Capillary electrophoresis with laser-induced fluorescence detection for natively fluorescent analytes

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Laser-induced fluorescence (LIF) detection systems for capillary electrophoresis (CE) are nowadays widely available. Many classes of analytes show native fluorescence, but often only upon absorption of short-wavelength UV light. Unfortunately, most CE-LIF systems make use of visible laser lines so that they can only be applied to analytes that have been chemically derivatised with a suitable fluorescent label matching the excitation wavelength. This paper illustrates how the applicability of CE-LIF can be broadened to include natively fluorescent analytes by employing either UV laser systems or multiphoton-excitation. Several pulsed and continuous wave (CW) lasers were compared in terms of analytical sensitivity and selectivity; in particular the CW 275-nm line from an adapted Ar^+ laser proved very suitable. For identification purposes emission spectra were recorded on-line. The usefulness of such systems in environmental chemistry is shown for amino- and hydroxy-substituted naphthalenesulphonates in river water samples. Extra spectral selectivity can be obtained by performing CE-LIF under flurorescence line-narrowing conditions using a cryogenic interface. The introduction of high-repetition rate, femtosecond Ti:sapphire lasers paved the way for two- and three-photon excited LIF detection of a wide array of biologically relevant fluorophores.

Introduction

Capillary electrophoresis (CE) has gained popularity in analytical chemistry, in particular because of its extremely high separation power. The technique is well suited for the separation of charged compounds, but it can also be applied to neutral molecules by using micelles in the running buffer, a technique known as micellar electrokinetic chromatography (MEKC). Detection limits (in terms of mass) that can be achieved by using commercially available UV absorption detectors can be as low as 600 fg [1]. However, even in favourable cases limits of detection in terms of concentration units are at best in the 10^{-6} M range, since the injection volumes that can be used in CE without affecting the separation performance are typically a few nanoliters only. To date, most applications of CE with absorption detection concern biological and pharmaceutical samples, that is, analytical problems in which the challenge is related to sample complexity rather than low analyte concentrations.

So far, CE has not been used extensively in environmental trace analysis. Many organic priority pollutants are neutral compounds (*e.g.*, first generation pesticides, PCBs), and suitable analytical schemes have been developed on the basis of sample enrichment followed by gas or liquid chromatography. For these compounds, CE could be useful if only very small sample volumes are available. However, organic environmental analysis increasingly focuses on ionic and ionogenic compounds, such as modern biocides and their (bio)degradation products. CE techniques have a high potential in this field. The main problem to be overcome is the poor sensitivity of detection. Analysis of micropollutants in environmental samples often has to be performed at much lower concentration levels than amenable by CE with absorption detection. Presumably the coming decade will see a breakthrough in this field once CE can be combined with appropriate sample handling procedures (including analyte preconcentration) and laser-based detection techniques, especially laser-induced fluorescence (LIF) detection. Below, recent achievements in the field of LIF detection are discussed that support this expectation.

Fluorescence techniques are generally much more sensitive than absorption-based methods, and lasers are easily focused onto the (sub)nanoliter detection volumes typical for CE. The LIF technique provides impressive concentration detection limits indeed; for standard solutions of analytes exhibiting native fluorescence, limits down to typically 10^{-12} M have been reported [2]. It should be noted, however, that for real samples this potential sensitivity is rather difficult to achieve. First of all, in many cases chemical derivatisation with a flurorescent or fluorigenic label is required, not only for non-fluorescent analytes, but also for fluorophores

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that cannot be excited at the wavelength of the available laser system. In general, the kinetic limitations of derivatisation reactions give rise to problems at low analyte concentrations (10^{-9} M, sometimes already at the 10^{-7} to 10^{-8} M level). Furthermore, co-migrating interferences may also be labelled and will have absorption/emission characteristics virtually indistinguishable from that of the analyte in question. In such cases it is the chemistry that precludes further improvement of detection limits [3].

