Quantification of Riboflavin and Pyridoxine in a Mixed Solution at a High Concentration by Fluorescence Spectrophotometry

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Summary: A new method of quantifying the contents of riboflavin (RF) and pyridoxine (PY) in their mixed solution was introduced in this study. A mathematical model was established to calculate the actual concentration of PY (*Z*) based on the apparent concentrations of PY (*Y*) and RF (*X*), which were quantified directly when RF and PY were mixed together. First, a linear relationship was found between Y and Z with a high coefficient, which defines fluorescence quenching efficiency. Second, a curvilinear equation was established between the apparent concentration of X and the fluorescence quenching efficiency (*k*) of PY. The actual concentration of PY could be obtained by using the two equations. The established mathematical model was verified, and the relative error of the calculated PY value was below 2.5%. The upper limit of fluorescence spectrophotometry quantification was up to 20 μg/mL for both RF and PY. Compared with RP-HPLC, this method is convenient in terms of sample pretreatment, as well as saves organic solvents and time.

Key words: Quantification, Riboflavin, Pyridoxine, High concentration, Fluorescence spectrophotometry.

Introduction

B vitamins constitute an important type of vitamins. Despite their low content in the human body, B vitamins are essential in maintaining human health [1]. The lack of B vitamins can cause numerous vitamin deficiencies, such as beriberi, growth retardation, anemia, neurological deficits, anorexia, or certain adaptive disabilities [2-4]. Pyridoxine (PY) and riboflavin (RF), which are known as vitamins B6 and B2, respectively, play important roles in cell metabolism. Vitamin B6, which is mainly in the form of pyridoxine, pyridoxal, and pyridoxamine, is abundant in meat, nuts, and whole grain products[5]. Pyridoxine is the precursor of pyridoxal phosphate, which acts as a coenzyme to catalyze the transamination of amino acids[6], therefore, PY is typically used in drug formulations as multivitamin supplements[7]. However, studies have shown that excessive amounts of PY cause peripheral sensory neuropathy, which is characterized by the loss of both muscle and cutaneous afferents in adult mammals[8]. RF can develop into flavin dinucleotide (FAD) and flavin mononucleotide (FMN), which are exclusive in wide varieties of enzymes of catalyzed oxidation and reduction reactions[9]. Riboflavin phosphate plays an important role in treating progressive keratoconus, a common degenerative disorder characterized by corneal ectasia[10]. However, PY metabolism is largely dependent on the presence of RF; both vitamins should be supplied as nutrient supplements simultaneously[11]. Therefore, the amounts of RF and PY in food or vitamin supplements should be simultaneously quantified.

Reversed-phase high-pressure liquid chromatography (RP-HPLC) coupled with ultraviolet spectrophotometric or fluorometric quantification is commonly used to quantify the concentrations of RF and PY in a mixed solution[12-14]. However, the preparation of the mobile phase is complicated, and the RP-HPLC analysis procedure requires a long time, whereas fluorescence spectrophotometry can quantify the concentration of RF and PY rapidly and sensitively[15]. Thus, fluorometry, which offers advantages of simplicity, broad applicability, and high sensitivity, can also be used to quantify RF and PY, because both compounds are endogenous fluorophores[16, 17]. Given that RF is a fluorescence quencher of PY, RF can affect the quantification of PY at a high concentration and not at a low concentration.

This study explored the influence of RF at different concentrations (2–20 μg/mL) on the quantification of PY. In addition, a mathematical model was established to calculate the actual concentration of PY based on the apparent concentrations of PY and RF when mixed together.

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Experimental

Reagents and Apparatus

RF and PY were purchased from Sigma (St. Louis, MI, USA). Deionized water (18.2 MΩ) was produced by Millipore Simplicity Water Purification System (Millipore, USA). A fluorescence spectrophotometer (Varioskan Flash) was sourced from Thermo Fisher (USA). Fluorescence and UV absorption spectra were obtained by Hitachi F-7000 (Japan) and Perkin Elmer instruments (USA), respectively. Costar Assay Plate (96-well) was purchased from Corning (USA). Precision electronic balance (AC 220V) was from AD (Japan).

Determination of relative fluorescence units (RFUs)

The fluorescence spectrum and the value of RFUs were collected by Varioskan Flash. The wavelengths used to quantify PY and RF before the two vitamins were mixed were $\lambda_{ex}/\lambda_{em}$ (nm) = 320/420 [18] and $\lambda_{ex}/\lambda_{em}$ (nm) = 452/516 [19], respectively. In the mixed solution, the condition of fluorescence quantification was adjusted as follows: wavelengths of PY (apparent concentration), $\lambda_{ex}/\lambda_{em}$ (nm) = 300/400; RF, $\lambda_{ex}/\lambda_{em}$ (nm) = 480/530.

