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ORIGINAL PAPER

Acidichromism and Ionochromism of Luteolin and Apigenin, the Main Components of the Naturally Occurring Yellow Weld: A Spectrophotometric and Fluorimetric Study

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Abstract Luteolin and apigenin, extracted from *Reseda luteola* L., were spectrophotometrically and fluorimetrically studied. The spectra were investigated as a function of pH in methanol/water solutions (1/2, *v*/*v*) in the 2–12 pH range. The absorption spectra markedly shifted to the red by increasing the pH. Three acid–base dissociation steps were detected for luteolin (pK_a=6.9; 8.6; 10.3) and two for apigenin (pK_a=6.6; 9.3). Fluorescence emission was very weak or undetectable ($\Phi_F<10^{-4}$) in acidic solution, but increased in intensity with increasing the pH. Both molecules exhibited a great propensity towards complex formation with metal ions, with association constants on the order of 10^5–10^7 for the first complexation step; in the presence of excess Al^{3+} ions, multiple equilibria were detected. A marked fluorescence enhancement was observed upon complexation with Al^{3+} ions ($\Phi_F \sim 1$ for luteolin and $\sim 10^{-2}$ for apigenin).

Keywords Luteolin · Apigenin · UV-visible spectrophotometry · UV-visible fluorimetry

Introduction

Dyes from natural sources were used in the past mainly for dyeing textiles. Presently, despite the wide development of industries for synthetic colourants, natural dyes are still appreciated to achieve particular colour nuances. They are also stimulating scientific interest in the field of the restoration and conservation of works of art where they have been mainly used, such as tapestries, carpets, miniatures, or only used for marginal details, as in paintings with different techniques.

In this paper an investigation is carried out on colourants from weld (*Reseda luteola* L.), a weedy plant originating from the Middle East, North Africa, and the Mediterranean area, which has been widely used because it is cheap, quick and easy to grow, and suitable for dying textiles. Metal ions are used as mordants to fix and stabilize the colour on the fibre; the metal salt most frequently in use has been alum, but other salts were also used to obtain different shades. With alum mordant, weld produces a yellow-orange colour on wool and silk, with copper it generates a greenish yellow colour, with iron it becomes olive [1, 2]. Weld was also used as a "lake" (on chalk or hydrated alumina) in painting and in medieval manuscripts [3].

The colour of weld is due to flavonoids; the principal of them are luteolin (LU, 5,7,3′,4′-tetrahydroxyflavone) and apigenin (AP, 5,7,4′-trihydroxyflavone), Scheme 1. The luteolin/apigenin ratio has been reported to be approximately 9/1 [4]; however, since weld is a natural product, the relative amounts of the two compounds depend on the origin of the plant and the dye extraction procedure used.

Works of art are subject to deterioration because of aging and, also, due to atmospheric pollutants and solar or artificial light irradiation. In dyed textiles, the interactions with metal ions, used as mordants, can significantly influence hue and fastness of the colour. It is important to know which chromatic changes they undergo when the environment changes. UV-visible spectrophotometry and fluorimetry are suitable techniques for the diagnosis of dyes

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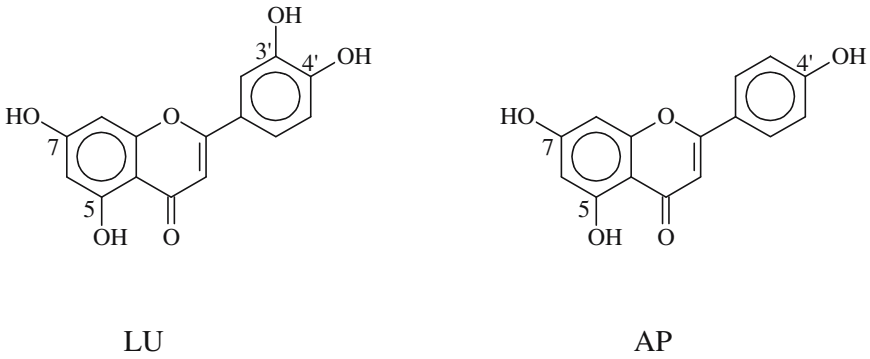
Scheme 1 Luteolin and apigenin



present in works of art and also for investigating the processes which could be responsible for the degradation of the dye and consequently for changes in colour [5–9].

In this work a spectrophotometric and fluorimetric investigation has been carried out on the two main components of the yellow colour extracted from weld. The effect of pH on their absorption and fluorescence spectra has been investigated; as a first approach to understand the interactions of the dyes with the mordant, their spectral properties have been investigated in methanol in the presence of aluminum ions.

We will demonstrate that both of them exhibit acidichromic and ionochromic effects; moreover, deprotonation and chelation transform non fluorescent or weakly fluorescent molecules in efficient fluorophores, thus improving lightfastness of the dyed textiles.

