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Talanta 64 (2004) 970–974
Talanta
www.elsevier.com/locate/talanta

Analysis of riboflavin in beer by capillary electrophoresis/blue light emitting diode (LED)-induced fluorescence detection combined with a dynamic pH junction technique

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Received 27 February 2004; received in revised form 10 April 2004; accepted 15 April 2004
Available online 2 June 2004

Abstract

A simple, inexpensive and reliable method for the routine analysis of riboflavin in beer by capillary electrophoresis–light emitting diode (CE–LED) induced fluorescence detection is described. A simple and straightforward sample preparation is involved and the method is based on an inexpensive blue LED as the light source combined with an on-line sample concentration technique. For this detection system, using a normal micellar electrokinetic chromatography (MEKC), stacking-MEKC and dynamic pH junction techniques, the detection limits were found to be 480, 20 and 1 ng mL^{-1}, respectively. In addition, the number of theoretical plates for riboflavin was determined to be 3.8×10^4 by means of a dynamic pH junction and this was improved to 3.2×10^6 when the dynamic pH junction-sweeping mode was applied. The concentrations of riboflavin in 12 samples of different types of commercial beer were found to be in the range of 130–280 ng mL^{-1}.

Keywords: Riboflavin; Capillary electrophoresis; Blue light emitting diode (LED); Dynamic pH junction; Dynamic pH junction-sweeping

1. Introduction

Light-emitting-diodes (LEDs), developed since the 1960s, constitute an exceptionally stable light source and ultra-high intensity LEDs at a variety of wavelengths in the visible spectrum have became commercially available in mid-1990s and are currently in use in many fields. Such LEDs can be operated with battery power, so that the stability of the output is significantly better than that of currently used lasers, such as the He–Cd (325/442 nm), argon ion (488/514.5 nm) and He–Ne (543.5 nm) lasers. Tong and Yeung were first reported on the application of an absorption detection system for a CE separation based on red LEDs [1]; red and green LEDs were also applied to CE and microchip separations by Collins and Yu [2,3]. Although the beam quality (beamwidth, intensity, coherence and monochromaticity) of a LED is still not superior to that of a laser, our earlier studies have shown that the use of a combination of on-line sample concentration techniques in conjunction with CE–LED induced fluorescence detection can overcome this drawback, clearly indicating further potential uses [4]. Moreover, traditional chromatographic methods, such as HPLC, GC or SFC, require complicated steps to prepare and pre-concentrate (liquid–liquid or solid-phase extraction) the analytes. Such procedures are usually time consuming. In CE separation, an open column can be used and this makes carrying out an on-line sample concentration a simple task. Recently, a series of reports on sample concentration techniques in CE separations appeared, concerning the so called "stacking" and "sweeping" techniques [5–9]. Such techniques doubtless have opened a new field for the investigation of compounds that are present at low levels, even in the parts per billion (ppb) range. A dynamic pH junction method represents one of the on-line sample concentration techniques [10–13]. The principal mechanism of this takes advantage of velocity-difference induced focusing, in which an analyte migrates differentially within two distinct segments of the background electrolyte, resulting in the compression of the

Abbreviations: LED, light emitting diode; CE, capillary electrophoresis; SDS, sodium dodecyl sulfate

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doi:10.1016/j.talanta.2004.04.011

analyte into a narrow zone prior to its reaching the detector [12].

We previously demonstrated the utility of a blue LED for the detection of riboflavin in urine [4]. However, in the stacking-micellar electrokinetic chromatography (MEKC) method, it is necessary to dissolve the sample in a low-conductivity medium in order to concentrate the analyte through the amplified electric field distributed in the sample zone. This is difficult for actual samples, such as urine or blood samples, if the conductivity is too high. This also makes a stacking technique difficult because the conductivity is difficult to control. In this study, we report on a simple and highly sensitive method using an inexpensive blue LED source (instead of a laser) in conjunction with pH junction techniques for the detection of riboflavin in beer. Several electrophoretic parameters such as buffer pH, SDS and sodium tetraborate concentration, and the sample injection length required for the separation were optimized and these data are reported herein.

2. Materials and methods

2.1. Reagents

Riboflavin and sodium phosphate were acquired from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) and sodium tetraborate were obtained from Acros (Belgium). Phosphoric acid was obtained from Shimakyu's pure chemicals (Osaka, Japan).

