Supplementary Material (ESI) for PCCP
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Protic Equilibria as the key factor of quercetin emission in solution. Relevance to biochemical and analytical studies.

Alberto Mezzetti [a, b, *], Stefano Protti [a, b, c, *], Christine Lapouge [b] and Jean-Paul Cornard [b].

[a] *Laboratoire de Photocatalyse et Biohydrogène, SB2SM, CNRS URA 2096, CEA-Saclay, DSV/iBiTecS, 91191 Gif-sur-Yvette cedex, France.*

[b] *LASIR CNRS UMR 8516, Université Lille 1, Bat C5, Cité Scientifique, 59655 Villeneuve d'Ascq cedex, France*

[c] *Department of Chemistry, University of Pavia, Via Taramelli 10, 27100 Pavia, Italy.*

*Email: prottistefano@gmail.com, alberto.mezzetti@cea.fr

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ELECTRONIC SUPPLEMENTARY INFORMATION

1. Overview on previously reported fluorimeteric studies

Table S1. Selected biological and physicochemical studies where quercetin is employed as a fluorophore. Abbreviations: BSA: Bovine Serum Albumin; HSA: Human Serum Albumin; HST: Human Serum Transferrin; SDS: Sodium dodecyl sulphate; HDMAB: Hexadecylmethylammonium Bromide.

Reference	**Conditions**	**λ_{em}, nm**	**λ_{abs} nm**	**λ_{exc}, nm**
S1	Phosphate Buffer, pH = 7.4, BSA	530 (λ_{ex} = 450 nm)	/	~460
S2	Hepes buffer, pH = 7, BSA	526 (λ_{ex} = 370 nm)	/	/
S3	Hepes buffer, pH = 7, HSA	~440, ~505 (λ_{ex} = 370 nm)	~430	~460
S4	Phosphate buffer, pH = 7.4 HSA	~490, ~515 (λ_{ex} = 420 nm)	420, sh. ~460	/
S2	Soy glycinin	~540 (λ_{ex} = 370 nm)	/	/
S5	DNA Gyrase B (fragment)	530 (λ_{ex} = 370 nm)	/	/
S6	pH = 6.8, Tubulin	535 (λ_{ex} = 385 nm)	/	/
S7	pH = 7.4, HST	454, ~530 (λ_{ex} = 360 nm)	/	/
S8	Tris buffer, pH 7.4, DNA	~460, ~535 (λ_{ex} = 365 nm)	380	/
S9	Lipid nanocapsule	550 (λ_{ex} = 370 nm)	375	~415
S10	Water, β-cyclodextrines	~540 (λ_{ex} = 440 nm)	/	/
S11	Water, TritonX-100	~530 (λ_{ex} = 370 nm)	375	/

S12	SDS micelles	~525 (λ_{ex} = 370 nm)	~375	/
S12	HDMAB micelles	~550 (λ_{ex} = 370 nm)	~390	/
S13	Chitosan nanoparticles	530 (λ_{ex} = 481 nm)	~480 (sh).	481

From the UV-Vis absorption, emission, and fluorescence excitation spectra in Table 1 in the main text (reported above as Table S1) it appears that in all the mentioned systems the specific binding to a biomolecule or the localization in a given microenvironment induce partial formation of (one or more) species that absorb(s) at higher wavelength and/or fluoresce(s) with higher quantum yield than neutral quercetin. Where available, fluorescence excitation spectra are consistent with partial formation of one (or more) anionic form(s) of quercetin. This seems to be the case when quercetin is bound to HSA [S3, S4; see also S14] and BSA [S1], to soy glycinin [S2], to DNA-gyrase [S5]. Two other mechanisms suggested in the literature can in principle explain modification of absorption spectra and increased fluorescence of quercetin, namely distortion or breaking of the intramolecular hydrogen bond between the 5-OH and the oxygen of the C=O moiety [S15], and formation of a pyrylium cation by protonation of the C=O group [S1, S14]. Indeed, these two mechanisms have been proposed to explain the photophysics of quercetin bound to HSA [S3] and to BSA [S1].

In several situations reported in table S1, partial anion formation seems more realistic, given that other hydroxyflavones are partially anionic under same or very similar conditions [S16-S21]. It should also be noticed that quercetin with a “broken” intramolecular hydrogen bond between 5-OH and the carbonyl has a fluorescence excitation spectrum blue-shifted compared to “normal” quercetin [S15] whereas all the available fluorescence excitation spectra reported in table 1 have a peak red-shifted (in some cases to a great extent) compared to “normal” quercetin. In addition, it

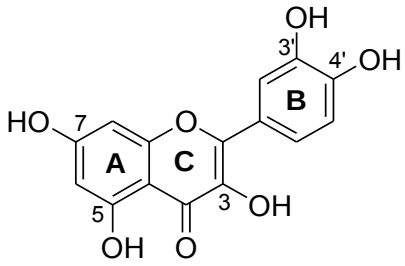
should be noticed that pyrylium cation formation has been reported only in very acidic conditions (in 1 M HBr in acetic acid [S1]; in sulphuric acid solution [S22]).

