Ultrasensitive Fluorescence Detection of Polycyclic Aromatic Hydrocarbons in Capillary Electrophoresis

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Capillary electrophoresis with UV-laser-excited native fluorescence has been employed for ultrasensitive determination of polycyclic aromatic hydrocarbons, including anthracene, phenanthrene, benz[a]anthracene, methylanthracene, pyrene, fluoranthene, and perylene. The separation is based on solvophobic association of the analytes with tetraalkylammonium ions in mixed acetonitrile/water solvent. With both near-UV (325-nm) and deep-UV (257-nm) laser excitation, background fluorescence from the fused silica capillary and the nonaqueous electrolyte was the limiting factor in detection sensitivity. Effective rejection of the intense capillary wall fluorescence was achieved by the use of a high-numerical-aperture microscope objective coupled with an adjustable precision slit in a confocal configuration. The achieved mass detection limits were in the range of (3-15) × 10^{-20} mol, with linear fluorescence response spanning over 4 orders of magnitude. This sensitivity is expected to be sufficient for analyzing chemical carcinogens, anticancer drugs, and their metabolites in individual mammalian cells.

INTRODUCTION

The ability to perform trace chemical analysis in small volumes is of vital importance for gaining a deeper understanding of many biological and medical processes at the molecular level. Indeed, microscale separation techniques, such as microcolumn liquid chromatography (micro-LC)[1] and capillary electrophoresis (CE),[2] have allowed the chemical assay of individual snail neurons,[1] bovine adrenomedullary cells,[3] and human erythrocytes.[4] Submicrometer-sized optical sensors[5,6] and chemically modified microelectrodes[7-9] have also been used for spatially and temporally resolved chemical analysis of single biological cells. Of particular fundamental interest and practical significance in this analytical frontier is the ultrasensitive determination of chemical carcinogens and therapeutic drugs in small-volume situations, such as in the picoliter-sized volumes of 10-μm biological cells and in the femtoliter-sized volumes of 1-μm airborne particulates.

The methodology required for small-space chemical measurement is demanding because of the need to be not only ultrasensitive in detection limits but also compatible with extremely small sample sizes. The current repertoire of techniques for small-volume measurement includes resonance ionization mass spectrometry,[10] laser microspectrofluorometry,[11] submicrometer optical sensors,[5,6] microelectrodes,[7-9] and capillary electrophoresis.[2] Among these, capillary electrophoresis is particularly powerful and well-suited for analyzing complex microsamples because of its high separation efficiency. The CE analysis of chemical carcinogens and polycyclic aromatic hydrocarbons (PAHs),[12] however, has two significant challenges. First, the electrophoretic separation of these compounds, which are often neutral and highly hydrophobic, is difficult by conventional CE or micellar electrokinetic chromatography (MEKC);[13] second, the small amounts of materials available for analysis demand mass detection sensitivity in the subattomole level. Previously, two methods were developed for the electrophoretic separation of PAHs: (i) solvophobic interaction of the analyte with tetrahexylammonium (THA^+) ions in conventional CE[14] and (ii) use of organic additives/modifiers (i.e., γ-cyclodextrin and methanol) in MEKC.[15-17] The reported detection limits (femtomoles), however, were insufficient by several orders of magnitude for ultrasensitive small-volume measurements. Consequently, detection sensitivity has become the "bottleneck" in CE analysis of chemical carcinogens and PAHs in small-volume situations.

This work reports the use of an ultrasensitive confocal detection scheme based on UV-laser-induced native fluorescence (UV-LIF). A significant advantage of the CE/UV-LIF approach is that it does not require analyte derivatization, which is difficult in small volumes or at low concentrations. CE/UV-LIF is thus well-suited for ultrasensitive measurement in small volumes, which was amply demonstrated by Lee and Yeung[4] in the determination of hemoglobin and carbonic anhydrase in the picoliter-sized human red blood cells. To effect electrophoretic separation of PAHs, we employed a mixed water/acetonitrile solvent with a tetraalkylammonium electrolyte. Native LIF detection was carried out with both near-UV and deep-UV laser excitation, and effective rejection of the intense capillary wall fluorescence was achieved by the

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use of a high-numerical-aperture (N.A.) microscope objective coupled with an adjustable precision slit in a confocal configuration. With this system we achieved subattomole detection limits in the CE analysis of PAHs.

