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Violet light emitting diode-induced fluorescence detection combined with on-line sample concentration techniques for use in capillary electrophoresis

The first application of a violet light-emitting diode (LED) for fluorescence detection in capillary electrophoresis (CE) is described. The utility of violet LED (peak emission wavelength at 410 nm, ~ 2 mW) for fluorescence detection is demonstrated by examining reserpine and dopamine-labeled NDA (naphthalene-2,3-dicarboxaldehyde), respectively. The detection limit for reserpine was determined to be 2.5×10^{-6} M by normal micellar electrokinetic capillary chromatography (MEKC) and this was improved to 2.0×10^{-9} M and 2.0×10^{-10} M when sweeping-MEKC and cation-selective exhaustive injection (CSEI)-sweep-MEKC techniques were applied, respectively. In addition, the detection limit of NDA-labeled dopamine was determined to be 6.3×10^{-6} M by means of normal MEKC and this was improved to 3.0×10^{-8} M when the sweeping-MEKC mode was applied.

Keywords: Capillary electrophoresis / Dopamine / Reserpine / Violet light-emitting diode
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1 Introduction

The use of laser-induced fluorescence (LIF) leads to a remarkable improvement in the sensitivity of detection compared to the use of a conventional incoherent light source. However, for analytes which are either naturally fluorescent or derivatized by reaction with a fluorescent label, it is necessary to select readily available lasers that can be matched to their spectral properties. Currently, conventional lasers, such as the He-Cd laser (325/442 nm), argon ion (488/514.5 nm) and He-Ne (543.5 nm), are generally expensive, relatively bulky and have short lifetimes (~3000 h); whereas diode lasers are much less costly, compact, have good output stability, longer lifetimes and require little or no maintenance. Thus far, applications of diode LIF detection in CE have been reported at available wavelengths such as 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]. However, only a few suitable labeling dyes are excited in these specific regions [10]. Lucy and Melanson [6] reported a detection limit of 10 nM for amino acids labeled with naphthalene-

2,3-dicarboxaldehyde (NDA) in which a 5 mW violet diode laser (405 nm) was used. This suggests that the violet diode laser might be ideally suited for LIF detection for molecules that can be labeled with NDA, such as amino acids or amines. The current cost of a violet diode laser is still expensive (US\$ ~2000, depending on the output power and lifetime), while the current price of diode lasers emitting in the IR and visible ranges are reasonably priced.

Light-emitting diodes (LEDs), developed since the 1960s, constitute an exceptionally stable light source and ultra-high intensity LEDs at a variety of wavelengths (blue-blue green-green-yellow-orange-super red-bright red) have become commercially available in mid-1990s and are currently in use in many fields. Furthermore, LEDs can be operated with battery power, so that the output stability is significantly better. This property also suggests that the LEDs would be ideally suited for absorption detection. Yeung and Tong [11] first reported on the application of an absorption detection system for a CE separation based on red LEDs. Later, red and green LEDs were also applied to CE for microchip separations by Collins and Lu [12, 13]. Although the beam quality (beamwidth, intensity, coherence, and monochromaticity) of an LED is still not superior to that of a laser, the use of a combination of sample concentration techniques in conjunction with CE-LED induced fluorescence detection can overcome this drawback and clearly indicates further potential uses. A series of reports on sample concentration techniques appeared by Terabe *et al.* [14–18], as well as by other groups [19–23], concerning the so-called “stacking” and “sweeping” techniques. Such techniques doubtless have opened a new field of

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Abbreviations: CSEI-sweep-MEKC, cation-selective exhaustive injection-sweep-micellar electrokinetic chromatography; LED, light-emitting diode; NDA, naphthalene-2,3-dicarboxaldehyde

investigation of compounds that exist in low levels, even in the parts per billion (ppb) range. We previously demonstrated the utility of a blue LED (467 nm, 3 mW) for the detection of riboflavin in urine. The detection limit was determined to be 20 ng/mL when a stacking technique was applied [24]. In this study, we report on an investigation of a violet LED (410 nm, 2 mW) in conjunction with various sample concentration techniques, including stacking, sweeping-MEKC (sweeping-micellar electrokinetic capillary chromatography) and cation-selective exhaustive injection (CSEI)-sweep-MEKC for the detection of reserpine and dopamine-labeled NDA. Several electrophoretic parameters such as buffer pH, SDS concentration, and the injection length required for the separation were optimized and these data are reported herein.

