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UV Light-Emitting Diode-Induced Fluorescence Detection Combined with Online Sample Concentration Techniques for Capillary Electrophoresis

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The application of an ultraviolet (UV) light-emitting diode (LED) to on-line sample concentration/fluorescence detection in capillary electrophoresis (CE) is described. The utility of a UV-LED (peak emission wavelength at 380 nm, ~2 mW) for fluorescence detection was demonstrated by examining both a naturally fluorescent (riboflavin) compound and a non-fluorescent compound (tryptophan), respectively. The detection limit for riboflavin was determined to be 0.2 ppm by the normal MEKC mode, which was improved to 3 – 7 ppb when dynamic pH-junction technique was applied. On the other hand, the detection limit of the tryptophan derivative was determined to be 1.5 ppm using the MEKC mode, which was improved to 3 ppb when the sweeping-MEKC mode was applied. In an analysis of an actual sample, the concentrations of riboflavin in beer, and tryptophan in urine and milk samples were determined, respectively.

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Introduction

The most common fluorescence excitation source in current use in capillary electrophoresis (CE) is lasers, involving the so-called laser-induced fluorescence (LIF), which leads to a remarkable improvement in the sensitivity of detection compared to the use of a conventional incoherent light source. However, typical lasers used in CE, such as the He-Cd (325/442 nm), argon ion (351/488/514.5 nm) and He-Ne (543.5 nm) lasers are generally expensive, relatively bulky and have short comparatively lifetimes. In contrast, diode lasers are much less costly, compact, have good output stability, longer lifetimes and require little or no maintenance. Thus far, applications of diode laser-induced fluorescence detection in CE have been reported for wavelengths in the near-IR (785/780 nm),[1,2] red (635/670 nm),[3-5] green (532 nm) and InGaN-based violet (405 nm) regions.[6-9] Even though, only a few suitable labeling dyes are excited in these specific regions for all of the previously used diode lasers.[10]

Light-emitting diodes (LEDs), developed since the 1960s, are exceptionally stable and intensive light sources. LEDs emitting at a variety of wavelengths have become commercially available since the mid-1990s. Yeung *et al*. were the first to report on the application of an absorption detection system for a CE separation based on red LEDs.[11] Red and green LEDs were also subsequently applied to CE and microchip separations by Collins *et al*.[12,13] Macka *et al*. also reported on an indirect detection technique for inorganic anions using a chromate background electrolyte involving UV-LED (emission maximum at 379.5 nm).[14] Hillebrand *et al*. reported on the application of a pulsed (500 Hz) UV-LED (370 nm; $\Delta\lambda$ = 12 nm) for the detection of fluorescence-derivatized bradykinin and lysine at the femtomole level.[15] Although the UV-LED (375 nm, 2 mW; NSHU550A) is commercially available, the Nichia Corporation (http://www.nichia.co.jp) reported on the successful development of high-output ultraviolet LEDs of 380 nm (85 mW; Model, SIRIUS) and 365 nm (100 mW; Model, i-LED), respectively. More recently, Dupuis *et al*. reported on an AlGaN/AlGaN quantumwell UV-LED and a ternary AlGaN UV-LED grown on sapphire, with peak emission wavelengths at 341 nm and 302 nm, respectively, with a narrow line width of $\Delta\lambda$ = 10 nm that can be used at room-temperature.[16] Given the pace of development in this area, it is possible that an UV-LED may replace some of the currently used UV CW-lasers, such as the He-Cd laser (325 nm) and the argon ion laser (351 or 257 nm), because a beam quality (narrow beam-width, high intensity, good coherence and monochromaticity) of a laser is not absolutely necessary, if it is used only for fluorescence excitation. At this point, a laser could be replaced by a LED as a light source for fluorescence detection if on-line sample concentration techniques (such as the so-called "stacking" and "sweeping" techniques[17-26]) can be applied.

We previously demonstrated the utility of violet (410 nm) and blue (476 nm) LEDs for fluorescence detection by examining a naturally fluorescent compound and a non-fluorescent compound, respectively.[27-29] In this study, riboflavin (naturally fluorescent) and tryptophan (non-fluorescent) were selected as model compounds. The use of a UV-LED in conjunction with on-line sample concentration techniques for the detection of these two analytes as well as their presence in actual samples is described. Several electrophoretic parameters, such as the buffer pH, SDS concentration, and the injection length required for the separation, were optimized; these data are reported herein.

