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Natively fluorescent isoflavones exhibiting anomalous Stokes' shifts

Eva de Rijke [a], Hem C. Joshi [b], Huib R. Sanderse [a], Freek Ariese [a], Udo A.Th. Brinkman [a], Cees Gooijer [a,*]

[a] *Department of Analytical Chemistry and Applied Spectroscopy, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands*

[b] *Institute for Plasma Research, Bhat, Gandhinagar 382428, India*

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Abstract

The fluorescence behaviour of 19 flavonoids was studied. Three isoflavones (formononetin (F), ononin (FG) and daidzein (D)) were found to exhibit large Stokes' shifts, possibly due to a change of the structure of the molecule from non-planar in the S_0 state to planar in the S_1 state. Lifetime measurements were carried out using time-correlated single photon counting spectroscopy to further characterise the mechanism. These large shifts provide a high selectivity, so that fluorescent isoflavones can be readily detected in plant samples by means of reversed-phase LC with fluorescence detection. Attention has to be paid to possibly fluorescent impurities in flavonoid standards, as was observed for daidzin (DG), which is not fluorescent itself, but has a fluorescent isomer. To distinguish between the two compounds, LC with fluorescence and MS detection was used to separate and identify the impurity.

Keywords: Fluorescence; LC; Stokes' shift; Lifetime measurements; Flavonoids; Isoflavones

1. Introduction

Flavonoids are one of the most characteristic groups of compounds in higher plants and are present not only in flowers and leafs but also in roots [1]. They are known for their UV-B protecting properties in plants, are used as food supplements and some of them, the isoflavonoids, are known to be phytoestrogens [2]. The ultimate objective of our present research programme is to use flavonoid profiles in wetland plants as chemical indicators for stress in wetland ecosystems. Obviously, this requires the use of various analytical methods, primarily gradient LC–UV, LC–MS and LC–MS/MS. In addition, it is of interest to find out whether fluorescence spectroscopy, known for its inherent selectivity, can be used as an alternative detection technique, either stand-alone or in combination with LC.

To the best of our knowledge, there is no report of a systematic and comprehensive study of the native fluorescence of flavonoids, though individual members of this class received much attention. This holds especially for 3-hydroxyflavone (3HF) and some of its derivatives. 3HF has been studied since the early 1980s [3,4] and is still receiving interest from various sides [5–7]. Its fluorescence behaviour is fascinating

* Corresponding author. Tel.: +31-204447540; fax: +31-204447543.
E-mail address: gooijer@chem.vu.nl (C. Gooijer).

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because it shows an extremely large wavelength difference between excitation and emission which is strongly dependent on solvent polarity observations that have been interpreted by the excited state intramolecular proton transfer (ESIPT) mechanism. After excitation, extremely fast (~30 fs) proton transfer takes place and emission of the tautomeric form is observed [7].

The present study deals with 19 representative flavonoids, i.e. flavones characterised by a chromone skeleton and a phenyl substituent at the C_2 position (3HF belongs to this group), isoflavones with the phenyl substituent at the C_3 position of the chromone group and some flavones in which the C_2–C_3 bond in the chromone skeleton is saturated. It will be shown that within this series of 19 flavonoids—apart from 3HF—only a few isoflavones show native fluorescence, characterised by very large Stokes' shifts that cannot be attributed to ESIPT.

2. Experimental

2.1. Materials

Daidzein (D), genistin, genistein, naringin, naringenin-7-glucoside, naringenin, formononetin (F) and ononin (FG) were purchased from Roth (Karlsruhe, Germany); 3HF, biochanin A, sissotrin and puerarin from Indofine Chemical Co. (Somerville, NJ, USA); rutin trihydrate and kaempferol from Fluka Chemie (Buchs, Switzerland); and quercetin, hesperedin, hesperitin and ammonium formate from Sigma–Aldrich (Steinheim, Germany). Daidzin (DG) was obtained from both Roth and Indofine Chemical Co. Methanol and formic acid were purchased from J.T. Baker (Deventer, The Netherlands). Ultrapure water was prepared with a Millipore-Academic system (Etten-Leur, The Netherlands). For the fluorescence experiments at different pH values, all solutions were prepared by diluting stock solutions in methanol with phosphate buffer, except for the pH 13 solution, to which extra 4 M NaOH was added to obtain the correct pH.