2.2. Sample preparation method

The beer samples were purchased directly from markets in Taipei. Aliquots of beer samples were filtered through a 0.45 μm filter prior to use. The pH of the beer was ~3.9 and was adjusted the pH to 5.8 (to obtain the most strong fluorescence intensity) by adding 250 mM sodium phosphate prior to the analysis.

2.3. CE apparatus

The CE set-up was fabricated in-house and is identical to that described previously [4]. 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. (total length/effective length: 64/59 cm) fused silica capillary (J&W Scientific, CA, USA) was used for the separation. The sample was hydrodynamiclly injected by raising the reservoir 30 cm relative to the exit reservoir (at this height, the flow rate for the sample injection was 0.6 mm s^{-1}) to provide the injection length (depending on the specific situations). A blue LED (Part No. SLR-05PNW40-030X46DA, Sharlight Electronics Co. Ltd.; applied voltage, +4 V; price, US$ <1.5) with a luminous intensity of 6000 mcd (operating current: 20 mA; viewing angle: $2\theta_{1/2} = 15^{\circ}$; peak emission wavelength: 467 nm; spectral half width: 30 nm) was purchased on the Taipei electronic market. A microscope objective (40×) was used for focusing on the capillary. Fluorescence emission was collected by means of a microscope lens (10×), passed through an orange–red cut filter and a slit (0.3 mm), focused by a second microscope lens (10×), and then detected by a photomultiplier tube (Hamamatsu-R928). The analog signal was converted to a digital signal by an A/D converter (ADAM-4012 module, Advantech Co., Ltd., Taiwan). Electropherograms were collected at a speed of 1 point s^{-1} with a data acquisition system connected to a personal computer.

3. Results and discussion

3.1. Separation conditions and calibration curve

Fig. 1 (frames, A and B) shows typical CE electropherograms of a riboflavin standard (100 ppb) separated by the dynamic pH junction and the dynamic pH junction-sweeping

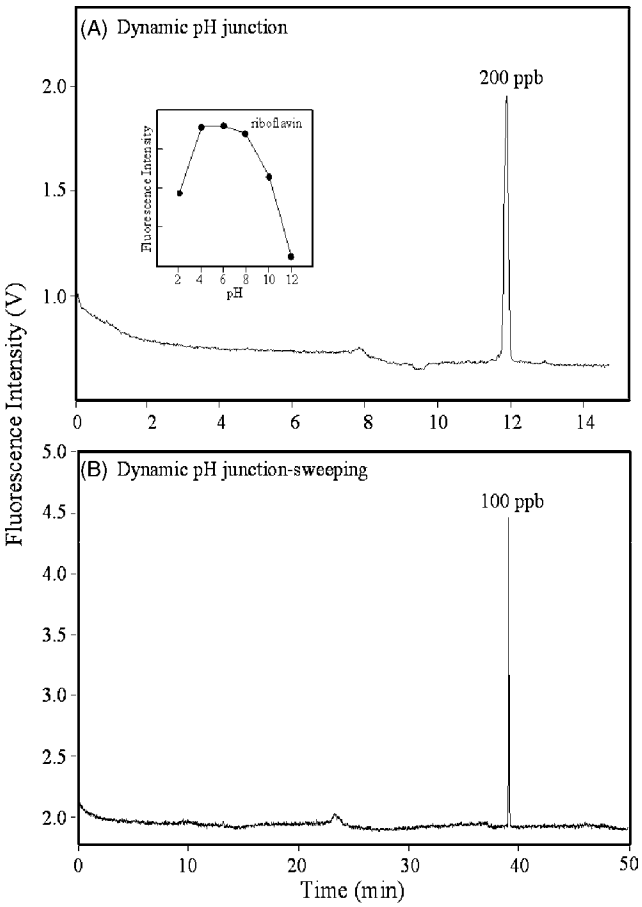

Fig. 1. CE electropherograms of a riboflavin standard separated by: (A) the dynamic pH junction mode and (B) the dynamic pH junction-sweeping. The inset shows the fluorescence intensity of riboflavin in different pH buffers (pH values, 2–12). CE conditions are summarized in Table 1.

