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Determination of riboflavin in urine and beverages by capillary electrophoresis with in-column optical fiber laser-induced fluorescence detection

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Abstract

A simple, rapid and sensitive method was developed for routine analysis of riboflavin in beverage, green tea and urine by capillary electrophoresis with in-column optical fiber laser-induced fluorescence detection (LIF). The difference between the present detector in the study and others is that an optical fiber was adopted in the former, which can guide the excitation light into the capillary right at the detection window. The linearity of the method ($r^2 = 0.998$) was good over the concentration range from 0.05 to 20 μM for riboflavin. The limit of detection (LOD) was determined using linear regression analysis and was found to be 3.0 nM. The percent recoveries of riboflavin in beverage, green tea and urine samples were 95.3 ± 2.9, 105.5 ± 3.9 and 94.3 ± 1.7, respectively. These results of quantitative analysis of riboflavin in beverage and green tea samples is in agreement with that of obtained by the AOAC of fluorometric method. In the analysis of urine samples, all electropherograms of urine samples and corresponding concentrations of riboflavin in the period of 13 h after orally administrating the ingestion of vitamin B_2 tablets were illustrated.

Keywords: Capillary electrophoresis; Laser-induced fluorescence detection; Optical fiber; Riboflavin; Urine; Beverage

1. Introduction

Riboflavin (RF), commonly called vitamin B_2, is a water-soluble vitamin. It is very stable during thermal processing, storage, and food preparation but is susceptible to degradation on exposure to light [1]. RF is the general term for the fluorescent isoalloxazine derivative [2], and is a natural fluorophore. It may participate in enzymatic processes as coenzymes or their precursors. As an essential vitamin in human nutrition, RF plays a crucial role in certain metabolic reactions [3]. RF cannot be synthesized in human body therefore has to be obtained dietary, such as liver, cheese, milk, meat, eggs, wines, and tea [4], therefore vitamin B_2 depleted food results in health problem [5]. Symptoms of RF deficiency include fatigue, slowed growth, digestive problems, cracks and sores around the corners of the mouth, swollen magenta tongue, soreness of the lips, mouth and tongue [6]. To construction an accurate method for the determination of RF is essential for assessing nutritional metabolic requirements, for the metabolism of the given vitamin and for dietary intake. However, it is quite difficult to measure RF in metabolite and foodstuff because of the complexity of the biological matrix and the extremely low concentration of these compounds.

The most widely used techniques for the determination of RF are high-performance liquid chromatography (HPLC) [7,8] and capillary electrophoresis (CE) [9–11]. However, as a valid separation method, HPLC requires long analysis time and large solvent consumption of the mobile phase. Capillary electrophoresis has the following advantages: relatively short analysis time, low sample and reagent consumption, ease of automation, high separation efficiency, and flexible applications. Capillary electrophoresis has become a versatile analytical tool for the routine determination of a great variety of compounds. In recent years, CE methods for the determination of RF in food and biological matrix have been reported. Philip and co-workers has reported that dynamic pH junction-sweeping CE with LIF detection was applied as a robust single method to analyze trace amounts of three flavin derivatives in complex biological sam-

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ples [12]. Su and co-workers used LED-induced fluorescence detection for the separation and identification of riboflavin in urine [13]. Qin and co-workers detected the flavin derivatives by microchip with LIF-CE intensified charge-coupled device detectors [14]. Various detectors currently are available for CE to meet different requirements on measurement, such as UV–vis [15], light emitting diode (LED)-induced fluorescence [16], laser-induced fluorescence (LIF) [17], electrochemical [18], chemiluminescent (CL) [19], and mass spectroscopy [20,21] detections. UV–vis detection is carried out in most CE systems because it is easy to fabricate; however, its limit of detection and selectivity are poor. LED-induced fluorescence detector is of low cost and little volume, but its broad emission wavelength range results in high background noise [22]. With electrochemical detector, the high separation voltage could interfere with the detection of the electrochemical signal, and the contamination of electrode surface might also pose problems in the analysis of real sample [23]. The CL detector suffers from the limited application due to lack of CL reaction for many compounds. MS detector has a higher sensitivity, but it is expensive and requires complex instrumentation. LIF detector is recognized to be an extremely sensitive detection method for capillary electrophoresis, which is about 1000 times more sensitive than the traditional UV–vis detector [24]. If the laser excited analytes from outside of the capillary at the detection window directly, the intensive light reflection, scattering and refraction would produce on the capillary surface or wall. Thus, it will result in high background noise and low detection sensitivity. To avoid these shortcomings, an optical fiber was inserted directly into the tail end of capillary and was settled right at the detection window in the new CE-LIF detection system. The excitation light was guided into the capillary directly and then could be utilized completely to excite analytes [25–27].