Sample preparation

Water used here was purified with a Mill-Q system (Millipore Corporation, Boston USA), its resistance is 18.2MΩ. Stock solutions of RF and PY were 100 μg/mL and 1 mg/mL, respectively. A series of solutions with different concentrations of RF and PY was prepared by mixing the stock solution and water to an appropriate volume (10 mL). About 70 μL of each sample was quantified by Varioskan Flash and replicated three times. Samples of RF (10 μg/mL), PY (10 μg/mL), and their mixed solutions (10 μg/mL) were prepared for spectral scanning.

Statistical analysis

Statistical analyses were performed using SPSS package (SPSS 22.0 for Windows, SPSS Inc., Chicago, IL, USA). Statistical significance was established at $p < 0.05$. Graphs were prepared using ORIGIN 8.0 program (OriginLab Inc., Northampton, MA, USA).

Results and discussion

Quantification of RF affected by PY

The adjusted detection wavelength ($\lambda_{ex}/\lambda_{em}$ (nm) = 480/530) of RF was selected because of the effect of eliminating the PY spectrum (Fig. 1A). The concentrations of RF were quantified when a series of PY concentrations was mixed in the solution. The concentrations of both RF and PY were below 20 μg/mL. As shown in Fig. 1B, PY slightly influenced RF quantification under the wavelengths of $\lambda_{ex}/\lambda_{em}$ (nm) = 480/530.

Establishment of a mathematical model

Quantification of PY affected by RF

The fluorescence emission spectrum of RF, PY and their mixed solution (10 μg/mL) were obtained at a constant excitation wavelength (300nm). As shown in Fig. 2A, partial overlapping was observed between the fluorescence emission spectrum of PY and UV absorption spectrum of RF. This overlap resulted in lower fluorescence intensity of PY detected in the mixed solution compared with the actual value, indicating that the PY concentration detected by fluorescence was inaccurate under the presence of RF.

The RFUs of all samples were quantified under the previously described wavelengths. The PY concentration was calculated using a standard curve. The quantified PY concentration showed a significant difference from the actual PY concentration. All the points above the auxiliary line (y = x) suggested that the apparent PY concentration was smaller than the actual concentration when PY was mixed with RF. High RF concentration indicated a large difference between the apparent concentration and actual concentration of PY (Fig. 2B). Moreover, a linear relationship was found between the actual and apparent concentrations of PY at a given constant RF concentration, thereby suggesting that RF remarkably influenced PY quantification.

Three parameters were defined as follows: *X* is the RF concentration, *Y* is the apparent PY concentration, and *Z* is the actual PY concentration. At a series of concentrations of RF, the relationship between the actual PY concentration (*Z*) and apparent PY concentration (*Y*; shown in Fig. 2B) could be digitized in Table-1. The ratio of *Z* to *Y* (*Z*/*Y*) was defined as reflecting fluorescence quenching efficiency (*k*).