2 Materials and methods

2.1 Apparatus

The CE setup was fabricated in-house and is identical to that described previously [24]. Briefly, a high-voltage power supply (model RR30-2R; 0–30 kV, 0–2 mA; Gamma, Ormond Beach, FL, USA) was used to drive the electrophoresis and a 75 μm ID (total length/effective length: 105/98 cm) fused-silica capillary (J&W Scientific, Folsom, CA, USA) was used for the separation. The sample was hydrodynamically injected by raising reservoir to a certain height (depending on the specific situation) to provide the injection length. A violet LED (InGaN; type No. M053UVC; Monarchal Electronics; price: < 2.0 US\$) with a luminous intensity of 300 mcd (operating current: 20 mA; viewing angle: $2\theta_{1/2} = 30$ deg; peak emission wavelength: 410 nm; spectral half width: 15 nm) was purchased on the Taipei electronic market. A microscope objective (40 \times) was used for focusing on the capillary. Fluorescence emission was collected by means of a microscope eyepiece (10 \times), passed through a green-yellow cut filter and a slit (0.3 mm), focused by a second microscope eyepiece (10 \times), and then detected by a photomultiplier tube (Hamamatsu-R928, Shizuoka, Japan). The analog signal was converted to a digital signal by an A/D converter (Adam-4012 module; Advantech, Taipei, Taiwan). Electropherograms were collected at a speed of 1 s/point with a data acquisition system connected to a personal computer. A liquid chromatograph equipped with a triple-quadrupole mass spectrometer (Finnigan TSQ-700; San Jose, CA, USA) was used for the analysis of the purity of dopamine-labeled NDA derivative.

2.2 Reagents

All chemicals used were of analytical grade. Reserpine ($\text{C}_{33}\text{H}_{40}\text{N}_2\text{O}_9$; 3,4,5-trimethoxybenzoic acid ester) and dopamine ($\text{C}_8\text{H}_{11}\text{NO}_2$) were purchased from Sigma (St.

Louis, MO, USA). NDA was obtained from Aldrich (St. Louis, MO, USA). SDS, sodium tetraborate, sodium phosphate, methanol, and phosphoric acid were purchased from Acros (Geel, Belgium). Acetonitrile and ammonium acetate were obtained from Alps Chem (Taipei, Taiwan), and RdH Laborchemikalien (Seelze, Germany), respectively. Glacial acetic acid was obtained from Union Chemical Work (Sin-Chu, Taiwan).

2.3 Derivatization procedure of dopamine-labeled NDA

The derivatization procedure was modified from the literature description [25]. To a 4 mL containing 0.7 mL of sodium tetraborate aqueous buffer (0.1 M, pH 9) was added 0.1 mL of dopamine (10^{-3} M in MeOH) and the same volume of KCN (10^{-3} M in a tetraborate aqueous buffer). The reaction was initiated by the addition of 0.1 mL of NDA (10^{-3} M in MeOH) and the final concentrations were [Dopamine] = 10^{-4} M, [CN] = 10^{-4} M, and [NDA] = 10^{-4} M. After mixing, the reaction solution was allowed to stand at room temperature in a dark environment for 20 min. The derivative of dopamine-labeled NDA was selectively removed by liquid-liquid extraction with ethyl acetate (2.0 mL). The upper layer of the organic fraction was transferred to a glass vial and evaporated to dryness. The residue, containing the dopamine-labeled NDA derivative, was dissolved in 1.0 mL of sample matrix (50 mM sodium phosphate in an acetonitrile-water solution, 1:2 v/v) for the subsequent CE separation.

2.4 Methodology

2.4.1 CZE stacking

Reserpine: The CE buffers were aqueous solutions, containing 50 mM ammonium acetate (pH 6.8; conductivity = 4.7 mS/cm). The samples (4.1×10^{-6} – 2.1×10^{-7} M) were prepared in matrix solutions, obtained by diluting the CE buffer to 1/100 (pH 7.8; conductivity = 53.9 $\mu\text{S/cm}$). Hydrodynamic injection was achieved by raising the sample reservoir to a height of 32 cm relative to the exit reservoir, thus generating a flow rate of 0.60 mm/s. 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.*, so-called “stacking”. Once the ions reach the running buffer boundary, the field decreases and the migration becomes slower. When the ions reach the running buffer boundary, the field decreases and the migration becomes slower. This results in the concentration of the sample.