Benefiting from intrinsic fluorescent nature, RF can be directly detected at very low amounts using CE-LIF. A novel method for the routine analysis of RF was described by CE with in-column optical fiber LIF detection. To avoid the light reflection and scattering from capillary surface and decrease the background noise, the exciting light was taken directly into the separation capillary to excite the analytes in the detection window. It presents a rapid, simple, reliable and very selective one-step method for the detection of RF in beverage, green tea and urine samples.

2. Experimental

2.1. Apparatus

The experimental apparatus used to perform CE-LIF analysis is schematically illustrated in Fig. 1. Briefly, the home-built CE system consisted of a high-voltage supply (0–30 kV, Beijing Cailu Science Instrument Company, Beijing, China), a 55 cm (effective length)× 100 μm i.d. (365 μm o.d.) uncoated fused-silica capillaries (Hebei Optical Fiber, China), and a laser-induced fluorescence detector. The detection window was made by removing off a 5 mm section of polyimide coating on the capillary with about 2.0 cm distance to the tail end of the capillary. The separation capillary, laser-induced optical fiber and a grounded Pt wire are fixed on a plastic chip, and the laser-induced optical fiber was inserted into the capillary about 2 cm. In this way, the whole set-up could be more conveniently and three-dimensionally adjusted [25,26]. A solid bare optical fiber with a diameter of 40 μm (Beijing Glass Institute, Beijing, China) was used as the excitation light transmission. A 474 nm diode-pumped solid state blue laser (Changchun New Industries Optoelectronics Tech, Co., China) was used as the excitation light source.

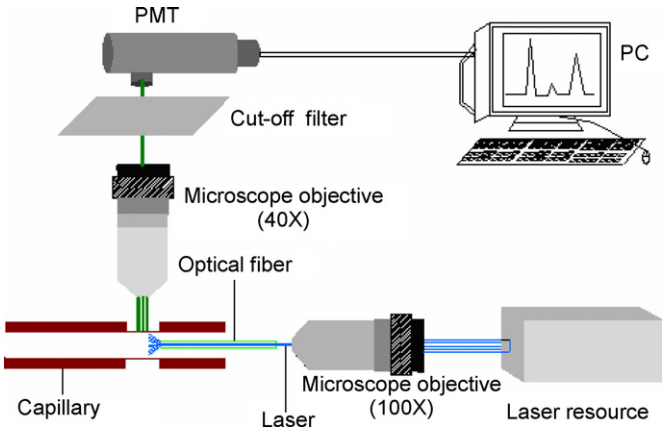

Fig. 1. Schematic representation of the capillary electrophoresis with in-column optical fiber laser-induced fluorescence detection.

The light emanating from the laser resource was focused by a 100× microscope objective (Olympus, Japan) before coupling into one end of the light-guiding optical fiber. The other end of the fiber was inserted into the separation capillary for introducing the exciting light to the detection window. Fluorescence emission from analytes was collected by a 40× microscope objective (Olympus, Japan) and focused on a spatial filter (0.3 mm) and passed through a cut-off filter (maximum wavelength at 515 nm) before reaching the PMT (CR105 equipped with a socket assembly, Beijing Hamamatsu Photon Techniques Inc., Beijing, China). The output signal was recorded and processed with a computer using in-house written software.

2.2. Chemicals and reagents

All chemicals and organic solvents used were of analytical-reagent grade. Milli-Q water was used throughout and solutions were filtered through a 0.45 μm membrane filter. RF (98% purity) was purchased from Shanghai Biochemical Reagents Company (Shanghai, China).