Experimental

Materials

Dried weld (*Reseda luteola* L.), leaves, stems and flowers, were supplied by Zecchi (Firenze, Italy). Reference reagents for HPLC analysis of weld extract were luteolin (99%) and apigenin (96%) supplied by Fluka (Buchs, Switzerland), luteolin-7-glucoside (99%) and apigenin-7-glucoside (99%) from Roth (Karlsruhe, Germany). For spectrophotometric and fluorimetric determinations commercial apigenin was purified by preparative HPLC, whereas luteolin was isolated from the dried plant. Methanol and acetonitrile for UV-visible spectroscopy were supplied by Fluka (Buchs, Switzerland). Quinine sulphate, used as a fluorescence standard, was from Baker (Phillpsburg, New Jersey), $AlCl_3{\cdot}6H_2O$ and $CaCl_2$ were from Carlo Erba (Milano, Italy). The solvents used for chromatographic separations (water and acetonitrile) were purchased from BDH and trifluoroacetic acid (TFA) was from Aldrich (Milwaukee, WI, USA).

Extraction from *Reseda luteola* L. and purification of the dyes

The natural yellow colour was extracted from the dried plant, cut into pieces and finely chopped into a mortar, with methanol. After refluxing for one hour, the solution was filtered and evaporated under vacuum at 30°C. The dry residue was dissolved in methanol and filtered. The dye components were detected at 350 nm and identified according to the retention time and the UV-visible spectrum, based on commercially available compounds used as standards. Besides luteolin, apigenin and their glucosides, the weld extract contained minor components showing absorption spectra typical of flavonols, which were not identified. Luteolin was then isolated from the extract using a preparative HPLC system. Apigenin, used for the spectroscopic investigations, was purified from the commercial product by an analogous HPLC separation. Preparative HPLC guaranties sample purity better than 98%.

Equipment and measurement conditions

Absorption spectra were recorded using a Perkin Elmer Lambda 16 spectrophotometer. Fluorescence lifetime, fluorescence emission and excitation spectra were obtained using a Spex Fluorolog-21680/1 spectrofluorimeter, controlled by the Spex Datamax spectroscopy software. For fluorimetric measurements, sample concentrations were adjusted in order to keep the absorbance at the excitation light not higher than 0.1–0.15 to avoid self-absorption of the fluorescence emission. The emission quantum yields were obtained by measuring and comparing corrected areas of the sample and of the standard (standard quinine sulphate in 1 M H_2SO_4, Φ_F=0.546 [10]) using the formula in Eq. 1 which accounts for the differences between absorbance and the refraction index of the sample (A_S, n_S) and standard (A_{ST}, n_{ST}) solutions.

$$\Phi_F = \Phi_{ST} \times (A_{ST}/A_S) \times (\text{area}_S/\text{area}_{ST}) \times \left(n_S^2/n_{ST}^2\right) \quad (1)$$

The fluorescence lifetimes were determined by using the Pockel's cell of the Spex spectrofluorimeter accessory which is based on the phase-shift method (time resolution ca. 30 ps). The light scattered by an aqueous glycogen solution (undetectable fluorescence) was used as the standard. The accuracy of the data obtained depended on the intensity of the emission. The data were processed using the Global Unlimited™ 3.0 software, which allows the analysis of multiple decays of up to four components.

The pK_a measurements were carried out in methanol/water (1/2, *v*/*v*), using Britton buffer solutions (pH 2–12) at low ionic strength (0.01) prepared with re-distilled water. Measurements of pH were made in the aqueous medium with an Orion SA 720 pH-meter using a combined pH glass electrode Metrohm AG 9101 Herisau. The pK_a values were determined from the absorbance versus pH curves at the wavelengths of maximum difference in absorbance of the equilibrating species. For AP more accurate pK_a values than for LU could be obtained by using the Thamer and Voigt equation [11]. Fluorimetric titrations were carried out in the basic pH region by exciting at an isosbestic point between the spectra of the equilibrating species. Given the low concentration of the solutions, ~10^{-5} mol dm^{-3}, the activity coefficient ratio of the acid–base couple was considered unitary.

For chelation studies methanol solutions containing dye concentrations of the order of 10^{-5} mol dm^{-3} and different concentrations of aluminium ions (10^{-6}–10^{-3} mol dm^{-3}) were used.

For chromatoghraphic determinations, an HPLC system, equipped with Waters 600 pump and controller, 2,487 dual λ absorbance and photodiode array detector, was used. For analytical purposes, a Jupiter C_{18} 300 A column (Phenomenex, 250×4.6 mm, 5 μm) and a security guard cartridge C_{18} 300 A (Phenomenex, 100×10 mm, 5 μm particle size) were used. For preparative purposes, a Jupiter C_{18} 300A column (Phenomenex, 250×21.20 mm, 10 μm particle size) was used.

All measurements were performed at room temperature 20±2 °C, using freshly prepared solutions.

Results and discussion

General spectral features of weld extract, luteolin and apigenin

After separation and drying, the absorption, fluorescence and fluorescence excitation spectra of purified apigenin (Fig. 1), luteolin (not shown here) and the weld extract (Fig. 2) were recorded in methanol.

