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Determination of riboflavin in urine by capillary electrophoresis–blue light emitting diode-induced fluorescence detection combined with a stacking technique

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Abstract

A simple, inexpensive and reliable method for the simultaneous, routine analysis of riboflavin in urine by capillary electrophoresis–light emitting diode (LED)-induced fluorescence detection is described. Using a blue LED as the light source, the detection limit of riboflavin was determined to be 0.48 μg/ml and was improved to 20 ng/ml when a stacking technique was applied. In the analysis of an actual sample, various concentrations of riboflavin were distributed in the urine samples over a period of 9 h after the ingestion of a vitamin B_2 tablet.

Keywords: Blue light emitting diode; Riboflavin

1. Introduction

Laser induced fluorescence (LIF) leads to a remarkable improvement in the sensitivity of detection compared with the use of a conventional incoherent light source. In particular, LIF detection combined with capillary electrophoresis (CE) has achieved detection limits that approach the level of a single molecule [1]. However, for analytes which are either inherently fluorescent or derivatized using a fluorescent label, it is necessary to choose readily available lasers that can be matched to their spectral properties. Currently, conventional lasers, such as argon ion and He–Cd lasers, are generally expensive, relatively bulky and have short lifetimes (~3000 h). In contrast, semiconductor or diode lasers are much less costly, compact, have good output stability and require little or no maintenance. Applications of CE–DIO–LIF detection have been reported at available wavelengths such as the near-IR (785/780 nm) [2,3], red (635/670 nm) [4–6], green (532 nm) and violet (405 nm) regions [7,8], even though only a few suitable labeling dyes are excited in these specific regions. Light-emitting diodes (LEDs) constitute an exceptionally stable light source and ultra-high intensity LEDs at a variety of wavelengths (red to blue) have recently become commercially available. Those commercially available LEDs, which cover the entire visible spectrum and extend into the near infrared (420–950 nm), are currently in us as a light source in many fields. Yeung et al. [9] were first reported the application of an absorption detection

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system for CE separation based on a red LED. Later, a red and green LED has also been applied to CE and microchip separations by Collins and co-workers [10,11].

Some relevant CE–LIF methods for flavin analyses been reported in the literature [17–19]. Although LIF detection has already became one of the most sensitive detection methods in CE, the small injection volume is sometime a major drawback because of the poorer S/N ratio. To improve the limit of detection, a series of reports appeared by Terabe and co-workers [12–16], as well as other groups, concerning the so-called ''stacking'' and ''sweeping'' technique for on-line sample concentration.

In this study, we report on a simple and highly sensitive method using a blue LED in conjunction with the stacking technique, for the detection of riboflavin in urine. Several electrophoretic parameters such as buffer pH, SDS concentration, and injection length required for the separation were optimized and these data are reported herein.

2. Materials and methods

2.1. Chemicals

Acetonitrile was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Riboflavin was acquired from Sigma (St. Louis, MO, USA). A vitamin B_2 tablet (each tablet contains 10 mg of riboflavin per tablet) was purchased from China Biological and Chemical Laboratories (Taiwan) for use. Sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), dioctyl sulfosuccinate sodium salt (DOSS) and sodium cholate (SC) were obtained from Acros (Belgium). The urine samples were centrifuged and then filtered through a 0.45-μm filter and were used directly without any further pre-treatment.

2.2. CE apparatus

The CE set-up was fabricated in-house and is shown in Fig. 1. Briefly, a high-voltage power supply (Model RR30-2R, 0–30 kV, 0–2 mA, Gamma, FL, USA) was used to drive the electrophoresis and a 75-μm I.D. fused-silica capillary (J&W Scientific, CA, USA) was used for the separation (71 cm in length/65 cm to the detector). A blue LED (part no. SLR-05PNW40-030X46DA, Sharlight Electronics; applied voltage, 4 V; price, ~1.5 US dollars) purchased on the Taipei electronic market with an intensity of ~3 mW (peak wavelength ~467 nm, spectral half width ~30 nm) was used. A microscope objective (40×) was used for focusing on the capillary. Fluorescence emission was collected by a microscope eyepiece (10×), passed through an orange cut filter and a slit (0.3 mm),