Standard stock solutions of RF were prepared by dissolving the calculated quantity in water to give a concentration of 50 μM. Standard solutions of RF were prepared by diluting the stock solution in water to give concentrations in the range of 1 nM to 50 μM. All solutions were stored in amber glass bottles in a refrigerator at 4 °C. The running buffer solution was prepared by dissolving 0.3814 g $Na_2BO_4{\cdot}10H_2O$ in 100 ml water (10 mM borate) and adjusted to pH 9.6 with 0.1 M NaOH solution.

2.3. Samples preparation

2.3.1. Beverage and green tea samples preparation

Tang (a kind of non-alcoholic beverages, Tianmei Food Co., Tianjing, China.) and Zhuyeqing (a kind of green tea, Emei Mountain Tea Co., Sichuan, China.) were purchased from local markets. Green tea samples were obtained by extracting 10 g green tea in 100 ml boiling tap water for 10 min. Then the samples were centrifuged and filtered through a 0.45 μm filter to produce a final solution. Beverage samples were prepared by dissolving 2 g in 50 ml Milli-Q water then centrifuged and filtered through a 0.45 μm filter for use.

2.3.2. Urine samples collection and storage

Urine samples (5–10 ml) were collected from a healthy volunteer (female, 50 kg). The blank (control) samples were collected before and then after oral administration of 20 mg (four tablets) of vitamin B_2 at 1, 2, 3, 4, 5.5, 7, 8.5, 10, 11.5 and 13 h, respectively. Fresh urine samples were immediately stored at 4 °C in a refrigerator after a preliminary centrifugation step (8000 rpm, 15 min) and filtered through a 0.45 μm filter. All urine samples were injected after dilution with water.

The concentrations of urinary RF vary greatly depending on dietary intake, nutritional supplement use, health and physical condition. To decrease the influence of food, the volunteer was not permitted to take food and beverage in which RF was abundant such as liver, milk, tea, egg and meat, etc. five days before the oral administration of vitamin B_2 tablets and during the test time. At the same time, the volunteer's drinking was controlled (drinks 100 ml pure water at 1 h intervals) and allowed to function in a normal manner during the test time. After controlling the intake, the urine without RF was used to be blank matrix.

2.4. CE procedure

Before use, a new capillary was flushed with 1 M NaOH for 30 min, and then extensively rinsed with water and running buffer for 3 min, respectively. Between two consecutive injections, the capillary was rinsed sequentially with 0.1 M NaOH, water and running buffer for 3 min each. The sample solution was injection into the capillary by hydrodynamic flow at a height differentia of 20 cm for 10 s. After the introduction of sample solution, 18 kV was applied across the capillary. All separations were performed on an uncoated fused-silica capillary (55 cm × 100 μm i.d.) with running buffer. Fluorescence was excited by a laser with a maximum wavelength at 474 nm and detected after passing through a 0.3 mm spatial filter and a 515 nm cut-off filter. The electropherogram was recorded and RF quantification was achieved by measuring the CE peak height. All CE procedures were conducted at room temperature.

Calibration curve was obtained by using different standard concentrations of RF in triplicates. Each sample was analyzed five times.

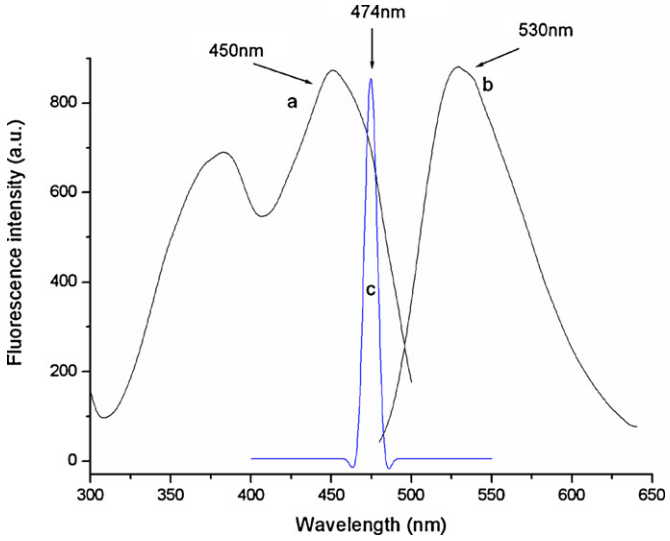

Fig. 2. Typical excitation (a) and fluorescence spectra (b) of RF in water. The dashed line spectrum (c) shows the wavelength range of the laser used in this study.