EXPERIMENTAL SECTION

Apparatus. The CZE apparatus constructed for this work is similar to that described previously.[18] Except when noted otherwise, a 60-cm-long, 25-μm-i.d., and 150-μm-o.d. fused silica capillary (Polymicro Technologies, Inc., Phoenix, AZ) and a positive high-voltage power supply (Glassman High Voltage, Inc., Whitehouse Station, NJ) were used throughout this work. Separations were performed at 20 kV with an effective separation capillary length of ~40 cm. The separation capillary was pressure-flushed with purified water (18.2 MΩ) and 0.1 M sodium hydroxide solution for 5 min each.

UV-LIF Detection. Deep-UV laser radiation was provided by a prototype intracavity frequency-doubled argon ion laser (Coherent Laser Group, Santa Clara, CA). This laser is capable of generating stable CW outputs in the 10–100-mW power range at several UV wavelengths (229, 238, 244, 248, 257, and 264 nm). The 257-nm line was primarily employed in this work. Its continuous-wave nature and high beam quality allow tight beam focusing and also alleviate the problem of ground-state depletion often encountered with pulsed UV lasers.[19] Near-UV excitation at 325 nm was obtained by using a He/Cd laser (Liconix, Santa Clara, CA). A short-focal-length quartz lens (f = 1.9 cm, d = 0.65 cm) (Oriel Corp., Stratford, CT) mounted on an X–Y stage (Newport Corp., Irving, CA) was used to achieve precise focusing at the detection zone, which was created by removing a 5-mm section of the polyimide coating on the fused silica tubing. Fluorescence was collected with a right-angle scattering geometry by using a high N.A. microscope objective (40×, N.A. 0.85, WD 0.37 mm; minimal autofluorescence and high UV transmission, Nikon Instrument Group, Melville, NY). After passing through an interference filter (bandpass width: 70 nm, center wavelength 400 nm; Corion Research Inc., Boston, MA), the collected fluorescent light was focused on a variable slit (Oriel Corp.), which was precisely adjusted to match the analyte fluorescence image size. With an estimated laser beam waist of ~10 μm, the slit size was 1.0 mm long by 0.4 mm wide. Efficient rejection of silica wall fluorescence was achieved by positioning the sample and the slit exactly at the two focal points of the high N.A. objective (confocal arrangement). A photomultiplier tube (PMT) (Model R928, Hamamatsu Corp, Bridgewater, NJ) operating at 800 V was used for fluorescence detection. The data acquisition system consisted of a PMT readout device (Acton Research Corp., Acton, MA), a plug-in data acquisition board (Chrom-1, Omega Engineering, Stamford, CT), and commercial software (Galactic Industries Corp., Salem, NH) run on a personal computer (IBM PC-AT).

Injection Calibration. Throughout this work, thin-wall and small-i.d. capillaries were used for accurate and reproducible injections.[20] The capillary was first filled with the electrophoretic medium (no PAH); the injection end was then placed in the analyte vial (containing PAH) that was raised to a certain height. The exact injection volume was calculated by measuring the time for the analyte to flow from the injection end to the detection zone under gravity. With the injection time set at 20 s and height at 15 cm, the measured injection volume was ca. 0.5 nL with a 60-cm, 25-μm-i.d. capillary and the mixed water/acetonitrile electrophoretic medium.[21] To minimize solvent evaporation, the buffer reservoirs were specially designed with covers and the capillary inlet was transferred between the buffer and analyte reservoirs in 1–2 s.

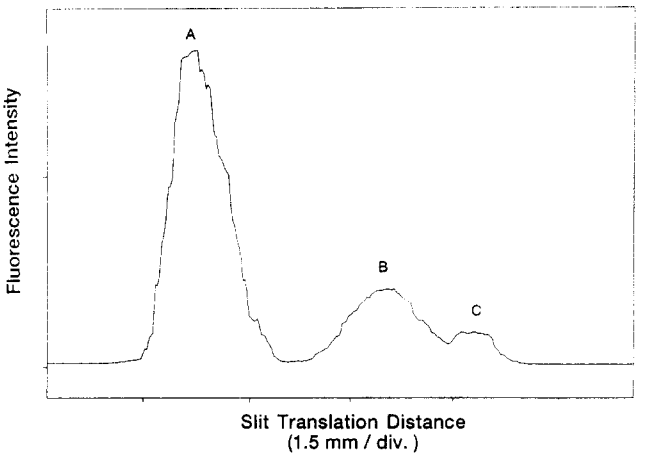

Figure 1. One-dimensional profile of UV-laser-induced fluorescence image: (A) fused silica (rear wall, laser exiting); (B) analyte; (C) fused silica (front wall, laser entering). Laser power at capillary 10 mW; laser wavelength 325 nm. See text for discussion.