The lowest energy transition, π,π^* character, observed in the absorption spectrum is located at 333 nm for AP (Fig. 1) and at 349 nm for LU. No fluorescence emission was detectable in methanol for LU ($\Phi_F<10^{-4}$), whereas AP exhibits an exciting wavelength dependent double emission. The two emission maxima at 430 nm (λ_{exc}=300 nm) and 534 nm (λ_{exc}=357 nm) are shown in Fig. 1. The band at 430 nm corresponds to a value reported in the literature [12]. The origin of the double emission lies in the fact that the π,π^* state of these molecules possesses a certain degree of charge transfer character, due to excited state intramolecular proton transfer (ESPT) from the phenolic 5-OH, more acidic in the excited state than in the ground state, to the carbonyl oxygen, more basic in the excited state. The proton transfer occurs with change in molecular geometry, in agreement with the large Stokes' shift (~7,000 cm^{-1}) observed. The emission quantum yield is $\Phi_F = 4 \times 10^{-4}$ and the fluorescence lifetime 0.8 ns. From these values, the radiative rate constant, $k_F=\Phi_F/\tau$, results 5×10^5 s^{-1} and the radiationless deactivation constant, $k_{NR} = (1/\tau) - k_F \approx 10^9 s^{-1}$. That is, the main relaxation paths for the excited singlet state are non-radiative.

The absorption spectrum of the extract (Fig. 2, spectrum 1) is closer to that of LU, as expected being LU the main weld component, whereas the fluorescence emission (Fig. 2, spectrum 2, λ_{max}=435 nm) and the excitation spectrum (Fig. 2, spectrum 3) resemble the emission and absorption of AP, being only AP fluorescent in methanol. Very interestingly, a weak and sharp absorption band was detected at 665 nm. In correspondence, a resonance fluorescence emission was observed at 671 nm. The intensity ratio of both these bands with respect to those of the yellow dye changed with the extraction batch. In the insert of Fig. 2, the spectra, obtained from an extraction batch where this red absorbing component was present in a significant amount, are shown. By comparison with literature data [13, 14], they are assigned to chlorophyll a,

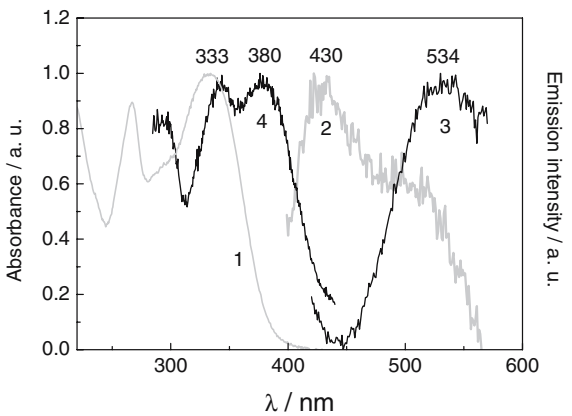


Fig. 1 Normalized absorption (*1*), fluorescence (*2*, λ_{exc}=300 nm; *3*, λ_{exc}=357 nm) and fluorescence excitation (*4*, λ_{em}=540 nm) spectra of AP in methanol

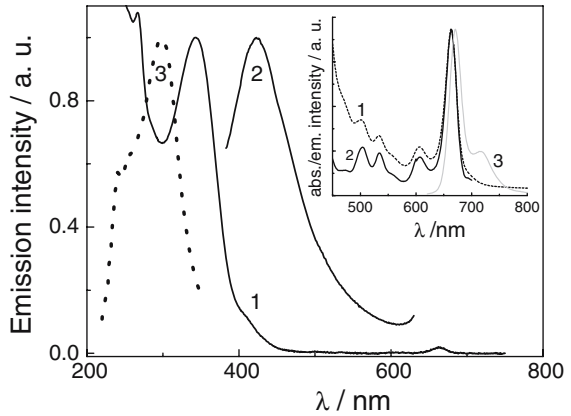


Fig. 2 Normalized absorption (*1*), fluorescence (*2*, λ_{exc}=330 nm) and fluorescence excitation (*3*, λ_{em}=410 nm) spectra of the extract in methanol. *Insert*: normalized absorption (*1*) excitation (*2*, λ_{em}= 720 nm) and fluorescence (*3*, λ_{exc}=600 nm) spectra of a different extraction batch

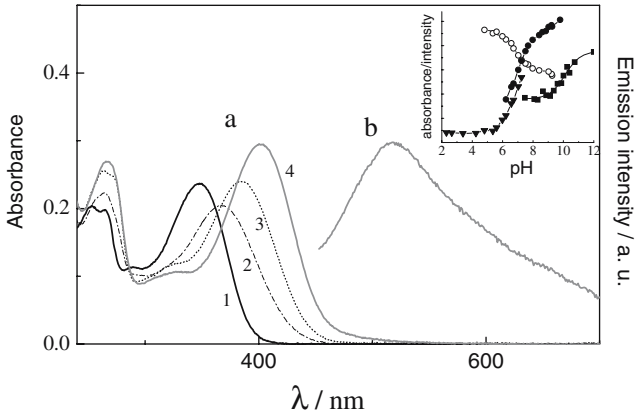


Fig. 4 Absorption spectra (*a*) of LU extracted from *Reseda luteola* L. in MeOH–water (1/2, *v*/*v*) at pH=2 (*1*), pH=7 (*2*), pH=9 (*3*), pH=12 (*4*), and fluorescence spectrum (*b*) in alkaline solution. *Insert*: spectrophotometric [$\lambda_{analysis}$=395 (*filled triangle*), 347 nm (*open circle*) and 402 nm (*filled circle*)] and fluorimetric (λ_{exc}=384 nm and λ_{em}=521 nm, *filled square*) titration curves

whose contribution changes with the percentage of green leaves present in the material used for the extraction.

