

# A Comparison Study on Fluorescence Properties of Formononetin and Ononin

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## Research Article

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# Abstract

In this work, reasons for the spectral difference between two isoflavones, Formononetin (F) and ononin (FG), are explained in the viewpoint of molecular structure through a comparison study of the fluorescence features of the two. The fluorescence enhancement of FG in hot alkaline condition is reported for the first time. For F, there was almost no fluorescence under acidic conditions, but when  $\text{pH} > 5$ , its fluorescence began to increase with increasing pH due to the proton ionization of 7-OH. In the range of pH 9.3-12.0, the anion form of F produced a fairly strong and stable fluorescence with maximum excitation wavelength ( $\lambda_{\text{ex}}$ ) of 334 nm and emission wavelength ( $\lambda_{\text{em}}$ ) of 464 nm, its fluorescence quantum yield (Yf) was measured to be 0.042. And for FG, its aqueous solution fluoresced weakly in a wide pH range until it was placed under hot alkaline conditions, which was presumed to the cleavage reaction of the  $\gamma$ -pyrone ring in FG by observing a significant fluorescence at  $\lambda_{\text{ex}} / \lambda_{\text{em}} = 288 / 388\text{nm}$ , and Yf was determined to be 0.020. The fluorescence sensitization methods of F and FG both exhibit low limits of detection ( $2.60 \text{ ng}\cdot\text{mL}^{-1}$ ,  $9.30 \text{ ng}\cdot\text{mL}^{-1}$ ) and wide linear ranges ( $0.0117\text{-}1.86 \mu\text{g}\cdot\text{mL}^{-1}$ ,  $0.0146\text{-}2.92 \mu\text{g}\cdot\text{mL}^{-1}$ ). Although the structural relationship between F and FG is glycoside and aglycone, FG cannot be translated to F by glucoside hydrolysis under hot alkaline condition, the fluorescence enhancement mechanisms of the two are essentially different. The fluorescence difference between the two under different experimental conditions lays the foundation for future fluorescence quantitative analysis.

## 1. Introduction

Isoflavones are a class of compounds derived from isoflavone (3-phenylchromone), which widely exist in foods and drugs, and are also the active ingredient of many traditional Chinese medicine (TCM). Formononetin (F) and ononin (FG) are a pair (glycosides and aglycone) of isoflavones (Scheme 1), and also major types of flavonoids found in various Chinese herbal medicines, such as astragali radix (the root of *Astragalus membranaceus var. mongholicus* or *A. membranaceus*) and red clover (*Trifolium pratense* L.) [1–4]. Anti-neoplastic [1–3, 5], antioxidative [6], anti-inflammatory [7, 8], hypo-lipidemic [9] and other pharmacological effects of F and FG have been reported. Generally, the content of F and FG in TCM or biological samples are measured by liquid chromatography combined with ultraviolet and fluorescence or mass spectrometry detector [10–13].

Eva de Rijke et al. had tested the native fluorescence of 19 kinds of flavonoids, observed that only some flavonols and isoflavones, such as, daidzein, formononetin and ononin had fluorescence [14]. They studied the effects of pH and different solvents on the fluorescence of F and FG, but did not discuss the relationship between fluorescence properties and their molecular structure. The authors had studied the fluorescence properties of some flavonoids and found that some flavonoids and flavonoids will undergo fluorescence enhancement reactions under hot alkaline conditions, which is attributed to the cleavage reaction in the C ring [15, 16]. These methods and ideas have expanded the scope of fluorescence analysis of flavonoids to some extent.

In this paper, based on the existing research results, fluorescence properties of F and FG, and the relationship with the molecular structure were studied. Focused on the effect of pH on the fluorescence spectrum, the authors studied the reaction of C ring cleavage reaction or oxygen glycosides hydrolysis reaction in hot alkaline conditions and the fluorescence spectrum of their reaction products. The possible existing forms (molecular form, ionic form, cleavage product or hydrolyzate) of the two compounds were explained from the intrinsic characteristics of molecular structure and spectral information by analyzing the spectral information of fluorescence wavelength and fluorescence intensity under different experimental conditions. The results showed that the molecular form of F had no fluorescence in the aqueous solution, and the anion form could exhibit strong fluorescence due to the proton ionization of 7-OH under weak alkaline conditions. FG aqueous solution was weakly fluorescent, but could produce strong fluorescence by C ring cleavage reaction in strong alkaline solution. These results provide some new experimental basis for the establishment of fluorescence analysis methods of F and FG, and also open up a new way to expand the fluorescence analysis of isoflavones.

## 2. Materials And Methods

### 2.1 Materials

Formononetin (F, CAS No.: 485-72-3) was purchased from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ononin (FG, CAS No.: 486-62-4) was got from TianJin Yifang Technology Co., Ltd (Tianjin, China). Stock solution was prepared by dissolving F or FG with methanol (HPLC grade, Tedia Company, Inc. USA) and kept in refrigerator at 4 °C for later use. Quinine sulphate, produced in Chemical Limited company of Bodi Plant(Tianjin,China) was diluted to  $1.00 \times 10^{-5}$  M with 0.1 M  $H_2SO_4$  when it was used. L-Tryptophan ( $C_{11}H_{12}N_2O_2$ , biochemical reagent, chromatographic grade, molecular weight: 204.33) purchased from the Institute of Microbiology, Chinese Academy of Sciences (Beijing, China), was dissolved in the water and made its concentration into  $1.00 \times 10^{-4}$  M. Britton-Robinson buffer solution was a mixture of phosphoric acid, boric acid, and acetic acid (each 0.2 M), prepared for pH adjustment by adding NaOH solution (1 M). All other reagents and solvents were analytical reagent grade and used without further purification unless otherwise noted. The water used throughout the study was doubly-deionized and verified to be free from fluorescence.