Experimental

Apparatus

The CE set-up (Fig. 1) was fabricated in-house and is similar to a previously described unit.[28] A high-voltage power supply

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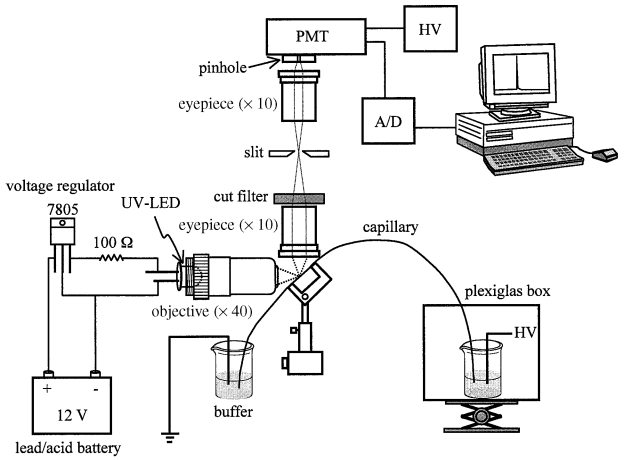

Fig. 1 CE/LIF system. Schematic of a LED-based capillary electrophoresis system.

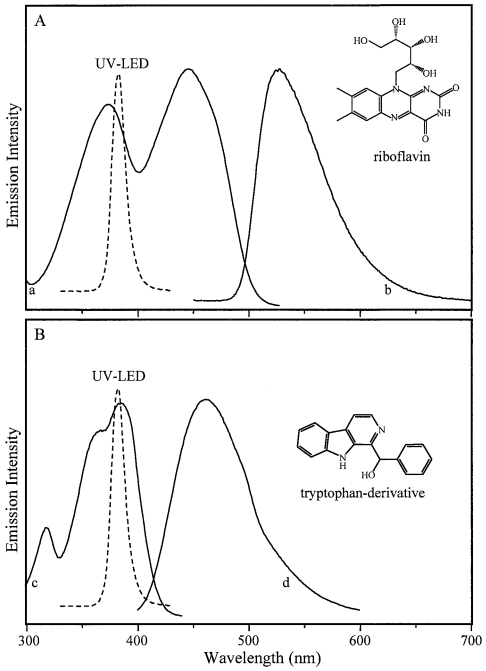

Fig. 2 Excitation and fluorescence spectra. Frames A and B are typical excitation (spectrum a) and fluorescence (spectrum b) spectra of riboflavin and tryptophan-derivative, respectively. The inset shows their molecular structures. The dashed lines show the emission region of the UV-LED.

(Model RR30-2R, 0 – 30 KV, 0 – 2 mA, Gamma, FL, USA) was used to drive the electrophoresis and a fused-silica capillary (J & W Scientific, CA, USA) was used for separation. The effective and total length of the capillary was 64 and 70 cm; 75 μm i.d.. The sample was hydrodynamically injected by raising the reservoir 46 cm relative to the exit reservoir (at this height, the flow rate for the sample injection was 0.91 mm/s) to provide the injection length (depending on the specific situations). A UV-LED (Model NSHU590A, Nichia Co., Ltd.) with a luminous intensity of 1.2 – 2.0 mW (forward voltage and current, 4.0 V/20 mA; peak emission wavelength, 380 nm; spectral half width, 14 nm) was purchased from Sander Electronic Co., Ltd. (http://www.sanderled.com.tw; Taipei, Taiwan). A lead/acid battery was used to drive the LED power *via* a connection with a voltage regulator (7805 device; a +5 voltage regulator) and a 100 Ω resistance. A microscope objective (×40) was used for focusing on the capillary. Fluorescence emission was collected by means of a microscope eyepiece (×10), passed through a long-pass cut filter (cut below 450 nm), and a slit (0.3 mm), focused by a lens (f = 2.5 cm), and then detected by a photomultiplier tube. The analog signal was converted to a digital signal by means of an A/D converter (ADAM-4012 module, Advantech Co., Ltd, Taiwan). Electropherograms were collected with a data-acquisition system connected to a personal computer.

