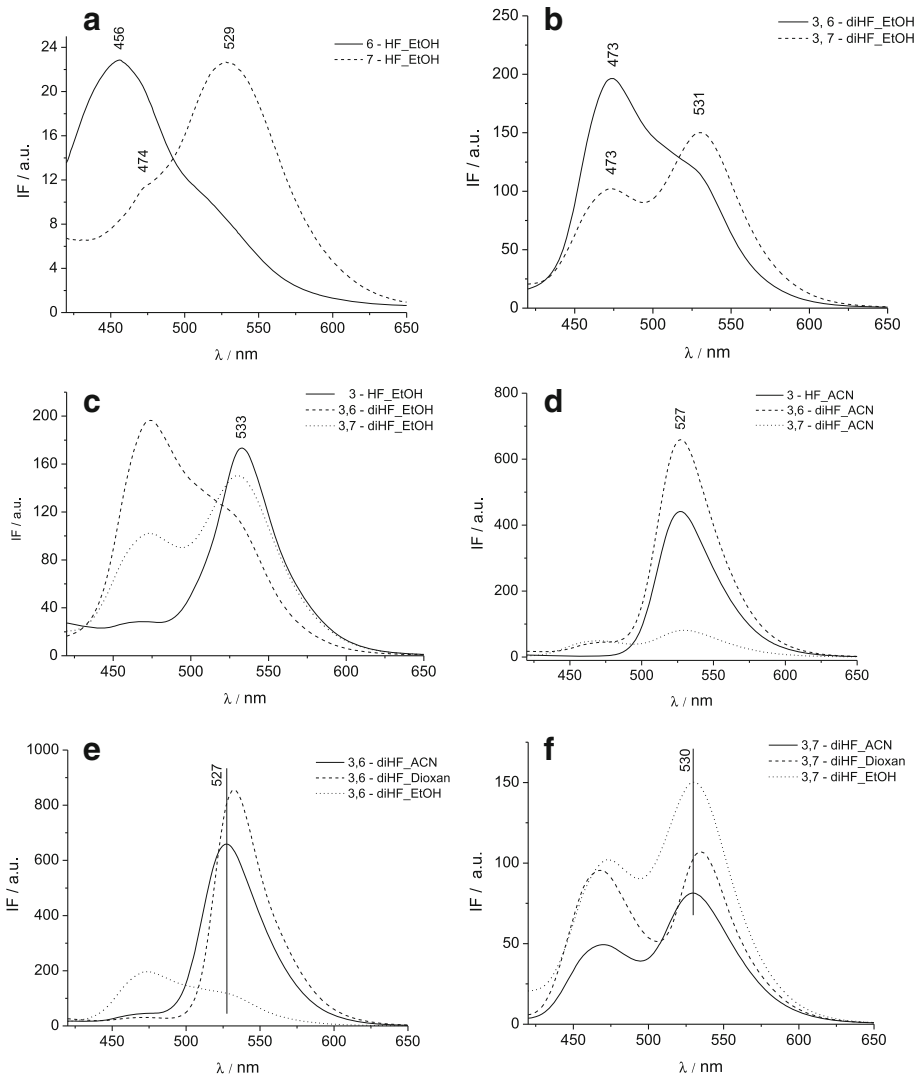


Fig. 4 Fluorescence emission spectra of 6 and 7-HF in ethanol (**a**) and of 3, 6 and the 3, 7 -diHF in ethanol (**b**); Fluorescence emission spectra of 3 - HF and 3,6 - diHF and 3,7- diHF in ethanol (**c**) and in acetonitrile (**d**); Fluorescence emission spectra of the 3, 6 - diHF (**e**) and 3, 7 – diHF (**f**) in different solvents; λ_{ex}=365 nm

Table 2 Fluorescence lifetimes (ns), preexponential factors and fractional intensity of the flavonols in different solvents; λ_{em}=530 nm

Probe	Solvent	a_1	f_1	τ_1	a_2	f_2	τ_2	<τ>	χ^2
3-HF *(6× 10^{-5}M)*	ACN	0.093	88.77	0.756	0.003	11.23	2.799	0.97	1.189
	EtOH	0.141	69.39	0.369	0.01	30.61	2.265	0.94	1.162
	Dioxan	0.09	96.05	0.856	7.5E-4	3.953	4.222	0.99	1.111
3, 6-diHF *(4.8× 10^{-5}M)*	ACN	0.071	87.87	1.156	0.003	12.13	3.486	1.42	1.088
	EtOH	0.036	21.55	0.798	0.034	78.45	3.045	2.56	1.101
	Dioxan	0.056	76.98	1.379	0.012	23.02	2.001	1.53	1.114
3, 7-diHF *(4.8× 10^{-5}M)*	ACN	0.033	21.77	0.878	0.033	78.23	3.083	2.59	1.098
	EtOH	0.027	13.6	0.704	0.041	86.4	3.024	2.72	1.156
	Dioxan	0.058	30.06	0.641	0.026	69.94	3.406	2.59	1.012