### 2.2 Apparatus

Fluorescence measurements were performed on a Hitachi (Tokyo, Japan) F-7000 fluorescence spectrophotometer equipped with a xenon lamp and 1 cm quartz cell. The excitation and emission slits (band pass) 5 nm/5 nm were used throughout the work. Absorption spectra were recorded using a Shimadzu (Kyoto, Japan) UV-2501PC recording spectrophotometer with 1 cm quartz cell. An Orion (Beverly, USA) 868 pH/ISE meter was used for pH measurement. Five-digit analytical balance, up to 0.01mg.

### 2.3 Methods for spectral measurement

Effects of pH on fluorescence: Added F or FG solution into a series of 10 mL volumetric flasks, added B-R buffer solution and NaOH solution to adjust different pHs, then diluted to volume with water and mixed well. After setting aside for 20 min, the two-dimensional fluorescence spectrum was measured at room temperature.

Effects of solvent on F: Determined pH = 9.3, F solution and different volumes (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 mL) of MeOH were added into 10 mL volumetric flasks, separately. The mixtures were diluted to the mark with water and mixed well. After setting aside for 20 min, the two-dimensional fluorescence spectrum was measured at room temperature.

Effects of temperature on fluorescence: Prepared series of 10 mL volumetric flasks containing 1.0 mL FG and 2.0 mL NaOH solution. The mixtures were diluted to the mark with water and mixed well, then set for a 30 min at room temperature (about 20 °C), 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C and 100 °C, respectively. Then scanned the spectrum after cooling to room temperature.

Effects of heating time on fluorescence: Added 1.0 mL FG solution and 2.0 mL 1.0 M NaOH solution into 10 mL volumetric flasks. The mixtures were diluted to the mark with water and mixed well, then heated in a 100°C-water bath. Took out one every 10 minutes, then cooled to the room temperature and added water to the scale, scanned the two-dimensional fluorescence spectra, and investigated the influence of heating time on fluorescence.

## 2.4 Measurement of fluorescence quantum yield

The fluorescence quantum yield (Y<sub>f</sub>) was measured according to the reference method [17], selected quinine sulfate (Y<sub>f</sub> = 0.58 in 0.1 M H<sub>2</sub>SO<sub>4</sub>) and L-Tryptophan (Y<sub>f</sub> = 0.13 in water) as the standards separately [18]. Appropriate quantities of quinine sulphate solution were prepared into a 25 mL flask and diluted to the scale with 0.1 M H<sub>2</sub>SO<sub>4</sub>, mixed well. Add a moderate amount of L-Tryptophan into a 25 mL flask and diluted with water. Prepared suitable amounts of F or FG into a 25 mL flask, diluted to the mark with water and mixed well. Recording absorption and fluorescence spectra, calculated quantum yields.

The relative Y<sub>f</sub> of the sample is represented by Y<sub>u</sub>, and the corresponding Y<sub>s</sub> represents the known Y<sub>f</sub> of the standards. The calculation formula is:

$$Y_u = [(F_u A_s) / (F_s A_u)] \times Y_s$$

Wherein, A is the absorbance of the solution, F is the integrated area of the emission peak, s is the standard, and u is the sample. The prerequisite for the application of this formula is that the solubility of the solution should not be too large, and it is generally controlled that the absorbance is close to and not more than 0.05.

## 3. Results And Discussions

### 3.1 Fluorescence spectrum of F aqueous solution

The fluorescence spectra of F aqueous solution at different pHs were measured, as shown in Fig. 1. F hardly emitted fluorescence in the range of pH 2.0–5.0. The fluorescence peak of F was significantly enhanced at the excitation wavelength ( $\lambda_{\text{ex}}$ ) of 256 nm and 334 nm and the emission wavelength ( $\lambda_{\text{em}}$ ) of 464 nm in weak acidic and weak basic conditions (pH > 5.0). Fluorescence intensity would reach its peak and remain stable in pH range of 9.3–12.0, and then quench rapidly as the pH continued to increase (pH > 12.0), but the wavelength of excitation and emission remained the same throughout the progress.

The reason why the fluorescence spectrum changed with pH is that F molecules contain ionizable hydroxyl protons. There is a hydroxyl group at position 7 (7-OH) in the F molecule as shown in Scheme 1. The proton dissociation of 7-OH caused the change of fluorescence spectrum when the pH of the solution changed from near neutral to weakly alkaline. The ionization constant of 7-OH proton was determined to be  $\text{p}K_{\text{a}} = 7.31 \pm 0.03$  using the pH-F data from the Fig. 1(c) and based on pH-Fluorescence method [19].

