Research Article

A simple light-emitted diode-induced fluorescence detector using optical fibers and a charged coupled device for direct and indirect capillary electrophoresis methods

We constructed a simple fluorescence detector for both direct and indirect CE methods using a blue light-emitted diode (470 nm) as excitation source, a bifurcated optical fiber as a waveguide, and a CCD camera as a detector. The connection of all the components is fairly easy even for nonexperts and the use of a CCD camera improves the applicability of this detector compared to the others using PMTs because it permits the recording of 2-D electropherograms or phosphorescence measurements. This detector provides a compact, low cost, and rapid system for the determination of native fluorescence compounds which have high quantum yields by CE with direct fluorescence detection, showing an LOD of $2.6 \times 10^{-6}$ M for fluorescein; the determination of fluorescence derivative compounds by CE with direct fluorescence detection, showing an LOD of $1.6 \times 10^{-7}$ M for FITC-labeled 1,6-diaminohexane; and non-fluorescence compounds by CE with indirect fluorescence detection with an LOD of $2.7 \times 10^{-6}$ M for gallic acid.

Keywords: Capillary electrophoresis / Derivatized fluorescence detection / Indirect fluorescence detection / Light-emitted diode-induced fluorescence / Native fluorescence detection DOI 10.1002/elps.200500701

1 Introduction

CE has developed into a versatile and powerful tool in the area of separation science [1, 2]. Various detection methods, including absorption, fluorescence, electrochemistry, and MS have been used for CE [3]. Among all of these detection methods, fluorescence-based systems present the highest sensitivity and are used in the field of routine biotechnology analysis [4].

The discharged light sources (e.g., xenon lamp), widely used in conventional fluorometers, present wide optical spectrum output to match the maximum excitation wavelength of the target analyte [5], but LIF leads to a remarkable improvement in the sensitivity of detection compared with the use of conventional incoherent light sources [6]. However, for analytes which are either inherently fluorescent or derivatized using a fluorescent label, it is necessary to choose readily available lasers that can be matched to their spectral properties. In addition, conventional lasers are generally expensive, relatively bulky, and have short lifetimes (~3000 h). Laser diodes (LDs) are much less costly, compact, have good output stability, longer lifetime, and require little or no maintenance, but the current cost of a violet LD is still expensive (US$ ~2000, depending on the output power and lifetime). In contrast, light-emitted diodes (LEDs) are reasonably priced and they can be operated with battery power, so the output stability is significant better than that of currently used laser and they have reasonably high intensity, very small size, and very long lifetime. In addition, LEDs at a variety of wavelengths in the UV-visible-near infrared have become commercially available (between 350 and 950 nm). The combination of all these good characteristics makes LEDs an exceptionally attractive stable excitation light source for luminescence detection in CE [7].
Tong and Yeung [8] first reported the application of a red LED (660 nm) to develop an absorption detection system for a CE separation. The first detector based on an LED source which could be used in CE separations was proposed by Dasgupta et al. [9]. Later, Kist et al. [10] reported the performance of a fluorescence detector in CE using an LED as excitation source for analyzing peptides. Lin et al. [11, 12] used a blue LED (467 nm) for the direct detection of riboflavin in urine and in beer by LED-induced fluorescence (IF) detection and a violet LED (410 nm) for the determination dopamine and NDA-labeled reserpine mixtures by CE [13], suggesting that LEDs have great potential for use as new light sources in CE separations for naturally fluorescent compounds as well as for derivative ones.

In addition, Chen et al. [14] developed a method for the simultaneous determination of cationic and anionic solutes by LED-based indirect fluorescence detection in CE, amplifying the applicability of the LED-CE to indirect determinations.

All these LED-IF-CE detectors use plastic lenses or microscopes to focus the light from the LED to the capillary and also to collect the fluorescence emission from the capillary to the detector (which is usually a photomultiplier). It is not easy to focus the light into the capillary or transport the emitted light to the detector but the use of optical fiber for transporting the excitation light and collecting the emission fluorescence makes the optical system compact, simple, and flexible. Therefore, Yang and Guan [15] proposed the use of lenses to focus the emanating light from the LED into the capillary and an optical fiber which has a spherical end to develop an LED-IF detector for CE [15]. However, they demonstrated the difficulties for aligning the capillary with the excitation slits and the optical fiber and designed a T-style detection cell to align the optical fiber with the capillary.