Methodology: pH-junction

The background electrolyte buffer was an aqueous solution, containing 140 mM sodium tetraborate, the pH (8.5) of which was adjusted by the addition of H_3PO_4; riboflavin standards were prepared in 75 mM Na_2HPO_4 (pH = 6.0, adjusted with NaH_2PO_4). In the initial step, the capillary was filled with the background electrolyte buffer and the sample solution was then injected to a certain length (16 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. In an acidic solution, riboflavin (pK_a = 10.2) is almost a neutral species, but it acquires a negative charge if the solution is alkaline. Because of this property of riboflavin, when a high voltage is applied, a discontinuous electrolyte zone is created. Meanwhile, OH^- and $B(OH)_4^-$ ions move toward the sample zone, but the EOF migrates in the reverse direction (toward the background electrolyte zone). As a result, the analyte is focused on the boundary of the pH-junction.

Reagents

All chemicals used were of analytical grade. Sodium dihydrogen phosphate was purchased from Sigma (St. Louis, MO, USA). Riboflavin (vitamin B_2), phenylglyoxal hydrate (PGO-hydrate), tryptophan, SDS (sodium dodecyl sulfate), sodium tetraborate, hydroxypropyl-β-cyclodextrin (HP-β-CD) and sodium hydrogen phosphate were purchased from Acros (Geel, Belgium). Phosphoric acid was obtained from J. T. Baker (Phillipsburg, NJ, USA).

Sample preparation

A beer sample (Taiwan Beer) was purchased directly from a market in Taipei. Aliquots of beer samples were sonicated and filtered through a 0.45 μm filter prior to use for the detection of riboflavin. In the case of a urine sample for detecting tryptophan, urine was collected from 4 adult volunteers and was used for analysis. Each 1.0 mL aliquot of urine sample was first derivatized by a reaction with phenylglyoxal-hydrate (PGO).[30] The derivatization procedure is described below. The derivative was then diluted to 1/20 with the non-micelle buffer for a subsequent CE experiment. A milk sample (Kuang-Chuan Dairy Co., LTD.) was also obtained from a supermarket. A 1.0 mL aliquot of the milk sample was centrifuged, filtered, derivatized by a reaction with PGO, and then directly used without any further pretreatment.

Derivatization procedures

PGO: A 0.075-mL (80%, w/v) of H_3PO_4 solution was added to a 1.0-mL urine sample. A 0.036-g of PGO was dissolved in a 0.075 mL MeOH solvent. A mixture of the two above solutions-mentioned was heated in a hot water bath (100˚C) for 40 min.[30] After the reaction, the solution was centrifuged and

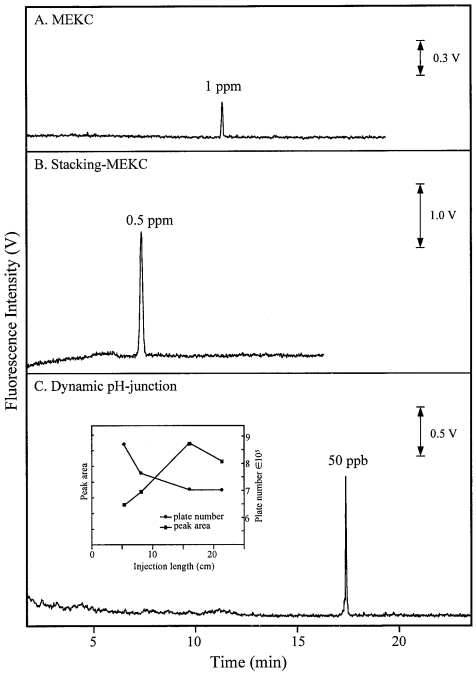

Fig. 3 Electropherograms of a riboflavin standard. A, MEKC; B, stacking-MEKC; C, dynamic pH-junction mode, by the UV LED. The applied voltage and current were 16 kV and ~50 μA, respectively. The inset in frame C shows the relationship between the injection length and the corresponding signal intensity and theoretical plate numbers when the dynamic pH-junction mode was applied.

filtered. The organic phase (0.5 mL) was collected, and used in subsequent CE separations.