2.2. Instrumentation

Absorption spectra were recorded on a Varian Cary 50 Bio UV–VIS absorption spectrophotometer over the wavelength range of 200–500 nm. Fluorescence absorption and emission spectra were recorded on LS-50-B spectrofluorimeter (Perkin-Elmer, Beaconsfield, UK). LC–MS was performed on a Shimadzu (Princeton, NJ, USA) LC system, consisting of two LC-10A LC pumps, a DGU-14A degasser, a SIL-10AD auto-injector, a SCL-10A system controller unit and a SPD-10A UV detector (set at 265 and 290 nm), coupled to a ThermoQuest Finnigan (San Jose, CA, USA) LCQ deca ion trap mass spectrometer. The mass spectrometer was provided with an electrospray interface. Mass spectra were acquired in the positive and negative ion mode in the *m*/*z* range of 150–650. The capillary temperature was maintained at 275 °C. MS/MS spectra were acquired by using collision-induced dissociation at 30% activation energy. The LC effluent was split, 0.2 ml/min being led to the mass spectrometer.

For LC–UV–FLU, LC was performed on a Hewlett-Packard (Palo Alto, CA, USA) series 1090 LC system with a UV/DAD detector; fluorescence data were obtained with an Applied Biosystems (Foster City, CA, USA) model 980 programmable fluorescence detector. Excitation was at 250 nm and two emission filters were used; a 350 nm bandpass emission filter or a cut-off filter of >450 nm. A 250 mm × 4.6 mm i.d., 5 μm Zorbax SB-C_{18} column was used. The eluent was a mixture of methanol and aqueous 10 mM ammonium formate buffer, pH 4.0. The LC gradient used is shown in Table 1. All solvents were filtered and degassed with helium before use. The flow was 1.0 ml/min and the injection volume was 10 μl. The optimisation of the LC gradient and detection parameters in LC–UV–FLU and LC–MS was described in earlier work [8].

Lifetimes were measured using the time-correlated single photon counting technique [9]. The excitation

Table 1
LC gradient used for flavonoid analysis

Time (min)	Percentage of methanol	Percentage of buffer
0	30	70
5–10	45	55
15	50	50
25	55	45
30	60	40
35–40	99	1
42	30	70

source was a Coherent Mira 900 Ti-sapphire laser with a pulse width of about 3 ps. The output from the laser was frequency tripled to obtain an excitation wavelength of 250 nm. The energy was about 2 nJ per pulse, at a repetition rate of 76 MHz. Fluorescence was collected at a right angle from the sample cell and dispersed by a monochromator on a MPC–PMT (Hamamatsu R3809U-50) detector. Decay data were recorded with the help of an SPC-630 (Becker–Hickl) module and analysed using Fluofit software (Picoquant). For a good fit, χ^2 should be close to unity and residuals should be distributed randomly [10]. The accuracy of the instrument was checked by recording the lifetimes of some standard compounds. The precision of the lifetimes was around 50 ps. The temperature was controlled and measured by a home-built system; the precision of the temperature measurements was ±1 K.