UV absorption spectra of F at different pH conditions were studied to verify the 7-OH proton ionization of F, and the results are shown in Fig. 2. In the pH range of 2.2–11.2, three absorption peaks appeared in the absorption spectra, which were located at 255, 303 and 334 nm, respectively. As the pH increased, the absorption peaks of 255 and 334 nm increased, while the absorption peak at 303 nm decreased, in this way, three isochromatic points located at 245, 285 and 312 nm were formed. The spectral characteristics showed that the molecular form of F changed into ionic form under weak alkaline conditions. According to pH-A data in Fig. 2 (b), the 7-OH proton ionization constant of F ( $\text{p}K_{\text{a}}$ ) was calculated by pH-spectrophotometry [20], the result  $\text{p}K_{\text{a}} = 7.34 \pm 0.01$  was consistent with the  $\text{p}K_{\text{a}}$  value obtained by the fluorescence method. In addition, there were isosbestic points in Fig. 2, but no isofluorescent points in Fig. 1, which showed that both the molecule and ionic form of F can absorb light in the ultraviolet region, but only the ionic form can produce fluorescence.

As shown in Fig. 1, the fluorescence intensity of F increased in the pH regions of 6.0–10.0 and reduced rapidly when pH > 12.0. This was due to the cleavage reaction of pyrone ring in F with product of the o-hydroxyl-phenyl-benzyl ketone derivative [21], as exhibited in Scheme 2. This product had no fluorescence under experimental conditions.

### 3.2 Fluorescence spectrum of FG and its Cleavage product

Different from F, the fluorescence of FG was very weak in the acidic, neutral or weak alkaline aqueous solutions at room temperature (only weak fluorescence of FG was observed in the literature [14]). The absence of ionizable hydroxyl protons, and the presence of 7-position oxoside (7-OGlu) prevents FG from producing fluorescence like F, therefore, the spectral properties did not change in varying pHs .

But in the experiment, we found that FG aqueous solution would produce strong fluorescence when heated under alkaline conditions, as shown in Fig. 3. The fluorescence intensity of the FG aqueous solution was still very weak after heating in the pH range of 1.0–8.8, but when pH > 8.8, the fluorescence

peak located at  $\lambda_{\text{ex}}/\lambda_{\text{em}} = 243 / 388$  nm enhanced significantly, until the fluorescence intensity reached the maximum at pH 12.5, then dropped sharply as the pH increased (pH > 12.5).

As the experimental results shown in Fig. 3, we can conclude that, chemical changes must have occurred in FG and produced new fluorescent substances under hot alkaline conditions. According to the molecular structure of FG, it can infer that there are two possible reactions: one is the ring-opening reaction of  $\gamma$ -pyrone ring, as shown in Scheme 2, and the other was the hydrolysis reaction of 7-OGLu.

We inferred which kind of chemical reaction happened in FG combining with the fluorescence characteristics of the reaction product. If the hydrolysis reaction of the 7-oxo-glycosidic bond had occurred, the reaction product should be like the anionic form of F after 7-OH proton ionization, as the fluorescence spectrum was shown in Fig. 1, the  $\lambda_{\text{ex}}$  is located at 256 nm and 334 nm, and the  $\lambda_{\text{em}}$  is located at 464 nm. However, the  $\lambda_{\text{ex}}$  (243 nm, 288 nm) and  $\lambda_{\text{em}}$  (388nm) of the actual product shown in Fig. 3 were significantly different from the wavelength position in Fig. 1. These results indicated that no hydrolysis reaction occurred under hot alkaline conditions. Another speculation, if FG had undergone the ring-opening reaction of the  $\gamma$ -pyrone ring, the cleavage product should have a lower degree of conjugation in the molecular structure compared to the anionic form of F, and moreover, should have shorter-wavelength fluorescence peaks according to the theory of fluorescence, the theoretical speculation was in good agreement with the results shown in Fig. 3. Therefore, it could be inferred that the ring-opening reaction of the  $\gamma$ -pyrone ring occurred under hot alkaline conditions, and the cleavage product is a fluorescent. But due to the hydrolysis reaction of the cleavage product, its fluorescence quenched rapidly with the increased pH when pH > 12.5, the hydrolyzate was the same as the cleavage product of the anion form of F in Fig. 2.

In summary, the fluorescence properties of F and FG were quite different, although the two are the relationship between glycoside and aglycone from the molecular structure. In the experiment, FG cannot be converted to F by the cleavage of the glycosidic bond under hot alkaline conditions, instead, the  $\gamma$ -pyrone ring cleavage reaction occurred first to produce fluorescence, and then the glycosidic bond hydrolysed under strong alkaline conditions, resulting in fluorescence quenching.

### 3.3 Effect of Solvent on Fluorescence of F

The effect of solvent (the volume fraction of methanol in aqueous solution) on the fluorescence of F was investigated under weak alkaline conditions (pH 9.3). The results in Fig. 4 show that when the volume fraction of methanol in the aqueous solution was changed, the fluorescence wavelength was almost unchanged, but the fluorescence intensity was significantly enhanced. Therefore, in this study, the volume fraction of methanol in the aqueous solution was controlled to 10% or less when studying the effect of other experimental conditions on the fluorescence.

### 3.4 Effect of Heating Temperature on Fluorescence of FG

As shown in Fig. 5, fluorescence of FG is extremely weak under the strong alkaline solution (pH 12.5) at room temperature, then increases along with the heating temperature increases, indicating that the

temperature accelerated the cleavage reaction. When the temperature is greater than 70°C, the fluorescence reaches the maximum, showing that the cleavage reaction is basically completed. The fluorescence spectra were similar to those in Fig. 3 (a, b), and had no changes during the entire temperature-changing progress.

### 3.5 The stability of fluorescence

The experiments revealed that, the fluorescence of F was basically stable when placed in weakly alkaline solution at room temperature, and also had no changes when the solution was continuously irradiated by the xenon lamp.