Reagents. Tetrahexylammonium perchlorate (THAP) and tetrahexylammonium acetate (THAA) were obtained from Alfa Products (Danvers, MA) and Sigma Chemical Co. (St. Louis, MO), respectively. Polycyclic aromatic hydrocarbons, anthracence, 2-methylanthracene, 9-methylanthracene, phenanthrene, benz[a]anthracene, fluoranthene, pyrene, and perylene, were all purchased from commercial sources. Spectrophotometric grade acetonitrile and dimethylformamide (DMF) were obtained from Aldrich Chemical Co. (Milwaukee, WI) and were used without further treatment. Water was purified with an Ultra-Pure water system (Millipore, Bedford, MA). Stock solutions were prepared by first dissolving the PAHs in minimal amounts of DMF and then adding acetonitrile. The required PAH concentrations were obtained by serial dilution of the stock solutions in the electrophoretic medium (25 mM tetrahexylammonium in 3:1 (v/v) mixed acetonitrile/water).

RESULTS AND DISCUSSION

Minimization of Background Fluorescence. A serious problem in CE/UV-LIF is the exceedingly high level of background fluorescence observed with near-UV and deep-UV laser excitations. This interference is caused primarily by UV-induced luminescence of the fused silica wall. Buffer impurity fluorescence also tends to be higher with UV excitations, and the scattered UV light may induce autofluorescence in the collection lens (microscope objective). With fixed fluorescence quantum yield and collection/detection efficiencies, the detection sensitivity is limited by the high fluorescence background.

For visible-laser excitation, Hernandez et al.[22] employed a confocal fluorescence system for zeptomole (zepto = 10^{-21}) detection of fluorescein-derivatized amino acids in capillary zone electrophoresis, and Mathies and co-workers[23,24] reported the use of a confocal fluorescence scanner for enhanced detection of fluorescently derivatized DNA in slab and capillary gel electrophoresis. For UV-laser excitation in this study, we combined a high N.A. microscope objective (minimal autofluorescence and high UV transmission) with a precision adjustable slit to minimize the background problem (see Experimental Section). Figure 1 shows a typical one-

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(21) The injection volume can also be theoretically calculated by using the Poiseuille equation: $\Delta V = [(P_1 - P_2)\pi R^4/8\eta l]\Delta t$, where ΔV is the injection volume, Δt is the injection time, $(P_1 - P_2)$ is the injection pressure, R is the capillary radius, η is the viscosity of electrophoretic medium, and l is the capillary length. For $\eta = 1.0 \times 10^{-3}$ kg m^{-1} s^{-1} (viscosity of water at 25 °C), ΔV = 0.47 nL. The theoretical result is thus in general agreement with the measured injection volume (0.5 nL), although the exact viscosity of the electrophoretic medium may be slightly smaller than that of water.
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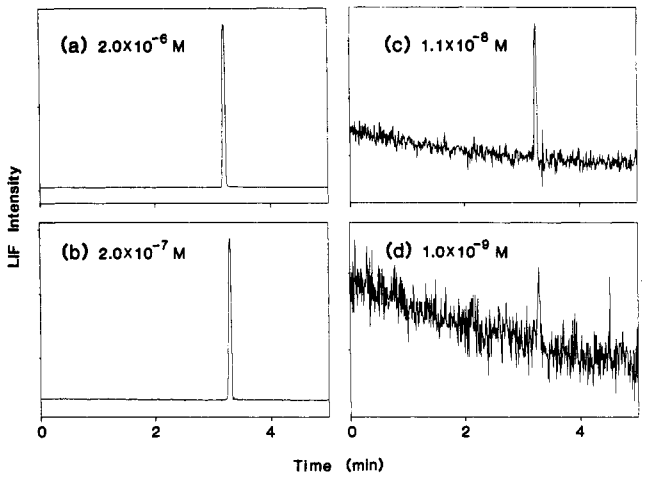

Figure 2. Electropherograms of anthracene with near-UV-LIF detection: laser power at capillary 10 mW; laser wavelength 325 nm; injection volume 0.5 nL.