Secondly, it should be realized that LIF detection – compared to conventional fluorescence detection with lamp excitation sources – is only advantageous if the background noise is not increased by the same factor as the analyte signal. The high irradiance (I) provided by a laser only leads to better signal-to-noise ratios in LIF as long as detector dark current or shot noise is the dominating contributor to the background. Under shot noise conditions the recorded signal intensity is proportional to I and the noise to $I^{1/2}$. Unfortunately, in certain systems laser power instability (flicker noise) is as large as 1 % so that shot noise conditions are easily violated [4].

In the case of single-channel fluorescence detectors, the emission signal does not provide any structural information on the analytes and the observed migration time is the only analyte identification parameter. In practice, more information is often required for identification purposes. Spectroscopic identification of separated peaks is particularly important in CE since migration times and separation parameters depend critically on experimental conditions that are difficult to control, such as pH, temperature, ionic strength and the condition of the capillary walls [5].

In this paper – which does not intend to present an exhaustive review, but rather to discuss the state-of-the-art and future trends in CE-LIF – we will focus on analytes that exhibit native fluorescence. Aspects to be addressed are the recent introduction of lasers emitting in the deep-ultraviolet region, the on-line recording of emission spectra for identification purposes, cryogenic interfaces for high-resolution spectroscopic identification, and the use of multiphoton-excitation to perform LIF. The emphasis is on the potential of CE as a tool in modern environmental and bioanalytical chemistry.

Laser systems

Continuous wave (CW) lasers

The most popular continuous wave (CW) laser in analytical chemistry is undoubtedly the argon-ion laser [6]. Small-frame Ar^+ lasers provide only visible lines in the region from 458-530 nm, with the strongest emission at 488 and 514 nm. Only *discrete* lines are produced (which can be considered as extremely monochromatic, typical spectral bandwidth 0.001 nm for an argon-ion laser), so that tunability (full freedom of wavelength choice) is a major point of analytical concern. Large-frame, water-cooled systems provide additional lines in the near-UV, *i.e.*, at 334, 351 and 364 nm. Under normal conditions these CW lasers do not provide output in the deep-UV, which seriously limits their applicability to LIF detection. In an attempt to overcome this problem, various fluorescent labels have been synthesised that match the excitation wavelengths of existing laser systems.

As will be further outlined below, some interesting developments within this context can be observed. First of all, diode lasers have been introduced that feature very low output power fluctuations (typically 0.01 %), are very small in size (down to 1 mm), and combine a low price with an extremely long life span (50,000 h). The best-known example is the Ga:Al:As laser emitting in the infrared at 780 nm; its 5-mW version can be considered as the workhorse of the compact disk industry. Interestingly, also diode lasers emitting in the visible range of the electromagnetic spectrum have entered the market, as for instance the In:Ga.Al:P laser providing 10 mW at 670 nm and 3 mW at 635 nm. It can be expected that shorter-wavelength diode lasers will become readily available; at present blue-violet-emitting diodes are already being produced [7].

A second important development is the introduction of laser systems providing CW output in the deep – and mid – UV region. Large-frame argon-ion lasers can be adapted to provide laser lines at 275, 300, 302 and 305 nm [8], as will be outlined below. Especially the 275 nm line is very interesting. It can be used, for instance, for LIF detection of native-fluorescent proteins in single cells, a subject that has recently been reviewed [9]. Here, the high sensitivity of CE-LIF is required because of the extremely small samples (the cells) of typically 1 picoliter. Peptides and proteins are intrinsically fluorescent when they contain the aromatic amino acids tryptophane or tyrosine, or signal transduction chemicals such as catecholamines. The difference in emission characteristics of these moieties can be used to distinguish different types of proteins when using wavelength-resolved CE-LIF [10]. Their extinction coefficients are usually rather poor, but the high laser intensities ensure efficient excitation [11]. Examples of proteins analysed in single cells are insulin [12] and several haemoglobin variants in single erythrocytes by CZE [13,14], or capillary isoelectric focusing (cIEF) [15]. Furthermore, various biopharmaceuticals [16], catecholamines [17], and nucleic acids [18] were analysed by CE with UV-LIF detection, and – last but not least – it can also be applied to a variety of organic compounds of environmental interest, as will be shown below. Another interesting line of development concerns intracavity frequency doubling of the visible lines of the Ar^+ laser, producing for instance CW output at 257 nm. The performance of CE-LIF at 257 nm is illustrated in a recent paper from Sweedler's group [19].