Effect of pH on the absorption and emission spectra of AP and LU

Flavonoids have several ionisable hydroxyl groups with pK_a values relatively close to each other. Therefore, the accurate determination of the dissociation constants is a difficult task. Deprotonations are accompanied by colour changes: for both molecules, a red spectral shift of the absorption spectrum was observed by increasing the pH of the solution. The emission was very weak or undetectable in acidic solution but was observed at increasing intensity as the solution became alkaline. The dissociation constants were determined by a spectrophotometric method. Fluorimetric titration were also carried out only in the basic pH range where the fluorescence emission is easily detectable.

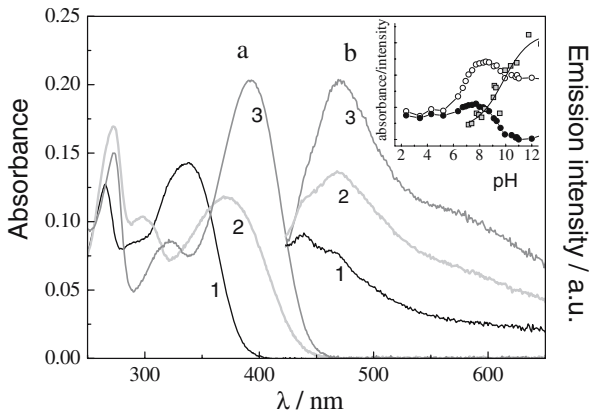


Fig. 3 Absorption (*a*) and fluorescence (λ_{exc}=357 nm, isosbestic point) (*b*) spectra of AP at pH values that correspond to the neutral form (*1*, pH=2), to the monoanion (*2*, pH=8) and to the di-anion (*3*, pH=12) in methanol–water (1/2 *v*/*v*) solutions. *Insert*: spectrophotometric [$\lambda_{analysis}$=273 (*open circle*) and 298 (*filled circle*) nm] and fluorimetric [λ_{exc}=357 nm and λ_{em}=472 nm (*filled square*)] titration curves

The absorption and emission spectra of AP at different pH values are shown in Fig. 3. The red shift of the lowest energy band (from 337 to 437 nm) causes the solution to turn from pale to intense yellow colour. The fluorescence intensity, which was very weak in neutral solution, increases with increasing the pH and the emission maximum shifts from 440 to 470 nm. The Stokes' shift is larger for the neutral form ($\Delta\nu$~6,800 cm^{-1}) while decreases in alkaline solution ($\Delta\nu$~4,200 cm^{-1}).

Absorbance ($\lambda_{analysis}$=273 and 298 nm) and fluorescence ($\lambda_{analysis}$=472 nm) titration curves for AP are shown in Fig. 3, insert. From the two inflection points in the absorbance-pH trends, two acid–base equilibration steps are recognised, the pK_as, determined using the Thamer–Voigt equation [11], are pK_{a1}=6.61±0.07 and pK_{a2}=9.31± 0.07 for the first and second deproptonation respectively. From the fluorescence titration curve, only the latter deprotonation step could be determined from sigmoidal fit of the experimental points (pK_{a2}=9.9, χ^2=0.84).

The absorption spectrum of LU, shown in Fig. 4, exhibits a parallel red shift (from 348 nm at pH=2 to 402 nm at pH=12) as AP with increasing the pH. Four species, characterized by distinct absorption spectra, were detected in the pH range explored (pH=2–12). Each of them was present within a limited pH interval and was the precursor for the one formed successively as the pH increased. The species were assigned to the neutral form (pH 2), mono-anion (pH 7), di-anion (pH 9) and tri-anion (pH 12). For this molecule the Voigt equation could not be applied, due to the absence of either maximum or minimum

Table 1 Absorption and fluorescence spectral properties as a function of pH and pK_a values for LU in MeOH–water (1/2 *v*/*v*)

Species	Absorption		Fluorescence	
	λ_{max}/nm	pK_a	λ_{max}/nm	τ/ns
Neutral (pH<5)	348			
		6.9±0.1		
		(7.3)[a]		
		(6.85)[b]		
Mono-anion (pH~7)	367		518	1.4–0.18
		8.6±0.2		
		(8.5)[a]		
		(8.1)[b]		
Di-anion (pH~9)	384.5		522	0.16–6.8
		10.3±0.3		
		(9.3)[a]		
		(8.95)[b]		
Three-anion (pH~12)	402		518	1.3
		(10.8)[a]		

[a] Data from [16]
[b] Data from [17] (in dioxane/water mixture, 3/7; *v*/*v*)

points in the titration curves (Fig. 4, insert). The pK_as were approximately determined from the inflection points of the absorbance/pH plots (see insert) and averaged over several analysis wavelengths. The spectral characteristics and pK_a values are collected in Table 1. The fluorescence was only detectable in alkaline solutions (see spectrum b in Fig. 4), where the fluorimetric titration yielded pK_{a3}=10.1 (Fig. 4, insert), corresponding to the third deprotonation step. Fluorescence lifetime measurements lead to emission decays which fitted a bi-exponential kinetic law well, with contributions from the two species that were pH dependent (Table 1), due to the impossibility to isolate the single ionic species. The two distinct lifetimes indicate that acid–base equilibration does not occur within the excited state lifetime. The di-anion is the longest-lived species (τ=6.8 ns). The Stokes' shift at pH 12 is $\Delta\nu$~5,600 cm^{-1}.