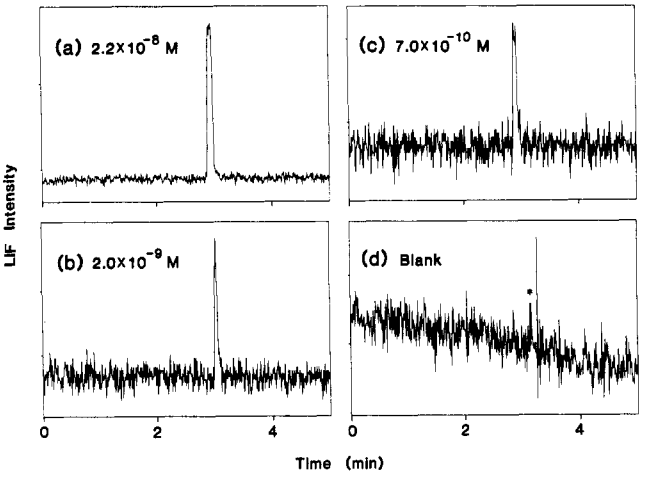

Figure 3. Electropherograms of benz[*a*]anthracene with near-UV-LIF detection: laser power at capillary 10 mW; laser wavelength 325 nm; injection volume 0.5 nL. The electropherograms were obtained with a single sample vial (20 mL) in the reverse order: the blank first, followed by sequential addition of the stock benz[*a*]anthracene solution. This procedure was necessary because the acetonitrile/water mixture solvent was able to dissolve certain impurities associated with a new glass vial (with plastic cap). A minor contamination peak (designated by *) was still observed in the blank.

dimensional profile of the fluorescence image at the slit, which was obtained by translating the slit in the horizontal direction. It indicates that the silica wall fluorescence is the dominant source of background. By adjusting the slit to exactly match the image size, the intense capillary wall fluorescence was efficiently separated from the analyte signal. An almost complete elimination of the silica wall fluorescence was achieved with this confocal arrangement.

It is surprising, however, that the fluorescence image at the slit is not symmetrical in our system; the rear capillary wall (laser beam exiting) exhibits much higher fluorescence than the front wall (laser beam entering). We are investigating the possible causes of this phenomenon, such as light reflection/diffraction at the silica/air and silica/liquid interfaces, and laser beam profile in the capillary.

Detection Sensitivity. With the low fluorescence background achieved, excellent detection limits for PAHs can be expected because of their generally high fluorescence quantum yields and photostability. Figure 2 shows a series of electropherograms of anthracene obtained with 0.5-nL injection at various concentrations. The limit of detection (defined as the amount of analyte injected to give a peak height that is three times the standard deviation of the baseline noise) was $\sim 1.5 \times 10^{-19}$ mol of anthracene with 10-mW near-UV excitation. This detection sensitivity is remarkable in view of the low optical absorption of anthracene in the near-UV ($\epsilon_{325\ nm} = 3 \times 10^3$) and its maximum absorption in the deep-UV ($\epsilon_{252\ nm} = 20.8 \times 10^3$). Further improvement in detection sensitivity should be possible with a better match between excitation wavelength and optical absorption. This prediction was confirmed by a study of larger PAHs (i.e., benz[*a*]-anthracene, pyrene) with near-UV excitation and smaller PAHs (i.e., anthracene, phenanthrene) with deep-UV excitation. Figure 3 presents electropherograms of benz[*a*]-anthracene (including a blank) obtained with near-UV excitation. The optical absorption of benz[*a*]anthracene in the near-UV is higher ($\epsilon_{325\ nm} = 6.5 \times 10^3$) than that of anthracene, and the achieved mass detection limit (5.3×10^{-20} mol) is considerably better. Similar detection limits ($\sim 5.0 \times 10^{-20}$ mol) were also achieved for pyrene and fluoranthene with near-UV laser excitation. The effect of excitation wavelength is further illustrated in Figure 4, which compares the electropherograms of phenanthrene obtained with near-UV and deep-UV laser excitations. It is clear that a close match of excitation wavelength and optical absorption leads to significant improvement in the achievable detection limits. In fact, this study has achieved a detection limit as low as 3.0×10^{-20} mol or 18 000 molecules for 2-methyl- and 9-methylanthracenes (λ_{max} = 254-nm) by using deep-UV laser excitation at 257 nm. It should be pointed out that the best detection sensitivity for a particular PAH can only be achieved under experimental conditions that are optimized on the basis of its photochemical and photophysical properties. It is also interesting to note that solvophobic interactions between the analytes and tetraalkylammonium ions may affect the