2.4.2 Sweeping-MEKC

Reserpine: The background solution consisted of 100 mM SDS and 30 mM H₃PO₄ in an aqueous solution (pH 1.6; conductivity = 6.72 mS/cm). Reserpine was dissolved in the same solution (without SDS) resulting in a nonmicelle buffer. When the injection was completed, +20 kV was applied to power the CE separation. This procedure permits the SDS-anionic surfactant micelles (in the inlet reservoir) to enter the sample zone. Thus, along the capillary axis, the samples were being swept and concentrated near the junction between the sample solution and the background solution. As in the following step, the samples were separated by the MEKC mode.

Dopamine-labeled NDA: The background solution consisted of 100 mM SDS and 30 mM H₃PO₄ in a mixed acetonitrile-water solution (15:85 v/v) the pH of which was 1.5 (conductivity = 6.63 mS/cm). Subsequent procedures were the same as described above.

2.4.3 CSEI-sweep-MEKC

Reserpine: The background buffer consisted of only 30 mM H₃PO₄ in an aqueous solution (pH 2.2; conductivity = 4.64 mS/cm). The capillary was initially filled with this background buffer, followed by the injection of a high-conductivity buffer (~ a 37 cm length of capillary with 100 mM H₃PO₄ solution), and lastly by the injection of a short water plug (~3 mm). By electrokinetic injection at +20 kV, the cationic analytes were injected for a period of 5 min. Meanwhile, the cationic analytes could be concentrated at the interface between the water zone and the buffer zone. Finally, the cationic analytes focus or stack at the interface between the water zone and the high conductivity buffer. The injection was then stopped and micellar background solutions were placed at both ends of the capillary. Following this, by quickly shifting the voltage to -20 kV, the high voltage of negative polarity permitted the entry of micelles from the inlet vial into the capillary to sweep the stacked and to introduce the analytes to the narrow bands. The separation was performed using the MEKC mode within the next ~25 min.

3 Results and discussion

3.1 Application to fluorescent reserpine

Reserpine, obtained from the root of the rauwolfia (*Rauwolfia serpentina*), is frequently used in the treatment of hypertension. We selected reserpine as the test sample because it can be excited in the violet region and emits a green-yellow fluorescence. Figure 1A shows the excita-

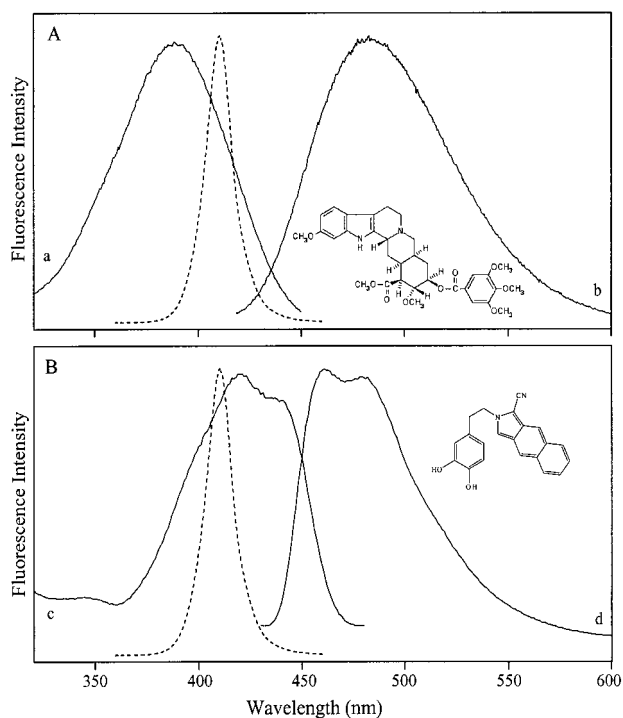


Figure 1. Typical excitation spectra (a and c) and fluorescence (b and d) spectra (A) of reserpine and (B) dopamine-labeled NDA. The dashed line spectrum shows the wavelength range of the violet LED used in this study.

tion (spectrum a) and fluorescence (spectrum b) spectra of reserpine in an acetic acid-water (1:9 v/v) matrix. The inset shows the molecular structure of reserpine. The dashed line shows the wavelength range of the violet LED used in this study. It appears that this light source is particularly well matched to excite reserpine. For a comparison of the luminous intensity, a laser power meter (model No. 45-545; detecting range: 20 mW–20 μ W; Metrologic Instruments, Blackwood, NJ, USA) was used directly and the finding shows that the output power of this violet LED was ~2 mW. For CE separation, based on CZE, CZE stacking, normal MEKC, sweeping-MEKC, and CSEI-sweep-MEKC modes, the calibration curves for various concentrations of reserpine were constructed and these results are shown in Table 1. For the CZE mode, a 4.1×10^{-6} M detection limit was obtained; whereas an obvious improvement of up to 1.6×10^{-8} M was found when the CZE stacking mode was applied. The linearity of these methods for reserpine was also fairly good. In order to investigate the effects of injection length when the stacking technique was used, under exactly the same experimental conditions, 29, 37, 44, and 51 cm column lengths of reserpine solution were injected into the capillary. The finding shows that a 37 cm column length provides optimal results.

