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Pulsed lasers versus continuous light sources in capillary electrophoresis and fluorescence detection studies: Photodegradation pathways and models

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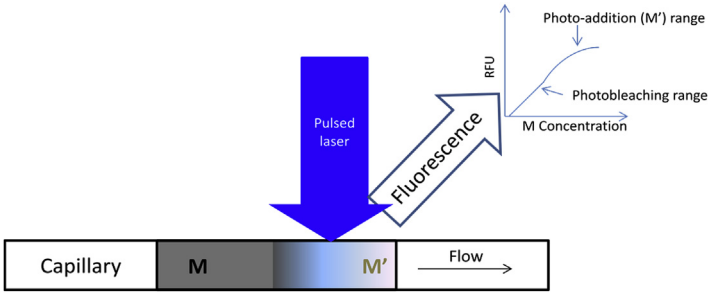
HIGHLIGHTS

- No linear calibration curves are obtained in CE/Pulsed-LIF detection.
- Photodegradation and photodimerisation are responsible of this non linearity.
- A mathematical model of this phenomenon is presented.
- 7 hydroxycoumarin in CE/LIF is used to verify the mathematical model.
- UV LEDs used in continuous light mode are a good alternative to UV pulsed lasers.

GRAPHICAL ABSTRACT



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ABSTRACT

Pulsed lasers are widely used in capillary electrophoresis (CE) studies to provide laser induced fluorescence (LIF) detection. Unfortunately pulsed lasers do not give linear calibration curves over a wide range of concentrations. While this does not prevent their use in CE/LIF studies, the non-linear behavior must be understood. Using 7-hydroxycoumarin (7-HC) (10–5000 nM), Tamra (10–5000 nM) and tryptophan (1–200 μM) as dyes, we observe that continuous lasers and LEDs result in linear calibration curves, while pulsed lasers give polynomial ones. The effect is seen with both visible light (530 nm) and with UV light (355 nm, 266 nm). In this work we point out the formation of byproducts induced by pulsed laser upon irradiation of 7-HC. Their separation by CE using two Zeta LIF detectors clearly shows that this process is related to the first laser detection. All of these photodegradation products can be identified by an ESI-/MS investigation and correspond to at least two 7HC dimers. By using the photodegradation model proposed by Heywood and Farnsworth (2010) and by taking into account the 7-HC results and the fact that in our system we do not have a constant concentration of fluorophore, it is possible to propose a new photochemical model of fluorescence in LIF detection. The model, like the experiment, shows that it is difficult to obtain linear quantitation curves with pulsed lasers while UV-

Abbreviations: 7HC, 7-hydroxycoumarin; LIF, laser induced fluorescence; CE, capillary electrophoresis.

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LEDs used in continuous mode have this advantage. They are a good alternative to UV pulsed lasers. An application involving the separation and linear quantification of oligosaccharides labeled with 2-aminobezoic acid is presented using HILIC and LED (365 nm) induced fluorescence.

1. Introduction

Pulsed lasers have been used for laser induced fluorescence capillary electrophoresis (CE/LIF) for fifteen years [1,2]. The most commonly used method employs a 266 nm pulsed laser, and this method can be used for native fluorescence measurements of amino acids and proteins [3,4]. Other pulsed lasers are available. For example in the UV range, at 248 nm, it has been demonstrated that sensitivity depends on the laser light frequency [5]. Sub-picomolar limits of detection were obtained for Rhodamine B isothiocyanate derivatives of amino acids with a 532 nm (visible light) laser [6]. An identical laser was used for the determination of four red food colorants [7] and for doxorubicin and epirubicin detection in cancerous cells [8].

Some authors show that the limitation of LIF sensitivity is due to the photodegradation that takes place during the flowing of the fluorophore through the illuminated zone. Many factors must be considered in optimizing fluorescence detection systems using continuous lasers. White and Stryer [9] presented a theory addressing laser induced photoalteration of dyes in flowing streams of different velocities. They predicted that excitation intensity must change with flow velocity to obtain equivalent photodegradation at all flow velocities. Indeed, it is well known that in CE, a compound passes the detection window at a velocity inversely proportional to the migration time [10]. Shear et al. [11] proposed to compensate the different peak velocities by decreasing both the data-digitization rate and the excitation intensity when separation time increases. Laser induced photodegradation of the sample along the detection was also reported by Mathies, Johnson and Bayle [12–14], each noted the importance of the velocity of the sample in the capillary. Rodat et al. described limits to sensitivity, when using a capillary containing a bubble as detection cell [15].