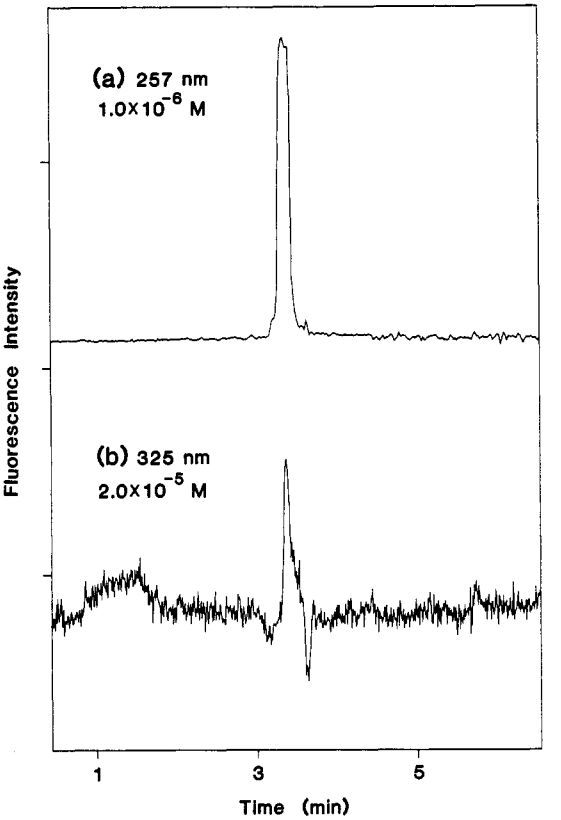

Figure 4. Comparison of detection sensitivity for phenanthrene between near-UV and deep-UV laser excitations. (a) Electropherograms of 1.0×10^{-6} M phenanthrene with deep-UV-LIF detection: laser wavelength 257 nm; laser power 10 mW; injection volume 0.5 nL. (b) Electropherograms of 2.0×10^{-5} M phenanthrene with near-UV-LIF detection: laser wavelength 325 nm; laser power 10 mW; injection volume 0.5 nL. Note the 20-fold concentration difference.

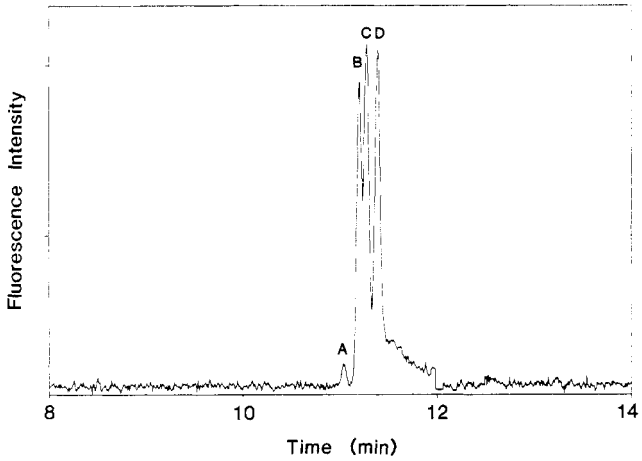

Figure 5. Capillary electrophoresis separation of four polycyclic aromatic hydrocarbons with UV-LIF detection: (A) perylene; (B) benz-[*a*]anthracene; (C) pyrene; (D) anthracene. Total capillary length 100 cm; effective separation length ~80 cm; total injected PAHs 250 amol. Note the use of a longer capillary, which led to slightly better separation (~200 000 theoretical plates), and the reduced detection sensitivity for perylene (peak A), which was caused primarily by a mismatch of the interference bandpass filter (center wavelength 400 nm) and the fluorescence emission maximum (~440 nm) of perylene.

fluorescence properties of PAHs, and such an effect needs to be explored for best detection sensitivity.

The mass detection limits achieved in this study are substantially better than those in previous HPLC/micro-LC studies with UV-LIF detection.[25] By using a windowless droplet design for detection, Diebold and Zare[26] first reported a detection limit of ~2.5 fmol for carcinogenic aflatoxins in HPLC. Sepaniak and Yeung[27] designed a fiber optic flow cell and achieved a detection limit of 10–15 amol for antibiotic drugs in micro-LC. Further improvements in detector design allowed the detection of 100–120 amol of pyrene and fluoranthene in micro-LC.[28,29] However, Gluckman et al.[29] reported that the fluorescence response became nonlinear below 2.76 pg or ~14 fmol of injected pyrene in micro-LC; this conclusion may be questioned because no background subtraction appears to have been made. A careful analysis of our CE/UV-LIF data (with background subtraction) indicates linear fluorescence response (linearity coefficient R = 0.98) for PAHs from 2×10^{-6} M to the detection limits ($(6–30) \times 10^{-11}$ M).