Pulsed lasers

The appropriateness of pulsed lasers for CE-LIF detection depends greatly on the repetition rate, *i.e.* the number of pulses provided per second. Repetition rates are typically up

to 100 Hz for XeCl excimer lasers (providing pulses of 15 ns at 308 nm) and 10 Hz for first-generation Nd:YAG lasers (providing pulses of 5-10 ns at 1064 nm, which can be doubled, tripled and quadrupled to 532, 355 and 266 nm, respectively). Such laser systems are still very expensive and often require a special laboratory infrastructure. We are currently evaluating a relatively cheap frequency-quadrupled Nd:YAG laser, emitting narrow pulses of only 0.3 ns at a repetition rate up to about 10 kHz ant 266 nm (not tunable). Its average power of only 5 mW is amply sufficient to obtain good CE-LIF results for PAH metabolites [20].

Pulsed laser systems typically have extremely low duty cycles, in other words, they are "off" some 99.999 % of the time. The laser pulse powers can be extremely high (10-100 MW), but in CE-LIF with its small detection volumes only a negligible fraction of the available laser output can be used in order to avoid absorption saturation and/or analyte photodecomposition. A potentially interesting aspect of such high peak powers is that LIF detection can be performed using multiphoton-excitation processes. This approach will be discussed in more detail below.

With pulsed lasers, wavelength tunability tends to be less of a problem than in the case of CW lasers, although it is far from being fully tackled. Pulsed lasers are better suited for pumping dye lasers, yielding typically a 50 nm tuning range per dye. The dye laser output can subsequently be frequency-doubled to reach the UV region. Within this context we should mention the introduction of high-repetition rate Ti:sapphire lasers which (in combination with frequency-doubling and tripling techniques) provide a broad tunability range from the deep-UV to the NIR. These solid-state lasers, pumped by Nd:YAG ou Nd:YLF lasers, do not require the laborious change of laser dyes, and emit pulses of, for instance, a few ps or sub-ps pulsewidth at a repetition rate up to 100 MHz [21,22].

Environmental and bioanalytical applications

Analysis of naphthalenesulphonates, wavelength-resolved detection

Recently we explored the potential of CE in the field of environmental analysis by using it for the separation and identification of naphthalenesulphonates (NS) in river water [23]. This is a challenging task: typical concentration levels in river samples are of the order of 0.1-1 μg l^{-1}, *i.e.* 10^{-8}-10^{-9} M.

Obviously, in order to handle such concentration levels, LIF is the detection method of choice, although preliminary studies have indicated that the complementary technique of phosphorescence detection provides perspectives as well [24,25]. NS, *i.e.* naphthalenes carrying SO_3^-, OH and/or NH_2 substituents, are used extensively as intermediates in dye manufacturing, and they can also be formed during dye degradation by microorganisms. Furthermore, large amounts of NS, both as such and as formaldehyde polymers, are used as plasticizers in concrete. These compounds can be determined using ion-pair liquid chromatography (LC) but their charged nature makes them well-suited for separation by CE: 23 NS including various isomers could be separated [8,26-28].

NS exhibit native fluorescence in aqueous buffer solutions upon excitation in the UV-region. The emission spectra, though exhibiting few details, are nevertheless characteristic for the individual NS since they are strongly influenced by the $–NH_2$ and –OH groups. This does not apply to the compounds without –OH and $–NH_2$ groups, as the sulphonate groups hardly have any effect on the spectral features.