For three of the situations reported in table S1 molecular modelling studies are available and can help to better understand how quercetin interacts with its microenvironment, in particular to investigate the formation of intermolecular hydrogen bond interactions. It should be kept in mind that formation of such hydrogen bonds are physicochemical processes that make possible any of the three mechanisms mentioned above (distortion of the intramolecular hydrogen bond between the oxygen of the C=O moiety and the 5-OH; formation of the pyrylium cation; anion formation). For quercetin bound to human serum transferrin, molecular modelling studies [S7] foresee the formation of the following hydrogen bonds: between the 3-OH group of quercetin (donor) and AspA63 (acceptor); the 7-OH group of quercetin (donor) and GluA83 (acceptor); the 3'-OH group of quercetin (donor) and GluA15 (acceptor); the 4'-OH group of quercetin (donor) and GluA15 (acceptor); GlnA206 (donor) and the 5-OH group of quercetin (acceptor). These interactions could make possible anion formation (by deprototonation of OH groups at positions 7, 3, 4' if just the most acidic sites are concerned; it should however be pointed out that a) deprotonation at the 3' site could also be possible, and b) formation of polyanionic species cannot be excluded). The involvement of 5-OH of quercetin in a hydrogen bond as an acceptor could also have an effect of the intramolecular hydrogen bond between 5-OH and the oxygen atom of C=O (which is responsible for the high internal conversion and consequent very low fluorescence of flavonoids with a 5-OH group [S15]) leading to the formation of a fluorescent species with dual emission, as reported in ethanol glasses at 77 K under continuous illumination [S15]. The rise of a 454 nm emission band of quercetin upon binding to human transferrin along with the presence of the 530 nm emission band [S7] could be interpreted as a sign of dual emission from quercetin (of the same kind of the well-known dual fluorescence of 3-hydroxyflavone and related compounds [S23, S24 and references therein]). It should however be noticed that the observed weak enhancement of the 530 nm band [S7] could mean the formation of a significant fraction of quercetin anion.

Also for quercetin bound to HSA, molecular modelling studies [S25] foresee the formation of hydrogen bonds between quercetin and the protein; in particular, hydrogen bonds are established between the 3-OH group of quercetin (donor) and the nitrogen atom of Lys195 (acceptor) and between the 4'-OH group of quercetin (donor) and Asp451. Both interactions could in principle lead to anion formation. It should be noticed no clear indication is mentioned [S25] concerning the hydrogen bonding state of the carbonyl group and the 5OH group of quercetin.

For quercetin bound to DNA-gyrase [S5], according to molecular modelling studies, the following hydrogen bonds would be established: 7-OH of quercetin (donor) and Thr165 (acceptor); 3'OH of quercetin (donor) and Asn46 (acceptor); OH of Tyr 109 (donor) and C=O of quercetin (acceptor). In addition, a weak hydrogen bond should be formed between 5-OH of quercetin (donor) and the OH of Tyr 109 (acceptor). In this situation, deprotonation ot the 7-OH site and possibly at the 3'-OH site could be possible; an intriguing feature is however represented by the involvement of 5-OH as a donor in a hydrogen bond, which would be consistent with a strong perturbation of the intramolecular hydrogen bond between 5-OH and the C=O. It should however be noticed that the experimental fluorescence spectrum of quercetin bound to a fragment of DNA-gyrase does not show any indication for a dual emission as conversely observed for quercetin after long illumination in EtOH glassy solutions at 77 K [S15]. This fact suggests that in the case of quercetin bound to the fragment of DNA-gyrase the fluorescent specie is not neutral quercetin with a "broken" intramolecular hydrogen bond between 5-OH and the carbonyl.

Table S2: order of acidity of the different OH groups of quercetin proposed in the literature



Reference	Method	Order of acidity
S26	^{13}C NMR (solid state) and spectrophotometric titration	7-OH > 4'-OH > 3-OH
S27	Spectrophotometric titration	(3'-OH or 4'-OH) > 7-OH
S28	Capillary electrophoresis	7-OH > 4'-OH
S29	Spectrophotometric titration	7-OH > 4'-OH
S30	Spectrophotometric titration	3-OH considered as the first deprotonation site
S31	Survey of literature data on a series of polyhodroxyflavones	4'-OH > 7-OH
S32	^{1}H NMR	3-OH > 7-OH > 4'OH
S33	Survey of literature data on mono and polyhydroxyflavones	4'-OH > 7-OH

2. UV absorption, emission and excitation spectra of quercetin in water at different pH.

Spectroscopic measures at different pH have been performed on solutions of quercetin 5 x 10^{-5} M in water containing 10%$_{v/v}$ of methanol. All solutions have been freshly prepared and degassed by nitrogen bubbling, in order to avoid decomposition of quercetin that occurs in alkaline solution. Hydrochloric acid and sodium hydroxide were used to adjust the pH at the desired value. Raman contribution of the solvent can be observed as a pronounced peak (centred at ~423 nm in the emission spectra when the excitation is carried out at 370 nm).

As previously reported in the main text, the dual emission (a consequence of the excited-state intramolecular proton transfer (ESIPT) process) is a peculiar property of flavonols lacking the 5-OH group such as 3-hydroxyflavone, whereas for flavonols with a 5-OH group fluorescence is reported to be absent [S34], almost absent, [35] or weak [36]. Although quercetin has a 5-OH group, for pH up to 5 a weak dual fluorescence deriving from the neutral form of quercetin and its tautomer (formed by ESIPT) can be observed (weak bands in the 450-460 nm and in 510-521 nm regions) .

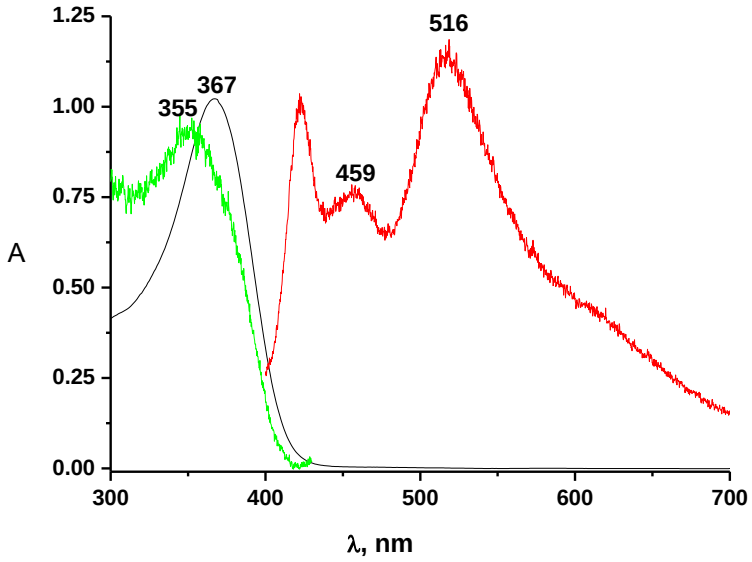


Figure S1: Absorption (black), excitation (green, λ_{em} = 530 nm) and emission (red, λ_{ex} = 370 nm) spectra of quercetin at pH = 1.