In this paper, we propose a simple LED-IF CE-detector based on an LED as excitation source, an optical fiber probe to transport the excitation light form the LED to the capillary and collect the fluorescence emission and a CCD camera as a detector to improve its applicability compared to others using photomultiplier tubes (PMTs). All the components of the proposed LED-IF-CE detector are commercially available and reasonably priced. The connection of all the components is fairly easy and can be performed even by nonexperts. To demonstrate the applicability of the LED-IF-CE detector, it was tested for separating and determining native fluorescence compounds and fluorescence-labeled compounds (direct CE methods) and nonfluorescence compounds (indirect CE method).

2 Materials and methods

2.1 Materials and CE system

Fluorescein (FLU) and FITC isomer I (FITC) were obtained from Scharlau (Barcelona, Spain). FLU and FITC stock solutions (1.0 \times 10^{-2} M) were prepared by dissolving these compounds in acetone. Agmatine, 1,6-hexanediamine, cadaverine, and putrescine were obtained from Sigma (St. Louis, MO, USA). Stock standard solutions of different concentrations of a mixture of all analytes were prepared in doubly deionized water. Other groups of analytes studied were organic acids (fenulic, gallic, and protocatechuic acids), obtained from Sigma (Barcelona, Spain). Stock standard solutions of different concentrations of a mixture of all analytes were prepared in doubly deionized water. All the solutions were kept in a refrigerator at 4°C and fresh solutions were prepared daily.

Sodium carbonate/bicarbonate was purchased from Panreac (Barcelona, Spain) used for the derivatization procedure while a sodium borate solution from Panreac was used for the separation methods. Isopropanol and acetone HPLC grade were obtained from Scharlau.

All solutions were filtered through a 0.45 \mu m Millipore (Bedford, MA, USA) membrane filters before injection into the capillary. Doubly distilled water was obtained by Milli-Q water purification system.

A Prince CE system (Prince Technologies, Emmen, The Netherlands) was used with 75 \mu m inner diameter; uncoated capillaries were purchased from Composite Metal Services (Worcester, England) with a total length of 57 cm and an effective length of 50 cm. The system comprises a 0–30 kV high-voltage built-in power supply.

2.2 Optical system

A bifurcated optical fiber probe was used to focus the light emitted by the LED to the capillary and also to collect the fluorescence emission from the capillary to the CCD camera. Figure 1a shows the optical arrangement of the LED-IF-CE detector.

A LS-450 Blue-LED (Ocean Optics, Europe, www.oceanoptics.com) pulsed light source which produces either pulsed or continuous output at 380, 395, 470, 518, 590, or 640 (depending where the LED was placed into the device) was used as excitation source. The illumination system was also equipped with a low-pass filter at 500 nm and an SMA connector to screw one of the branches of the optical fiber probe. The LED light used was the LED-470 (Ocean Optics, Europe, www.oceanoptics.com) which emits light at a maximum of 470 nm.
A 1/2” Industrial Fluorescence Probe (from Avantes BV, www.avantes.com) was used to transport the excitation and emission lights (see Fig. 1b). A branch of the probe which has 12 UV/VIS fibers of 200 μm-external diameter (called the illumination branch) was connected via SMA-905 with the LS-450 Blue-LED. The other branch, which is equipped with a 600 μm-external diameter fiber and is called the detection branch, was also connected via SMA-905 with the CCD spectrometer. The end of the probe consists of a stainless steel cylinder, 1/2” diameter, containing a 10 mm diameter x 1 mm thick sapphire windows with antireflection coating and the 12 illuminating 200 μm-fiber round the single 600 μm-fiber which is in the middle of the tip (see Fig. 1b).

The Fiber Optic Spectrometer AvaSpec-2048 (Avantes BV) is based on the AvaBench-75 symmetrical Czerny-Turner design with 2048 pixel CCD detector array. The spectrometer has an SMA fiber-optic entrance connector, a collimating and focusing mirror and a UB (UV/VIS: 200–800 nm) diffractional grating with a blaze at 0 nm and a slit of 500 μm. The CCD detector is connected to an electronics board with a 14-bit AD converter and USB/RS-232 interface. A 15-pin digital IO connector enables external triggering and control of shutter and pulsed light sources.