3. Results and discussion

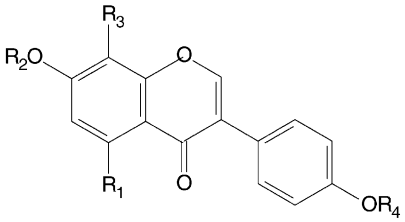
3.1. Natively fluorescent flavonoids: pH effects

Out of the 19 flavonoids studied (see Fig. 1), only 3HF and three of the soflavones (FG, F and D) showed native fluorescence in methanolic and aqueous solutions. Typical excitation and emission spectra are shown in Fig. 2; since the spectra of D are quite similar to those of F, they are not depicted separately. Fig. 2 shows that there is, for both F and FG—and the same holds for D—an exceptionally wide gap between excitation and emission, i.e. an exceptionally large Stokes' shift. Usually, such large shifts are related to excited state deprotonation processes—as, for instance, in 3HF [3–7]—which will, of course, be pH-dependent. For this reason, excitation and emission spectra were recorded over the pH range from 2 to 13. As can be seen in Fig. 2A, in the excitation spectrum of F, an extra band at 340 nm starts to come up at a pH of about 6. Above pH 6, the pK_a value of F, the molecule is predominantly in its anionic form. Simultaneously with the change in the excitation spectrum, the maximum in the emission spectrum of F shifts to slightly shorter wavelengths, i.e. from 495 nm (the emission maximum of F) to 470 nm (the emission maximum of F^-). As is to be expected, no pH effect is observed for FG (Fig. 2B) because, due to the glucose group in the C_7 position, there are no ionisable groups.

3.2. FG as a model system

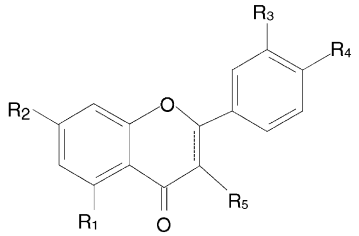
Since protonation/deprotonation effects apparently do not account for the Stokes' shifts in natively fluorescent isoflavones—very large Stokes' shifts are also observed for FG and F^-—other excited state processes, such as molecular rearrangements have to be considered. Since such processes might be influenced by solvent polarity, the proton donating/accepting character of the solvent or solvent viscosity, the fluorescence of FG was studied in various solvents. FG was used as the test compound since its fluorescence in an aqueous environment is not influenced by the pH (see Fig. 2B). The shape of the spectra which were obtained in protic solvents were found to be similar, apart from shifts in the wavelengths of the emission and excitation maxima. For instance, on going from methanol to ethanol, the emission maximum shifted substantially, i.e. from 492 to 456 nm. The decrease of the Stokes' shift on going from methanol to ethanol is in line with the difference in polarity of these solvents, as expressed by their $E_T(30)$ values, a frequently used solvent polarity scale based on a single parameter [11,12]. For ethanol, $E_T(30)$ is 51.9, significantly smaller than for methanol, which has an $E_T(30)$ of 55.4.

Contrary to the results obtained for protic solvents, in the aprotic solvents acetonitrile, dichloromethane and methylcyclohexane hardly any fluorescence was observed. Such a strong solvent influence is known from the literature if two close excited states of $n\pi^*$ and $\pi\pi^*$ character play a role, as, for instance, in 2-naphthylaldehyde [13]. However, a detailed theoretical treatment of the molecule at hand is beyond the scope of this paper. In fact, the weak fluorescence in acetonitrile could not be attributed to FG itself. The shapes of the associated excitation and emission spectra were completely different compared to those of FG in water (Fig. 3A); the emission maximum was found at the short wavelength of 350 nm and the corresponding excitation maximum at 230 nm. Presumably, in acetonitrile we are dealing with another fluorescent species, probably a decomposition product of FG. This is in line with the fact that the absorption spectra in acetonitrile and water were also found to be distinctly different (Fig. 3B) and, furthermore, with the fact that the shapes of the excitation and absorption spectra in acetonitrile are not identical. Here, it should be added that LC–FLU of FG did not



Isoflavones

Name	Acronym	R_1	R_2	R_3	R_4
Daidzein	D	H	H	H	H
Daidzin	DG	H	7-O-β-D-glc	H	H
Puerarin	PG	H	H	glc	H
Genistein	G	OH	H	H	H
Genistin	GG	OH	7-O-β-D-glc	H	H
Formononetin	F	H	H	H	CH_3
Ononin	FG	H	7-O-β-D-glc	H	CH_3
Biochanin A	B	OH	H	H	CH_3
Sissotrin	BG	OH	glc	H	CH_3