3. Results and discussion

3.1. Effect of laser resource

Fig. 2 shows the excitation (spectrum (a)) and fluorescence (spectrum (b)) spectra of RF in a water matrix. The blue line (spectrum c) shows the laser resource used in this study. As can be seen, the maximum excitation wavelength and the maximum fluorescence emission wavelength of RF were 450 and 530 nm, respectively. A diode-pumped solid state blue laser with a maximum wavelength at 474 ± 10 nm was adopted. To decrease the interference of the laser resource, a 515 nm cut-off filter was used in the experiment. Obviously, the maximum excitation wavelength of the RF was 450 nm. If a laser source with the maximum excitation wavelength of 450 nm has been selected to excite the analytes containing RF, the higher fluorescence intensity would be obtained at the maximum emission wavelength of 530 nm. As is well known, to obtain a laser source with 450 nm maximum wavelength is impossible since there is no commercial diode-pumped solid state laser with maximum wavelength at 450 nm in our area. Instead, a diode-pumped solid stated laser with maximum wavelength at 457 nm is commercially available. However, the 457-nm laser source is much expensive than a 474–nm laser source and is not commonly available in common analytical laboratory. Although the fluorescence intensity, excited with 474 nm light source, was about 74% compared with that obtained with 450 nm as shown in Fig. 2, the present results demonstrated that the sensitivity was satisfactory for the determination of the concentrations of RF in food samples and biological matrixes when a common 474-nm laser source was applied.

3.2. Separation conditions

In order to improve the resolution, two kinds of running buffers of 20 mM Na_2HPO_4–NaH_2PO_4 and 10 mM borate at pH

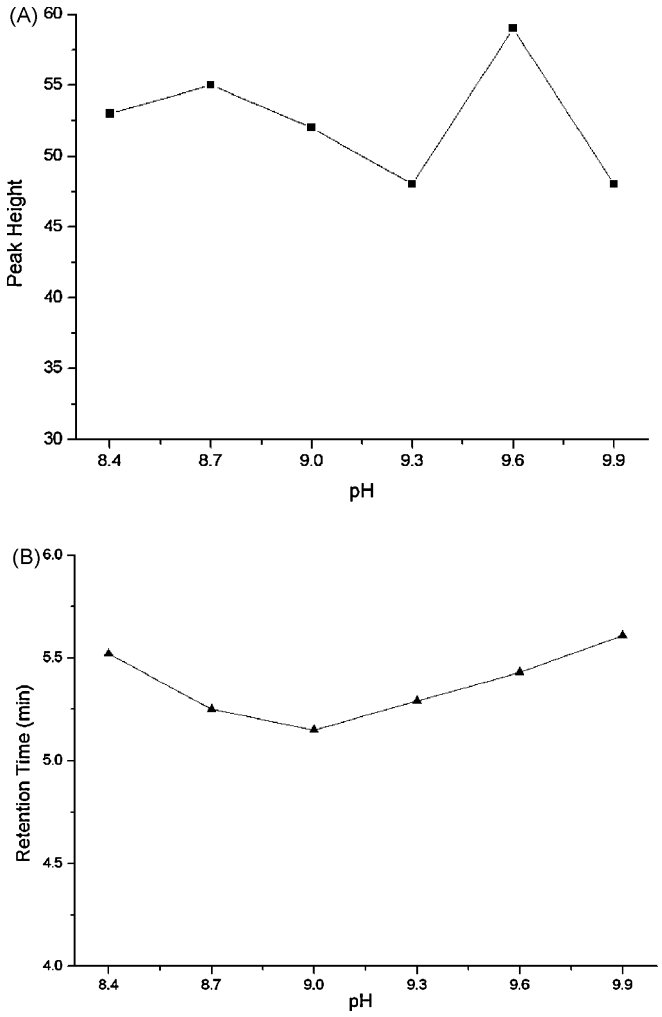

Fig. 3. Effect of the pH of the running buffer on peak height (A) and retention time (B) provided by 0.1 μM of RF standard solution. Running buffer was 10 mM borate buffer (at pH 8.4, 8.7, 9.0, 9.3, 9.6 and 9.9).