Table 1. Limit of detection (LOD) values, linearity of peak area, coefficient of variation and plate numbers for reserpine and dopamine-labeled NDA with various sample concentration techniques

Method	Equation of line Coefficient of variation	Concentration range	LOD (S/N = 3)	Plate number
Reserpine				
CZE	$Y = 55212x - 0.2499$ $R^2 = 0.9995$	$1.6 \times 10^{-4} - 1.6 \times 10^{-5}$ M	4.1×10^{-6} M	1.5×10^5
CZE-stacking	$Y = 1E+07x + 0.132$ $R^2 = 0.9997$	$4.1 \times 10^{-6} - 2.1 \times 10^{-7}$ M	1.6×10^{-8} M	8.1×10^4
MEKC	$Y = 71285x - 0.0747$ $R^2 = 0.9992$	$8.2 \times 10^{-5} - 6.7 \times 10^{-6}$ M	2.5×10^{-6} M	5.4×10^4
Sweeping-MEKC	$Y = 3E+08x - 2.0886$ $R^2 = 0.9972$	$1.6 \times 10^{-7} - 8.2 \times 10^{-9}$ M	2.1×10^{-9} M	2.4×10^5
CSEI-sweep-MEKC	$Y = 3E+08x - 0.1204$ $R^2 = 0.9915$	$1.6 \times 10^{-8} - 1.6 \times 10^{-9}$ M	2.1×10^{-10} M	2.7×10^6
Dopamine-labeled NDA				
MEKC	$Y = 48741x - 0.1302$ $R^2 = 0.9997$	$5.0 \times 10^{-5} - 6.3 \times 10^{-6}$ M	6.2×10^{-6} M	5.7×10^4
Sweeping-MEKC	$Y = 8E+06x + 0.0006$ $R^2 = 0.9983$	$5.0 \times 10^{-7} - 3.1 \times 10^{-8}$ M	3.1×10^{-8} M	3.0×10^5

On the other hand, a normal MEKC was performed with 100 mM SDS and the detection limit was found to be 2.5×10^{-6} M; whereas the detection limit was dramatically improved to 2.0×10^{-9} M and 2.0×10^{-10} M when the sweeping-MEKC and CSEI-sweep-MEKC techniques, respectively, were applied. The plate number obtained by CSEI-sweep-MEKC mode was $\sim 10^6$. This suggests that the electrokinetic injection method provided a narrower sample zone, a benefit for high-efficiency separation. In order to examine the effects of surfactants, different concentrations of 50, 100 and 150 mM SDS were tested and the optimum concentration of SDS was found to be 100 mM. For investigating the effects of injection length when the sweeping-MEKC technique was used, under exactly the same experimental conditions, 33, 37, 40, and 44 cm column lengths of sample solution were injected into the capillary. The finding shows that a 37 cm column length is optimal. In order to examine the effects of electrokinetic injection times when the CSEI-sweep-MEKC technique was used, under exactly the same experimental conditions, 3, 5, 7, and 9 min of sample injection were performed. The finding shows that a 5 min electrokinetic injection gave the best result. For comparison, Fig. 2 shows three of these electropherograms (CZE, MEKC and sweeping-MEKC, respectively). The concentrations of reserpine used were 8.2×10^{-5} M, 8.2×10^{-5} M and 8.2×10^{-8} M, respectively. In the case of the CZE mode, the signal intensity was only 0.7 V, whereas the MEKC mode provided better results

(1.1 V). However, after applying a sweeping-MEKC method, the detection limit of reserpine dramatically improved (2.7 V). Compared to the CZE and MEKC modes, a 3800–2500-fold improvement was obtained.