The fluorescence intensity of FG increased with the extension of heating time and became stable after 1.5 h, and did not change significantly when continuously exposed to the xenon lamp for 200s, indicating that the fluorescence properties were basically stable.

### 3.6 Fluorescence Quantum Yield

Y<sub>f</sub> of F in a weak alkaline solution (pH 9.3) was measured to be 0.042, and that of FG heating pyrolysis product in alkaline solution (pH 12.5) was 0.020. Though the values of Y<sub>f</sub> were not very high, we can analysis them using modern fluorescent instruments with high sensitivity.

### 3.7 Relationship between concentration and fluorescence intensity

Based on the above experimental results, a series of solutions containing different amounts of F under pH = 9.27, from 0.0117  $\mu\text{g}\cdot\text{mL}^{-1}$  to 1.86  $\mu\text{g}\cdot\text{mL}^{-1}$ , were prepared and their fluorescence spectra were scanned, as shown in inset in Fig. 6(a). A working curve of fluorescence intensity,  $I_F$  ( $\lambda_{\text{ex}}/\lambda_{\text{em}} = 334 \text{ nm} / 464 \text{ nm}$ ), *versus* concentration of F,  $c_F$ , was drawn, as shown in Fig. 6(a). In the range of 0.0117–1.86  $\mu\text{g}\cdot\text{mL}^{-1}$ ,  $I_F$  has a linear relationship with  $c_F$ . The regression equation is  $I_F = 21.9 + 2188.8c$ , with the correlation coefficient  $R^2 = 0.999$  ( $n = 14$ ). The blank signal was scanned and the lowest limit of detection for F was found to be 2.60  $\text{ng}\cdot\text{mL}^{-1}$  ( $9.69 \times 10^{-9} \text{ mol}\cdot\text{L}^{-1}$ ).

Similarly, Fig. 6(b), a working curve of  $I_F$  ( $\lambda_{\text{ex}}/\lambda_{\text{em}} = 288 \text{ nm} / 388 \text{ nm}$ ) *versus*  $c_{\text{FG}}$  was drawn by controlling the pH of the solution to 12.5, heating in a boiling water bath for 1 hour, and scanning the fluorescence spectra (as shown in inset of Fig. 6b) after cooling to room temperature. The results show that the linear relationship between  $I_F$  and  $c_{\text{FG}}$  is good in the range of 0.0146- 2.92  $\mu\text{g}\cdot\text{mL}^{-1}$ , the regression equation is  $I_F = 18.9 + 611.6c$ ,  $R^2 = 0.998$  ( $n = 15$ ), and the lowest limit of detection for FG was found to be 9.30  $\text{ng}\cdot\text{mL}^{-1}$  ( $2.16 \times 10^{-8} \text{ mol}\cdot\text{L}^{-1}$ ).

It can be seen from the above results that when a sample contains both F and FG, although the structures of the two compounds are similar, the difference in fluorescence between the two can be used for quantitative analysis, such as controlling the sample solution to weakly alkaline conditions for the

analysis and determination of F, and the sample solution can be placed under strong alkali heating conditions when determining the content of FG.

## 4. Conclusions

In summary, F is weakly fluorescent under acidic and neutral conditions, and its weakly basic solution can produce fluorescence at  $\lambda_{\text{ex}}/\lambda_{\text{em}}=339/465$  nm, which due to the proton ionization of 7-OH. Under strong alkaline conditions, the cleavage of the  $\gamma$ -pyrrole ring leads to fluorescence quenching. Since there is no ionizable protons in the molecular structure of FG, its fluorescence in aqueous solutions is quite weak under normal temperature conditions and regardless of the pH of the solution. But under high-temperature alkaline conditions, FG undergoes  $\gamma$ -pyrone ring cleavage reaction, resulting in increased fluorescence with  $\lambda_{\text{ex}}/\lambda_{\text{em}}=288/388$  nm. When heated under strong alkaline conditions, the pyrolysis products of FG will further undergo glycosidic bond hydrolysis, causing fluorescence quenching. Although the structural relationship of F and FG is glycoside and aglycon, in the experiment, the glycoside will not be converted into the aglycone, so the fluorescence enhancement mechanisms of the two are essentially different. The fluorescence difference between F and FG under different experimental conditions lays the foundation for future fluorescence quantitative analysis.

## Declarations

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### Conflicts of interest

There are no conflicts to declare.

### Ethics approval

Not applicable.

### Consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Data availability

The data that support the findings of this study are available from the corresponding author on request.

## Code availability

Not applicable.

## Authors' contributions

Conceptualization: Wenhong Li, Yongju Wei; Methodology: Wenhong Li, Jinjin Cao; Formal analysis and investigation: Jinjin Cao, Fang Lv, Luchen Niu, Bocong Han; Writing - original draft preparation: Wenhong Li, Jinjin Cao; Writing - review and editing: Wenhong Li, Jinjin Cao, Ting Liu; Funding acquisition: Yongju Wei, Wenhong Li, Jinjin Cao; Resources: Yongju Wei, Wenhong Li, Jinjin Cao; Supervision: Yongju Wei, Wenhong Li, Yanhui Zheng.