In the exploratory phase of our study, a pulsed excimer-dye laser system with frequency doubling was used. Based

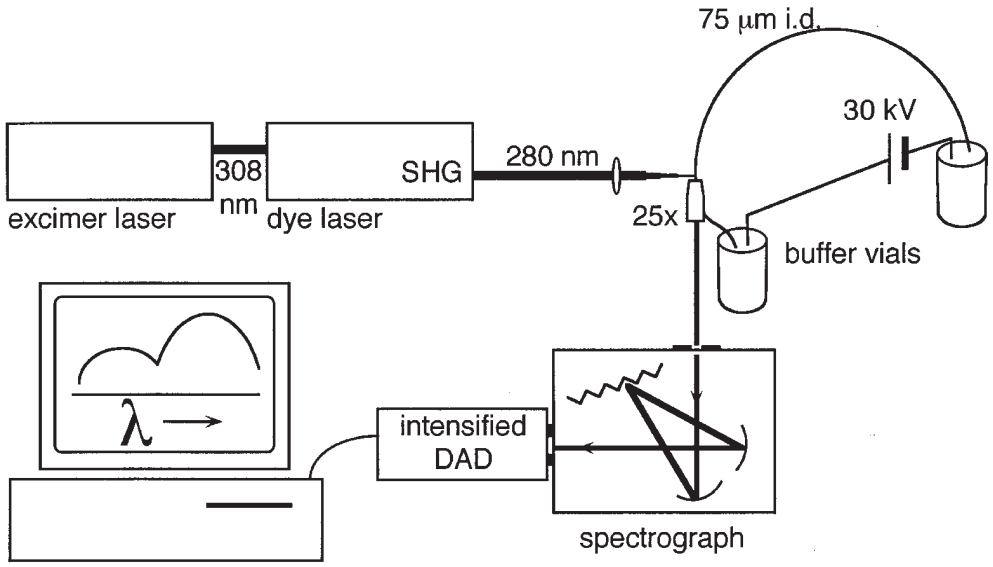

Figure 1. Schematic illustration of CE-LIF setup with pulsed UV excitation from a frequency-doubled XeCl excimer-dyelaser. The fluorescence is collected by means of a reflective microscope objective and coupled into a spectrograph with an intensified linear diode array detector for the on-line recording of emission spectra. From [23] with permission.