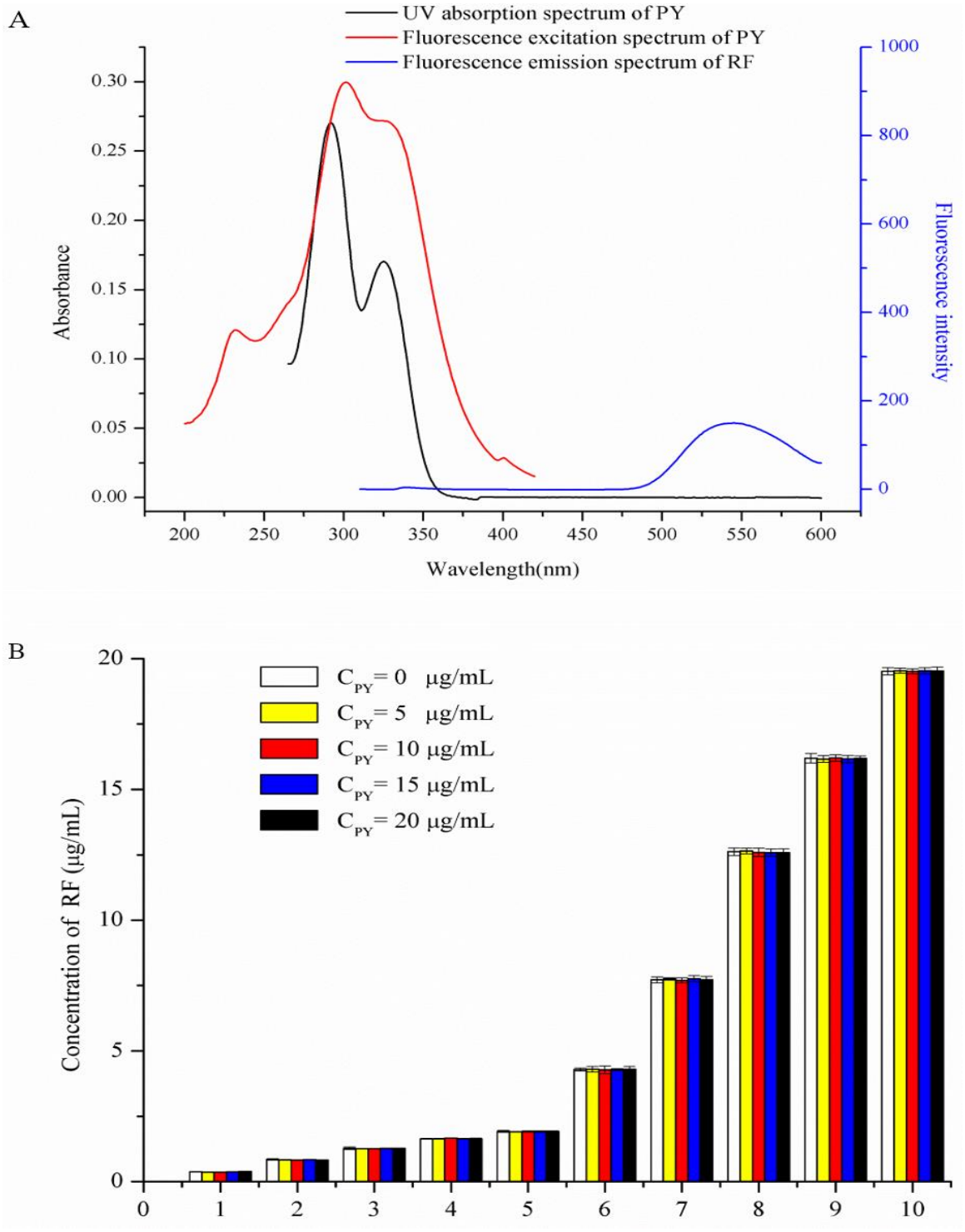

Fig. 1: Quantification of RF affected by PY.
A: The UV absorption spectrum(250-600nm) of PY(black), the fluorescence excitation spectrum of PY (red), and the fluorescence emission spectrum of RF(blue), (concentration, 10μg/mL); B: Detected concentration of RF with PY existence.