Table 1
Limit of detection (LOD) values, linearity of peak area, coefficient of determination and plate numbers for riboflavin by MEKC, stacking-MEKC, dynamic pH junction mode and dynamic pH junction-sweeping methods

Method	Equation of line coefficient of variation	Concentration range	LOD (S/N = 3) (ppb)	Plate number
MEKC	$y = 0.501x + 0.0058$, $r^2 = 1$	1.0–10.0 ppm	480	$(1.0–1.8) \times 10^5$
Stacking-MEKC	$y = 14.225x + 0.039$, $r^2 = 0.9999$	20–1000 ppb	20	$(2.9–3.9) \times 10^4$
Dynamic pH junction	$y = 0.1002x + 0.1623$, $r^2 = 0.9999$	6–500 ppb	1	$(3.7–4.0) \times 10^4$
Dynamic pH junction-sweeping	–	–	2	3.2×10^6
CE condition		Capillary		
MEKC	SDS: 80 mM; $Na_2B_4O_7$: 10 mM; conductivity: 4.25 mS cm^{-1}	Total/effective length = 60/55 cm; injection length: 1.8 mm		
Stacking-MEKC	Background solution: SDS, 80 mM; $Na_2B_4O_7$, 10 mM; conductivity: 4.25 mS cm^{-1}; sample matrix: $Na_2B_4O_7$: 0.05 mM; conductivity: 1.1 μS cm^{-1}	Total/effective length = 71/65 cm; injection length: 54 mm		
Dynamic pH junction	Background solution: $Na_2B_4O_7$: 100 mM; pH: 8.2 (adjusted by H_3PO_4); sample matrix: NaH_2PO_4: 80 mM; pH: 5.9	Total/effective length = 64/59 cm; injection length: 106 mm		
Dynamic pH junction-sweeping	Background solution: $Na_2B_4O_7$: 100 mM; SDS: 100 mM; pH: 8.5 (adjusted by H_3PO_4); sample matrix: Na_2HPO_4: 75 mM; pH: 6.0	Total/effective length = 65/59 cm; injection length: 67 mm		

modes, respectively. The inset shows the fluorescence intensity of riboflavin at different pH buffers (pH values, 2–12). It is obvious that riboflavin provides a stronger fluorescence intensity when the pH of buffer was adjusted at 6 ± 2. In frame A, the complete, optimal separation of riboflavin when the dynamic pH junction mode was applied was achieved using a sodium tetraborate buffer (100 mM); pH, 8.2 (adjusted by adding H_3PO_4). The riboflavin standard (100 ppb) was prepared in 80 mM sodium phosphate (pH 5.9). In the initial step, the capillary was filled with the background electrolyte buffer and the sample solution was then injected to a certain length (3.5–24.7 cm, in this work). Following this, the sample inlet was removed, the inlet of the background electrolyte buffer was reloaded and a high voltage was applied to drive the electrophoresis. The pK_as' of riboflavin are 1.7 (pK_{a1}) and 9.69 (pK_{a2}). In acidic solution (75 mM phosphate, pH 6.0), riboflavin is a neutral species [12], but it acquires a negative charge if the solution is alkaline. Because of this property of riboflavin, when a high voltage (+11 kV) was applied, the current changed from 200 to 300 μA (within ~12 min), resulting a discontinuous electrolyte zone. Table 1 summarizes the linear relationship of the calibration curve ($y = 0.1002x + 0.1623$, $r^2 = 0.9999$) in the 6–500 ppb range, in which a 3:1 signal-to-noise ratio was found for a concentration of ~1 ng mL^{-1}. In order to investigate the effects of the injection length of the sample zone, under exactly the same experimental conditions, 3.5, 7.0, 10.6, 17.7, and 24.7 cm column lengths of sample solution were injected into the capillary. It was obvious that when the injection length was longer than ~10.6 cm (up to 18% of the total capillary length) the peak became broader. Thus, we conclude that the optimum conditions for the analysis of riboflavin are 100 mM sodium tetraborate for the background buffer (pH 8.2), a 10.6 cm column length for sample (pH 5.9) injection and +11 kV for driving the electrophoresis. These conditions were applied to the analysis of riboflavin in samples of various beers, as described below.