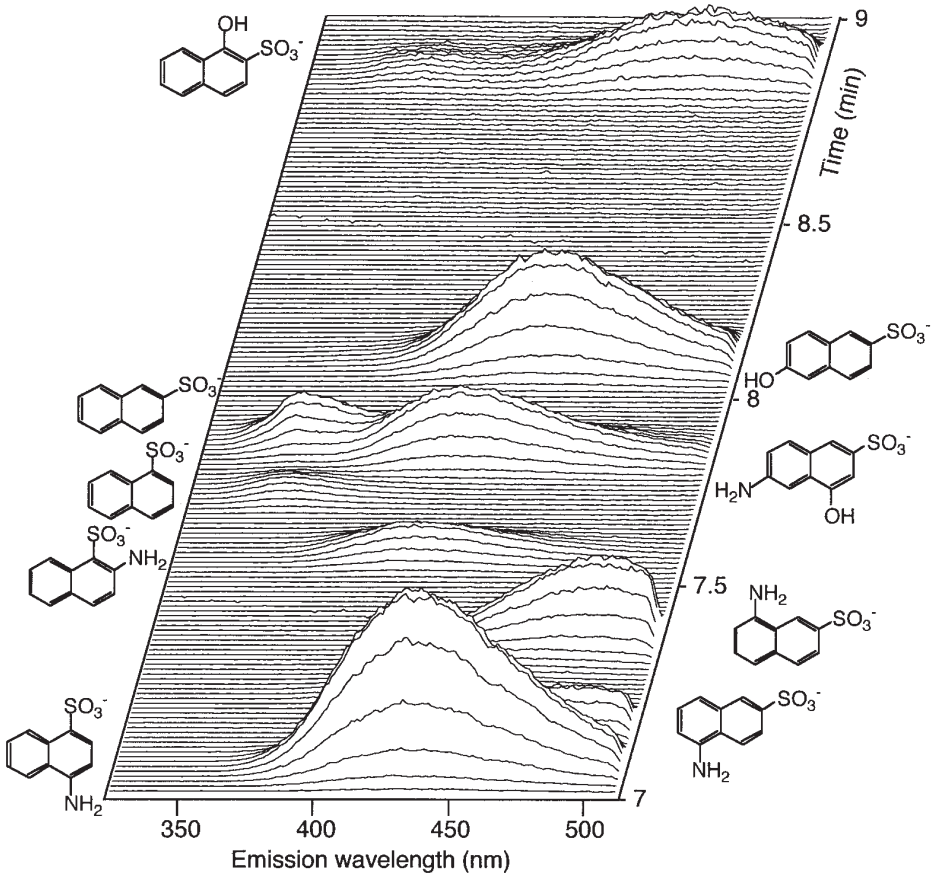

Figure 2. Threedimensional spectro-electropherogram of nine naphthalenesulphonates separated by CE using a borate buffer at pH = 8.75 and 100 mM SDS. For excitation a frequency-doubled XeCl excimer-dyelaser was used at 280 nm. From [27] with permission.

on the absorption characteristics of available NS calibration standards the following wavelengths were selected: 280 and 325 nm, the latter being less sensitive, but more selective for amino- and hydroxy-substituted NS [27]. As outlined above, in addition to electrophoretic migration times characteristic spectroscopic information is required for identification purposes. For this reason, a small spectrograph and an intensified diode-array detector were used to record on-line wavelength-resolved emission spectra. A schematic diagram of the instrumental setup is shown in figure 1. As an illustration of the extra selectivity offered by this setup, figure 2 shows a three-dimensional spectro-electropherogram of a separation of a standard mixture of monosulphonated naphthalenes. The fluorescence emission spectra are sufficiently different to enable tentative identification of the NS considered. For standard mixtures the NS detection limits obtained were in the 0.4-10 µg/l range. Although this level of sensitivity was promising, it was realized that background fluorescence and interferences often hamper detection in real samples. Hence, for spiked river water an off-line solid-phase extraction procedure was applied for trace enrichment of the NS and to remove matrix interferences. An enrichment factor of 30 could be achieved and the resulting detection limits were in the 0.1-2 µg/l range. Obviously these figures reflect both recoveries for the individual NS and differences in background interference from real samples compared to standard solutions. The procedure was successfully applied to river Elbe samples taken in Germany at different sites *i.e.* at 4 km, 475 km and 629 km from the Czech border. Three NS compounds could be identified based on migration time and comparison of their fluorescence spectra with available standard compounds [27].

CW excitation versus *pulsed excitation*

The conclusion of the above results was that the application of CE-LIF for trace analysis presumably requires CW excitation instead of pulsed lasers with a very low repetition rate. Therefore, in the next LIF study a 100 mW, frequency-doubled argon ion-laser was used, emitting at 257 nm. Optimum results were obtained by applying only a minor fraction of the available power, for instance 5 mW for the CW laser. A crucial factor was the use of a mirror-based microscope objective for emission collection, which (in contrast with the lens-based objectives that were also tested) did not produce impurity fluorescence background due to 257-nm scattered light. As expected, for standard solutions the CW laser performed significantly better than the pulsed system (20-fold lower detection limits). However, rather disappointingly, this improvement was not observed for river water samples. It was concluded that the wavelength applied was too short for these natural samples. At 257 nm a large number of interferences – presumably humic acids exhibiting native