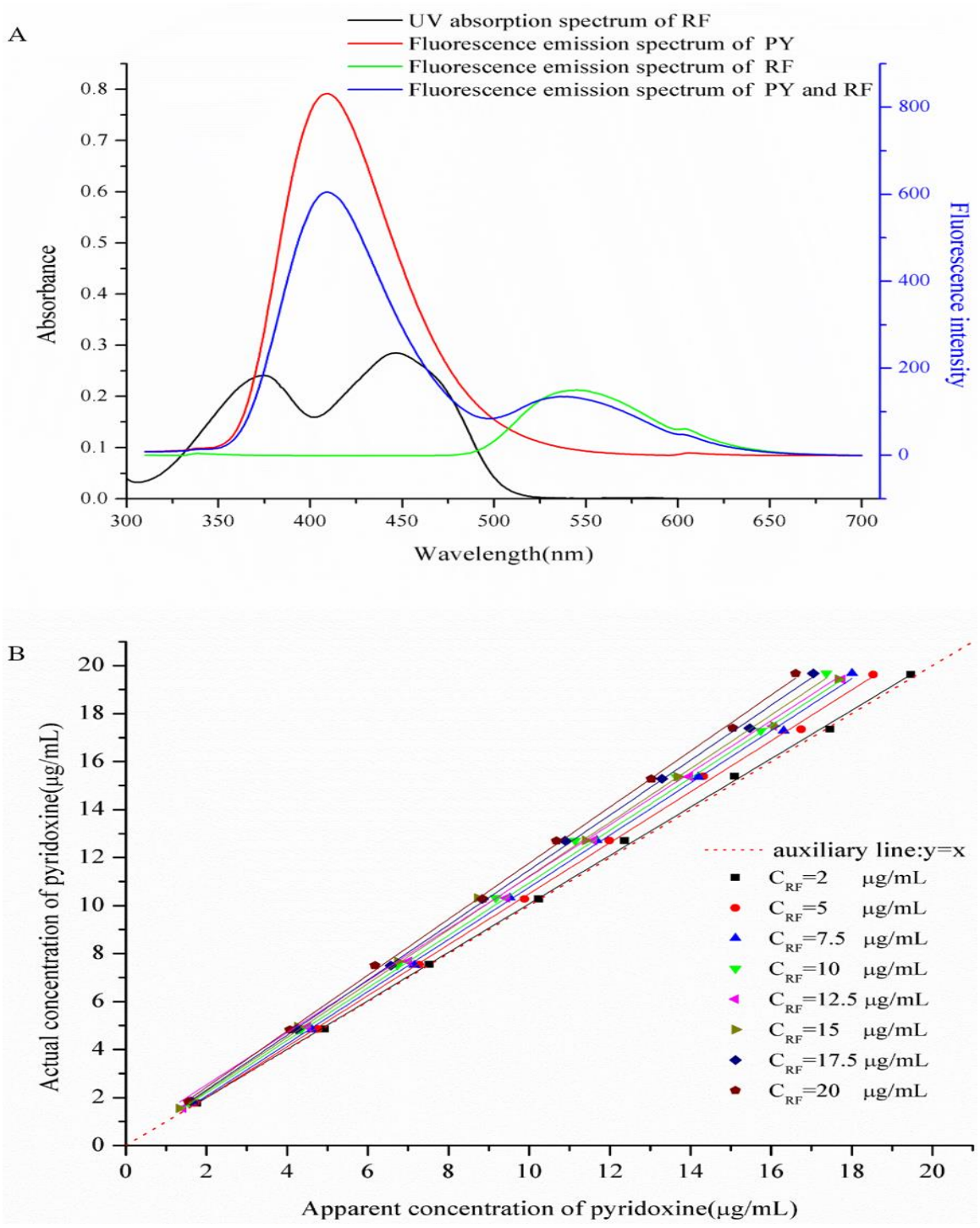

Fig. 2: Quantification of PY affected by RF.
A: The UV absorption spectrum(300-600nm) of RF(black), the fluorescence emission spectrum of PY(red), the fluorescence emission spectrum of RF (green), and the fluorescence emission spectrum of PY and RF (blue), (concentration, 10μg/mL); B: Detected concentration of PY with RF existence. The linear relationship between the detected and actual concentration of PY at a given RF.

Table-1: Formula of Z and Y at a given concentration of RF (μg/mL) in Fig 2B

RF	Formula	R^2	k
20	Z=1.1754Y	0.9992	1.1754
17.5	Z=1.1474Y	0.9993	1.1474
15	Z=1.1265Y	0.9993	1.1265
12.5	Z=1.1123Y	0.9975	1.1123
10	Z=1.0942Y	0.9994	1.0942
7.5	Z=1.0789Y	0.9991	1.0789
5	Z=1.0527Y	0.9991	1.0527
2	Z=1.0077Y	0.9994	1.0077

Relationship between the concentration of RF and fluorescence quenching efficiency

As shown in Fig. 3, the relationship between X and k was not linear. During curve estimation by SPSS 22.0, the curve conformed more to a cubic relation, and the equation between fluorescence quenching efficiency (*k*) and RF (*X*) was as follows (formula 1):

$$k = 0.966071 + 0.023662X - 0.001472X^2 + 4.066637*10^{-5}X^3 \quad (1)$$

$R^2=0.999$ ($p < 0.05$)

Finally, the actual PY concentration was obtained using the equation involving RF and the apparent PY concentration as follows (formula 2):

$$Z = (0.966071 + 0.023662X - 0.001472X^2 + 4.066637*10^{-5}X^3)*Y \quad (2)$$

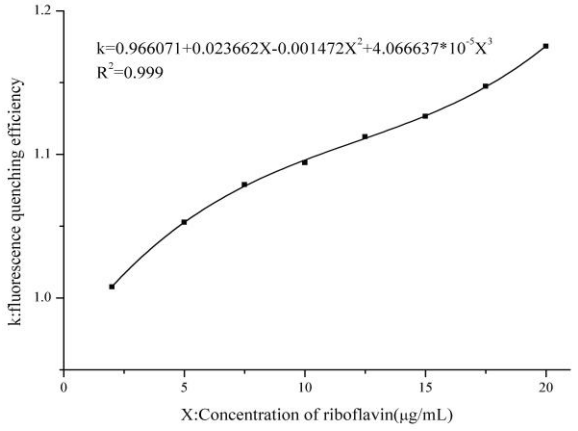

Fig. 3: The relationship between concentration of k and RF.