The dynamic pH junction-sweeping method, was first proposed by Terabe and co-workers [12], is similar to the dynamic pH junction method but additional surfactant (such as SDS in this study) is added to the background buffer. As a result, improved band focusing could be achieved. In frame B, the complete, optimal separation of riboflavin was achieved using a sodium tetraborate buffer (100 mM) containing 100 mM SDS; pH 8.5 (adjusted by adding H_3PO_4). The riboflavin standard (100 ppb) was also prepared in 75 mM Na_2HPO_4 (pH 6.0, adjusted by NaH_2PO_4). Compared to the peaks obtained by dynamic pH junction and dynamic pH junction-sweeping modes, an improvement in plate number of two orders of magnitude was obtained (3.8×10^4 was improved to 3.2×10^6). Table 2 summarizes the results of the relationship between injection length and peak width at the detector to quantitate riboflavin focusing using stacking-MEKC, dynamic pH junction and dynamic pH junction-sweeping modes, respectively. As a result, the dynamic pH junction-sweeping mode provided a sharper peak than the others. However, in this mode the migration time for separation is much longer than that of the dynamic pH junction. For this reason, we selected the dynamic pH junction mode for the following experiments.

Table 2
Bandwidth values to quantitate riboflavin focusing using stacking-MEKC, dynamic pH junction and dynamic pH junction-sweeping modes

Method	Injection bandwidth W_{inj} (mm)	Detected bandwidth W_{det} (mm)	W_{det}/W_{inj}	Sample buffer conditions
MEKC	1	10.3	10.3	–
Stacking-MEKC	54	23.0	0.43	50 μM $Na_2B_4O_7$, pH 8.9
Dynamic pH junction	106	16.2	0.15	80 mM NaH_2PO_4, pH 5.9
Dynamic pH junction-sweeping	67	3.5	0.05	75 mM Na_2HPO_4, pH 6.0

3.2. Analysis of riboflavin in beers

The vitamin riboflavin is found in beer, milk, mushrooms, sausage and other foods [14–17]. It serves as an enzyme cofactor in the vitalization of oxygen and the metabolism of amino acids, fatty acids, and carbohydrates. A shortage of this vitamin may manifest itself as cracks and sores at the corners of the mouth, eye disorders, inflammation of the mouth and tongue, and skin lesions. The concentrations of riboflavin in multivitamin capsules (∼10 mg/240 mg of capsule power) [18], Heineken beer (291.7 ng mL^{-1}) [14] and sour milk (156 μg/100 g) [15] have been reported. It is a naturally fluorescent compound (yellow–green at room-light) and is present at low levels in fluid samples, such as plasma, urine, wines and beers. Efforts have been made to increase the sensitivity of its detection including the use of HPLC–fluorometry (LOD: ∼1.1 ng mL^{-1}) [19] and MEKC–UV (LOD: ∼0.68 μg mL^{-1}) [20]. Some relevant CE-LIF methods for flavin analysis have also been reported in the literature [12,21–24]. Cataldi et al. reported on a calibration curve for riboflavin obtained by CZE–LIF (0.5 μg mL^{-1}), in which a 20 mW He–Cd laser (442 nm) was used [21]. Terabe and co-workers also reported on the picomolar delectability of a model mixture of flavins using the dynamic pH junction-sweeping method in conjunction with a 4 mW argon ion laser (488 nm) [12]. We also report herein on the content of riboflavin in beers by our CE–LED induced fluorescence detection system based on a dynamic pH junction technique. In Fig. 2, electropherograms a and b show typical CE electropherograms of Taiwan Beer and the same beer after spiking with 200 ng mL^{-1} of riboflavin, respectively. The CE conditions were the same as described above. A diluted, pH adjusted sample (pH 5.8) was used directly without any further pre-treatment. A comparison of electropherograms a and b, indicate that the peak (arrow) indeed increased. A few other native fluorescent compounds are also present which fluoresce in the orange–red wavelength (longer than 555 nm) range when excited at 467 nm. The qualitative and quantitative analysis of riboflavin in beer could be achieved by a comparison of the migration time and the peak area. We assigned this peak (arrow in electropherogram a) to riboflavin and its concentration was found to be 194.3 ± 4.4 ng mL^{-1} ($n = 3$). By applying this approach, 12 commercial beers were investigated and the results are summarized in Table 3. The method of calibration line was used for the quantitative determination. All of these beers were found to contain riboflavin at levels in the range of 130–280 ng mL^{-1}. To date, CE has proven to be a popular and 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 beer by CE/blue LED-induced fluorescence detection in a miniaturized system.