NDA: To 1.0-mL of solution containing 0.7-mL of aqueous sodium tetraborate buffer (0.1 M, pH = 9) was added 0.1-mL of urine and the same volume of KCN (10^{-3} M in a tetraborate aqueous buffer).[32] After mixing, the reaction solution was allowed to stand at room temperature in the dark for 20 min. The derivative was directly used in subsequent CE separation.

Results and Discussion

Figure 2 shows the excitation (spectra a and c) and fluorescence (spectra b and d) spectra of riboflavin and tryptophan-derivative, respectively. It appears that the UV-LED source is particularly well matched for excitation. Their molecular structures are shown in the insets.

Application to fluorescent (riboflavin)

Comparison of MEKC, stacking-MEKC and dynamic pH-junction modes. In Fig. 3, frames A – C show typical CE electropherograms of the riboflavin standard (test concentrations: 1 ppm, 0.5 ppm and 50 ppb for frames A – C, respectively) when the MEKC, stacking-MEKC and dynamic pH-junction modes were applied, respectively. In the case of the MEKC mode, the complete, optimal CE conditions for riboflavin were achieved using a sodium tetraborate buffer (10 mM) containing SDS (60 mM); pH = 9.3. The intensity of the detected peak was only ~0.3 V, corresponding to a detection limit of 0.2 ppm ($S/N = 3$). In order to improve this, several on-line sample concentration techniques were examined. Figure 3B shows a typical electropherogram of a riboflavin standard (0.5 ppm) performed using the stacking-MEKC mode. Herein, the CE buffers were aqueous solutions, containing 10 mM sodium tetrabarate and 60 mM of SDS (pH = 9.3; conductivity = 8.0 mS/cm). The sample was prepared in 0.01 mM sodium tetraborate (conductivity = 16 μS/cm). Hydrodynamic injection could also be achieved by raising the sample reservoir to achieve a 70 mm (in length) sample injection. After completion of the injection, upon the application of a voltage, a proportionally greater field developed across the sample zone, causing the ions to migrate faster, *i.e.*, so-call "stacking". Once the ions reached the running buffer boundary, the field decreased and the migration became slower. As a result, a linear relationship existed in the 50 ppb – 1 ppm range because of the stacking technique, in which a 3:1 signal-to-noise ratio was found at a level of ~50 ppb. The linearity of the method for riboflavin was also fairly good. These data are summarized in Table 1, including the equation for the calibration curve, the coefficient of variation value and the limit of detection for the above experiments. Although the detection limit was improved, this method (stacking-MEKC) provides a broader peak than the MEKC mode. This problem can be resolved by applying the dynamic pH-junction mode, which can provide a shaper peak.[29] Figure 3C shows the result obtained when the dynamic pH-junction mode was used; the sample concentration was 50 ppb (peak intensity, 1.36 V). In order to investigate the effects of the injection length and the corresponding signal intensity when the dynamic pH-junction mode was used, under exactly the same experimental conditions, various (5.3, 8, 16 and 21.3 cm) injection lengths of the sample solution were injected into the capillary and the results were plotted, as shown in the inset. Basically, the signal intensity increased with increasing injection length. The inset shows the relationship between the sample injection length and the plate numbers. It is clear that, using either the injection length, plate numbers of $7 - 9 \times 10^5$ can be obtained. Furthermore, when the injection length was 16 cm (~1/4 of the effective length), a ~60-fold improvement was obtained.

Application of UV-LED: determination of riboflavin in beer. Riboflavin, a vitamin, is a constituent of beer, milk, mushrooms, sausage and other foods. It is a naturally fluorescent compound (yellow-green at ambient conditions) and is present at low levels in many fluids, such as plasma, urine, wine and beer. In Fig. 4, electropherograms a and b show typical electropherograms of a Taiwan Beer and the same beer after spiking with 0.25 ppm of riboflavin, respectively, using the dynamic pH-junction mode. The CE conditions were the same as described above. In this analysis, the initial pH of the beer was ~3.9 and was adjusted to 6.0 by adding sodium phosphate prior to the analysis. The pH-adjusted beer sample was used directly without any further pretreatment. A comparison of electropherograms a and b indicate that the intensity of the peak (arrow) clearly increased. A few other native fluorescent compounds were also present, which fluoresce when excited at ~380 nm. We assigned this peak (arrow in electropherogram b) to riboflavin, and its concentration was determined to be 0.24 ppm.