Flavones, flavonoles and flavanones

Name	Acronym	R_1	R_2	R_3	R_4	R_5	C_2-C_3
Hesperedin	HG_2	OH	7-O-β-D-glcpyr	OH	OCH_3	H	single
Hesperitin	H	OH	OH	OH	OCH_3	H	single
Naringin	NG_2	OH	7-O-β-D-glcpyr	H	OH	H	single
Naringenin-7-glucoside	NG	OH	7-O-β-D-glc	H	OH	H	single
Naringenin	N	OH	OH	H	OH	H	single
Rutin trihydrate	RG_2	OH	OH	OH	OH	7-O-β-D-glcpyr	double
Kaempferol	K	OH	OH	OH	H	OH	double
3-Hydroxy-flavone	3HF	H	H	H	H	OH	double
Luteolin	L	OH	OH	OH	OH	H	double
Quercetin	Q	OH	OH	OH	OH	OH	double

Fig. 1. Structures and acronyms of the 19 flavonoids studied: glc, glucoside; glcpyr, glucopyranosyl.

reveal the presence of a fluorescent impurity. Since such decomposition in acetonitrile—which has not been reported in the literature—may well influence the results of conventional reversed-phase LC analyses, this phenomenon will be the subject of a more detailed study in the near future.

The fluorescence of FG was also studied at low temperature. In methanol:ethanol (1:1 (v/v)) cooled

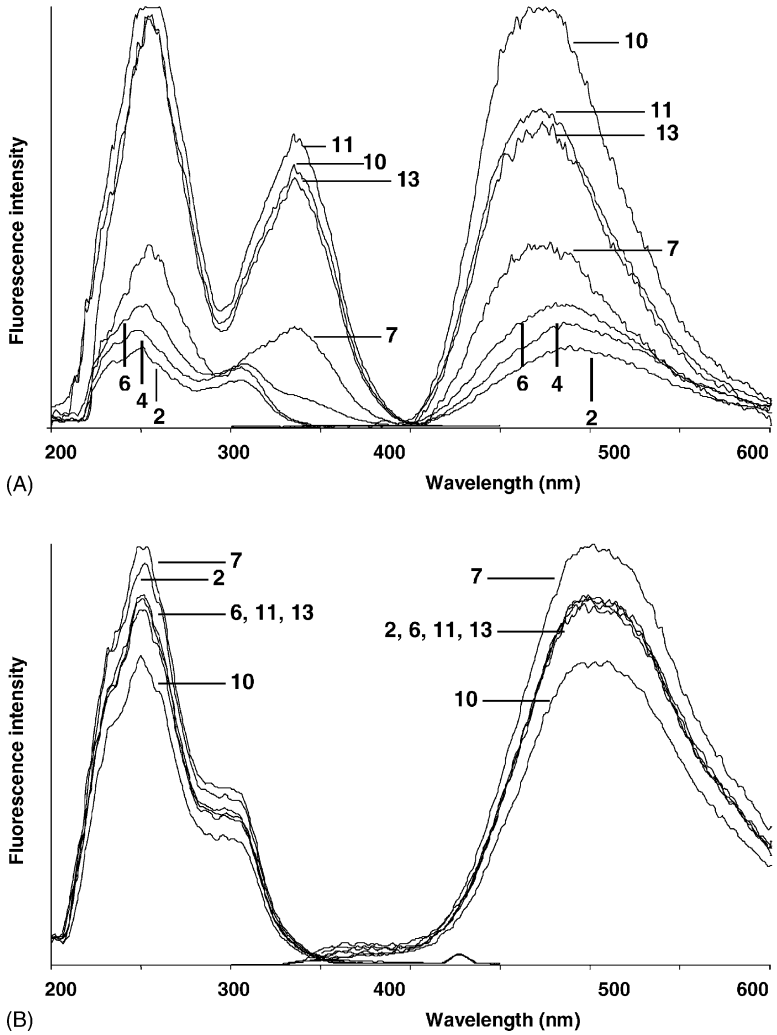


Fig. 2. Fluorescence excitation and emission spectra of (A) F and (B) FG in methanol:water (1:1 (v/v)) solutions at different pH values.