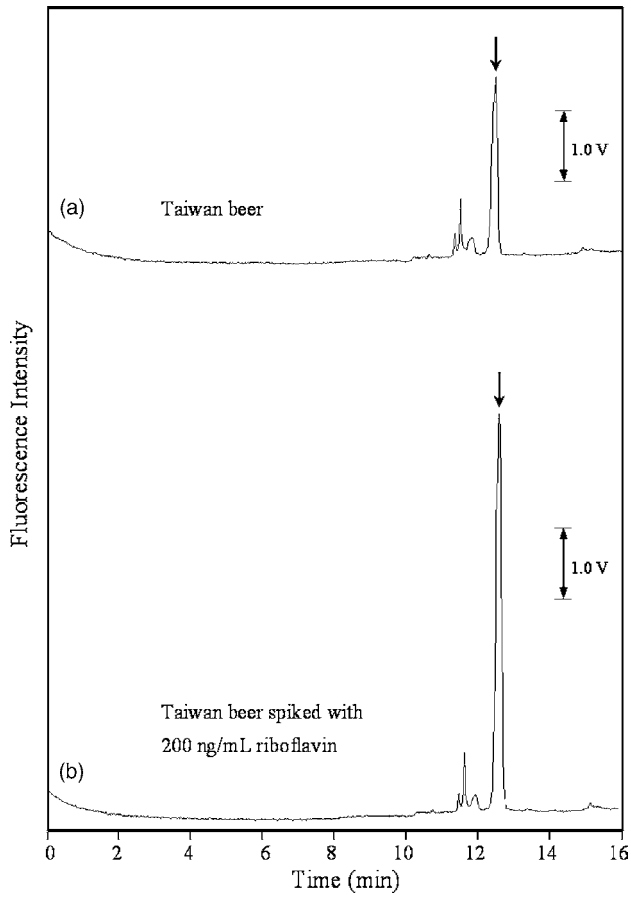

Fig. 2. CE electropherograms of Taiwan Beer (electropherogram a) and the same beer after spiking with 200 ng mL^{-1} riboflavin (electropherogram b). CE conditions: 100 mM sodium tetraborate for the background buffer (pH 8.2); 10.6 cm for the beer sample (pH 5.8) injection. The pH of the beer sample was adjusted to 5.8 and used directly without any further pre-treatment. Applied voltage: 11 kV (200–300 μA); light source: blue LED (467 ± 15 nm).

Table 3
Concentration of riboflavin in 12 commercial beers (2003)

Commodity (producing district)	Riboflavin (ng mL^{-1} ± S.D.)	CV (%); $n = 3$
Taiwan Beer (Taiwan)	194.3 ± 4.4	2.24
Kirin-Bar Beer (Japan)	132.6 ± 2.2	1.64
Heineken (the Netherlands)	282.4 ± 4.9	1.73
Kirin-Ichiban (Japan)	197.1 ± 3.1	1.55
Asahi Draft Beer-Karakuchi (Japan)	137.9 ± 1.5	1.11
Tsingtao Beer (China)	163.0 ± 1.4	0.86
Miller-High Life (USA)	243.0 ± 2.6	1.06
Yanjing Beer (China)	230.0 ± 3.8	1.81
Sapporo Draft (Japan)	225.2 ± 4.0	1.77
Miller-Premium Beer (USA)	166.4 ± 2.7	1.64
Busch Beer (USA)	154.4 ± 3.3	2.15
Essential Beer (Korea)	172.7 ± 1.9	1.09

4. Conclusions

Capillary electrophoresis/blue LED induced fluorescence detection can be successfully used for the separation and identification of riboflavin in beer samples. The optimum conditions for the analysis of riboflavin are 100 mM sodium tetraborate for the background buffer (pH 8.2) and 10.6 cm for sample (pH 5.9) injection. The samples were pH adjusted by adding sodium phosphate to ∼5.8 and used directly without any further pre-treatment. This method is a sensitive, accurate, rapid, simple, reproducible and economic technique. The use of a combination of on-line sample concentration techniques in conjunction with this method can clearly lead to further potential uses in the future.

Acknowledgements

This work was supported by a grant from the National Science Council of Taiwan under Contract no. NSC-90-2113-M-003-020.

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