Application to derivative (phenylglyoxal-hydrate derivatized-tryptophan)

Comparison of MEKC and sweeping-MEKC modes. Although tryptophan is weakly fluorescent, for the detection of a weak- or non-fluorescent sample, a derivatization procedure is normally recommended. The derivatization procedure for tryptophan was modified from a literature description.[30,31] The resulting derivative was identified by LC/MS. Figure 5 shows typical electropherograms of the derivative (test concentrations: 10 and

Table 1 Limit of detection (LOD) values, coefficients of variation and plate numbers for riboflavin and tryptophan-derivative for various sample concentration techniques

A. Riboflavin

Method	MEKC		Stacking-MEKC		Dynamic pH-junction	
Injected length	3 mm		7 cm		16 cm	
Concentration range	50 – 0.25 ppm		1000 – 50 ppb		10 – 250 ppb	
Equation of the line	$y = 1.262x + 0.0339$		$y = 0.0215x - 0.2793$		$y = 0.0659x - 0.0834$	
Coefficient of variation	$R^2 = 0.9999$		$R^2 = 0.9995$		$R^2 = 0.9992$	
LOD ($S/N = 3$)	0.2 ppm		50 ppb		5 ppb	
RSD (%); $n \geqq 3$	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
(a) migration time	1.73%	4.22%	1.30%	1.56%	3.91%	1.77%
(b) peak area	2.24%	4.61%	2.30%	1.56%	4.4%	2.48%
Plate number (N)	6.3×10^4		1.2×10^4		6.8×10^5	

B. Tryptophan-derivative

Method	MEKC		Sweeping-MEKC	
Injected length	1 mm		40 cm	
Concentration range	5 – 110 ppm		5 – 230 ppb	
Equation of the line	$y = 0.5263x + 1.8113$		$y = 0.0622x + 0.047$	
Coefficient of variation	$R^2 = 0.9955$		$R^2 = 0.9998$	
LOD ($S/N = 3$)	1.5 ppm		3 ppb	
RSD (%); $n \geqq 3$	Intra-day	Inter-day	Intra-day	Inter-day
(a) migration time	0.84%	1.43%	0.34%	1.06%
(b) peak area	9.85%	9.31%	7.63%	5.92%
Plate number (N)	6.8×10^4		9×10^5	

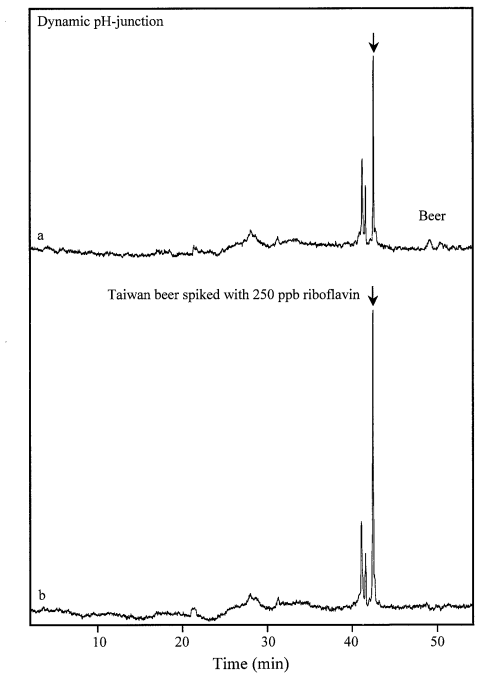

Fig. 4 Electropherograms of Taiwan Beer when the dynamic pH-junction technique was applied. Electropherogram: a, bear sample; b, the same beer sample after spiking with 0.25 ppm riboflavin. CE conditions: 140 mM sodium tetraborate for the background buffer (pH = 8.5); 7.8 cm for the beer sample injected. The pH of the beer sample was adjusted pH to 6.0 by adding Na_2HPO_4 and this solution was used directly without any further pretreatment. Applied voltage, +11 kV (200 – 300 μA); light source, UV-LED.

