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Sensitive absorbance detection method for capillary electrophoresis based on laser wave-mixing

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Abstract

Forward-scattering four-wave mixing is demonstrated as a sensitive absorbance detection method for capillary electrophoresis, using an argon ion laser operating at 457.9 nm. Since this four-wave mixing laser technique utilizes only two input laser beams, it offers important advantages, including ease of optical alignment, high wave-mixing efficiency and low excitation power requirements. In addition, since the analytical signal is a laser-like coherent beam, highly efficient optical signal detection can be performed with minimum optical background noise. Excellent detection sensitivity and short absorption path lengths, and hence, small detector probe volumes, are some of the useful features this absorbance detection method offers for on-column detection of both fluorescing and non-fluorescing analytes in capillary electrophoresis and liquid chromatography. Preliminary "detected" concentration detection limit of $8.5 \cdot 10^{-8}$ *M*, mass detection limit of 13 amol and an absorbance-unit detection limit of $1.35 \cdot 10^{-5}$ AU are determined for dabsyl-glycine using this absorbance detection method.

Keywords: Detection, electrophoresis; Laser wave-mixing; Amino acids, dabsyl derivatives

1. Introduction

Capillary electrophoresis (CE) offers many desirable features including excellent separation power, short analysis time, small detector volume, low solvent consumption and minimal sample requirements for many inorganic, organic and biological analytes [1,2]. However, the optical absorption path length in an on-column detection method is very short (i.e., diameter of the capillary column used), and hence, detection sensitivity available from conventional absorption detection methods is limited due to short optical path lengths, high stray radiation and poor light throughput. When a laser is employed as the excitation source, some of these problems are minimized since a laser beam can be focused into a very small spot to yield extremely small detection probe volumes. Sensitive laser-based detection techniques reported for capillary electrophoresis include laser-induced fluorescence detectors [3–5] and thermo-optical absorption detectors [6–8] that offer detection sensitivity improvements over non-laser optical methods. Although laser-induced fluorescence-based detectors offer excellent detection sensitivity, they are effective only for fluorescing analytes. In a thermal-lens detection method, divergence of a probe laser beam is monitored as an intensity loss at the center of the beam due to formation of a thermo-optical lens in the sample. Hence, the thermal-optical signal is based on the measurement of a

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small a.c. signal over a relative large d.c. background signal. In a laser wave-mixing detection setup, the signal is based on the measurement of an absolute positive signal against a very small or zero background (i.e., no laser-like signal beam is generated when the analyte is absent). In addition, many thermal-lens or thermal-optical detection methods use two independent lasers, one as the pump laser and the other as the probe laser, where as the wave-mixing absorbance detector uses a single laser.

Four-wave mixing is a versatile nonlinear laser technique increasingly used in optics and image processing fields, such as lensless imaging, real-time holography and for characterization of materials, such as third-order nonlinearity measurements [9–12]. Two basic wave-mixing optical configurations, a backward-scattering wave-mixing and a forward-scattering wave-mixing, have been used for generating the analytical signal. In a backward-scattering wave-mixing setup, a forward pump beam, a counter-propagating backward pump beam, and a probe beam, are mixed inside the analyte medium. The fourth beam (i.e., the signal beam) originates inside the analyte medium and retraces the probe beam path. In a forward-scattering wave-mixing setup, all input beams propagate in one direction (i.e., forward), and the signal beam propagates also in the same direction. Since the signal is a coherent laser-like beam, it can be collected and detected very conveniently with excellent optical collection efficiency (virtually 100%). This nonlinear laser spectroscopic technique offers many advantages including high spectral resolution (i.e., Doppler-free with backward-scattering wave mixing for gas-phase analytes), excellent detection sensitivity and ease of signal isolation from optical background noise. In addition, wave-mixing method could detect both fluorescing and non-fluorescing analytes with excellent (e.g., amol) detection sensitivity. Unlike many thermal-lens or thermal-optical detection methods, wave-mixing detection methods require only a single-laser setup, instead of two-color two-laser setups. Since the input laser beams can be mixed conveniently inside a small probe volume, this novel detection method is suitable for on-column detection in various capillary electrophoresis or liquid chromatography systems. We reported applications of backward-scattering wave-mixing as a Doppler-free high-resolution spectroscopic method using hollow-cathode discharge atomizers [13–15] and analytical flames [16–19]. We also demonstrated excellent detection limits for both forward- and backward-scattering wave-mixing detectors using liquid flow cells [20–22].