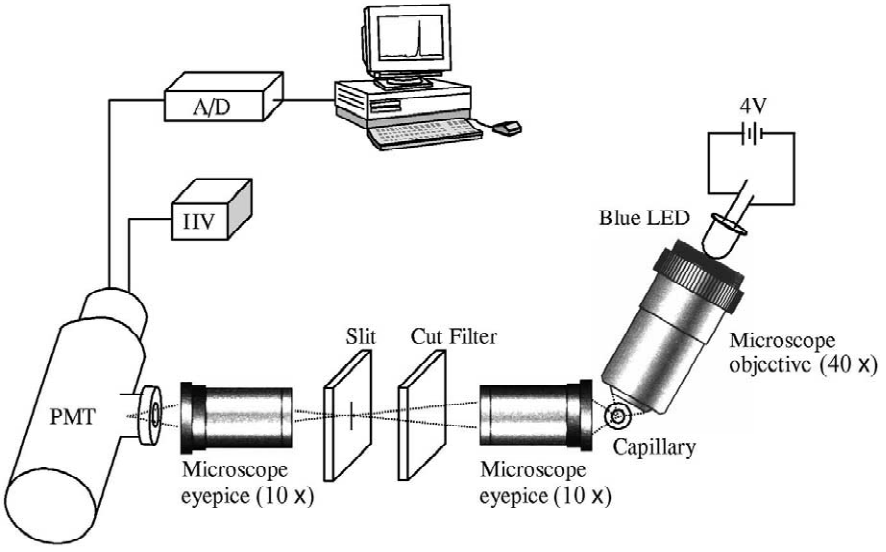

Fig. 1. A schematic apparatus of the capillary electrophoresis–blue light emitting diode (LED)-induced fluorescence detection.

focused by a second microscope eyepiece (10×), and then detected by a photomultiplier tube (Hamamatsu-R928). After an analog–digital converter, the electropherograms were collected with a data acquisition system, connected to a personal computer. For the measurement of excitation and fluorescence spectra (Fig. 2), the excitation source was selected by a monochromator (ARC, Acton Research; Model SP-150, 1200 grooves/mm grating) connected to Xe lamp (Muller Elektronik Optik, SVX/LAX 1450, 500 W). Fluorescence data were collected at a right angle to the light source and dispersed by another monochromator (ARC Model SP-300i, 2400 grooves/mm grating), followed by detection by a photomultiplier tube (ARC Model P2-R928, for 190–900 nm).

2.3. Administration of riboflavin to the volunteer

To obtain a valid urine sample, one vitamin B_2 tablet (10 mg of riboflavin) was given to a volunteer in the early morning. He was then allowed to function in a normal manner during the day with free access to food and water. All urine was collected over a period of 9 h at 30-min intervals. These samples represent the urine samples discussed below.

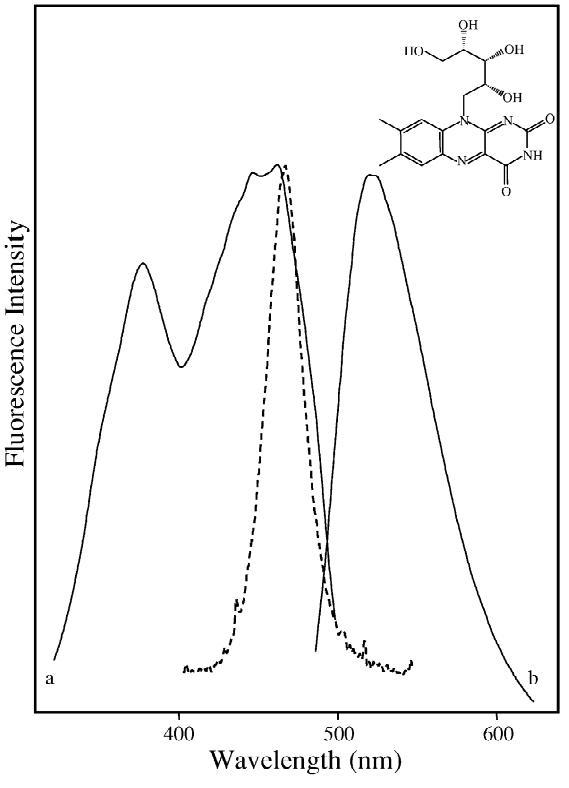

Fig. 2. Typical excitation and fluorescence spectra of riboflavin in water (10 μg/ml). The dashed line spectrum shows the wavelength range of the blue LED used in this study.