0.1 ppm for frames A and B, respectively) when the MEKC and sweeping-MEKC modes were applied, respectively. The inset in the Fig. 5A shows the mass fragmentation spectrum of the derivative. The presence of specific fragments permitted its characterization, and suggests that this derivative is identical with phenylglyoxal-hydrate derivatized-tryptophan, 1-(1-hydroxybenzyl)-β-carboline (β-HBC). In the MEKC mode, the CE buffer was a solution of water–acetonitrile (93:7, v/v), which contained 100 mM SDS and 30 mM phosphate buffer (pH, 1.9). The effective and total length of the capillary was 94/100 cm; 50 μm i.d. The sample (0.1 ppm) was dissolved in a non-micelle buffer (pH ~1.9); the sample injection length was 40 cm. Under these conditions, the intensity of the detected peak was improved to ~2.0 V, corresponding to a 3 ppb detection limit (S/N = 3). As a result, a ~500-fold improvement (electropherogram A, 10 ppm, 0.5 V; electropherogram B, 0.1 ppm, 4.0 V) was achieved. The detected peak was much sharper than that for the MEKC mode. In order to investigate the effects of the injection length and the corresponding signal intensity (voltage) when the sweeping-MEKC technique was used under exactly the same experimental conditions, various (25, 30, 35, 40 and 45 cm) column lengths of the sample solution were injected into the capillary, and the results were plotted, as shown in the inset of frame 5B. Basically, the signal intensity increased with increasing injection length. Furthermore, when the injection length was 40 cm (~2/5 of the effective length), a ~500-fold improvement was obtained. However, when the injection length was longer, the effective length became shorter. This might pose a problem for the separation of actual samples because of the complicated matrix. Using these results, calibration curves, the limit of detection, linearity and plate numbers were calculated, these data are summarized in Table 1.

Application of UV-LED: determination of tryptophan in urine and milk. Tryptophan is an essential amino acid in human nutrition. It is a neutral, genetically coded amino acid, that occurs naturally in food. In the brain, it is converted to 5-hydroxy-tryptophan (5-HTP), which in turn is converted to serotonin, which is a neurotransmitter essential for regulating appetite, sleep, mood and pain levels. In Fig. 6A,

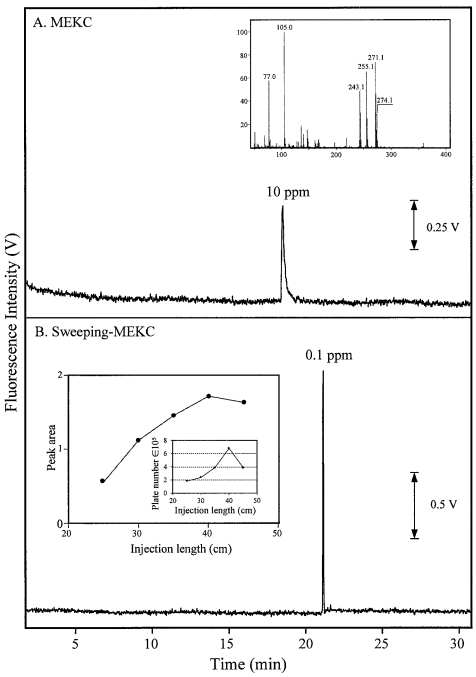

Fig. 5 Electropherograms of a tryptophan-derivative standard separated by the MEKC and sweeping-MEKC modes. Frames A and B show the results obtained from the MEKC and sweeping-MEKC modes, respectively, by the UV-LED. The inset in frame 5B shows the relationship between sample injection length and related signal intensity, and plate numbers for the sweeping mode.