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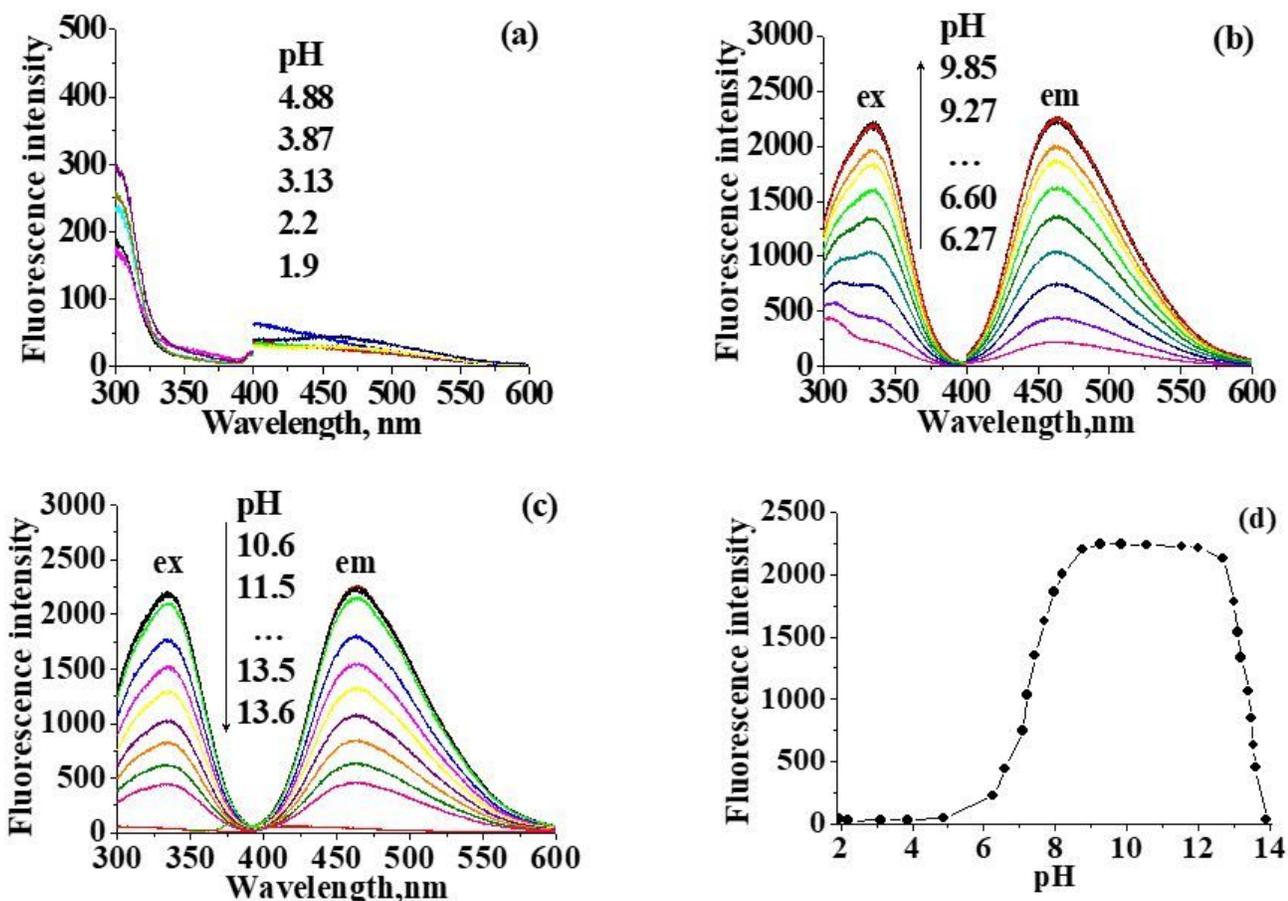
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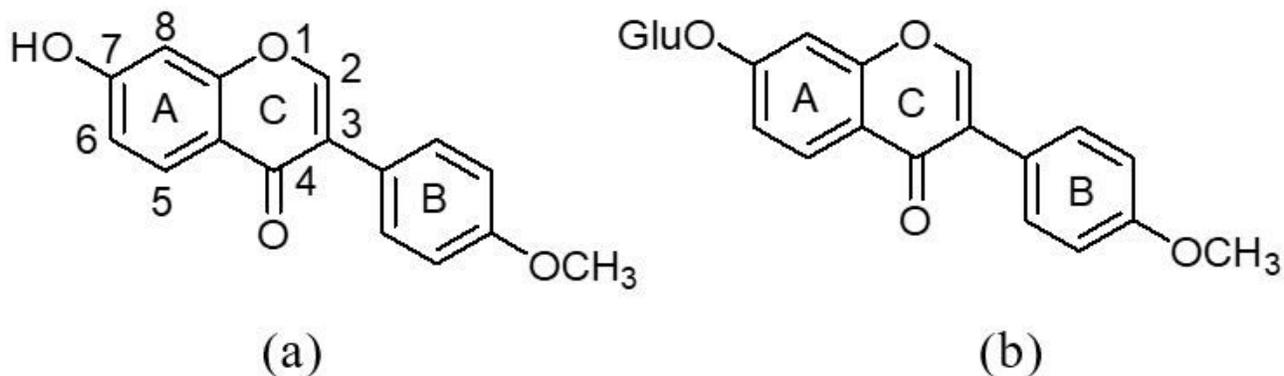
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## Figures