We believe that in both molecules the first ionization involves the OH in 7 position. This is the most acidic one due to the inductive electron-attracting effect of the 5-OH group (in relative *meta* position) which is stabilised by H-bonding to the carbonyl. The pK_{a1} of the two molecules have substantially the same value, due to the close similarity of the molecular environment of the acidic site, as also the spectra of the equilibrating species are very similar. In the mono-anion, the negative charge (O^- in 7 position) decreases the intrinsic acidity of the other OH groups in the molecule. Since the spatial distance between the two negative charges should be as large as possible to stabilize the di-anionic species, the second deprotonation probably occurs at the 4′-OH group in the 2-phenyl moiety. Its acidity is higher for LU where the inductive electron-attracting effect of an OH group on the nearby one increases the intrinsic acidity of both. This is in agreement with the experimental pK_{a2} lower for LU than for AP.

The proposed assignments are in line with literature data [15] reporting ionization constants of some flavonols (kaempferol, fisetin, morin and quercetin) which were determined by capillary zone electrophoresis comparing the values obtained with those calculated by a computational program. The LU pK_as are compared in Table 1 with those obtained by others, either indirectly by measurements on methyl-substituted compounds [16], or using potentiometric titrations [17]. Based on these data and the above considerations, the successive deprotonations occur in the order: 7-OH; 4′-OH; 3′-OH or/and 5-OH. The last change that could be detected for LU is probably due to superposition of the deprotonations of the 3′-OH and 5-OH.

Interaction with metal ions

The metal-dye interaction was followed from the changes in the absorption and emission spectra upon additions of Al^{3+} ions to methanol solutions of AP and LU.

In the case of AP (Fig. 5), a new band increased at 382 nm, which was assigned to formation of an Al^{3+}–AP complex. The spectral position of the complex absorption is close to that reported for the complex of 5-hydroxyflavone (λ=397 nm), where the unique chelation site corresponds to 5-hydroxy-4-keto groups [18, 19]. Three isosbestic points were clearly detectable and were maintained up to 8×10^{-5} mol dm^{-3} [Al^{3+}] concentration, indicating that the complex formation reaction is clean. Further additions of Al^{3+} (total Al^{3+} concentration, $[Al^{3+}]_0$, from 10^{-4} to 10^{-3} mol dm^{-3}) induced spectral changes in the UV region without significantly affecting the colour band. By comparing the obtained absorption spectra with those originated by varying the pH (Fig. 3a), it can be observed that deprotonation and chelation induce spectral changes which

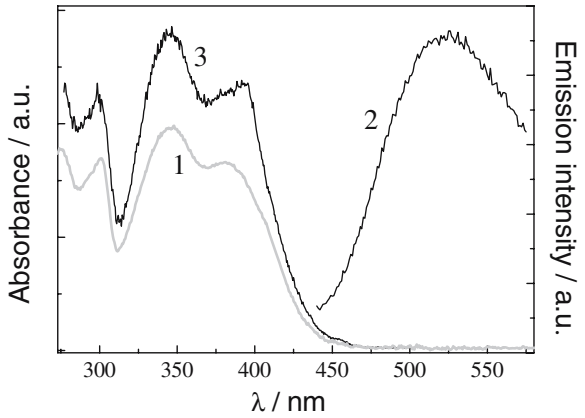


Fig. 5 Absorption (*1*), fluorescence (*2*, λ_{exc}=300 nm) and fluorescence excitation (*3*, λ_{em}=525 nm) spectra of a methanol solution of Al^{3+} and apigenin ([AP]=1.14×10^{-5} mol dm^{-3}, $[Al^{3+}]_0$=10^{-3} mol dm^{-3})

are qualitatively parallel. By assuming a 1:1 Al^{3+}–AP complex (C), the constant (K_1) for its formation reaction, occurring in the 2×10^{-6}–8×10^{-5} mol dm^{-3} $[Al^{3+}]_0$ range, was determined from the absorbance at the maximum of the complex, A (λ=382 nm), measured as a function of the added metal ions, Eq. 2.

$$Al^{+3} + AP \rightleftarrows C \qquad K_1 = \frac{[C]}{[AP] \times [Al^{+3}]} \qquad (2)$$

From the linear plot of $[Al^{3+}]_0/(A-A_0)$ vs $1/(A_\infty-A)$, Eq. 3, $K_1 = 2.8 \times 10^5$ dm^3 mol^{-1} is obtained from the slope (Fig. 6).

$$[Al^{3+}]_0 / (A - A_0) = 1/\Delta\varepsilon + (1/K_1)/(A_\infty - A) \qquad (3)$$

The constant for the second interaction (K_2) can be approximately estimated, following the absorbance changes at