Avasoft-Full software controls the spectrometer and saves the emission spectra. The software also permits the recording of eight different time-drive functions simultaneously, thus permitting the recording of the emission intensity at eight different wavelengths versus time, simultaneously, or seven wavelengths and a complete emission spectrum versus time, etc.

### 2.3 Detection cell

A homemade detection cell was developed to align the capillary with the optical fiber probe (see Fig. 2). This cell consists of a T-style detection cell similar to the cell proposed by Yang and Guan [15]. Two 1/2” diameter channels was perforated at 90° in a 25 mm x 25 mm x 13 mm piece of methacrylate and painted in black color. Two septums were fixed to maintain the correct position of the capillary in the middle of the inch channel. A detection window (about 1 cm length) was formed in the capillary by burning off the polyimide coating and this window was placed in front of the probe which was placed at 90° to the capillary.

One of the more important parameters to be in mind to make a detection cell is the distance between the tip of the probe and the capillary. In our case, the minimum
distance provides the best sensitivity. We observed that a
distance of 2 mm provides an LOD of 28 µM for FLU, and
when this distance is reduced at 0.05 mm, the LOD is
2.6 µM. Thus, the minimization of the distance between
the tip of the probe and the capillary decreases the LOD
ten times.

The distance between the tip of the probe and the capil-
lary was adjusted with a solution of 10^{-6} M FITC running
through the capillary by pressure flow. A screw was used
to fix the position of the probe.

2.4 Methods

At the beginning of each experimental session, the capil-
lary was rinsed with 0.1 M NaOH for 5 min, followed by
1 min with Milli-Q water. The capillary was equilibrated
with the separation buffer for 5 min before each sample
injection and rinsed with 0.1 M sodium hydroxide for
1 min and flushed with Milli-Q water for 1 min after each
run with a 1000 mbar pressure.

For the direct determination of FITC and FLU the separa-
tion was performed using a running buffer formed by
20 mM Na_2B_4O_7 (pH 9.2), samples were injected hyd-
dynamically during 10 s and detection was carried out by
on-column measurement at 520 nm with an integration
time of 200 ms and average of 5. The voltage applied was
25 kV. The capillary was conditioned before each run with
1 min sodium hydroxide 0.1 M, then for 1 min with water,
and finally for 3 min with the separation buffer.

The derivatization procedure for FITC-labeled amine was
described by our research group in a previous paper [16],
which described the optimization of all parameters that
may affect the FITC-labeled amine reaction. It consists in
the addition of 495 µL of 0.2 M carbonate buffer (pH 9),
1000 µL FITC solution (1 \times 10^{-2} M) and 1000 µL acetone

3 Results and discussion

We have developed three applications using different
compounds to demonstrate the potential of this config-
uration obtaining a considerably good LOD for each case.

3.1 Application to native fluorescent
compounds

FLU and FITC were used as models for native fluores-
cence compounds to evaluate the performance of the
detector. Figure 3 shows the excitation and fluorescence
emission spectra of 10^{-2} M FITC and 10^{-2} M FLU, both in
acetone matrix. FITC emits strong fluorescence at a
maximum wavelength of 486 nm when it is excited with a
maximum wavelength of 453 nm and FLU shows maxima
excitation and emission wavelengths at 511 and 529 nm,
respectively.

This figure also shows the emission spectra of the LED
used in this work (LED-470, Ocean Optics). It emits light
between 430 and 490 nm with maximum emission inten-
sity at 470 nm. It appears that this light source is well
matched to excite both FLU and FITC. So, using a 470 nm
blue-LED it is possible to excite both FLU and FITC and
the CCD detector can record the fluorescence emission
at 520 nm.
Figure 3. Excitation and emission spectra of (---) $10^{-6}$ M FLU and (–––) $10^{-6}$ M FITC in acetone/water (50:50 v/v). Scan rate 1200 nm/min, both excitation and emission slits width of 5 nm; detector voltage of 530 V. Also the emission spectra of the (· · · · · · ·) LED-475 which was used to obtain all the electropherograms (integration time 3 ms and average 10).