to 77 K with liquid nitrogen, rather unexpectedly no fluorescence was observed at all, but in pure ethanol at 77 K, which forms a glassy matrix, FG did show very weak fluorescence, with the emission maximum at about 400 nm. Apparently, cooling not only causes an extreme reduction of the fluorescence intensity—a phenomenon quite different from what is usually encountered in fluorescence studies where a temperature decrease often causes improved quantum yields—but also a large shift of the emission wavelength: from 456 to about 400 nm. One possibility is that the above temperature influence is related to the change in solvent viscosity. Therefore, the fluorescence spectra of FG were also recorded in ethylene glycol, a solvent with a viscosity of about 30 cP at room temperature, which is 30–50-fold higher than those of ethanol (1.07 cP) and methanol (0.63 cP), while its $E_T(30)$ value is 56.3, only slightly higher than that of methanol. If viscosity does not play a role at all, one would expect the emission maximum in ethylene glycol to be at a somewhat longer wavelength than that in methanol (493 nm). The emission maximum was found at 480 nm, i.e. at a slightly shorter wavelength, which may indicate a viscosity effect, but the shift is too small to be conclusive.

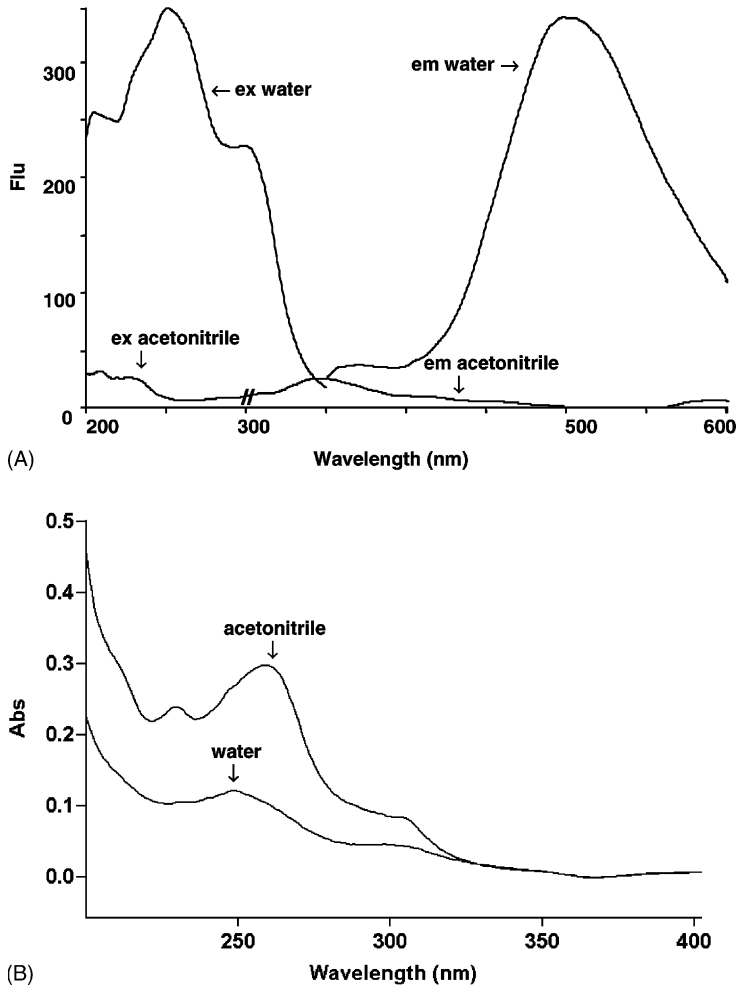


Fig. 3. (A) Fluorescence excitation (ex) and emission (em) spectra and (B) absorption spectra of FG in acetonitrile and in water (6 μM). Fluorescence ex and em slits were set at 15 nm.