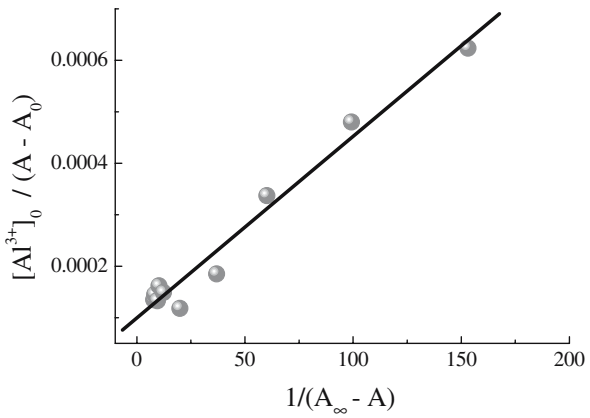


Fig. 6 Absorbance data at 381 nm for a methanol solution of AP treated according to Eq. 3: A_0 and A_∞ refer to the absence of Al^{3+} and total complexation, respectively

274 nm, using the simplified Eq. 4, which was obtained considering that the Al^{3+} concentration (2×10^{-4}–10^{-3} mol dm^{-3}) is much larger than that of the dye (10^{-5} mol dm^{-3}) and therefore can be considered constant. From the linear plot of $(A-A_0)/(A_\infty-A)$ vs $[Al^{3+}]_0$, $K_2 = 5 \times 10^3$ dm^3 mol^{-1} is obtained from the slope.

$$(A - A_0)/(A_\infty - A) = K_2 \times [Al^{3+}]_0 \qquad (4)$$

The association with Al^{3+} significantly enhances the fluorescence intensity of the dye, originating an emission band at 525 nm (spectrum 2 of Fig. 5), with a large Stokes' shift (~7,100 cm^{-1}). The excitation spectrum (spectrum 3 of Fig. 5) is quite similar to the absorption one (spectrum 1 of Fig. 5). The emission spectrum corresponds to that observed from the ESPT state in the pure AP methanol solutions (see Fig. 1), but the quantum yield, $\Phi_F = 1.2 \times 10^{-2}$, is almost two orders of magnitude larger than for pure AP($\Phi_F = 4 \times 10^{-4}$).

Some investigations carried out using calcium ions as chelating agents with AP showed that the dye is less prone to give complexes with calcium than with aluminium. Spectral changes only appeared when the Ca^{2+} concentration was approximately 10^3 times larger than that of AP. This scant affinity can explain the fact that weld lakes containing calcium salts are less stable than those on hydrated alumina [3]. Very interestingly, even for this complex the fluorescence (λ_{max}=475 nm) was markedly enhanced (Φ_F=0.014) compared with that of the free dye.

The spectral changes observed by adding Al^{3+} ions to a LU methanol solution are shown in Fig. 7. As can be argued from the spectra, where no isosbestic point is maintained over a wide concentration range ($[Al^{3+}]$: 5×10^{-6}–3×10^{-4} mol dm^{-3}), multiple interactions occur, leading to an overall bathochromic spectral shift (from 349 to 425 nm) of the colour band; even in the UV region marked variations are observed.

The spectral changes are quite similar, even if shifted to longer wavelengths, to those reported for the Al-complexation of 3′,4′-dihydroxyflavone, where only the two OH at the phenyl group can be involved in the chelation [19, 20] and 5-hydroxyflavone, where chelation occurs at the 5-OH and 4-CO.

It has been demonstrated [20, 21] that the stoichiometry composition of the chelate in methanol is 1:1. for 3′,4′-dihydroxyflavone as well for 5-hydroxyflavone [18, 21, 22]; however, there are also evidences for a 1:2 stoichiometry for Al–flavonole complexes [23]. In the present case, in the 10^{-6}–5×10^{-6} $[Al^{3+}]$ range, where isosbestic points were maintained, the equilibrium constant for a hypothesized 1:1 complex, Eq. 2 was approximately estimated on the order of 10^7 dm^3 mol^{-1}, in line with that determined for the 3′,4′-dihydroxyflavone, $K = 5 \times 10^6$ [20]. Analogies

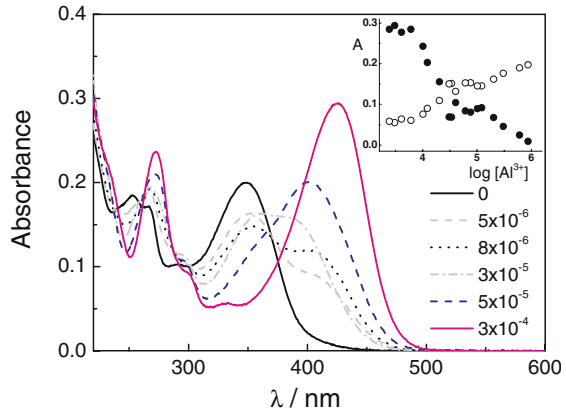


Fig. 7 Evolution of the absorption spectrum of LU in methanol (2.7×10^{-5} mol dm^{-3}) upon addition of Al^{3+} ions ($[Al^{3+}]$: $0–3\times10^{-4}$ mol dm^{-3}). *Insert*: absorbances at two selected wavelengths (*filled circle*, λ=425 nm and *open circle*, λ=348 nm) as a function of the negative $\log[Al^{3+}]_0$