Modes of Separation. Previous studies[14–17] on the electrophoretic separation of electrically neutral, highly hydrophobic compounds primarily employed optical absorbance detection at high analyte concentrations (e.g., 100 ppm). A recent report described the MEKC/γ-cyclodextrin separation of two PAHs with visible (415-nm) LIF detection.[30] The concentration detection limit achieved for benzo[*a*]pyrene was in the micromolar range; although the detection limit (~10^{-8} M) was better for aminoanthracene, this sensitivity is inadequate for ultrasensitive small-volume measurements. It is therefore important to demonstrate ultrasensitive detection of PAH mixtures separated with CE.

Figure 5 shows an electropherogram of four PAHs separated with the solvophobic method[14] and detected with near-UV-LIF. The total injected material was only 250 amol of PAHs. The speed of solute migration was consistent with that reported previously:[14] perylene > benz[*a*]anthracene > pyrene > anthracence. The larger and more hydrophobic the solutes, the faster they migrate because of stronger interaction with THA^+ ions. Despite the high efficiency (~200 000 theoretical plates) achieved with this mode of separation, we were unsuccessful in resolving PAH isomers, such as anthracence/phenanthrene, 2-methylanthracence/9-methylanthracence, and pyrene/fluoranthene. On the other hand, the MEKC-modifier method allowed PAH isomer separation, such as anthracence/phenanthrene and benz[a]anthracene/chrysene. It is of interest to note that two-step laser desorption/laser ionization mass spectrometry (L^2MS) is capable of detecting subattomole amounts of PAHs in meteorite and interplanetary dust particles,[31,32] but it lacks the ability to resolve isomers. CE/UV-LIF may thus be combined with L^2MS to provide both mass and structural information on PAHs with subattomole sensitivity.

Prospects and Applications. The ability to analyze polycyclic aromatic compounds in small volumes at the subattomole level opens many opportunities in studying the intracellular uptake, metabolization and action of chemical carcinogens and anticancer drugs. For example, detection and quantitation of PAHs and their metabolites in mammalian cells would certainly provide insights to the molecular mechanisms of carcinogenicity at the individual cell level, and ultrasensitive chemical analysis of "breathable" pollutant particulates may help delineate how the adsorbed PAHs are transported.

Ultrasensitive studies using microspectrofluorometry have revealed the nonuniform distribution of PAHs and the existence of multiple metabolic pathways that convert PAHs into active carcinogens (i.e., diol epoxides) in mammalian cells.[33] The active carcinogens may form adducts with proteins and DNA inside a cell. To elucidate such carcinogenic processes at the molecular level, it is essential to analyze the parent PAHs, the active carcinogens, and the adducts. This system is extremely complex and a particular species may exist only at the attomole level, as estimated by using micromolar carcinogens in a 10–20-μm cell.[34] Microspectrofluorometric techniques are unable to resolve such a complex mixture because of the broad-band nature of fluorescent emissions. While fluorescence line-narrowing spectroscopy at low temperatures allows the observation of vibrational lines,[35,36] its sensitivity is only at the femtomole level. Clearly, the separation of such a complex mixture by CE, followed by ultrasensitive UV-LIF detection, represents a powerful approach. Furthermore, many drugs (especially antipsoriatic and anticancer) are polycyclic aromatic compounds and are highly fluorescent upon UV-laser excitation.[33]

In conclusion, we have achieved subattomole detection sensitivity in CE analysis of polycyclic aromatic hydrocarbons by using the native fluorescence excited with near- and deep-UV laser radiation. The intense fluorescence background from the fused silica wall was effectively rejected through a confocal arrangement consisting of a high N.A. microscope objective and an adjustable precision slit. The detection limits for the PAHs were in the range of $(3–15) \times 10^{-20}$ mol, with

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linear fluorescence response spanning over 4 orders of magnitude. This sensitivity is comparable to or only slightly lower than that reported in the CE analysis of fluorescently derivatized molecules,[37,38] and further improvement in detector design (e.g., sheath flow)[39,40] may reduce the detection limits for PAHs to the zeptomole or subzeptomole level. The detection sensitivity achieved in this work, combined with the extreme separation efficiency and small sample sizes of CE, renders CE/UV-LIF a powerful approach in the chemical analysis of carcinogens and drugs in complex microenvironments.

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