Apparently, also in ethylene glycol at room temperature the excited state molecular rearrangement of FG is still too fast to allow its unambiguous establishment in steady state experiments.

In view of the above results, time-resolved experiments should be performed to try to monitor such an excited state molecular rearrangement. One may speculate that the molecule, after being excited to the S_1 state, has to undergo a structural rearrangement before being able to emit fluorescence, for instance, from non-planar (the configuration in the S_0 state) to planar; intermediate states will emit at shorter wavelengths and with a lower fluorescence quantum yield. Fluorescence lifetimes of FG in methanol:ethanol (1:1 (v/v)) were measured at two emission wavelengths, i.e. 440 and 480 nm, at room temperature and at 173 K; lower temperatures could not be handled in the time-correlated single photon counting experiment because of too strong a reduction in fluorescence yield. The wavelength of 480 nm reflects the red edge of the emission spectrum corresponding to the fully equilibrated excited state molecules. The wavelength of 440 nm reflects the blue edge; if, as assumed, molecular rearrangement processes are operative, the emission at this wavelength may be partly due to not yet fully rearranged S_1 state molecules,

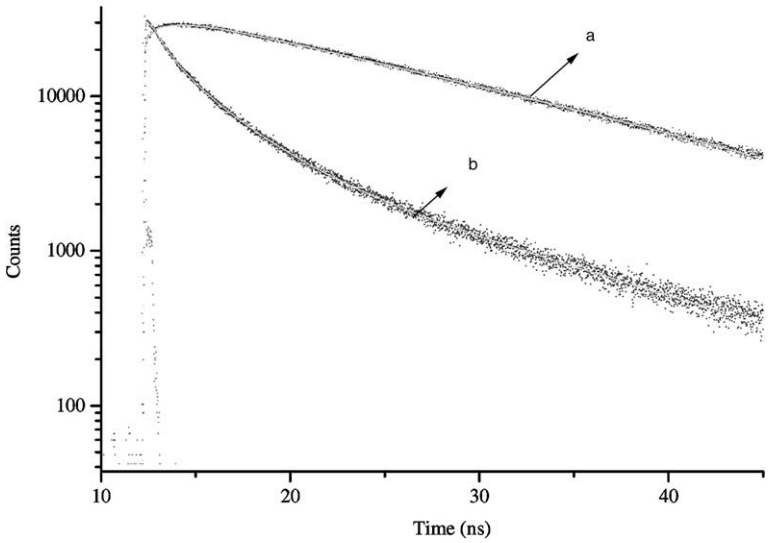


Fig. 4. Time-resolved emission of FG in methanol:water (1:1 (v/v)) at 173 K recorded at (a) 480 nm and (b) 440 nm.

for convenience denoted as FG††. The experimental results confirmed this interpretation: at room temperature the emission lifetimes at 440 and 480 nm were found to be the same, i.e. 3.2 ± 0.1 ns. Apparently, under these conditions full S_1 state equilibration is achieved before emission takes place. At 173 K, however, the red edge emission behaves significantly different from the blue edge emission (Fig. 4). The 480 nm curve was fitted with two mono-exponential functions, i.e. a decay function representing a fluorescence lifetime of 16.4 ± 0.5 ns (as expected, longer than the room temperature value of 3.2 ns) and also an in-growth function with a rise time of 1.5 ± 0.1 ns. The latter function represents the proposed molecular rearrangement process: it takes about 1.5 ns before the molecules, after being excited, arrive in the planar excited state. To describe the 440 nm curve, two mono-exponential decay functions were needed, a rapid one with a decay of 1.0 ± 0.1 ns reflecting the further rearrangement of FG†† and a slow one of 10.5 ± 0.5 ns, representing the fluorescence lifetime of FG††. The similarity of the rapid decay at 440 nm and the rise time at 480 nm points to an excited state conformational change as assumed above. They are not identical because most probably we are dealing with a continuous relaxation process starting at the configuration of the initially excited state to the relaxed excited state configuration. The spectra of FG†† and fully relaxed FG will partly overlap, both at 440 and 480 nm. We, therefore, conclude that the large Stokes' shifts observed for the fluorescent isoflavones, may be attributed to a structural change of the molecule from non-planar in the S_0 state to planar in the S_1 state, as was previously found for the S_0 state of similarly structured compounds [14].