with other hydroxyflavones and comparison of the spectra of the complex with those of the deprotonated forms do not allow the structure of the complex to be established with certainty. The firstly formed complex is involved in further interaction with the Al^{3+} ions added. In the $8\times10^{-6}–3\times10^{-5}$ mol dm^{-3} Al^{3+} concentration range, where the dye and the ion are present in comparable amounts, no isosbestic point was maintained and the global spectral pattern slightly shifted to the blue. A regular spectral variation started again when Al^{3+} reached a concentration on the order of 10^{-4} mol dm^{-3}. The final spectrum exhibits a bathochromically shifted, very intense band at 425 nm (Fig. 7), which does not show correspondence in the spectra detected for LU by increasing the pH, but is in the same spectral position as found for other flavonoids–Al complexes and assigned to 5-OH chelation [19]. Participation of the 5-OH in the last step of complexation of LU seems to justify the marked absorption shift to the red. Absorbances at selected wavelengths, corresponding to the most significant variations, that are reported in the insert of Fig. 7 as a function of the total Al^{3+} added (logarithmic scale), evidence the multiple-step complexations. Formation constants reported by others for Al(III)–luteolin interaction are not in satisfactory agreement with our findings [17], probably due to the different solvent used (dioxane/water vs methanol). Even the spectra of the complex were different: they did not show the most bathochromic band at 425 nm. However, the Al^{3+}–flavonoid stoichiometry remains an open problem [24].

The intensity of the fluorescence emission induced by Al^{3+} addition in methanol solutions of luteolin is exceptionally high. The emission maximum shifts from 570 nm ($[Al^{3+}]_0=10^{-6}$ to 10^{-5} mol dm^{-3}) to 540 nm ($[Al^{3+}]_0\sim3\times10^{-5}$ mol dm^{-3}) and again to the red (553 nm) upon further Al^{3+} additions (Fig. 8b). The excitation spectra follow a parallel trend (Fig. 8a). The Stokes' shift is 5500 cm^{-1} for $[Al^{3+}]_0=10^{-4}$ mol dm^{-3}. The emission quantum yield is ~1. That is, fluoresce is the unique relaxation path for the excited state which guaranties a very high lightfastness of the chelate [25]. The spectral behaviour of LU in the presence of Al^{3+} ions, indicates its potential as chromogenic and fluorogenic sensor for anions [26].

Conclusions

Two naturally occurring dyes, apigenin and luteolin, are here investigated by absorption and emission spectrometry. Even though luteolin has been widely investigated, information that can be found in the literature about the minor component of weld, apigenin, is very scarce. This study describes and compares the effects of pH changes and cation additions on the chromatic properties of both molecules for the first time in a systematic and unitary context. They behave as acidichromic and ionochromic molecules since in aqueous solutions their colour changes as the pH changes and in methanol solutions their colour changes upon metal ion additions. The absorption maximum moves to the red on increasing the pH; from spectrophotometric titrations two p*K*s were determined for AP and three for LU. In the neutral form (pH<6) AP is only weakly fluorescent, whereas fluorescence from LU is undetectable. By increasing the pH the fluorescence increases in both of them: deprotonation enhances the emission, probably because of the greater compactness of the ion, where the contribution to deactivation of the excited state through OH vibrations is weak.

Chelation with Al^{3+} ions has an effect parallel to deprotonation. It shifts the absorption spectrum to the red,

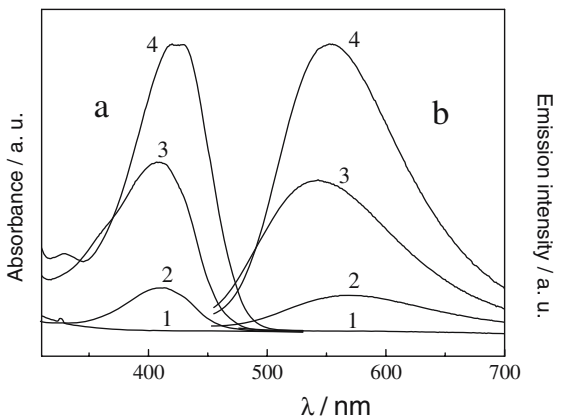


Fig. 8 Normalized emission excitation (*a*) and emission (*b*) spectra of the LU–Al^{3+} adducts at increasing concentrations of Al^{3+} ions: $[Al^{3+}]_0$= 0 (*1*); $[Al^{3+}]_0=5\times10^{-6}$ (*2*); $[Al^{3+}]_0=2.5\times10^{-5}$ (*3*) and $[Al^{3+}]_0=4\times10^{-4}$ (*4*) mol dm^{-3}

induces fairly intense fluorescence emission in LU ($\Phi_F \sim 1$) and significantly enhances the intensity of AP fluorescence (by almost 100 times). The appearance of emission when chelation occurs can be attributed to the lack of excited state vibrational relaxation through hydrogen bonding with the solvent, similarly to what happens for the deprotonated forms. The intense fluorescence induced by Al^{3+} ions can be used as a diagnostic probe for these flavonols. Moreover, important diagnostic information about the presence of weld, or even other colourants of vegetal origin, can be provided by the detection of the bathochromic absorption and emission bands typical of chlorophyll, that can be present at a different extent depending on the kind of plant and the extraction method applied.

It can be concluded that complexation transforms molecular systems which are not (or poorly) fluorescent and weakly yellow coloured into efficient fluorophores and intensely yellow coloured materials. The intense fluorescence explains the light-fastness of the colourant on textiles dyed using a mordant, since the absorbed light is given up as emission, thus reducing possible photochemical degradation processes.