3. Results and discussion

3.1. Separation condition and calibration curve

Fig. 2 shows the excitation (spectrum a) and fluorescence (spectrum b) spectra of riboflavin in a water matrix; the concentration was 10 μg/ml. The inset shows the molecular structure of riboflavin. The dashed line shows the wavelength range of the blue LED used in this study. It appears that this light source is particularly well matched to excite riboflavin. Based on a normal MEKC separation, a calibration curve (Fig. 3A) was constructed at various concentrations. Herein, the CE buffer was an aqueous solution, containing 10 m*M* sodium tetraborate and 80 m*M* of SDS (pH 9.2–9.3). The sample was prepared in water. Hydrodynamic injection was achieved by raising the sample reservoir 18 cm relative to the exit reservoir for a period of 3 s (~2 mm in length). When the injection was completed, 16 kV was applied to power the CE separation. A linear relationship exists in the 1–10 μg/ml range, in which a 3:1 signal-to-noise ratio (as shown in the inset) was found for ~0.48 μg/ml. The linearity of the method for riboflavin was fairly good, and can be described by the equation ($y = 0.501x + 0.0058$, $r^2 = 1$). Several techniques have been reported for enhancing sensitivity by on-capillary sample concentration immediately after sample injection. Fig. 3B shows the calibration curve after application of the stacking technique. Herein, the CE buffers were aqueous solutions, containing 10 m*M* sodium tetraborate and 80 m*M* of SDS (pH 9.2–9.3; conductivity: 4.25 mS/cm). The sample was prepared in 0.05 m*M* sodium tetraborate (conductivity: 1.1 μS/cm). Hydrodynamic injection could also be achieved by raising the sample reservoir for a period of 90 s (54 mm in length). After completion of the injection, upon application of the voltage, a proportionally greater field will develop across the sample zone causing the ions to migrate faster, i.e. the so call

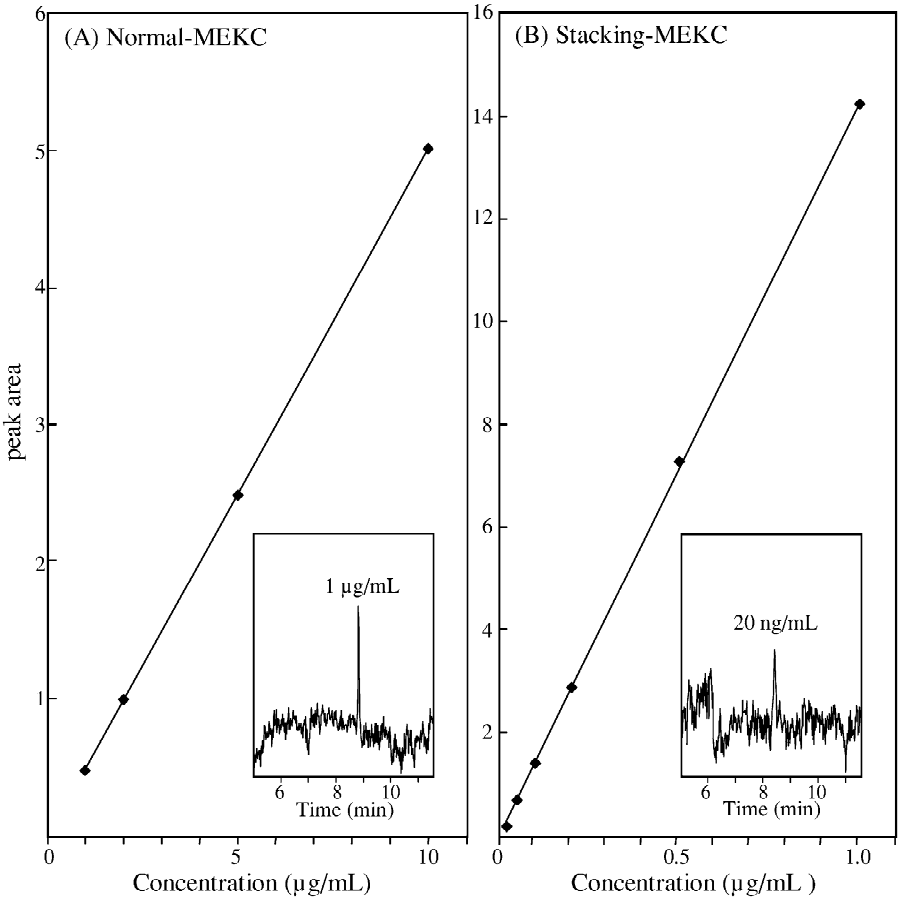

Fig. 3. Calibration graphs of riboflavin at concentrations between 1 and 10 μg/ml in normal-MEKC mode (A) and 0.02–1.0 μg/ml in stacking-MEKC mode (B). The insets show electropherograms at lowest concentrations of 1.0 and 0.02 μg/ml, respectively.