3.3. Daidzin impurity

One should be very careful in attributing a fluorescence emission to a particular flavonoid. In fact, a purity check, in which the LC–UV and the LC–FLU traces are compared, is crucial. Thus, it was found earlier [8] that the fluorescence displayed by a methanolic solution of a DG standard from Roth had to be assigned to an unknown isomer of DG present in low concentration. In fact, DG itself does not show any fluorescence at all. This conclusion was confirmed by the observation that for a DG standard from another company (Indofine Chemical Co.), no fluorescent impurity was found. It should be noted that, apart from the fluorescence behaviour, DG and the isomer in the Roth standard have almost identical properties.

When the methanolic solution of DG (Roth) was subjected to LC–ESI–MS in the positive mode, using

the same LC conditions as above, two peaks were observed in the mass trace, viz. at 6.7 and 8.7 min. In the mass spectrum of the first peak, *m*/*z* 255 and 417 show up and have rather similar intensities, while in the mass spectrum of the second peak only *m*/*z* 417 is observed and *m*/*z* 255 is essentially absent; *m*/*z* 255 is formed after loss of a glucose group. According to the literature [15], when a glucose moiety is bound to oxygen, as is the case for DG, both $[M + H]^+$ and $[M + H - glc]^+$ ions will be observed in the full-scan mass spectrum, but with a C-bound glycoside, only $[M + H]^+$ will be observed. That is, the early eluting peak is possibly an O-bound glycoside and the second (fluorescent) peak, a C-bound glycoside. Puerarin, the C_8-bound glucoside isomer of DG, was also subjected to LC–FLU, but it did not show any fluorescence. To check whether both peaks in the DG standard contain a glucose group, ESI–MS/MS was performed with *m*/*z* 417 as precursor ion in the positive mode. The MS/MS spectra of both peaks contain mass 255, which is indeed the aglycon moiety. Nonetheless, the structure of the isomer is not yet completely established. To the best of our knowledge, the presence of a fluorescent isomer of DG has not been reported in the literature.

3.4. LC–FLU analysis of real samples

Due to the large shifts between the excitation and emission wavelengths of the fluorescent isoflavones, high selectivity can be obtained in LC with fluorescence detection. This is particularly relevant if real samples have to be analysed. This was denoted for wetland plant extracts, viz. for two clover extracts. In Fig. 5, the (expected) gain in selectivity upon going