Figure 4 shows the separation of FITC and FLU ($3 \times 10^{-5}$ M) by LED-IF-CE-based on a normal CZE separation and fluorescence detection. Herein, the CE buffer was formed by 20 mM Na$_2$B$_4$O$_7$ at pH 9.2. The sample was prepared in water containing $3 \times 10^{-5}$ M of FLU and FITC. Hydrodynamic injection was achieved for a period of 10 s at 50 mbar. When the injection was completed, 25 kV was applied to power the CE separation and the current achieved was 52 µA. The fluorescence emission was recorded at 520 nm with an integration time of 200 ms and an average of 5.

Figure 5 shows the effect of the integration time (Fig. 5a) and average (Fig. 5b) versus the LOD of the method. Figure 5a shows that an increase of the integration time decreases the LOD between 10 and 100 ms. When the integration time is higher than 100 ms, the LOD is practically constant. Integration times higher than 250 ms cannot be used due to saturation of the CCD detector. Thus, an integration time of 200 ms was set.

Figure 5b shows that an increase of the average does not affect the LOD of the proposed method, but affects the error of the measurement. Thus, five averages were selected in order to decrease the error and to keep an appropriate number of points to draw the electrophoretic peaks (integration time 200 ms × 5 averages means 1 point by 1 s, so, 20 points to draw a peak).

In the range of $2.6 \times 10^{-6}$ and $8.0 \times 10^{-5}$ M, the linear relationships was good for FLU; the calibration coefficient $r$ was 0.994. The repeatability was tested by the detection of $10^{-5}$ M FLU and the RSD was 3.2%, which seemed to be related more to errors in the volume injected than the detection system. A LOD of $2.6 \times 10^{-6}$ M FLU was determined using the IUPAC method LOD = $3 \times s_0/b$, where LOD is the LOD, $s_0$ is the SD of ten replicates of blank solutions and $b$ is the slope of the calibration curve.

Although the LOD was worse than that usually achieved by LIF-CE and worse than that of the normal fluorometer using discharge light sources or similar designs [9, 15], the LED-IF detector provides several advantages such as...

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as being able to measure more than one emission wavelength simultaneously. It also permits the recording of the full emission spectra providing 3-D-electropherograms which shows intensity, emission spectra, and migration time in the same representation and permits the measurement of phosphorescence due to LED being pulsed.

The principal cause of low detection limit may be the low power of the LED. Two different aspects should be modified to improve the LOD. From an instrumental point of view, more powerful LEDs which can be easily implemented on commercially available fiber-optic couplers, and more sensitive CCD detectors and optical fiber probe which can collect more efficiently the fluorescence emission should improve the sensitivity of the proposed detector. On the other hand, from an experimental point of view, an increase of the injection times and the use of on-line pre-concentration techniques, such as staking techniques, should also increase it.

Therefore, the LED-IF detector which uses optical fiber as waveguides and a CCD camera could be used to analyze native fluorescence compounds which show high quantum yields and excitation wavelengths close to the available LEDs. New increased-power LEDs [17] and novel bifurcated fiber probes which show lower light loss are, also being studied to increase the sensitivity of the detector.

### 3.2 Applications to labeled fluorescence compounds

Although FITC can be determined by the proposed LED-IF-CE detector and this is one of the most frequently used labeled compounds for determining nonfluorescent amines, the proposed LED-IF-CE was used to determine biogenic FITC-labeled amines.

Four nonfluorescent biogenic amines were selected as representatives of this group because they are normal constituents of many foods (cheese, wine, beer, fishery products, and in bad-condition meat) [18, 19] and their presence in high amounts is associated with food deterioration [20].

Agmatine (2 × 10⁻⁶ M), 1,6-hexanediamine (4 × 10⁻⁶ M), cadaverine (2 × 10⁻⁶ M), and putrescine (1 × 10⁻⁵ M) before CE separation were derivatized following the procedure described in Section 2.4. The LED-475 was also used to determine biogenic FITC-labeled amines.

The analysis of a mixture of biogenic amines by LED-IF-CE is shown in Fig. 6. The CE buffer used consisted in a 50 mM borate at pH 9.2 with a 20% isopropanol. Hydrodynamic injection was achieved for a period of 10 s at 50 mbar. When the injection was completed, 25 kV was applied to power the CE separation. The fluorescence emission was recorded at 520 nm. The integration time and average were also optimized. The same results as in Fig. 5 were obtained. Thus, an integration time of 200 ms and an average of 5 were used.