Error estimation of the mathematical model

More solutions with different RF and PY concentrations were prepared. The RF concentration (*X*) and apparent PY concentration (*Y*) were quantified using the Varioskan Flash, and PY concentration calculated by the mathematical model was defined as *Z*. When PY was mixed with RF, the same volume of water was mixed with PY as contrast, and the value of quantified PY was defined as *C*, which was considered the intrinsic content of PY. The deviation was obtained by formula (3).

$$\text{Devitation} = \frac{|C-Z|}{Z} \times 100\% \quad (3)$$

As shown in Table-2, the deviations of all the samples were below 2.5%, suggesting that the mathematical model was suitable for obtaining the actual PY concentration in a mixed solution.

Table-2: Deviation of the detected value and the calculated value of PY (μg/mL).

RF (X)	Apparent concentration of PY (Y)	Calculated concentration of PY (Z)	Contrast (C)	Deviation
3.625	4.006	4.145	4.185	0.974%
3.625	16.363	16.930	16.803	0.753%
7.355	3.824	4.119	4.185	1.590%
7.355	15.592	16.796	16.803	0.044%
10.028	3.739	4.101	4.158	1.385%
10.028	15.482	16.980	16.651	1.977%
15.471	3.589	4.057	4.158	2.446%
15.471	14.708	16.624	16.651	0.163%

The concentration levels of PY can be calculated by the mathematical model mentioned above within the range of 2–20 μg/mL. When lower than the minimum detection value (2 μg/mL), the concentrations of RF and PY could be quantified directly because the experiment deviation did not exceed systematic error. When the RF concentration was more than 20 μg/mL, fluorescence could quench itself; a high fluorophore concentration resulted in quenching because of various interactions, such as radiative and non-radiative transfer and excimer formation[20]. The solution's pH was a key factor in the fluorescence intensity measurement of RF and PY, and the molecular structure of RF and PY was destroyed in alkaline solution [21, 22], but stable in acidic solution. Besides, numerous factors can influence fluorescence quantification, such as solvents [23], surfactants, and metal ions in solution [24, 25]. The mathematical model was suitable for quantifying the level of PY only when the fluorescent characteristics were undisturbed by environmental factors (e.g., pH and metal ion). For fluorescence measurements with 96-well microtiter plates, a well-to-well reproducibility of *ca.* 2% could be achieved under optimum conditions. Two experiments

were performed to evaluate the standard deviations of fluorescence measurements, and results showed that the SD was between 3% and 8%[26]. Therefore, the PY concentration acceptably varied below 2.5%. In other words, the present method was satisfactory for PY quantification.

Fluorescence quenching results in weakening or disappearing fluorescence signal [27], which mainly explains why PY quantification was affected by RF. The fluorescence intensity can be decreased by a wide variety of processes. Quenching can occur because of different mechanisms, such as energy transfer. Radiative energy transfer is due to the emission and reabsorption of photons and inner filter effects and not on the molecular optical properties of a sample [20]. Partial overlapping occurred between the fluorescence emission spectrum of PY and the fluorescence excitation spectrum of RF (Fig. 2A), such that PY fluorescence was probably absorbed by RF because of the radiative energy transfer caused by the inner filter effect, that is, absorption of light at both the excitation and emission wavelengths [28].

The extent of fluorescence resonance energy transfer (FRET) is determined by the distance (Förster distance) between the donor and acceptor, as well as the extent of spectral overlap. The efficiency of energy transfer is expressed as follows formula (4) [20]

$$E=\frac{R_0^{\,6}}{R_0^{\,6}+r^6} \qquad (4)$$

E is the efficiency of energy transfer, R_0 is Förster distance, and *r* is the distance between the donor (PY) and acceptor (RF).

Förster distances are typically in the range of 15–60 Å, and the donor and acceptor are coupled by a dipole–dipole interaction. The calculated minimum value of r is about 21 nm at the ideal environment, for example, at PY and RF concentrations of 20 μg/mL, individual solute molecules were uniformly distributed. This finding implied that efficiency of fluorescence resonance energy transfer could be ignored ($r_{min} > R_0$). PY fluorescence affected by RF may result from radiative energy transfer. When the concentration exceeded 20 μg/mL, energy transfer became more complicated and was no longer under quantification conditions; therefore, this model could not be used to calculate the exact concentration anymore.

Conclusion

This study established a model to quantify the concentrations of RF and PY in their mixed solution. When their concentrations were in the range of 2–20 μg/mL, the measured concentrations of RF and PY deviated less than 2.5% when quantified by fluorescence spectrophotometry and calculated by this model. Compared with RP-HPLC, this method is convenient in terms of sample pretreatment, saving organic solvents, and shortening analysis. This method markedly improves the efficiency of quantifying the content of RF and PY in food and beverages.

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