In this report, we present the use of forward-scattering wave-mixing as a simple one- color one-laser-based absorbance detector for capillary electrophoresis. An argon ion laser operating at 457.9 nm is used as the excitation light source, and amino acids derivatized with dabsyl (4-dimethylamino-azobenzene-4′-sulfonyl chloride) are used as analytes. Dabsyl chloride is a non-fluorescing compound with a molar absorptivity of approximately $3 \cdot 10^4$ M^{-1} cm^{-1} near the 457.9-nm argon laser line. Dabsyl-amino acids are relatively easy to prepare, and are stable under photon illumination [23]. Using our wave-mixing detector, preliminary "injected" detection limits (at $S/N=2$) of 7.2, 3.12, 2.0 and 1.8 fmol, respectively, are determined for dabsyl-labeled histidine, proline, glycine and glutamic acid, using a relatively low total laser power for all input beams of 30 mW. Assuming a 20-fold dilution at the detector end of the column, this corresponds to a preliminary "detected" concentration detection limit of $8.5 \cdot 10^{-8}$ M, a "detected" mass detection limit of 13 amol and an absorbance-unit detection limit of $1.35 \cdot 10^{-5}$ AU for dabsyl-glycine.

2. Experimental

Fig. 1 shows a typical forward-scattering wave-mixing detector interfaced to a capillary electrophoresis system. The excitation light source is provided by a continuous-wave argon ion laser (Coherent, Palo Alto, CA, USA; Model Innova 90-6) operating at the 457.9-nm line. The output of the laser beam is split by a beam splitter ($R:T$, 30:70) to form the input excitation light beams E_1 and E_2. Relative intensities of the two input beams arriving at the detector window, $I_1:I_2$ is 7:3. A 100-mm focusing lens is used to focus the input beams at the detection window. The diameter of the input beam spot on the detector window is 34 μm, and the two input beams intersect with an angle of 1.5°. Two signal beams, E_3 and E_4, are generated propagating

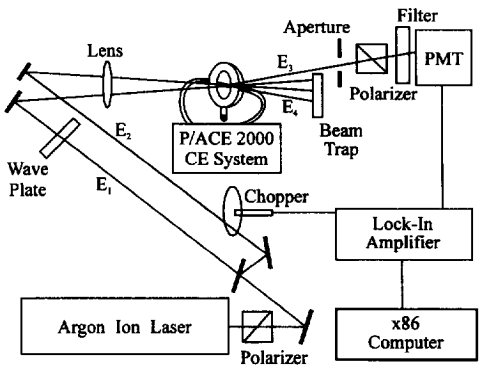

Fig. 1. Experimental arrangement for on-column CE detector based on forward-scattering four-wave mixing.

in the forward direction as that of the input excitation beams E_1 and E_2. The beam intensity of E_1 is made stronger than that of E_2, and hence, the signal beam E_3 is stronger than E_4 [22]. Only the stronger signal beam E_3 is monitored and detected in this study. In order to optimize the signal beam, the difference in beam path lengths (or distances traveled) for E_1 and E_2 beams is kept less than the coherence length of the laser (i.e., 5 cm for the argon ion laser used). A mechanical light chopper (Photon Technology International, Princeton, NJ, USA; Model 03-OC4000) is used to modulate the amplitude of the E_2 beam at 50 Hz. The signal beam is directed to a photomultiplier tube (Hamamatsu, Middlesex, NJ, USA; Model R928) after passing through a focusing lens (250 mm f.l.) and a 457.9-nm laser line filter.

A polarization discrimination scheme, which consists of polarizers, P1, P2 and a quarter-wave plate, is used to minimize the background noise. A small aperture is used in front of the photomultiplier tube to minimize background from the scattering of the E_1 beam. The output signal of the photomultiplier tube is then sent to a current-to-voltage converter and then monitored by a lock-in amplifier (Princeton Applied Research, Princeton, NJ, USA; Model 5207). The output voltage of the lock-in amplifier is sent to a strip-chart recorder and to a personal computer for digitization.