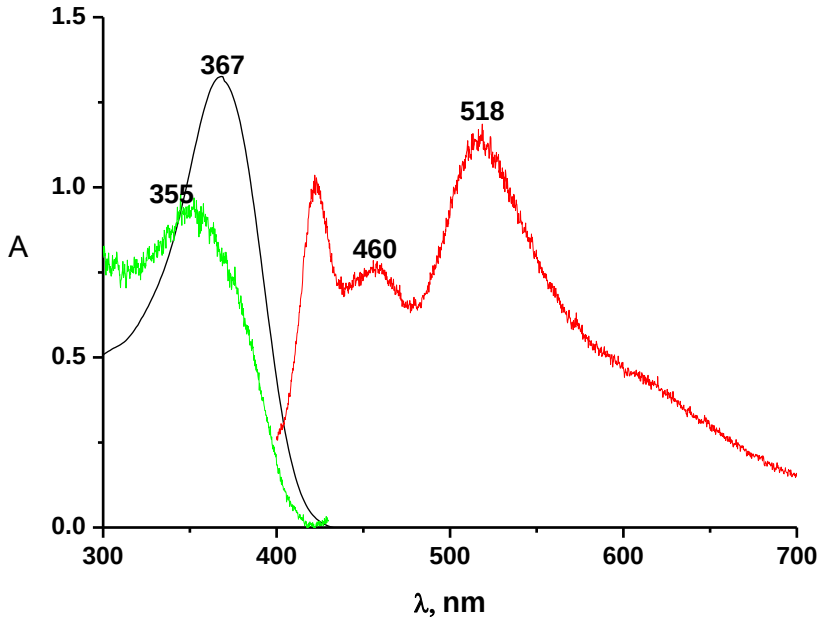


Figure S2: Absorption (black), excitation (green, λ_{em} = 530 nm) and emission (red, λ_{ex} = 370 nm) spectra of quercetin at pH = 2.

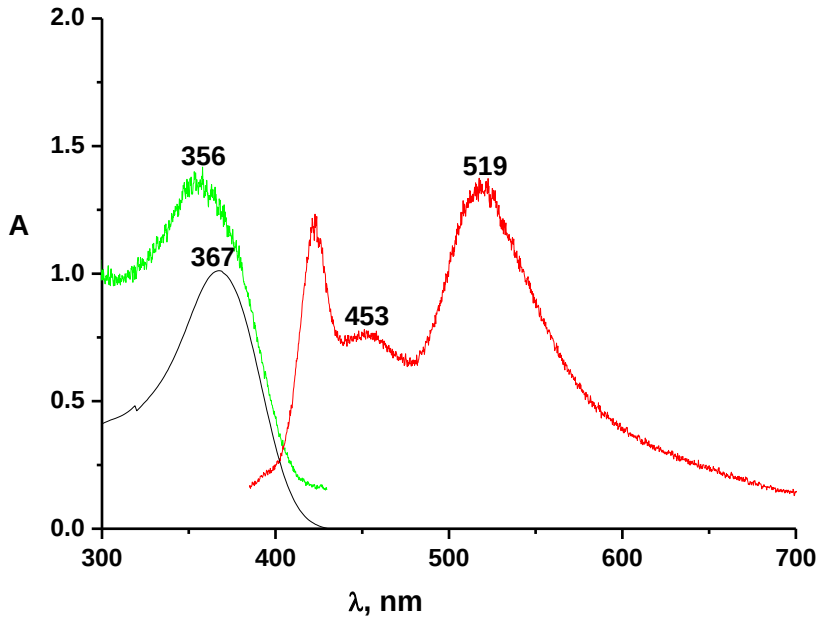


Figure S3: Absorption (black), excitation (green, λ_{em} = 530 nm) and emission (red, λ_{ex} = 370 nm) spectra of quercetin at pH = 3.

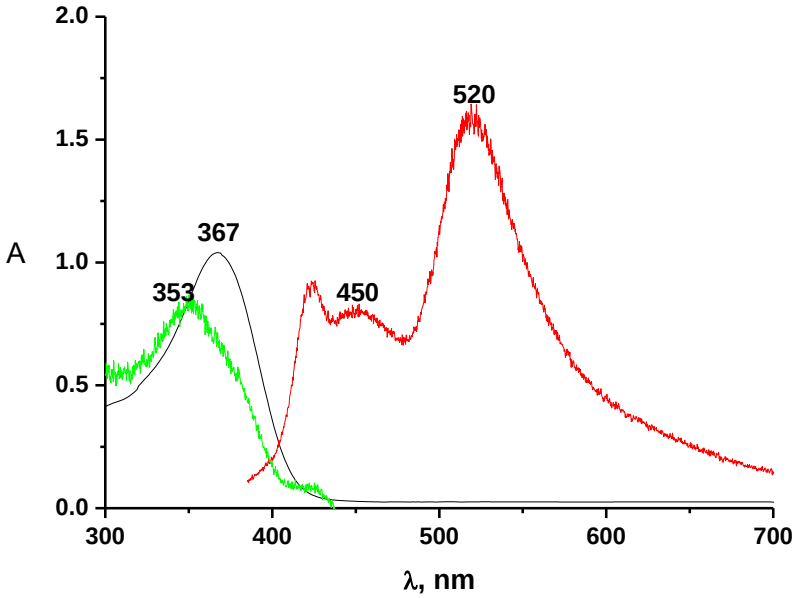


Figure S4: Absorption (black), excitation (green, λ_{em} = 530 nm) and emission (red, λ_{ex} = 370 nm) spectra of quercetin at pH = 4.