''stacking''. Once the ions reach the running buffer boundary, the field decreases and migration becomes slower. As a result, a linear relationship exists in the 0.02–1.0 μg/ml ranges as a result of the stacking technique, in which a 3:1 signal-to-noise ratio (as shown in the inset) was found at a level of ~20 ng/ml. The linearity of the method for riboflavin was also fairly good, and can be described by the equation ($y = 14.225x + 0.039$, $r^2 = 0.9999$). This is different from ''sweeping'' which involves an accumulation of analyte molecules by the pseudo-stationary phase that penetrates the sample zone. In this study, however, we found that the SDS micelle did not carry riboflavin well. When the injection length was longer than 20 mm, only a broad peak appeared and, for this reason the method of sweeping is not discussed. Based on Fig. 3A and B, for LODs for a 3-s (normal separation) and 90-s (stacking mode) are 0.48 μg/ml and 20 ng/ml, respectively. This makes sense, since a maximum of a 30-fold increase in sensitivity is possible using a 30-fold (3 versus 90 s) increase in sample volume assuming the same peak sharpness. Since sample stacking of large volumes of sample usually leads to broader peaks than that obtained for conventional injections, the increase in sensitivity would be expected for less than 30-fold.

In order to investigate the effects of injection length when the stacking technique was used, under exactly the same experimental conditions, 5, 9, 18,

36, 54, 89, and 143 mm column lengths (in times: 9, 15, 30, 60, 90, 150 and 240 s) of sample solution were injected into the capillary. Fig. 4A shows the relationship between the injection length and the related fluorescence intensity (peak area) in stacking-MEKC mode. Fig. 4B shows the relationship between injection length and peak width (cm) at detector [w_{det} (cm) = w_{det} (min) · L_d (cm)/t_{mig} (min)]. Although the fluorescence intensity increased linearly for longer injection lengths, the peak width at the detector also increased. Thus, this volume of 50 mm injection provides the best compromise between the two parameters which behave oppositely. For this reason, a 50-mm injection time was used. Furthermore, in order to investigate the effects of surfactants, different surfactants such as CTAB, DOSS and SC were also tested, but resulted in a poor sensitivity of the stacking of riboflavin. The optimum concentration of SDS was 80 m*M*.

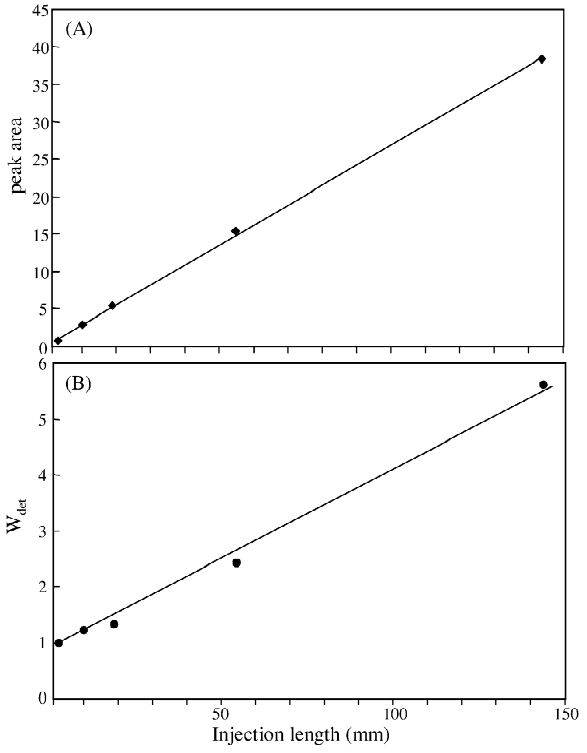

Fig. 4. Relationship between sample injection length and related fluorescence intensity (A) and peak width (cm) at detector (B) using the stacking-MEKC mode.