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References

1. Cerrato A, De Santis D, Moresi M (2002) Production of luteolin extracts from *Reseda luteola* and assessment of their dyeing properties. J Sci Food Agric 82:1189–1199
2. Hofenk de Graaff JH (2004) The colourful past. Abegg-Stiftung and Archetype, London, pp 215–219
3. Saunders D, Kirby J (1994) Light-induced colour changes in red and yellow lake pigments. Natl Gallery Tech Bull 15:79–97
4. Wouters J, Rosario-Chirinos N (1992) Dye Analysis of Pre-columbian Peruvian Textiles with High Performance Liquid Chromatography and Diode-Array Detection. J Am Inst Conserv 31:237–255
5. Miliani C, Romani A, Favaro G (1998) A spectrophotometric and fluorimetric study of some anthraquinoid and indigoid colorants used in artistic paintings. Spectrochim Acta Part A 54:581–588
6. Miliani C, Romani A, Favaro G (2000) Acidichromic effects in 1,2-di- and 1,2,4-tri-hydroxyanthraquinones. A spectrophotometric and fluorimetric study. J Phys Org Chem 13:141–150
7. Clementi C, Romani A, Miliani C, Favaro G (2006) In situ fluorimetry: a powerful non-invasive diagnostic technique for natural dyes used in artefacts. Part I: Spectral characterization of orcein in solution, on silk and wool laboratory-standards and a fragment of Renaissance tapestry. Spectrochim Acta Part A 64:906–912
8. Favaro G, Miliani C, Romani A, Vagnini M (2002) Role of protolytic interactions in photo-aging processes of carminic acid and carminic lake in solution and painted layers. J Chem Soc Perkin Faraday Trans 2:192–197
9. Shimoyama S, Noda Y (1994) Non-destructive determination of plant dyestuffs used for ancient madder dyeing, employing a three-dimentional fluorescence spectrum technique. Dyes Hist Archaeol 13:14–26
10. Meech SR, Phillips D (1983) Photophysics of some common fluorescence standards. J Photochem 23:193–217
11. Thamer BJ, Voigt AF (1952) The spectrophotometric determination of overlapping dissociation constants of dibasic acids. J Phys Chem 56:225–232
12. Wolfbeis OS, Begum M, Geiger H (1984) Fluorescence Properties of Hydroxy- and Methoxyflavones and the Effect of Shift Reagents. Z Naturforsch 39b:231–237
13. Seely GR, Jensen RG (1965) Effect of solvent on the spectrum of chlorophyll. *Spectrochim Acta* 21:1835–1845
14. Du H, Fuh RA, Li J, Corkan A, Lindsey JS (1998) Photochem CAD: A computer-aided design and research tool in photochemistry. Photochem Photobiol 68:141–142
15. Herrero-Martinez JM, Sanmartin M, Roses M, Bosch E, Rafols C (2005) Determination of dissociation constants of flavonoids by capillary electrophoresis. Electrophoresis 26:1886–1895
16. Wolfbeiss OS, Begun M, Geiger H (1987) The fluorescence properties of luteolins. Monatsh Chem 118:1403–1411
17. Karadag R (2003) Potentiometric and Spectrophotometric Determination of the Stability Constant of Luteolin (3′,4′,5,7-tetrahydroxyflavone) Complexes with Aluminium (III) and Iron (III). Chem Anal (Warsav) 48:931–937
18. Cornard JP, Merlin JC (2001) Structural and spectroscopic investigation of 5-hydroxyflavone and its complex with aluminium. J Mol Struct 569:129–138
19. Cornard JP, Merlin JC (2003) Comparison of chelating power in hydroxyflavones. J Mol Struct 651–653:381–387
20. Cornard JP, Boudet AC, Merlin JC (2001) Complexes of Al(III) 3′ 4′-dihydroxy-flavone: characterization, theoretical and spectroscopic study. Spectrochim Acta Part A 57:591–602
21. Porter LJ, Markham KR (1970) Aluminum(III) complexes of hydroxyflavones in absolute methanol. I. Ligands containing only one chelating site. J Chem Soc C: Organic: 344–349
22. Alluis B, Dangles O (1999) Acylated flavone glucosides: synthesis, conformational investigation, and complexation properties. Helv Chim Acta 82:2201–2212
23. Deng H, van Berkel GJ (1998) Electrospray Mass spectrometry and UV/Visible spectrophotometry studies of aluminium (III)-flavonoid complexes. J Mass Spectrom 33:1080–1087
24. Boudet AC, Cornard JP, Merlin JC (2000) Conformational and spectroscopic investigation of 3-hydroxyflavone-aluminium chelates. Spectrochim Acta Part A 56:829–839
25. Smith GJ, Thomsen SJ, Markham KR, Andary C, Cardon D (2000) The photostabilities of naturally occurring 5-hydroxyflavones, flavonols, their glycosides and their aluminum complexes. J Photochem Photobiol A: Chem 136:87–91
26. Sathish S, Narayan G, Rao N, Janardhana C (2007) A Self-Organized Ensemble of Fluorescent 3-Hydroxyflavone-Al(III) Complex as Sensor for Fluoride and Acetate Ions. J Fluoresc 17:1–5