9.0 have been selected for testing their effects on the separation of the analytes at the separation voltage of 18 kV, respectively. The experimental results showed that borate buffer gave not only the best resolution but also the highest sensitivity under the same conditions. So, borate buffer was chosen as the running buffer and the optimum pH.

In an attempt to find a suitable pH value of the running buffer, several pH values (in the range from 5.3 to 9.9) were tested using 10 mM borate buffer. When pH values were less than 8, the peak shape of RF electropherogrames was not well-formed. Thus, the influence of buffer pH values on the peak height and the retention time of RF was tested and is illustrated in Fig. 3. The highest fluorescence intensity of RF was obtained at pH 9.6 with the best peak shape. With increasing the pH value of the buffer, the charges on the capillary wall and the electroosmotic flow (EOF) were increased, which can be ascribed to the dissociation of surface silanol groups. It was generally accepted that the electroosmotic mobility strongly went up at pH 7–8 [28]. At pH 8–9, the increase of mobility was very slow because the dissociation of surface silanol groups gradually reached saturation. So the retention time of RF gradually decreased from pH 8.4 to 8.7 and was almost constant at pH 8.7 and pH 9.0 (Fig. 3 B). When pH value of the buffer was higher than 9.0, the effects of electroosmotic mobility from the ionic strength was stronger than that from the 'pure' pH. Increasing the pH values will result in an increase of the ionic strength and a decrease of the EOF. So, the retention times of RF were increased gradually with increasing pH values of the buffer. To obtain well-shape peak, stability and higher fluorescence intensity, a running buffer of pH 9.6 was chosen.

The concentrations of the buffer (5, 10, 20 and 50 mM) were tested at a constant pH of 9.6 and potential of 18 kV. Increasing the concentration of the buffer might decrease the EOF and increase the time of migration. At the same time, it might also result in excessive Joule heating that could ruin the resolution and repeatability. Compromised between the run time and the Joule heat generated inside the capillary, the borate buffer of 10 mM was the optimum condition.

The influence of separation voltage on the retention time of the analyte was also studied. The results indicate that increasing the separation voltage not only gave shorter retention time for all analytes, but also increased the baseline noise and decreased the resolution of the analytes. However, too low separation voltages would increase the analysis time considerably and cause peak broadening. Based on the experiments, 18 kV was selected as the optimum separation voltage to accomplish a good compromise.

Taking into account influence of injection volume on the sensitivity and the peak shape of RF, hydrodynamic injection of 20 cm for 10 s was performed in the experiment. The injection volume was about 3.0 nl according to the Hagen-Poiseuille equation [29].

Through the above-mentioned experiments, the optimum conditions for separating RF have been obtained. A 10 mM borate buffer (pH 9.6) was used as the running buffer at a separation voltage of 18 kV. The sample solution was injected into the capillary by hydrodynamic flow at a height differentia of 20 cm for 10 s. When a potential of 18 kV was applied for the separation, it resulted in a current of approximately 60 μA.

3.3. Linearity, limit of detection, and reproducibility

A linear relationship was evaluated over the concentration range of the analytical procedure. For this purpose, calibration curve (Fig. 4) was constructed at seven concentration levels in the range from 0.05 to 20 μM. The regression equation of standard curve was $y = 71.44 \pm 1.72x + 41.25 \pm 0.24$ with $r^2 = 0.998$ for 95% confidence interval of the regression equation, where y is the peak height (mV) of the fluorescence of RF and x is the concentration (μM) of RF. The retention time and the peak height of a 1.0 μM RF standard solution were 5.48 ± 0.07 min (RSD = 1.30%, $n = 5$) and 142.7 ± 3.7 (RSD = 2.98%, $n = 5$), respectively. Under the optimized conditions, limit of detection (LOD) of concentration of the method was 3.0 nM obtained with 99% level of confidence according to regression analysis, and a concentration of 8.0 nM RF could be identified in the electropherogram (Fig. 5). The LOD was relatively high when compared with that reported by Tommaso [10], Tomás