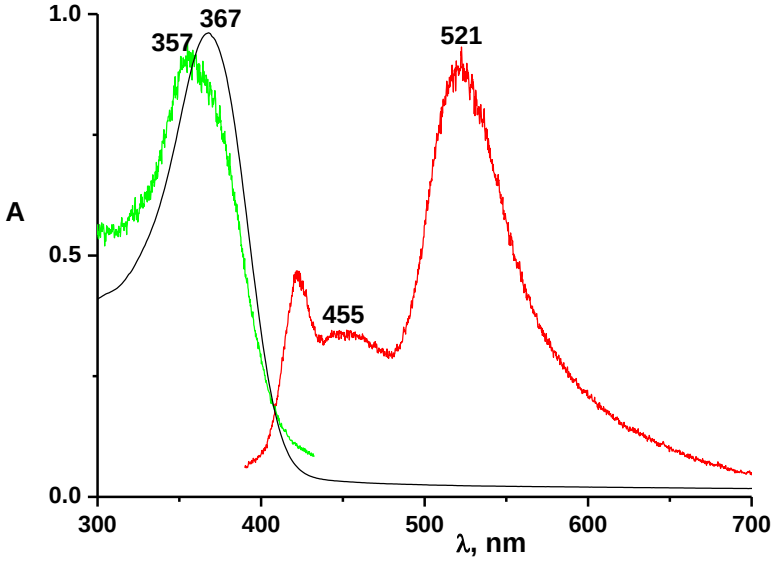


Figure S5: Absorption (black), excitation (green, λ_{em} = 530 nm) and emission (red, λ_{ex} = 370 nm) spectra of quercetin at pH = 5.

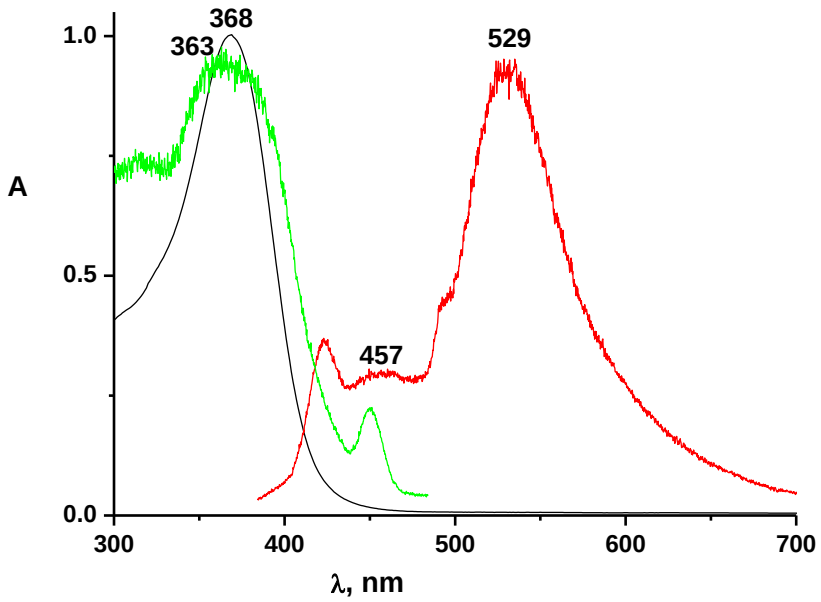


Figure S6: Absorption (black), excitation (green, λ_{em} = 530 nm) and emission (red, λ_{ex} = 370 nm) spectra of quercetin at pH = 6.

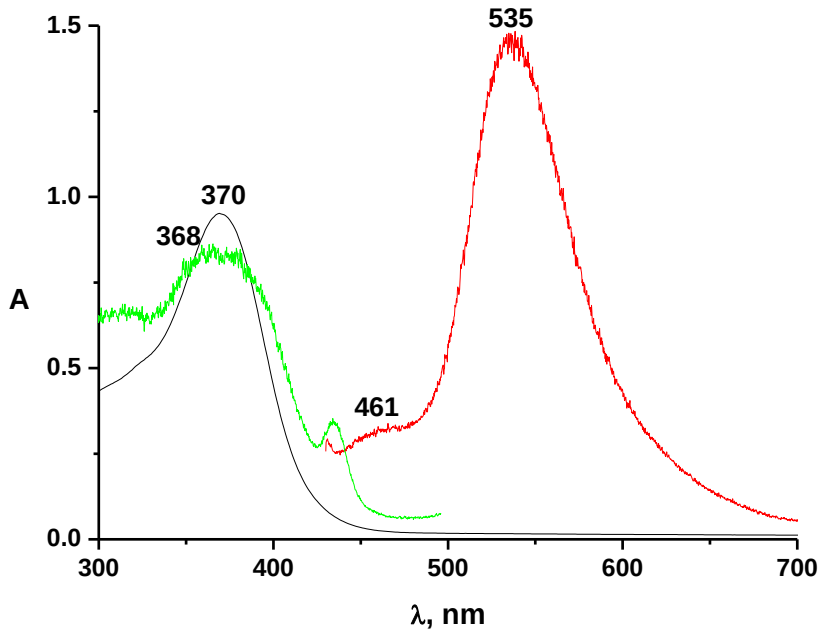


Figure S7: Absorption (black), excitation (green, λ_{em} = 530 nm) and emission (red, λ_{ex} = 370 nm) spectra of quercetin at pH = 7.