3.2 Application to derivative (dopamine-labeled NDA)

Although dopamine is weakly fluorescent, for the detection of a weak- or nonfluorescent sample a derivatization procedure normally is recommended. The derivatization procedure for dopamine is described in Section 2.3. This derivative was examined by LC-MS electron impact (EI mode, 20 eV) and the finding shows that a major fragment (m/z 328) is produced (data not show). This suggests that this derivative is identical with dopamine-labeled NDA, the so-called 1-cyano-2-substituted-benz[*f*]iso-indole (CBI). Figure 1B shows the excitation (spectrum c) and fluorescence (spectrum d) spectra of CBI; the molecular structure of CBI is shown in the inset. For CE separation, a normal MEKC was performed with 50 mM SDS and the detection limit was found to be 6.3×10^{-6} M; whereas the detection limit was dramatically improved to 3.1×10^{-8} M when sweeping-MEKC was applied. In order to determine the effects of injection length, 29, 34, 39, and 44 cm column lengths of sample solution were injected into the capillary. The finding

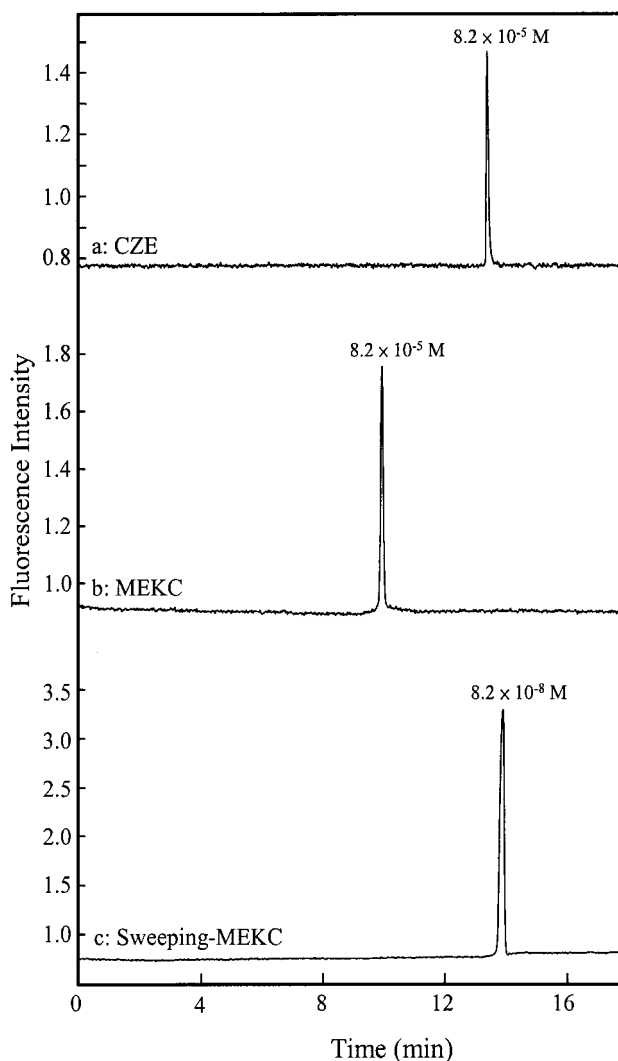


Figure 2. CE electropherograms obtained by using the CZE, MEKC, and sweeping-MEKC modes, respectively. CE conditions: (a) 50 mM ammonium acetate buffer, 20 kV, $\sim 60 \mu\text{A}$; (b) 30 mM ammonium acetate and 100 mM SDS buffer, 20 kV, $\sim 50 \mu\text{A}$; (c) background solution, 100 mM SDS and 30 mM H_3PO_4 in an aqueous solution (pH 1.6; conductivity = 6.72 mS/cm), reserpine was dissolved in the same solution (without SDS), -20 kV , $\sim -50 \mu\text{A}$.

shows that a 39 cm column length is optimal. Using these results, calibration curves were constructed, as shown in Fig. 3. The detection limit values, linearity and plate numbers were calculated and are summarized in Table 1. In general, the concentration of dopamine in adult urine is about 40–600 $\mu\text{g}/\text{day}$. This method provides sufficient sensitivity for the detection of dopamine.