Most of previous papers present theoretical studies on continuous lasers with light power bellow 100 mW, only few articles report on pulsed laser (having a very high instantaneous power, over 800 W) and on the signal to noise (S/N) ratio variation due to the migration rate or the light power. Schulze et al. [16] reported that an increase of the laser power leads to an enhancement of the S/N ratio at low power and then remains constant, and sometimes S/N decreases at higher power values. They used a 266 nm pulsed laser to study the fluorescence of aromatic species such as serotonin, propranolol, tryptophan and noted that a continuous laser gives better results than a pulsed one at the same wavelength. They indicate that the high excitation power during the pulses leads to fluorescence saturation and photobleaching effects.

Heywood and Farnsworth proposed a calculation model for the obtained fluorescence using the same 266 nm pulsed laser [17]. Theses authors postulated that the photobleached molecule does not fluoresce. It is generally assumed that the photobleaching reactions are not depending on concentration, i.e. the obtained calibration curve of an aromatic compound will be linear. It is noted that in many studies, the calibration curves were obtained using narrow and low calibration ranges (for example, 1–100 nM of tryptophan) [18].

In the present study we will show that in higher and larger concentration ranges, the calibration curves are not linear using pulsed lasers. The detection of products from an on-line illuminated hydroxycoumarin sample results in the identification in CE-LIF of both the fluorescent native molecule and fluorescent by-products. Then, we will adapt the calculations of Heywood and Farnsworth taking into account the formation of by-products upon the illumination of the sample. We show that experimental data fit perfectly with our theoretical calculations. This demonstrates that the non linearity of the calibration curve using pulsed laser, depends on second order kinetic [19,20].

LEDs are more and more used for CE/LEDIF studies, because they are stable, low cost, and powerful light sources [21,22]. Finally, a study using a 365 nm LED (continuous illumination) of a polysaccharide labeled with 2-aminobenzoic acid (2AB) separated by μHILIC (hydrophilic interaction liquid chromatography) will be presented. The linearity between concentration and fluorescence with the 365 nm LED will be demonstrated.

2. Material and method

2.1. Chemicals

7-Hydroxycoumarin (7HC), Tryptophan (Trp), 5-carboxytetramethylrhodamine (Tamra) 2-aminobenzoic acid (2AB), Dextran 5000, glucose, maltose, maltotriose, carbonate buffer, triethylamine, acetic acid, acetonitrile are from Sigma-Aldrich- Fluka (St Quentin Fallavier, France). HILIC column, TSK gel NH_2-100 150 × 2.0 mm, 3 μm, (Tosoh Bioscience, Interchim, Montluçon France).

2.2. Instrumentation

An Agilent Technologies CE7100 instrument (Waldbronn Germany) was equipped with a 75 μm id capillary, effective length 46 cm, total length 65 cm. Zetalif Laser and Zetalif LED detectors from Picometrics Technologies (Toulouse, France) can be equipped with different lasers and LEDs. In this study, for the experiments concerning hydoxycoumarin, we use a pulsed laser Horus 355 nm laser (47 kHz, pulse duration 0.5 ns, 44 mW mean power), and a continuous Cobolt Zouk 355 nm laser (10 mW). The fluorescence collection was achieved with a dichroic mirror 355 nm (bandwidth), and a highpass filter (370 nm–700 nm). CE experiments were realized with 25 mM sodium carbonate/bicarbonate pH = 9.6 as BGE at 20 kV with the 7HC diluted in the buffer at different concentrations, while in flow experiments different 7HC concentration samples were flushed (950 mbars) through the capillary. The relative fluorescence was recorded using the OpenLAB software. All the calculations were carried out using Excel (Microsoft).

266 nm pulsed laser (Teem photonics, France) and 280 nm LED (Picometrics Technologies) connected to the ZETALIF Laser detector were used to analyze Trp samples, 532 nm pulsed laser (Crysta Laser, USA) and continuous LED 530 nm (Picometrics Technologies) were used to analyze Tamra samples.

For the study of the photodegradation of 7HC, the double detection CE experiment used two detection windows on a capillary of 83 cm total length. The first one is localized at 19 cm from the injection end positioned in front of a first ZETALIF detector