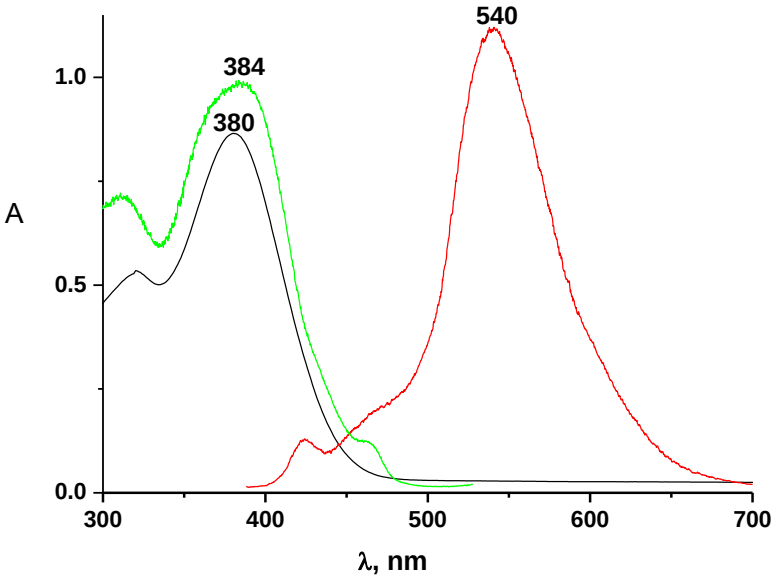


Figure S8: Absorption (black), excitation (green, λ_{em} = 550 nm) and emission (red, λ_{ex} = 370 nm) spectra of quercetin at pH = 8.

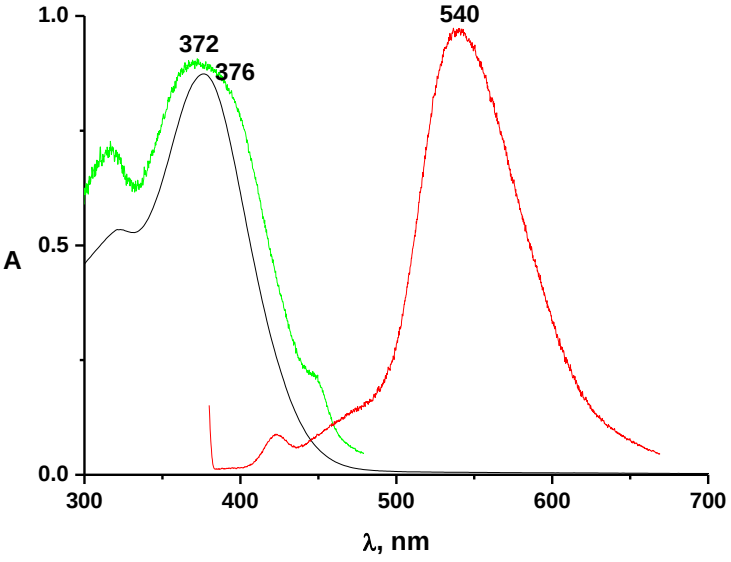


Figure S9: Absorption (black), excitation (green, λ_{em} = 550 nm) and emission (red, λ_{ex} = 370 nm) spectra of quercetin at pH = 9.

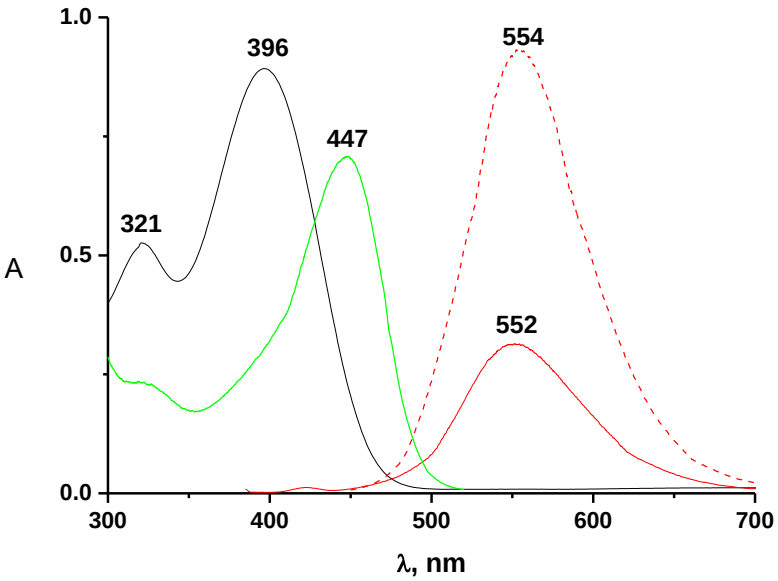


Figure S10: Absorption (black), excitation (green, λ_{em} = 550 nm) and emission (red, λ_{ex} = 370 nm, dotted line λ_{ex} = 430 nm) spectra of quercetin at pH = 10.

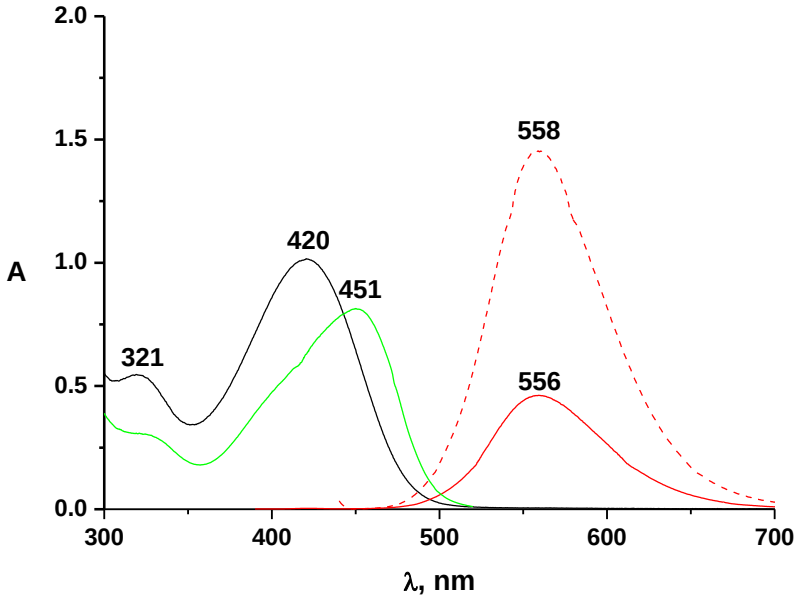


Figure S11: Absorption (black), excitation (green, λ_{em} = 550 nm) and emission (red, λ_{ex} = 370 nm, dotted line λ_{ex} = 430 nm) spectra of quercetin at pH = 11.