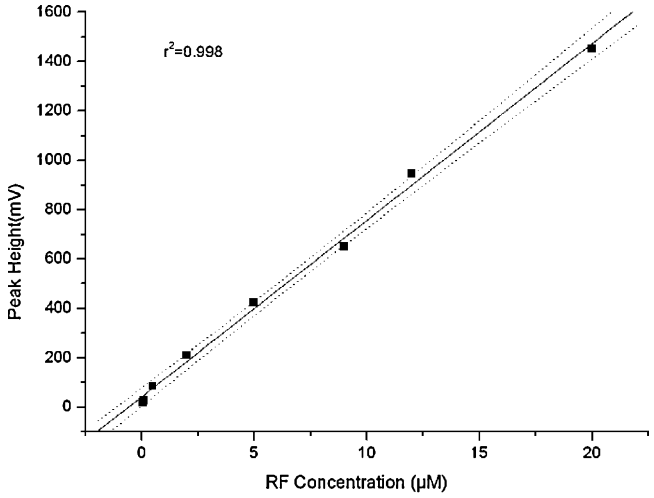

Fig. 4. Standard curve for RF, produced using standard solutions at concentrations of 0.05, 0.5, 2.0, 5, 9.0, 12.0 and 20 μM. The line was the linear regression symbol and correlation was $r^2 = 0.998$. The dot line was 95% confidence intervals symbol.

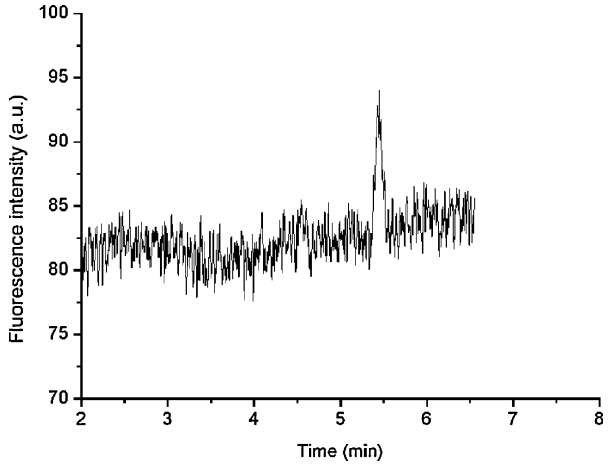

Fig. 5. Electropherogram recorded at RF concentration of 8 nM. Electrolyte composition was 10 mM borate buffer (pH 9.6). Capillary, 100 mm i.d., 55 cm in length; applied voltage, 18 kV; laser resource maximum wavelength, 474 nm.

[11] and their co-workers, where their LOD for RF were 1.3 and 0.23 nM. This can be ascribed to the relatively lower coupling efficiency between the laser light and the optical fiber. Clearly, optimizing the optical arrangements and using a 450-nm laser should improve the sensitivity of this method. In fact, the present system has adequate sensitivity for the routine determination of RF in some food and biological specimens. The maximum wavelength of laser resource at 474 nm was larger than the maximum wavelength of RF at 450. As a result, the fluorescence intensity of RF in excitation wavelength 474 nm was only about 74% compared with that in excitation wavelength 450 nm. If a 450-nm laser resource was used, a better LOD of RF would be obtained.

3.4. Comparison with the AOAC method

In order to verify the accuracy of the proposed method, the content of RF in beverage and green tea samples was analyzed by the fluorometric method of American Organization of Analytical Chemists (AOAC) [30]. Fluorescence of RF in the samples and different concentrations of standard solution have been detected by a fluorescence spectrophotometer (F4500, Hitachi, Tokyo, Japan). The results of RF obtained with the AOAC method and the proposed method are summarized in Table 1. The results of RF in beverage samples and green tea obtained by using the developed method showed a satisfactory agreement with that obtained by the fluorometric method of AOAC. However, RF would be destroyed by $KMnO_4$ and H_2O_2 in the procedure of sample preparation to remove the background in the AOAC method. It is also criticized for being tedious and time consuming. Additionally, the validated method has been reported as a procedure, which cannot determine the total RF by effective separation, and overestimates total RF content due to the presence of interfering artifacts [31]. On the contrary, sample preparation only required centrifugation and filtration by the developed method. It was also simple, rapid, selective and reliable, and the whole CE procedure was completed within 10 min.