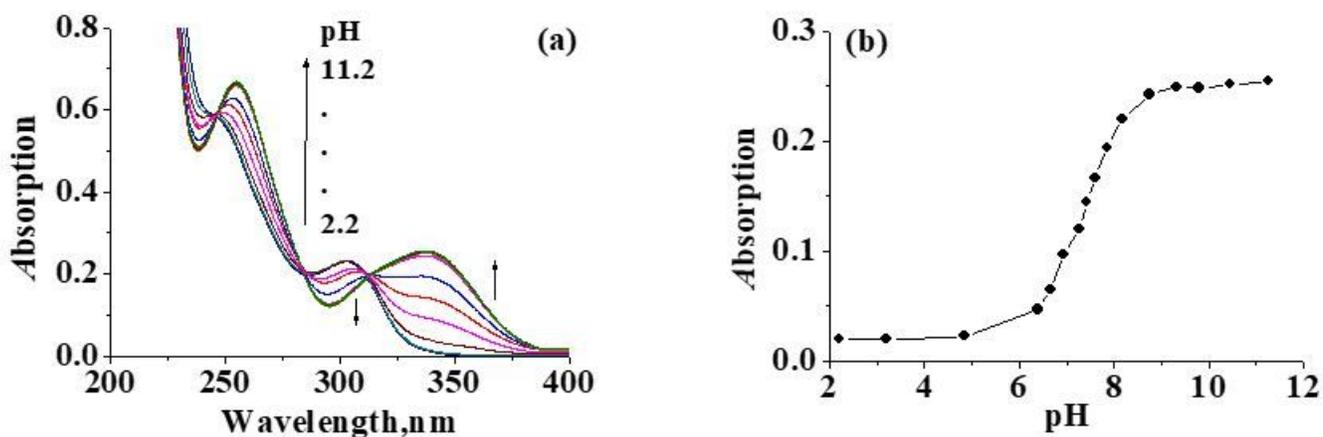
**Figure 1**

Fluorescence spectra ( $\lambda_{ex}/\lambda_{em}$ : 334/464 nm) of F aqueous solutions ( $0.972 \mu\text{g}\cdot\text{mL}^{-1}$ ) at different pHs (a, b, c) and relationship between fluorescence intensity and pH(d).



**Figure 2**

Molecular structures of F (a) and FG (b)



**Figure 3**

UV absorption spectra ( $\lambda$ :334 nm) of F aqueous solutions ( $5.05 \mu\text{g}\cdot\text{mL}^{-1}$ ) at different pHs (a) and relationship between absorption and pH (b).