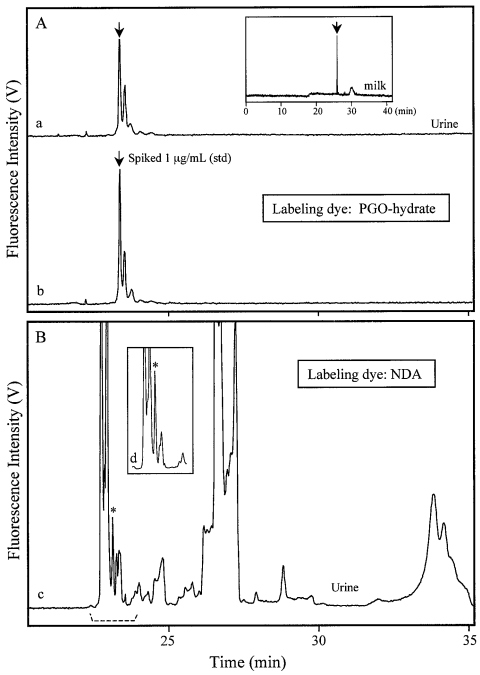

Fig. 6 Electropherograms of urine samples. A, the labeling dye was PGO-hydrate. CE electropherograms a and b, an adult urine-derivative sample (electropherogram a) and the same urine-derivative after spiking with 110 ppb of tryptophan-derivative standard (electropherogram b) when the sweeping-MEKC mode was applied. The inset in frame 6A shows a similar result obtained from a commercial milk sample. B, the labeling dye was NDA. CE conditions: solution, water-acetonitrile (93:7, v/v), which contained 30 mM of HP-β-CD, 100 mM of SDS and 30 mM phosphate buffer (pH ~1.9). Effective/total length of the capillary, 94/100 cm, 50 μm i.d. The urine sample was dissolved in a non-micelle buffer (pH ~1.9). The sample injection length was 25 cm. Applied voltage, –20 kV.

electropherograms a and b show typical sweeping-MEKC electropherograms of an urine-derivative sample from an adult volunteer, and the same urine-derivative after spiking with 0.1 ppm of the tryptophan-derivative, respectively. The CE conditions were the same as described above, but with additional HP-β-CD (30 mM) in the CE buffer. The present of HP-β-CD is important for completely separating the tryptophan-derivative from the other peaks. A comparison of electropherograms a and b indicate that the intensity of the peak (arrow) has clearly increased; a few other native fluorescent compounds were also present which fluoresce when excited by the UV-LED. We assigned this peak (arrow in electropherogram b) to tryptophan, and its concentration was determined to be 6.9 ppm. The inset shows a typical electropherogram of a milk-derivative sample. By comparing with a spiked electropherogram (data not shown), we assigned this peak (arrow in electropherogram b) to tryptophan, and its concentration was determined to be 1.5 ppm. This represents the successful detection of riboflavin in beer and tryptophan in urine and milk by CE/UV-LED-induced fluorescence detection in a miniaturized system. Thus, we conclude that this method provides sufficient sensitivity for the detection of tryptophan in either urine or milk samples. In general, a currently substance used for labeling with primary and secondary amines is NDA (naphthalene-2,3-dicarboxaldehyde), while PGO can react with tryptophan selectively. In order to realize a specific effect from the use of PGO-hydrate compared to that of NDA, the following experiment was carried out using another adult urine sample. The NDA derivatization procedure for amino acids was described above. Under the same CE conditions described in the caption (Fig. 6B), the results of the separation are shown in electropherogram c. It is clear that the detected peaks are complicated, compared to the use of PGO-hydrate. Electropherogram d in the inset of frame 6B shows the result after spiking. A comparison of electropherograms c and d indicate that the intensity of the peak (asterisk) has clearly increased. We assigned this peak (asterisk in electropherogram c) to tryptophan, and its concentration was determined to be 20.4 ppm. To date, CE has proven to be a popular and 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 the need for a smaller injection volume.

Conclusions

To successful apply UV-LED-induced fluorescence detection in CE is described. The results suggest that UV-LED has great potential for use as a new light source in CE separations, not only for naturally fluorescent compounds, but perhaps for derivatives as well. The method is a sensitive, accurate, rapid, simple, reproducible and economical technique. The use of a combination of on-line sample concentration techniques in conjunction with this method could clearly lead to further potential uses.

Acknowledgements

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