Table 1
Determination results of RF in beverage and green tea samples by proposing method and AOAC method, respectively

Sample	Proposed method		AOAC method	
	Mean ± SD (mg/100 g)	RSD (%) $n = 6$	Mean ± SD (mg/100 g)	RSD (%) $n = 6$
Beverage-1	0.61 ± 0.03	4.08	0.57 ± 0.01	2.12
Beverage-2	1.02 ± 0.05	4.99	1.13 ± 0.03	2.57
Green tea -1	0.54 ± 0.02	3.82	0.58 ± 0.02	3.06
Green tea-2	0.28 ± 0.01	4.12	0.24 ± 0.01	2.86

Table 2
Determination results of RF in beverage, green tea and urine samples (see Section 3.4 for CE conditions)

Sample	Concentration (μM) (average ± SD)	RSD (%) ($n = 5$)	Added (μM)	Found (μM)	Recovery (%) (average ± SD)	RSD (%) ($n = 3$)
Beverage	2.81 ± 0.05	1.86	5.0	4.76 ± 0.14	95.3 ± 2.9	3.09
Green tea	1.58 ± 0.06	2.82	7.5	7.91 ± 0.26	105.5 ± 3.3	3.10
Urine sample (2 h)	7.01 ± 0.32	4.51	2.5	2.36 ± 0.04	94.3 ± 1.7	1.76