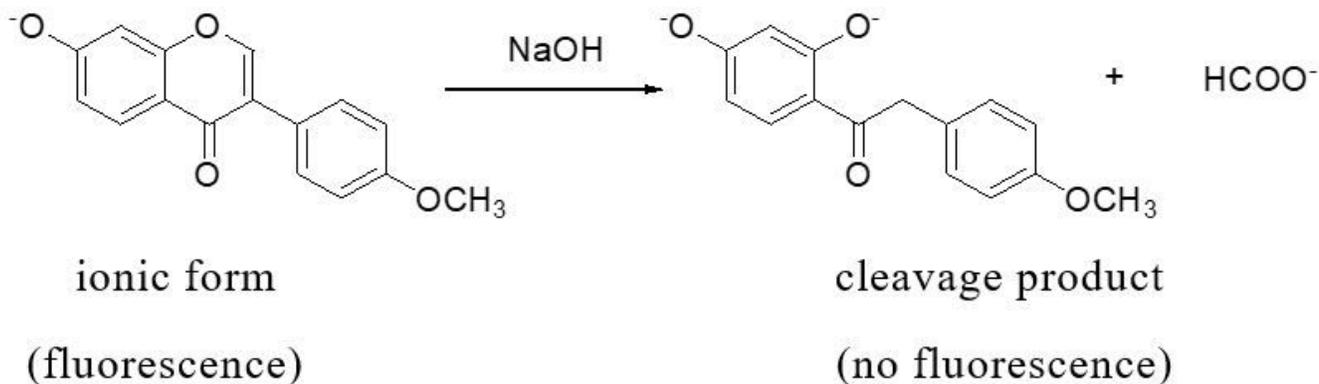


Figure 4

Cleavage reaction of F

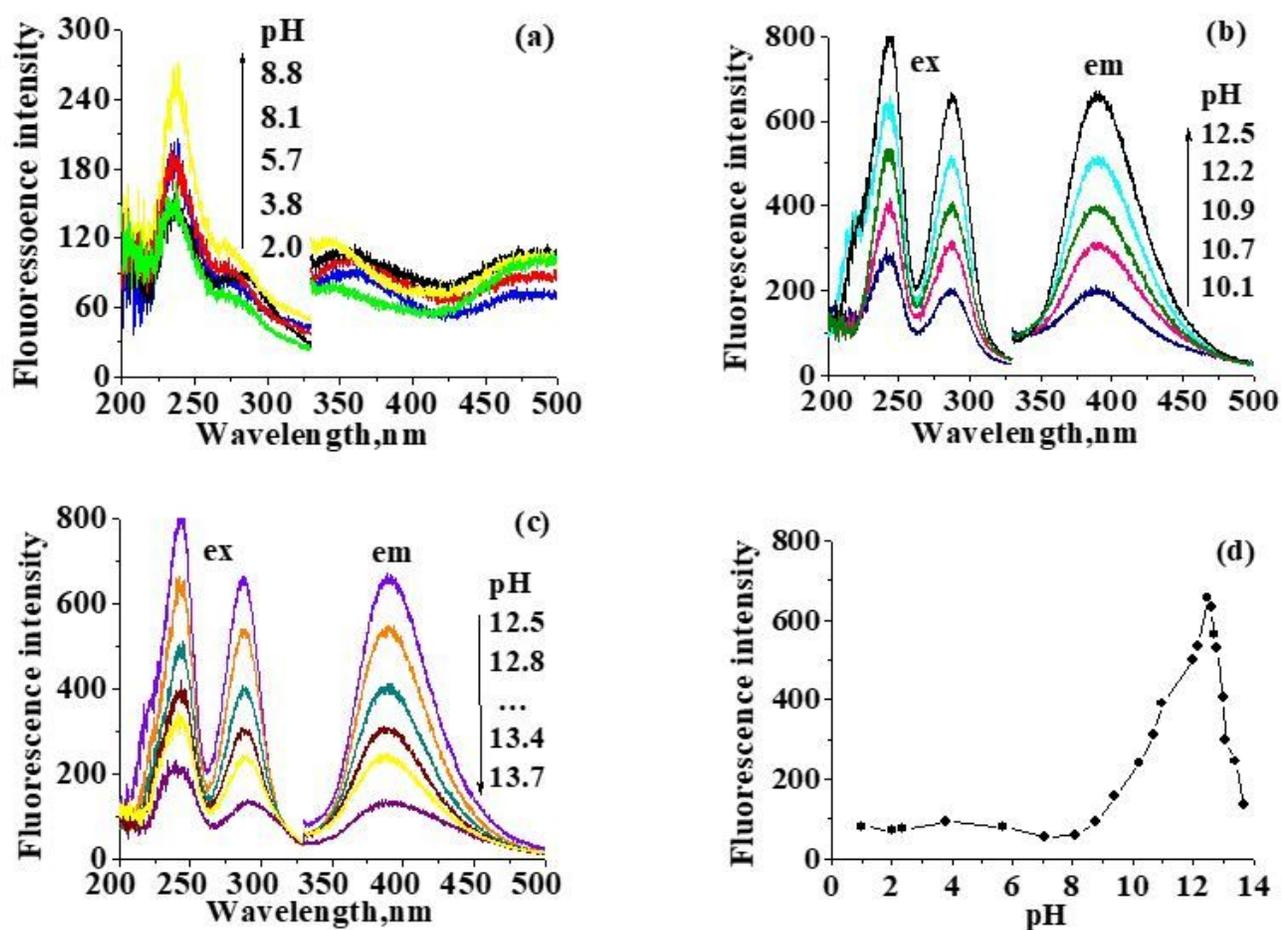
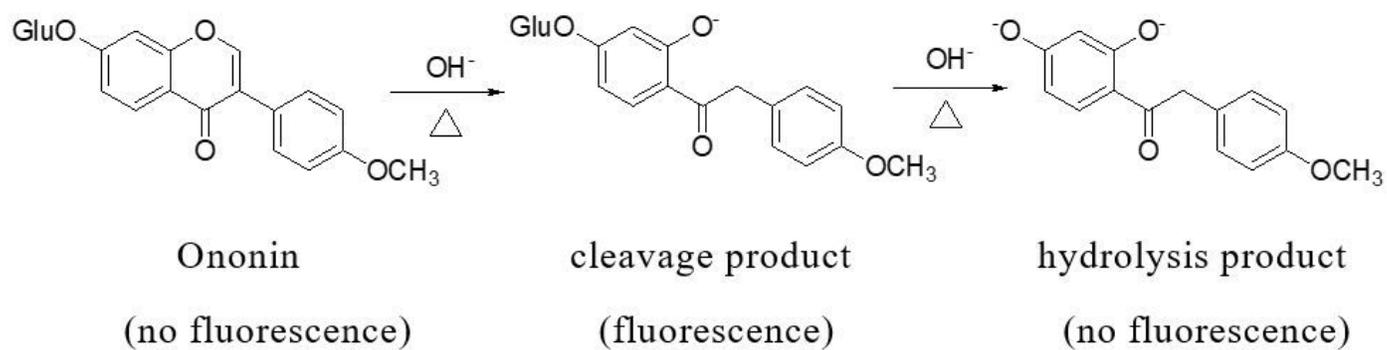


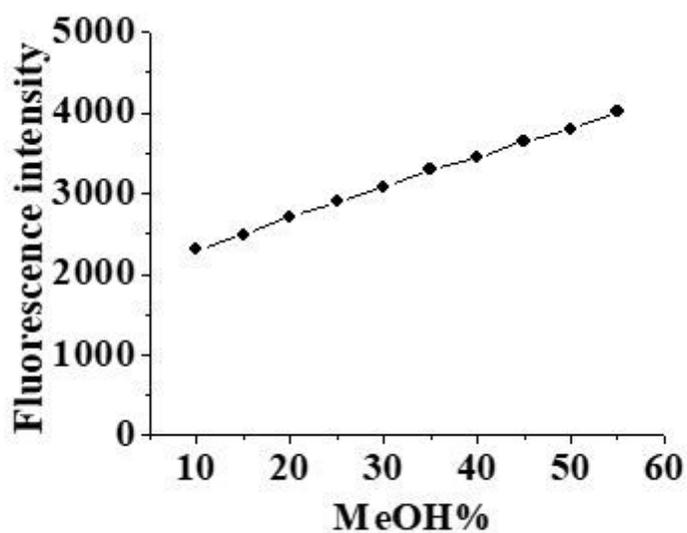
Figure 5

Fluorescence spectra ( $\lambda_{ex}/\lambda_{em}$ : 288/388 nm) of FG aqueous solutions ( $0.914 \mu\text{g}\cdot\text{mL}^{-1}$ ) after heating (heat for 1 h at  $100^\circ\text{C}$ ) at different pHs (a, b, c) and influence of pH on fluorescence intensity (c).