The system exhibited linear response for the concentration ranges between 1.6 × 10⁻⁷ and 1.3 × 10⁻⁴ M with a calibration coefficient, r of 0.995 and an LOD of 1.6 × 10⁻⁷ M for FITC-labeled 1,6-diaminohexane.

This result demonstrates the suitability of the described detection system for both qualitative and quantitative routine analyses and also suggests that the same system can be used as a simple IF detector not only for compounds which show native fluorescence but also for derivatives as well.

### 3.3 Applications to nonfluorescence compounds: indirect fluorescence determination

The third fluorescence-induced CE methodology which is able to determine nonfluorescence compounds is called induced indirect fluorescence-CE (IIF-CE) [21]. This methodology is based on the fluorescence measurement of a high-quantum yield fluorescent dye called “probe” which is displaced in the capillary during the separation of non-fluorescent compounds. Negative peaks are recorded when nonfluorescence compounds are crossing the detection cells whose areas or negative intensities are proportional to the concentration of the target analyte [22].

![Figure 6. CE analysis of a mixture of fluorescent-labeled compounds.](image)

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Although FITC can be determined by the proposed detector and can be used as a probe due to its very high quantum yield, another application consisting in the determination of nonfluorescence organic acids by LED-IIF-CE is proposed.

Three nonfluorescent phenolic acids (ferulic, gallic, and protocatechuic acid at $1 \times 10^{-6}$ M each one) were selected as representative compounds. Recent studies have shown that many dietary phenolic constituents derived for plants are effective antioxidants due to their redox properties. Therefore, fortification of diets with food materials rich in phenolic compounds was shown to impart antimutagenic, antiglycemic, and antioxidative properties [23].

The analysis of a mixture of carboxylic acids by LED-IIF-CE is shown in Fig. 7. The CE running buffer used consisted in a 60 mM borate (pH 8) and $2 \times 10^{-4}$ M FITC solution. Hydrodynamic injection was achieved for a period of 10 s at 50 mbar. When the injection was completed, 25 kV was applied to power the CE separation. The fluorescence emission was recorded at 520 nm illuminating the capillary with the LED-470. The integration time and average were also optimized. The same results as in the capillary with the LED-470. The integration time and average were also optimized. The same results as in Fig. 5 were obtained. Thus, an integration time of 200 ms and an average of 5 were used.

The system exhibited linear response for the concentration ranges between $2.7 \times 10^{-6}$ and $0.8 \times 10^{-4}$ M with a calibration coefficient, $r$ of 0.994, and an LOD of $2.7 \times 10^{-6}$ M for gallic acid. This result demonstrates the suitability of the described detection system for indirect fluorescence determination.

![Electropherogram of three carboxylic acids: (1) ferulic acid, (2) gallic acid, and (3) protocatechuic acid ($1 \times 10^{-6}$ M). CE conditions: 60 mM borate (pH 8) and $2 \times 10^{-4}$ M FITC buffer, 25 kV, and 10 s injection time. EOF: Electrosmotic flow. Fluorescence detection was carried out by on-column measurement at 520 nm with an integration time of 200 ms and average of 5.](image)

**Figure 7.** Electropherogram of three carboxylic acids: (1) ferulic acid, (2) gallic acid, and (3) protocatechuic acid ($1 \times 10^{-6}$ M). CE conditions: 60 mM borate (pH 8) and $2 \times 10^{-4}$ M FITC buffer, 25 kV, and 10 s injection time. EOF: Electrosmotic flow. Fluorescence detection was carried out by on-column measurement at 520 nm with an integration time of 200 ms and average of 5.

### 4 Concluding remarks

A simple LED-IF CE-detector based on an LED as excitation source, an optical fiber probe to transport the excitation light from the LED to the capillary and collect the fluorescence emission, and a CCD camera as detector to improve the applicability of this detector is presented in this paper. This detector provides a compact, low cost, and rapid system for the determination of native fluorescence compounds which have high quantum yields by CE with direct fluorescence detection; the determination of fluorescence derivatives compounds by CE with direct fluorescence detection; and nonfluorescence compounds by CE with indirect fluorescence detection. It also allows measuring more than one emission wavelength simultaneously and permits the recording of the full emission spectra providing 3-D electropherograms which shows intensity, emission spectra, and migration time in the same representation. In addition, the LED-IF-CE detector permits the measurement of phosphorescence due to LED being pulsed and the CCD being triggered with the excitation source.

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### 5 References