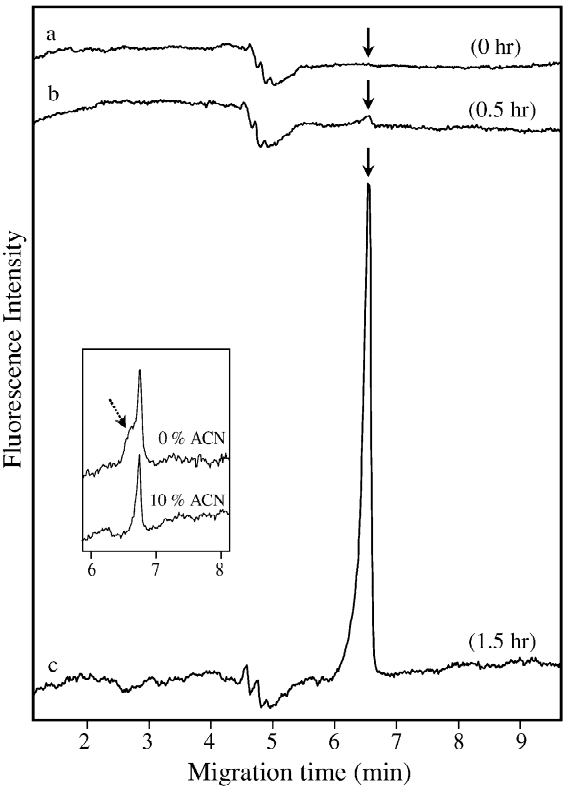

Fig. 5. CE electropherograms of urine samples from an adult volunteer after ingestion of a vitamin B_2 tablet for 0 (electropherogram a), 0.5 h (electropherogram b) and 1.5 h (electropherogram c), respectively. The CE conditions: 10 m*M* sodium tetraborate and 80 m*M* of SDS in water. The urine samples were used directly only after centrifugation and filtration. Applied voltage: 18 kV (~35 μA); light source: blue LED (~467 nm).

3.2. Analysis of riboflavin in urine samples

In Fig. 5, the electropherograms: (a) before dosing of a vitamin B_2 tablet, (b) after ingestion of a vitamin B_2 tablet for 0.5 h, and (c) after ingestion of a vitamin B_2 tablet for 1.5 h, show typical CE electropherograms of urine samples obtained from an adult volunteer (weight: 72 kg). Herein, the vitamin B_2 tablet was 0.4 g in weight and contained 10 mg of riboflavin. The CE buffer was a water–acetonitrile (9:1, v/v) solution, containing 10 m*M* sodium tetraborate and 80 m*M* of SDS (pH 9.25; conductivity: 3.5 mS/cm). The 10% acetonitrile is necessary for the separation of components in urine samples because it prevents overlap by unknown

compounds, as shown in the inset (broken arrow). Herein, the urine samples were used directly after centrifugation and filtering and, instead of sample stacking, a shorter sample injection (~20 mm) was applied. The reason for this is that the conductivities of the urine sample were high (10–30 mS/cm, depending on body conditions), the application of stacking is difficult unless a pretreatment is used to reduce the conductivity. As a result, only a few native fluorescent compounds are present which fluoresce in the orange wavelength (longer than 530 nm) range when excited at ~467 nm. We assigned these peaks (arrows in electropherograms b and c) to riboflavin and their concentrations were determined to be 0.16 and 9.4 μg/ml, respectively, after being ingested at 0.5 and 1.5 h. In order to investigate the distribution of riboflavin in urine, a volunteer lived a normal life without any food and water restricted, we sampled the urine every 0.5-h intervals. Fig. 6 shows the distribution of riboflavin in urine during the 9 h of monitoring. Each data point (for 19 urine samples) represents the average of three measurements. The coefficient of variance (C.V.) values of these points ranged from 1.8 to 6.2. It is clear that the concentration of riboflavin decreased approximately exponentially. The riboflavin passed through the body quickly and was excreted within ~3.5 h. By applying this approach, if the relationship between the drugs and their concentration in urine and various dosing times can be determined, it would be of use in determining the quantification of drugs ingested, after the passage of a period of time. This would be of value in pharmaceutical research. Thus, the proposed method not only permits the accurate analysis of riboflavin in urine samples, but also can be applied to any compounds that can be excited at a wavelength range of ~467 nm, such as flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). On the other hand, when derivatization methods are use, for example NBD-H (4-hydrazino-7-nitro-2,1,3-benzoxadiazole; Ex. 470 nm, Em. 530–570 nm) for aldehydes and ketones [27] and DBD-pro-COCl (4-(2-chloroformyl-pyrrolidin-1-yl)-7-(*N*,*N*-dimethlyaminosulfonyl)-2,1,3-benzoxadiazole)) for alcohols [28], this method has potential for use in a numbers of applications.