In conclusion, this work represents the first successful applications of violet LED-induced fluorescence detection in CE separations and suggests that the violet LED

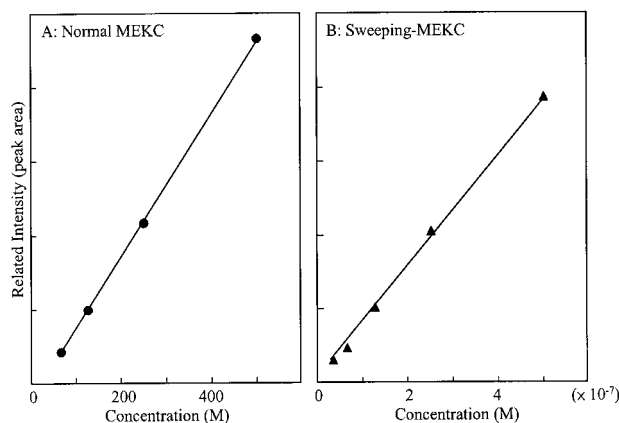


Figure 3. Calibration graphs for dopamine-labeled NDA at various concentrations in (A) the normal-MEKC and (B) sweeping-MEKC mode.

has great potential for use as a new light source in CE separations, not only for naturally fluorescent compounds (excited in violet region) but for derivatives as well. The method is a sensitive, accurate, rapid, simple, reproducible, and economic technique, and, numerous dyes either commercially available or laboratory-synthesized could easily be tuned to possess an excitation maximum close to the violet region. The use of a combination of a stacking or sweeping technique in conjunction with this method could clearly lead to further potential uses.

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4 References

- [1] Legendre, B. L. Jr., Moberg, D. L., Williams, D. C., Soper, S. A., *J. Chromatogr. A* 1997, 779, 185–194.
- [2] Gallaher, D. L. Jr., Johnson, M. E., *Analyst* 1999, 124, 1541–1546.
- [3] Rahavendran, S. V., Karnes, H. T., *Anal. Chem.* 1997, 69, 3022–3027.
- [4] Kaneta, T., Shiba, H., Imasaka, T., *J. Chromatogr. A* 1998, 805, 295–300.
- [5] Mank, A. J. G., Yeung, E. S., *J. Chromatogr. A* 1995, 708, 309–321.
- [6] Melanson, J. E., Lucy, C. A., *Analyst* 2000, 125, 1049–1051.
- [7] Melanson, J. E., Boulet, C. A., Lucy, C. A., *Anal. Chem.* 2001, 73, 1809–1813.
- [8] Major, A. L., Rose, G. S., Svaasand, L. O., Lüdicke, F., Campana, A., Van Gemert, M. J. C., *J. Photochem. Photobiol. B* 2002, 66, 107–114.

- [9] Ryder, A. G., Glynn, T. J., Przyjalowski, M., Szuzupak, B., *J. Fluoresc.* 2002, 12, 177–180.
- [10] Toyooka, T., *Modern Derivatization Methods for Separation Science*, John Wiley & Sons, New York 1999.
- [11] Tong, W., Yeung, E. S., *J. Chromatogr. A* 1995, 718, 177–185.
- [12] Collins, G. E., Lu, Q., *Anal. Chim. Acta* 2001, 436, 181–189.
- [13] Lu, Q., Collins, G. E., *Analyst* 2001, 126, 429–432.
- [14] Quirino, J. P., Terabe, S., *Science* 1998, 282, 465–468.
- [15] Quirino, J. P., Terabe, S., *Anal. Chem.* 1999, 71, 1638–1644.
- [16] Quirino, J. P., Terabe, S., *Anal. Chem.* 2000, 72, 1023–1030.
- [17] Kim, J. -B., Otsuka, K., Terabe, S., *J. Chromatogr. A* 2001, 932, 129–137.
- [18] Quirino, J. P., Terabe, S., Boček, P., *Anal. Chem.* 2000, 72, 1934–1940.
- [19] Marwah, A., Marwah, P., Lardy, H., *J. Chromatogr. B* 2001, 757, 333–342.
- [20] Ghulam, A., Kouach, M., Racadot, A., Boersma, A., Vantghem, M. C., Briand, G., *J. Chromatogr. B* 1999, 727, 227–233.
- [21] Miksik, I., Vylitova, M., Pacha, J., Deyl, Z., *J. Chromatogr. B* 1999, 726, 59–69.
- [22] Burgi, D. S., Landers, J. P., *Anal. Chem.* 2002, 74, 3931–3931.
- [23] Shihabi, Z. K., *J. Chromatogr. A* 2000, 902, 107–117.
- [24] Su, A.-K., Lin, C.-H., *J. Chromatogr. B* 2003, 785, 39–46.
- [25] Kawasaki, T., Higuchi, T., Imai, K., Wong, O. S., *Anal. Biochem.* 1989, 180, 279–285.