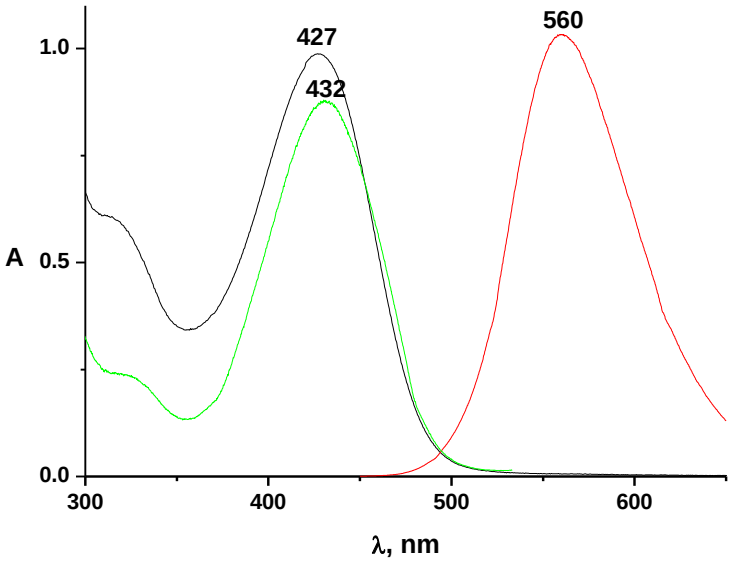


Figure S12: Absorption (black), excitation (green, λ_{em} = 550 nm) and emission (red, λ_{ex} = 440 nm) spectra of quercetin at pH = 12.

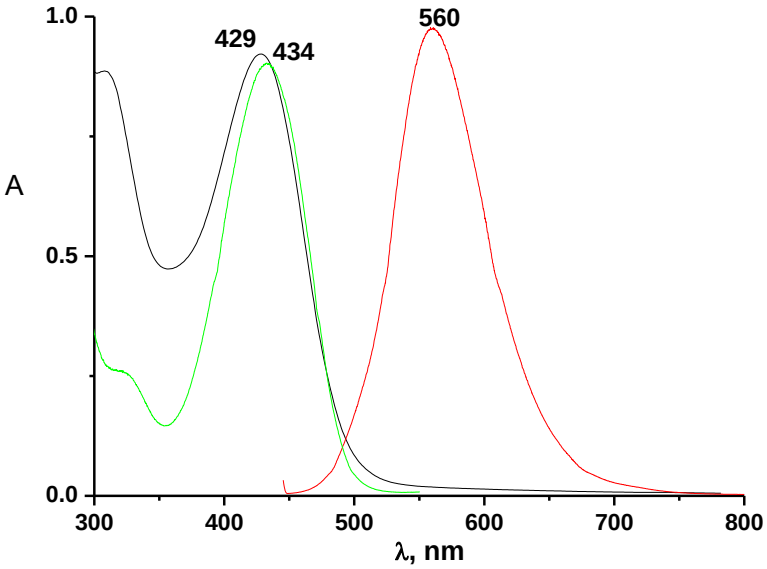


Figure S13: Absorption (black), excitation (green, λ_{em} = 550 nm) and emission (red, λ_{ex} = 440 nm) spectra of quercetin at pH = 13.

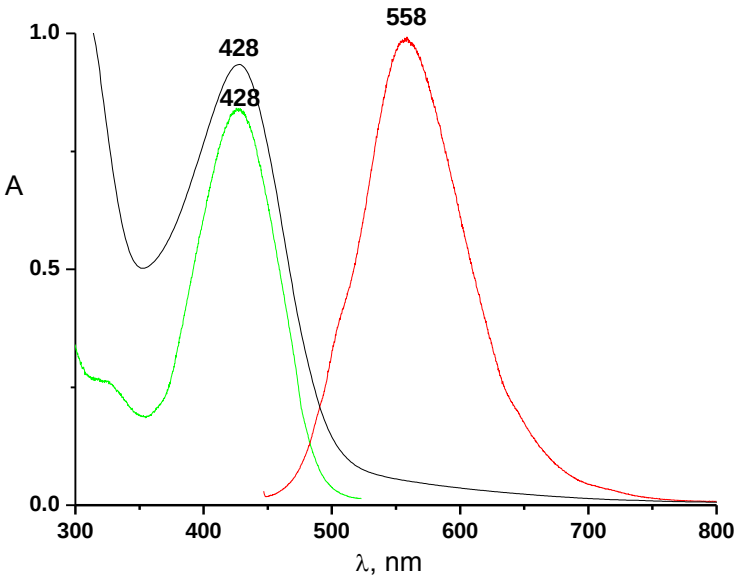


Figure S14: Absorption (black), excitation (green, λ_{em} = 550 nm) and emission (red, λ_{ex} = 440 nm) spectra of quercetin at pH = 14.