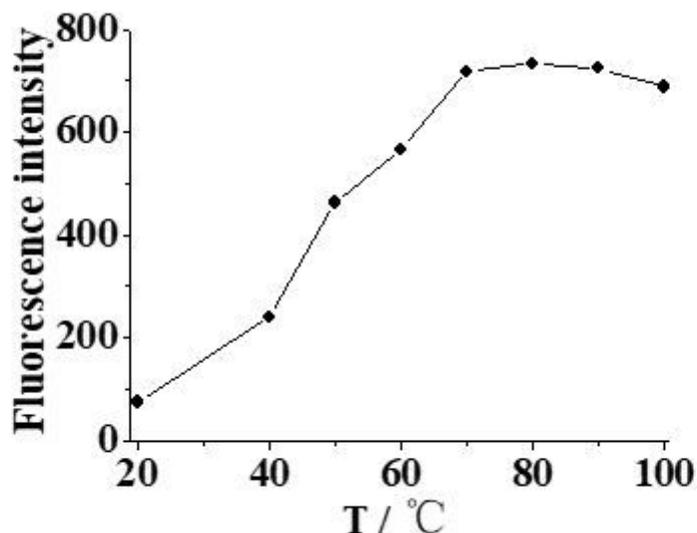
**Figure 6**

Cleavage and hydrolyzation reaction of FG



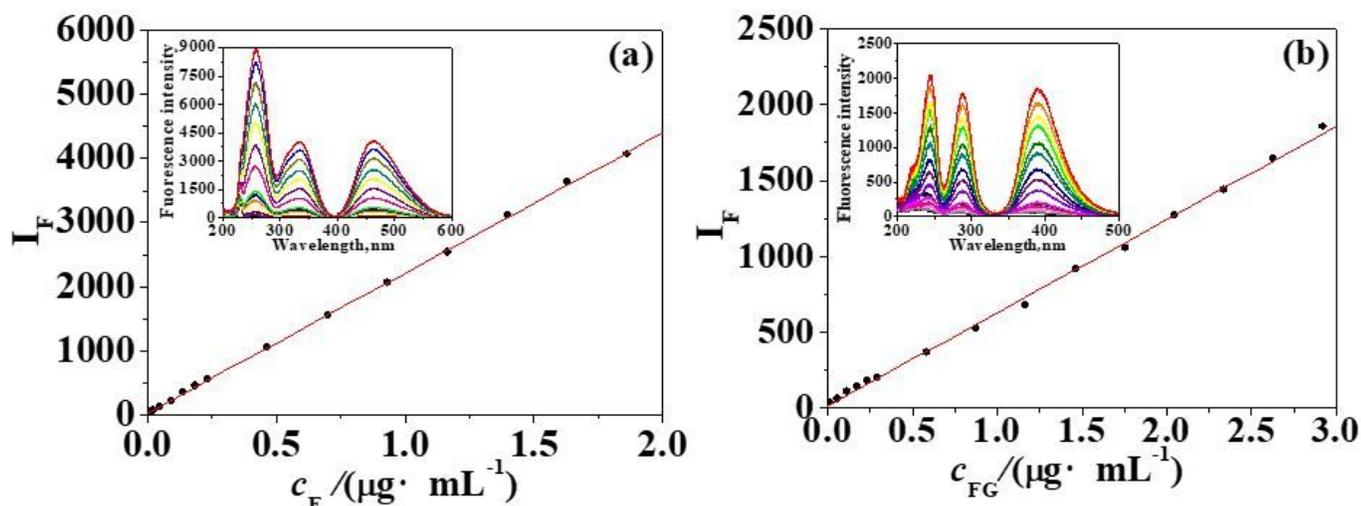
**Figure 7**

Influence of methanol on fluorescence intensity ( $\lambda_{ex}/\lambda_{em}$ : 334/464 nm) of formononetin( $0.972 \mu\text{g}\cdot\text{mL}^{-1}$ , pH 9.3).



**Figure 8**

Relationship between fluorescence intensity ( $\lambda_{ex}/\lambda_{em}$ : 288/388 nm) of ononin ( $0.914 \mu\text{g}\cdot\text{mL}^{-1}$ ) in alkaline solution (1.0M NaOH, 2.0mL) and heating temperature (heated for 1 h at 20,40,50,60,70,80,90,100°C).



**Figure 9**

(a) Relationship between fluorescence intensity and concentration of F ( $\lambda_{ex}/\lambda_{em} = 334 \text{ nm} / 464 \text{ nm}$ ). Inset in the fig. depicts fluorescence excitation and emission spectra of F (pH=9.27) at various concentrations ( $0.0117 \mu\text{g}\cdot\text{mL}^{-1}$  to  $1.86 \mu\text{g}\cdot\text{mL}^{-1}$ ). (b) Relationship between fluorescence intensity and concentration of FG ( $\lambda_{ex}/\lambda_{em} = 288 \text{ nm} / 388 \text{ nm}$ ). Inset in the fig. depicts fluorescence excitation and emission spectra of F (pH=12.5) at various concentrations ( $0.0146 \mu\text{g}\cdot\text{mL}^{-1}$  to  $2.92 \mu\text{g}\cdot\text{mL}^{-1}$ ).