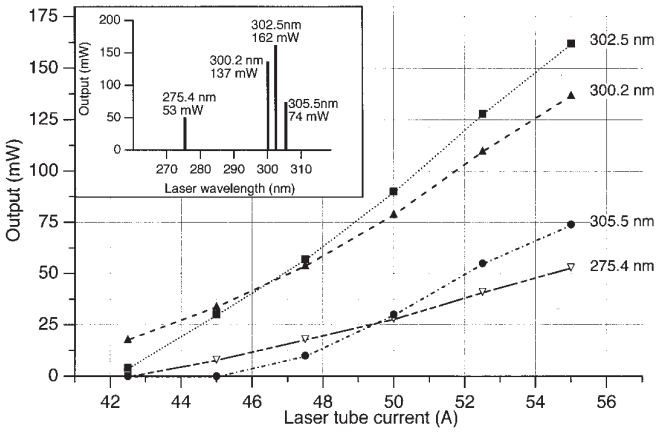

Figure 3. Deep-UV output from an Ar^+ laser after fitting a UV-coated mirror set; the lines were selected by means of a laser filter monochromator. The insert shows the total output spectrum at a maximum laser tube current of 55 A. From [8] with permission.

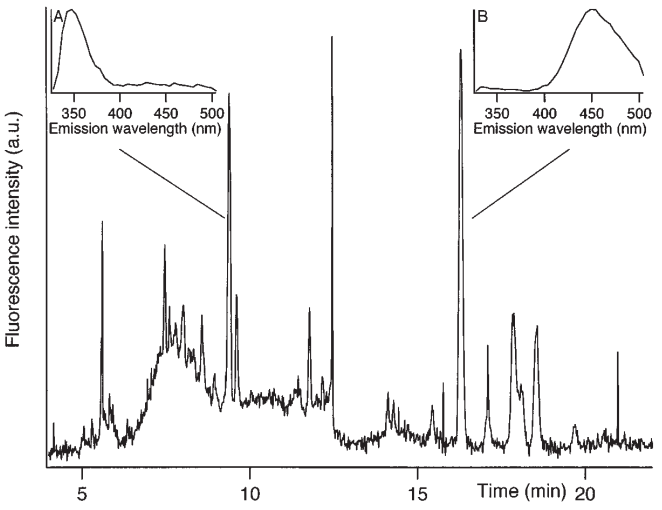

Figure 4. Electropherogram of a wastewater sample (borate buffer at pH = 8.75) after off-line SPE clean up, but without analyte enrichment. CW excitation at 275.4 nm. Two NS compounds were detected at 9.4 (A) and 16.3 min (B); their emission spectra are shown as inserts. From [8] with permission.

fluorescence – are efficiently excited, much more efficiently than at 280 nm (the shortest wavelength tested with the frequency-doubled XeCl-dye laser system).

In order to reduce the background fluorescence from interfering substances we decided to test a CW laser emitting at slightly longer wavelengths. Such laser lines can be generated from a standard, large-frame argon ion laser, as will be available in many laboratories [8]. A not-too-complicated modification was needed, *i.e.* the installation of a set of inexpensive, commercially available UV-coated laser mirrors. The modification included a thorough cleaning of the Brewster windows of the laser tube and a careful alignment. As shown in figure 3, four lines were generated in the deep UV, *i.e.* at 275.4, 300.2, 302.5 and 305.5 nm. Thus, the modified system increases the choice of available wavelengths, although real tunability is of course not provided.

As expected, using this laser system at 275.4 nm (3 mW) the 30-fold analyte preconcentration step previously required to detect NS in river water samples was no longer needed. Figure 4 shows the electropherogram of a wastewater sample featuring a large number of peaks. On the basis of their migration times and emission spectra two of these could be identified, *i.e.* a monosulphonated naphthalene at 9.4 min and a disulphonated aminonaphthalene at 16.3 min. These results underline the potential of CE for environmental analysis.