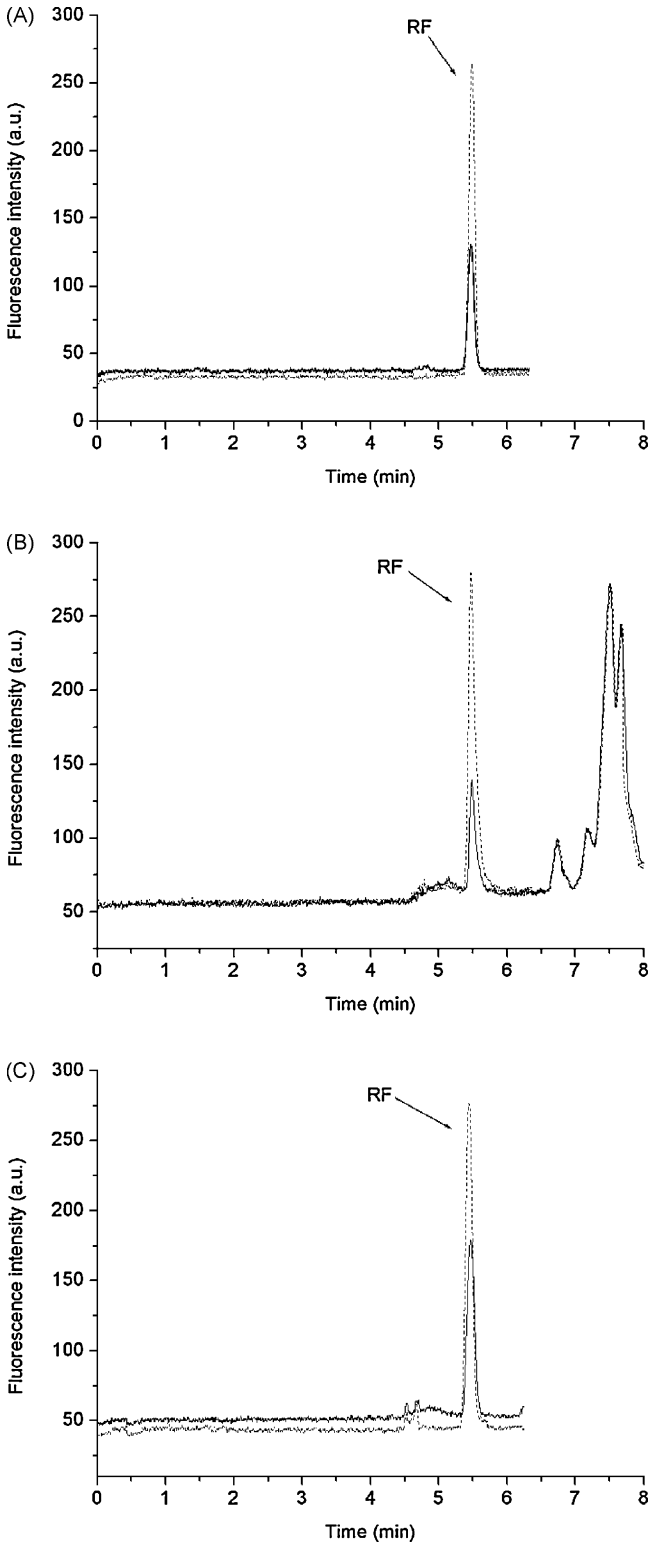

Fig. 6. Electropherograms were obtained from the separation of beverage (A), green tea (B), and urine (C) samples. Solid traces were obtained from the real samples; dotted traces were obtained from samples enriched standard RF solution with the final concentrations of 1.2, 1.5 and 0.9 μM, respectively. Running buffer was a water–acetonitrile (9:1, v/v) solution, containing 10 mM borate buffer at pH 9.6. Other CE conditions were as in Fig. 5.

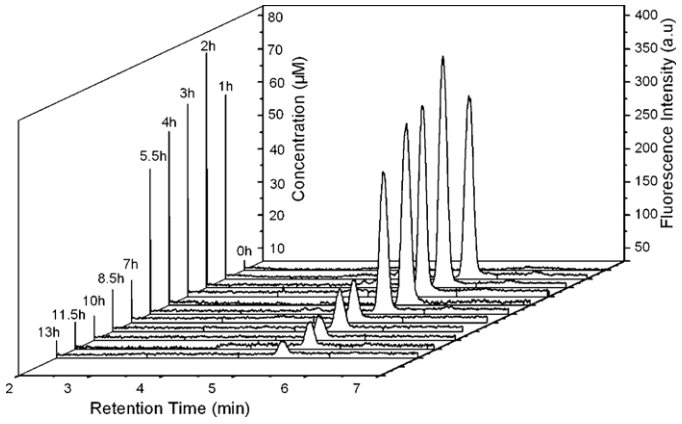

Fig. 7. Electrophoretograms of RF in urine samples during 13 h after the administration of vitamin B_2 tablets. The corresponding final concentrations of RF also were shown in the figure by the time after the administration.

3.5. Samples analysis

The CE buffer was a water–acetonitrile (9:1, v/v) solution, containing 10 mM borate buffer at pH 9.6. Acetonitrile is necessary for the separation of components in urine samples because it prevents overlap by unknown compounds. Well-shaped peaks were obtained by adding appropriate acetonitrile into the buffer solution according to Ref. [13]. Other separation conditions are described in Section 3.1. The electropherograms of the beverage, green tea and urine samples are shown in Fig. 6. Solid trace was obtained from samples analysis and the dotted trace was obtained from sample enriched with 20 μM standard RF solution. As can be seen, a few native fluorescent compounds are present in the green tea sample and urine sample when excited at 474 nm, and RF was separated from the compounds completely.

Using peak height, beverage, green tea and urine samples were analyzed, respectively. The external standard method was used to measure the RF concentration. Peaks monitored by fluorescence detection were identified by the retention time in comparison with the RF standard solutions and by standard additions. The recovery percentages are shown in Table 2.

In order to investigate the metabolism of RF in healthy body, all urine samples were collected as mentioned in Section 2.3. Electropherograms of the urine samples collected before and then after oral administration of vitamin B_2 are shown in Fig. 7. The RF concentration of urine was rapidly increased from blank to 55 μM within 1 h after ingesting RF. The riboflavin was maintained in the body for about 5.5 h and then decreased quickly.

Acknowledgments

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