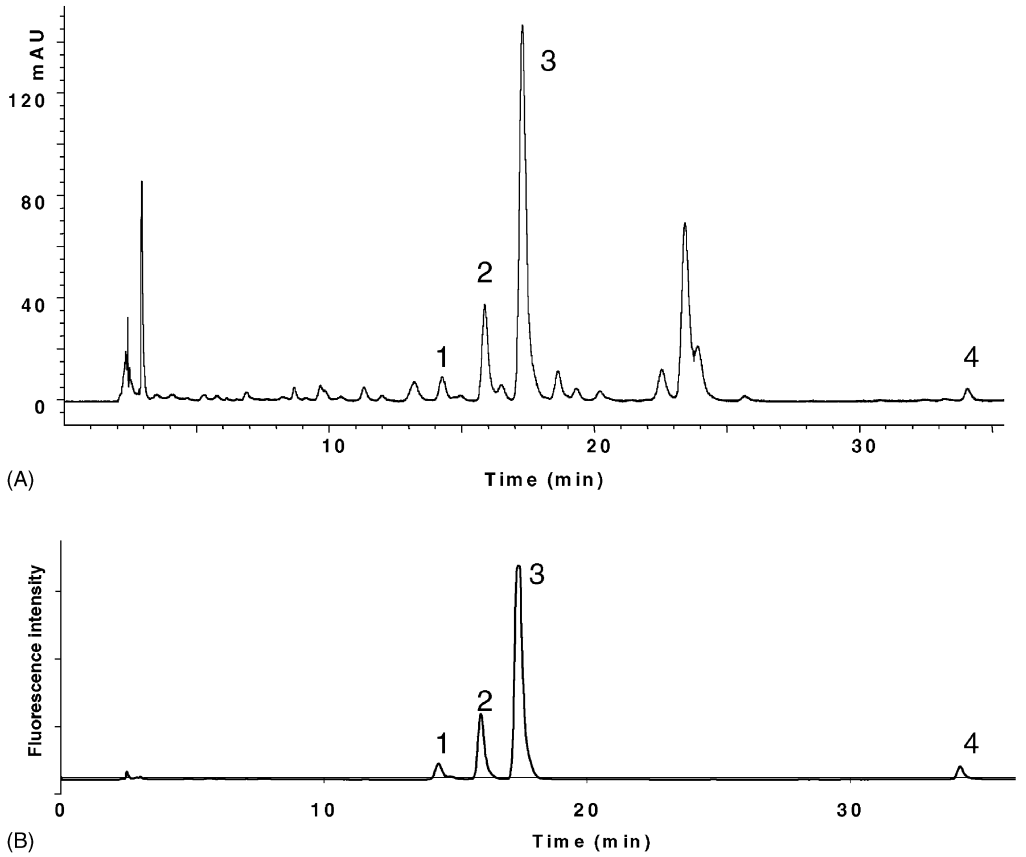


Fig. 5. (A) LC–UV_{265} and (B) LC–FLU trace of red clover extract. In (B), excitation at 250 nm, emission at >450 nm. For LC conditions, see Section 2.2. Peak assignments are given in Section 3.4.

from LC–UV to LC–FLU is immediately apparent upon comparing Fig. 5A and B, which show the results for a red clover extract. When the fluorescence detector was equipped with a 350 nm bandpass emission filter (a wavelength at which isoflavones hardly show fluorescence), no peaks were observed above 10 min (results not shown). If, instead, a cut-off filter of >450 nm is applied (Fig. 5B), four prominent peaks show up at retention times between 13 and 35 min. These can be attributed to the fluorescent isoflavones, formononetin-7-*O*-β-D-glucoside-6″-*O*-malonate (FGM; peak 3), an isomer of FGM (peak 1), FG (peak 2) and F (peak 4). Closely similar results were obtained with a white clover extract, for which no results are shown here. Limits of detection (LODs, $S/N = 3$) were determined for FG and F on both detectors; for the UV detector LODs were 1 and 0.5 mg/l, and for the fluorescence detector LODs were 0.1 and 0.05 mg/l, respectively. Repeatability of the method was satisfactory; R.S.D. values of the peak area of the 10 major peaks in the chromatogram were 1–5% ($n = 7$).

4. Conclusions

Large Stokes' shifts for fluorescent isoflavones are possibly due to a change of the structure of the molecule from non-planar in the S_0 state to planar in the S_1 state. This phenomenon deserves further spectroscopic study. From an analytical point of view, these large shifts provide a high selectivity, so that fluorescent isoflavones can be readily detected in real samples by means of reversed-phase LC with fluorescence detection, applying methanol:water gradients and a large wavelength difference between λ_{ex} and λ_{em}. Because of instability problems of at least some of the target analytes, acetonitrile should be avoided as a solvent.

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