Coupling of CE to fluorescence line-narrowing

An interesting new development that can significantly increase the selectivity of CE is the coupling with fluorescence line-narrowing spectroscopy (FLNS). Under normal, room temperature conditions fluorescence spectra typically consist of broad bands, as illustrated in figure 2. This lack of structural detail is due to inhomogeneous broadening: individual molecules in solution will experience different interactions with their surrounding solvent cages, leading to a broad distribution of electronic transitions. In FLNS a tunable laser is used to select a subset (isochromat) of molecules with identical $S_1 \leftarrow S_0$ transition energies. When the experiment is carried out at cryogenic temperatures this selection is maintained during the lifetime of the excited state and this subset will show line-narrowed fluorescence (see [29,30] for details on the method and applications). FLNS has been applied as a stand-alone technique, for the off-line identification of HPLC fractions, or for the off-line identification of TLC-separated spots [31,32]. Recently, however, Jankowiak and coworkers managed to apply the method to CE-separated analytes [33,34].

A capillary cryostat was designed consisting of a double-walled quartz cell with inlet and return lines for liquid helium. The small dimensions of the inner section ensure rapid cooling to 4.2 K. To allow the sequential characterisation of the separated analytes in the capillary, the cryostat is attached to a translation stage. Fluorescence is collected at a right angle with respect to the excitation laser beam. Further discrimination against scattered laser light and background fluorescence from the capillary walls is obtained by spatial filtering and time resolved detection. For screening purposes, a broadbanded spectro-electropherogram of CE-separated analytes is detected first at ambient temperature (or 77 K), using non-selective UV excitation. Subsequently, the temperature of the capillary is lowered in less than 1 min to 4.2 K and a dye laser is tuned to excite different regions of the $S_1 \leftarrow S_0$ transition for high-resolution FLNS characterisation of the bands of interest.

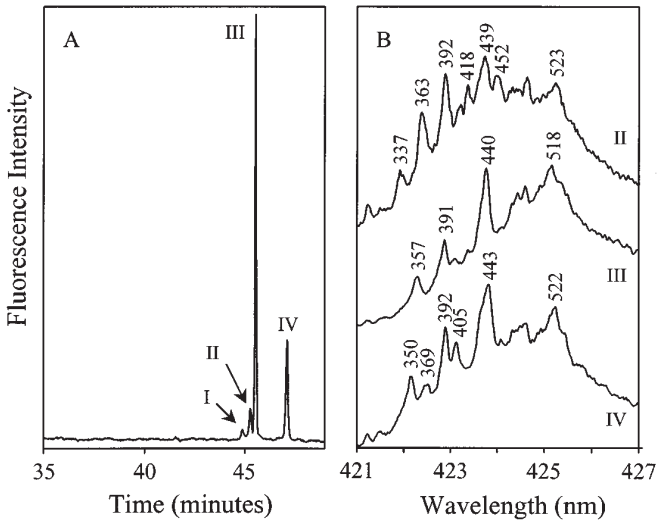

Figure 5. Frame A: CE separation of a mixture of DB[*a,l*]P one-electron oxidation adducts to adenine, detected using LIF at room temperature: (II) DB[*a,l*]P-10-N7Ade, (III) DB[*a,l*]P-10-N1Ade, and (IV) DB[*a,l*]P-10-N3Ade. Peak I is an unidentified compound. Frame B: FLN spectra of the three major peaks, obtained at 4.2 K using selective laser excitation at 416.0 nm. The FLN peaks are labeled with their S_1 excited state vibrational frequencies, in cm^{-1}. From [34] with permission.