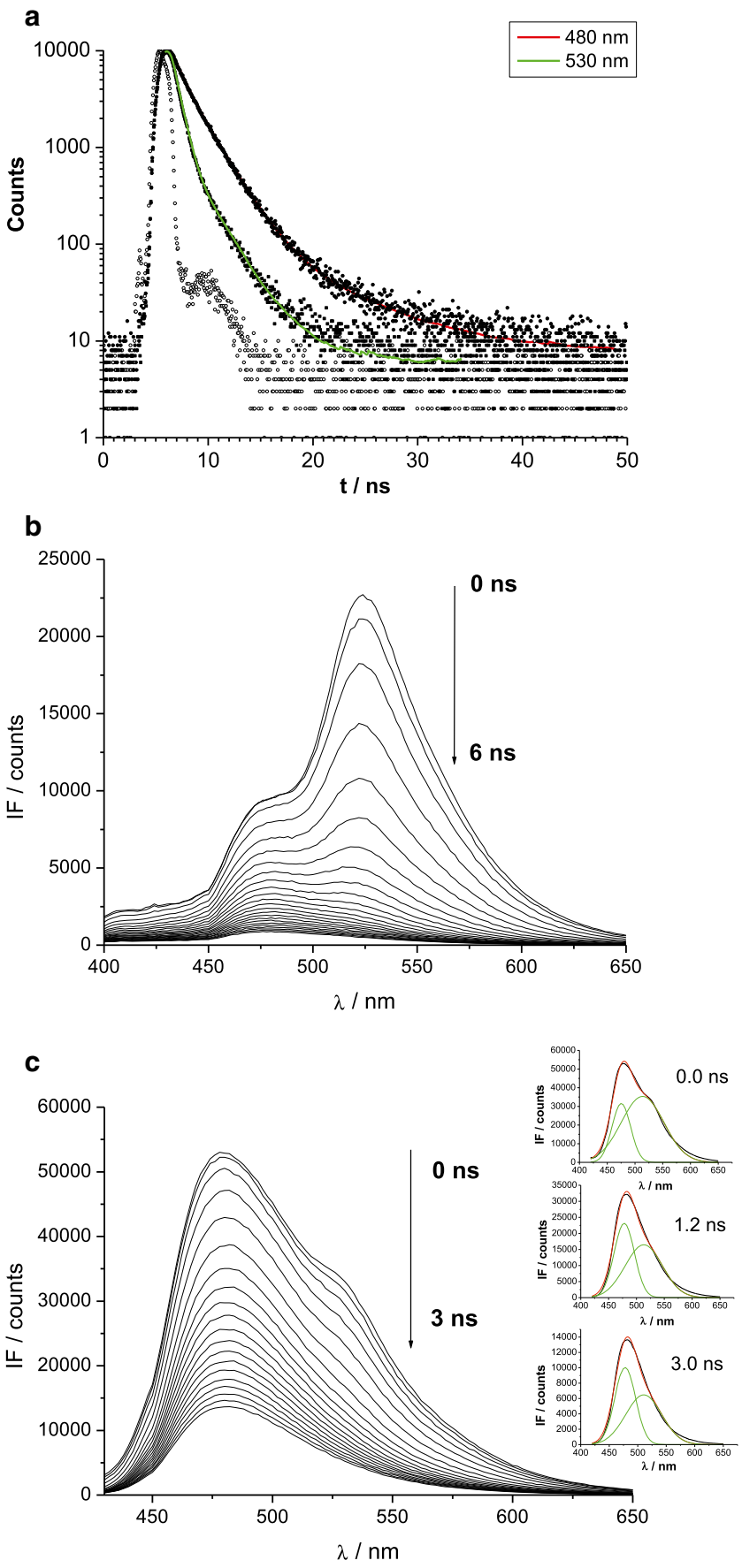


Fig. 5 Time-resolved emission data: **a** fluorescence decay of 3-HF in ACN at λ_{em} 480 (f_1=32.95; f_2=67.05) and 530 nm (f_1=69.39; f_2=30.61). (*white circle*) is the instrument response; (*black square*) 530 nm; (*black circle*) 480 nm. TRES spectra of 3-HF in **b** ACN and **c** EtOH. Deconvolution of some intermediate spectra as inset. The time after reaching maximum emission is noted

Conclusions

From the fluorescence data presented in this work, insight into the photophysical properties of some flavones probes developed, highlighting the following ideas:

Significant discrepancies in the data regarding proton transfer provide evidence for the presence of different tautomer configurations, especially of the 3, 7 - diHF. Moreover, the intramolecular proton transfer appears to be promoted by the solvent polarity as well as by the position and number of the -OH groups of the phenyl ring. Subsequently, an influence of the rate of the excited state proton transfer process can be noticed;

The dependence of the tautomer fluorescence properties on the - OH groups and on their position, 3, 6 and 3, 7 respectively, on the side phenyl ring reveals the influence of external hydrogen bonding perturbation on the internal hydrogen bond of the molecule;

The fluorescence quantum yield of 3, 6-diHF is higher than that of 3-HF and 3, 7-diHF. It is solvent dependent in the following: Dioxan (ϕ=0.207)>ACN (ϕ=0.183)>EtOH (ϕ=0.095);

The two states have different lifetimes, i.e. approx. 3 ns for the normal state and less than 1 ns for the ESPT state. Both lifetimes depend on the polarity of the solvent;

These excited-state relaxation phenomena are important for further studies to investigate the microenvironment of flavonols probes in a system of biological interest and especially in a typical protein environment.

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