3. Influence of the alcaline pH on the fluorescence quantum yield (Φ_F) of quercetin in aqueous solution

Table S4.

pH	Φ_F
11.6	0.0115
12	0.00991
12.48	0.0084
12.7	0.0081
13	0.00653
13.48	0.00414

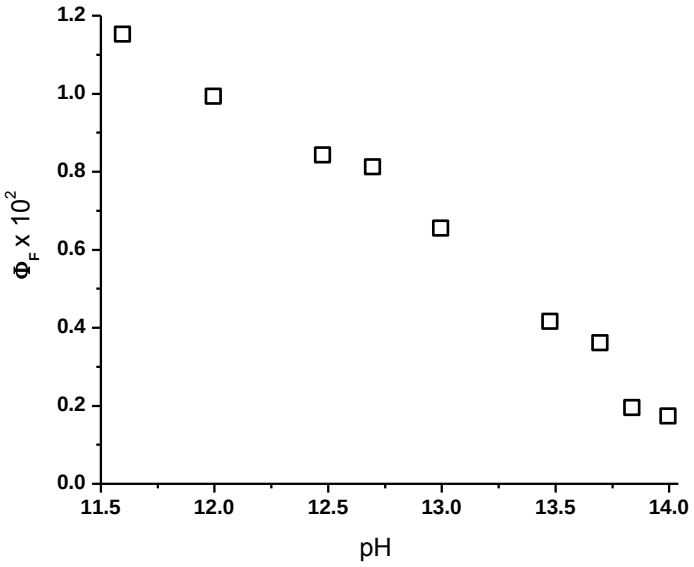


Figure S15. Fluorescence Quantum Yields (Φ_F) of an aqueous solution of quercetin at different alkaline pH (determined with respect to Eosin in basic ethanol (Φ_F = 0.67, [S37])

5. Emission/excitation spectra of quercetin in organic solvents.

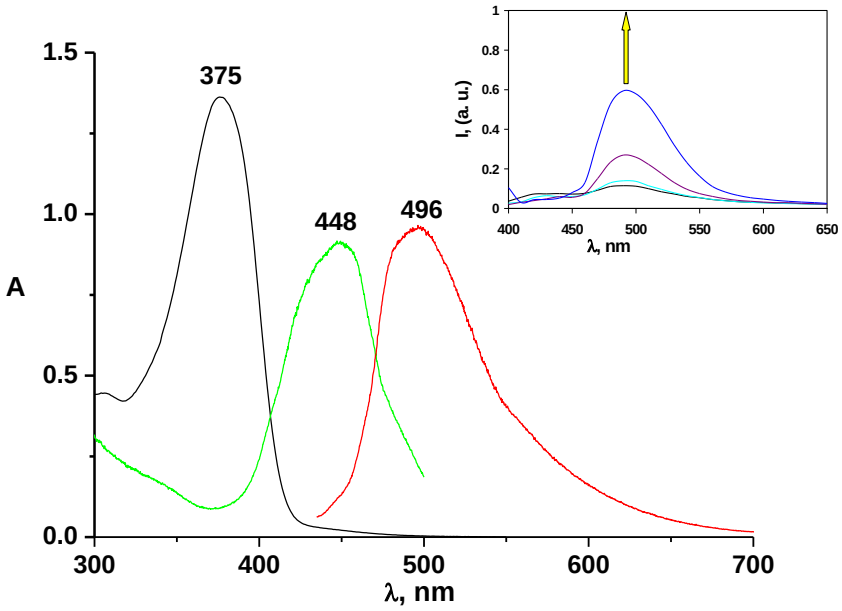


Figure S16: Absorption (black), excitation (green, λ_{em} = 505 nm) and emission (red λ_{ex} = 440 nm) spectra of quercetin (5 x 10^{-5} M) in DMF. Inset: Emission behaviour of quercetin in DMF varying λ_{ex} from 370 (black) to 445 nm (blue).

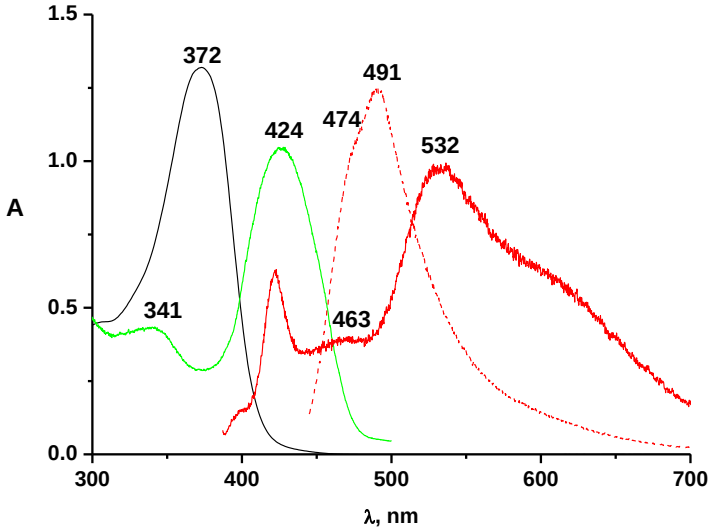


Figure S17: Absorption (black), excitation (green, λ_{em} = 500 nm) and emission (continuous red λ_{ex} = 375 nm, dotted red line λ_{ex} = 440 nm) spectra of quercetin (5 x 10^{-5} M) in methanol. It should be

noticed that the fluorescence spectra of quercetin in MeOH (λ_{ex} = 375 nm) has been multiplied by 10. Furthermore, in this spectrum a clear Raman band of the solvent is present as a pronounced peak centred at 423 nm.