The analytical potential of the CE-FLN technique was demonstrated for the analysis of DNA adducts of potent carcinogens such as benzo[*a*]pyrene and dibenzo[*a,l*]pyrene (DB[*a,l*]P). As an illustration, figure 5A shows the CE separation of three isomeric adducts of (DB[*a,l*]P), differing only in the binding position at adenine. The capillary was frozen and the immobilised adduct zones were subsequently analysed by FLNS, as shown in figure 5B. Based on differences between their S_1 excited state vibronic frequencies, peaks II, III and IV could be attributed to DB[*a,l*]P-10-N7Ade, DB[*a,l*]P-10-N1Ade and DB[*a,l*]P-10-N3Ade, respectively, by comparison with previously recorded FLN spectra of the pure individual compounds [34]. It should be stressed that under room temperature conditions the LIF spectra of the three adducts would be *indistinguishable* (compare the spectral resolution and the wavelength scale of figure 5B with that of figure 2).

Multiphoton excited LIF detection

A fascinating alternative to deep-UV excitation is the use of two or three-photon excitation processes: two or three visible or infrared photons are acting simultaneously to bridge the energy gap between the S_{0-} and S_1-electronic states of the analytes concerned. The applicability of this approach was explored some ten years ago for conventional-size LC, using the visible output of a XeCl excimer-pumped dye laser [35]. The results obtained were promising, even though the efficiency of two-photon excitation is rather low. With two-photon excitation the level of background interference is often substantially lower than in conventional, one-photon excited LIF because the fluorescence emission occurs at shorter wavelengths than the excitation light.

In general, muliphoton excitation requires that two or more photons are absorbed within the uncertainty times (~ femtoseconds) of virtual states, thereby achieving electronic excitation without passing through real states of intermediate energies. If ground-state depletion and photobleaching are insignificant, the fluorescence intensity is proportional to I^n, where I is the laser (peak) and n is the number of photons involved per transition. For this purpose Ti:sapphire lasers are very suitable. This was recently shown by the group of Shear [21,22,36] who used a mode-locked Ti:sapphire laser (~ 100 fs pulses produced once every 13 ns). The laser was operated at an average power of 100 mW and focused to 1 μm spot sizes, thus producing peak irradiances of 10^{12} Wcm^{-2}. By coupling this technique to CE a wide array of native biological fluorophores could be investigated. Samples containing serotonin (5-hydroxytryptamine), melatonin, flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NADH) were reproducibly analysed in extremely narrow capillaries (*i.e.* 5-μm and 1-μm i.d.). Detection limits in 5-μm capillaries ranged from 350 zmol (38 nM) for FAD to 27 amols (1.0 μm) for serotonin. Electropherograms could be recorded in the UV-channel (reflecting three-photon excited fluorescence, 740 nm/3) and the visible channel (two-photon excited fluorescence, 740 nm/2). Also zeptomole quantities of fluorogenically labelled neurotransmitters could be analysed using multiphoton excitation [22]. These results indicate the potential of CE-multi-photon excited fluorescence for single cell analysis to correlate levels of various peptides, proteins and monoamine neurotransmitters, a field that will undoubtedly receive extensive attention in the next decade.

Conclusions

The results above indicate that CE-LIF is certainly applicable to trace analysis in environmental and bioanalytical samples, provided that efficient UV lasers are available for selective excitation, preferably at various wavelengths. CW lasers or lasers with high duty cycles are preferred in order to avoid absorption saturation. Another possibility is the use of multi-photon excitation; the fact that fluorescence can be measured at wavelengths shorter than the (visible or near-IR) excitation light often leads to reduced backgrounds. The combination with wavelength-resolved detection is very important to enable (tentative) identification of natively fluorescent analytes in complex samples and to avoid false positive determinations based on migration times only. Fingerprint identification can be achieved when CE is coupled to fluorescence line-narrowing spectroscopy.

For many analytical laboratories the main obstacle for CE-LIF to become a routine tool for natively fluorescent analytes is the heavy laser equipment required (both

investment and running costs). Current experiments in our laboratory suggest that some of these problems may be overcome in the next couple of years. We refer to the use of the relatively inexpensive, rugged, small-size quadrupled Nd:YAG laser providing a 266 nm output of several mW and a 10 kHz repetition rate.

Acknowledgements

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