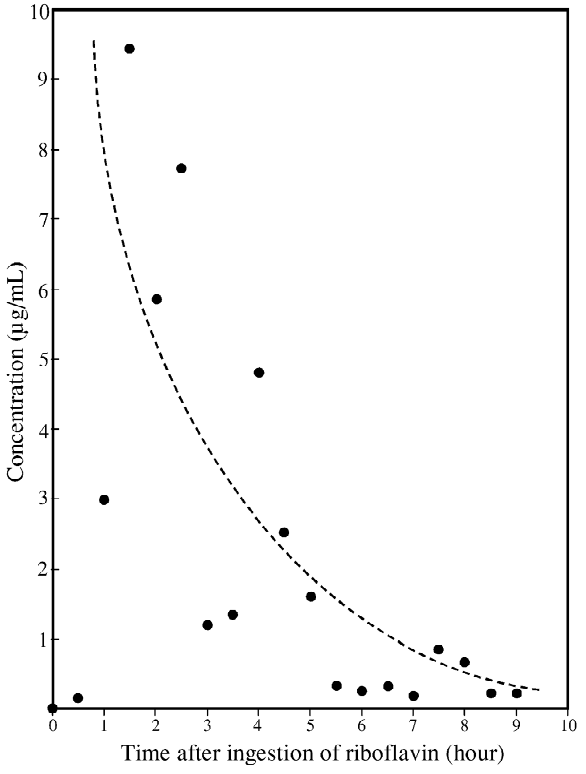

Fig. 6. Distribution of riboflavin in urine during 9 h of monitoring after the ingestion of one vitamin B_2 tablet (contains 10 mg of riboflavin).

Table 1 summarizes some analytical data relative to riboflavin in different samples and their detection methods. Each method has unique advantages and disadvantages with respect to sensitivity, precision and simplicity of use. Thus far, CE has become a popular technique and is a very useful method for the determination of drugs in body fluids because of its advantages in terms of speed, higher efficiency and resolution for separation, greater sensitivity and a smaller injection volume than is typically used for HPLC. This represents the first successful detection of riboflavin in urine by CE–blue LED-induced fluorescence detection in a miniaturized system.

4. Conclusions

Capillary electrophoresis–blue LED-induced fluorescence detection can be successfully used for the separation and identification of riboflavin in urine

Table 1
Analysis of riboflavin in different samples and detection methods used

Specimen	Method	LOD	Riboflavin	Ref.
Human urine	CE–LED	54 ng/ml	9.4–0.2 μg/ml	This study[a]
Multivitamin capsule	Voltammetry	–	~10 mg/240 mg of capsule powder	[20]
Plasma	HPLC–fluorometry	2.9 n*M*	~260 nM	[21]
Beer	HPLC–fluorometry	0.49 μg/l		[22]
Heineken			291.7 μg/l	
Forst Premium			235.8 μg/l	
Moretti			169 μg/l	
Forst Sixtus			507.9 μg/l	
Fruit juices	HPLC–fluorometry	0.49 μg/l		[22]
Pineapple			68.3 μg/l	
Orange			21.7 μg/l	
Grapefruit			39.2 μg/l	
Sausage	HPLC–fluorometry	0.015 mg/100 g		[23]
Sicilian mortadella			~0.118 mg/100 g	
Vitamined chopped			~0.664 mg/100 g	
Mushrooms	HPLC–fluorometry	0.0086 μg/ml	~6.24 μg/g	[24]
Milk	HPLC–fluorometry	1 ng/ml		[25]
Sour milk			156.0 μg/100 g	
Buttermilk			158.1 μg/100 g	
Acidophilus milk			167.8 μg/100 g	
Egg	HPLC–fluorometry	1 ng/ml		[25]
Raw egg white			326.6 μg/100 g	
Raw egg yolk			295.4 μg/100 g	
Sweet wort and beer	Fluorometric titration	~10 n*M*		[26]
Sweet wort			~1.4 μ*M*	
Unhopped beer			~0.81 μ*M*	
Hopped beer			~0.80 μ*M*	

[a] One vitamin B_2 tablet (10 mg of riboflavin) was given to a volunteer in the early morning. All urine was collected over a period of 9 h at 30-min intervals.

samples. This method is a sensitive, accurate, rapid, simple, reproducible and economic technique. Although the beam quality of LED is still not superior to that of a laser, the use of a combination of a stacking or sweeping technique in conjunction with this method can clearly lead to further potential uses in the future.

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