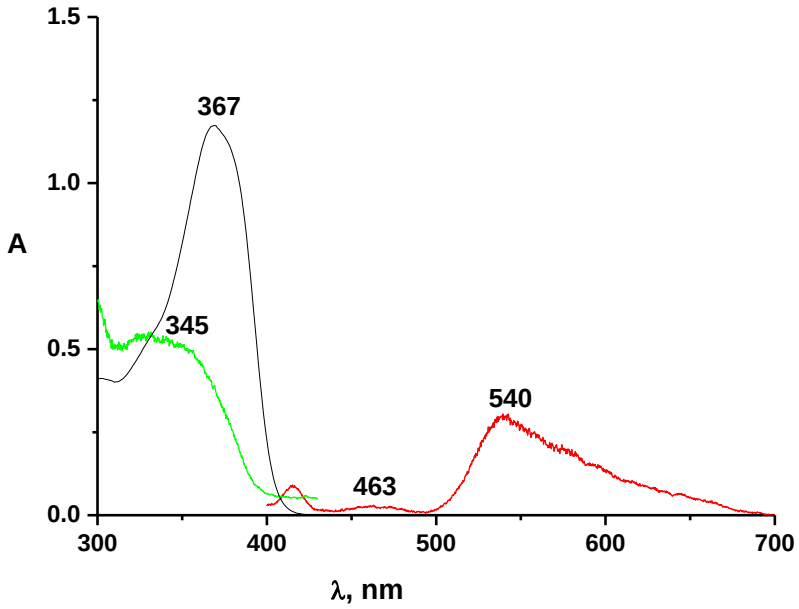


Figure S18: Absorption (black), excitation (green, λ_{em} = 550 nm) and emission (red, λ_{ex} = 370 nm) spectra of quercetin (5 x 10^{-5} M) in CH_2Cl_2. As reported for quercetin at low pH, a dual fluorescence can be observed as a broad emission at 540 nm and a very weak shoulder at 463 nm. Furthermore, a Raman signal deriving from the solvent is present as a pronounced peak centred at 415 nm in the emission spectra.

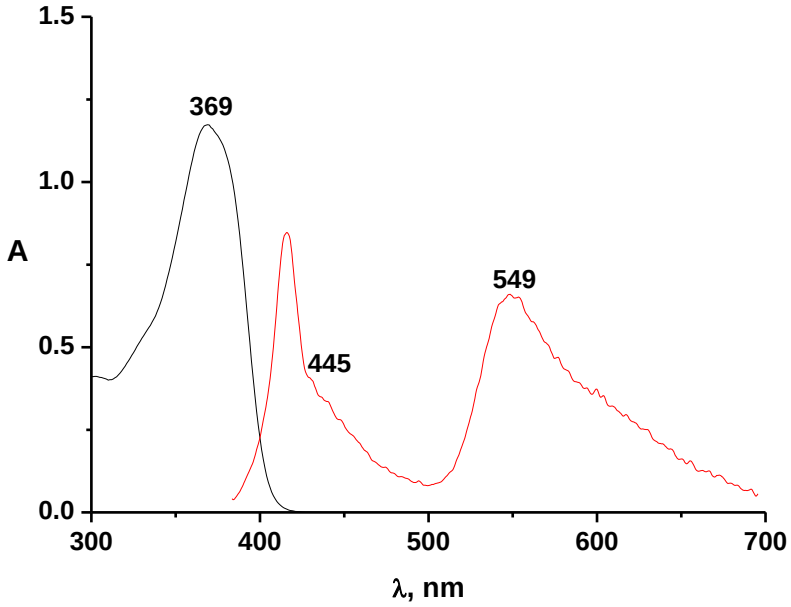


Figure S19: Absorption (black), and emission (red, λ_{ex} = 370 nm) spectra of quercetin (5 x 10^{-5} M) in Ethyl acetate. Notice the presence of two emission bands at 549 and 445 nm. The Raman contribution of the solvent is present as a pronounced peak centred at 416 nm in the emission spectra. It should be noticed that the measurement of the excitation spectrum has not been carried out because of the the weak intensity of the quercetin emission signal in these conditions.

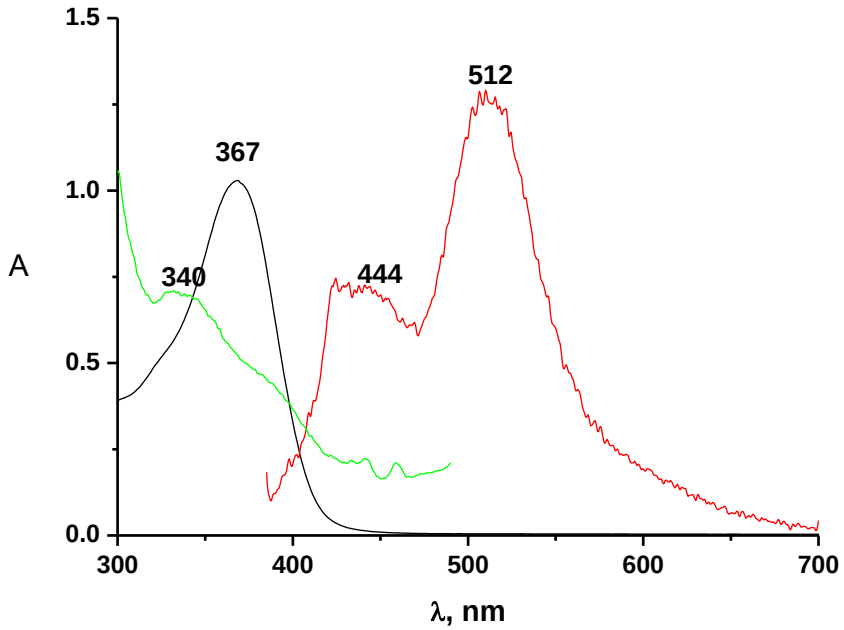


Figure S20: Absorption (black), excitation (green, λ_{em} = 550 nm) and emission (red, λ_{ex} = 370 nm) spectra of quercetin (10^{-5} M) in 2,2,2-trifluoroethanol (TFE). As reported above for the emission of quercetin at acidic pHs, in dichloromethane and ethyl acetate, the presence of the dual emission of neutral quercetin can be observed as two emission bands at 444 and 512 nm. A Raman band of the solvent is probably responsible of the ~421 nm peak in the emission spectrum. Note that because of the low fluorescence intensity of quercetin under these conditions it was not possible to obtain a well-defined excitation spectrum.

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