A modified Beckman P/ACE 2000 CE system (Beckman Instruments, Fullerton, CA, USA) is used to interface the wave-mixing detector. The Beckman CE cartridge is modified so that a small piece of the capillary tube is extended and mounted outside the cartridge housing. A small 5-mm section of the protective polyimide coating is removed from the fused-silica capillary tube (75 μm I.D., 360 μm O.D., Polymicro Technology, Phoenix, AZ, USA) and used as the laser illumination zone. This detector window is 44 cm from the cathode end, and hence, the effective length of the capillary tube (injection to detection) is 87 cm. The extended detection capillary loop is mounted firmly to protect the fragile uncoated portion of the fused-silica tube and to allow fine adjustment of the tube inside the laser beam path. Electrophoresis is performed at 28 kV and 17 μA after dabsyl-amino acids are electrophoretically injected at 5 kV for 5 s.

A pH 9.0 buffer solution (0.05 *M*) is prepared from Na_2CO_3 and $NaHCO_3$ in deionized water, and a pH 7 buffer solution (0.02 *M*) is prepared from NaH_2PO_4 and Na_2HPO_4 in deionized water. Amino acid stock solutions (1 μmol/ml) are prepared by dissolving a weighed amount of amino acid in the pH 9.0 buffer solution. Dabsyl chloride is prepared by dissolving 33 mg in 10 ml of acetone. The buffer solution is 50% acetonitrile and 50% phosphate buffer. Amino acids are purchased from Sigma and other chemicals are purchased from Aldrich.

Dabsyl-amino acids are prepared using a procedure reported previously [23]. 1 ml of amino acid stock solution and 1 ml of dabsyl chloride solution are mixed in a test tube, which is then stoppered and placed in a 80°C water bath until the reaction is completed (after 5–10 min). The reaction mixture is then evaporated by passing a nitrogen stream through it while heat is applied. The residue is then redissolved in 1 ml HPLC-grade solvent mixture and stored in the freezer. An extraction procedure used to separate the derivatized dabsyl-amino acids from unreacted dabsyl chloride [24,25] is also examined. After the derivatizing reaction between amino acids and dabsyl chloride is completed, acetone is evaporated from the reaction mixture with a gentle stream of nitrogen. The solution is then transferred to a separatory funnel and extracted twice with diethyl ether, and the organic layer is discarded. Solution pH is then adjusted to 3 using a 0.5 *M* HCl solution. The solution is then extracted five times with diethyl ether. Dabsyl-amino acids are then extracted into the diethyl ether layer at pH 3, and the organic layers are then combined and evaporated with nitrogen. The

dabsyl-amino acids are redissolved in 1 ml buffer solution mixture and stored in a freezer. Finally, the dabsyl-amino acid solutions are filtered through a 0.22-μm filter.

3. Results and discussion

In an absorbing liquid solution, wave-mixing signal is generated more significantly from thermally induced refractive-index gratings than from other nonlinear effects. The wave-mixing signal intensity I_s can be described as [22]

$$I_s \propto C\, I_{pump}^2\, I_{probe} \frac{\lambda^2}{\sin^4\theta}\left(\frac{dn}{dT}\right)^2 \frac{\alpha^2}{k^2} \quad (1)$$

where C is a constant, λ is the laser wavelength, θ is the angle between the two input beams, dn/dT is the temperature coefficient of the refractive index, α is the absorption coefficient of the analyte and k is the thermal conductivity. Eq. (1) describes several important characteristic properties of the wave-mixing signal, including its quadratic dependence on the refractive-index change due to temperature change, and its quadratic dependence on the absorption coefficient, and hence, on the analyte concentration. Eq. (1) also indicates that the wave-mixing signal has a linear dependence on the intensity of probe beam, a quadratic dependence on the pump beam intensity, and hence, a cubic dependence on the total intensity of both input beams.

Since only two input beams are used in this forward-scattering wave-mixing setup, as compared to three input beams in a backward-scattering wave-mixing setup, the optical alignment is unusually simple for a multiphoton method. In addition, the wave-mixing signal beam is strong enough to be visible to the naked eye even at trace- concentration levels, and hence, optical alignment and optimization is straightforward. After the two input laser beams, E_1 and E_2, are adjusted to overlay and mix with each other inside the detection zone, a beam blocker is used to trap the input excitation beams. A relatively high-concentration (e.g., $5 \cdot 10^{-4}$ M) dabsyl chloride "alignment" solution is injected first to generate a signal visible to the naked eye for easy optical and detector alignments. The mirrors are tuned carefully until the signal beam emerges from the capillary tube, and it is then maximized by adjusting the distance between the detector and the focusing lens. This visible signal beam is then conveniently directed to a photodetector. After the optical alignment is completed using this alignment solution, the analytes of interest are injected, separated and detected using the usual electrophoresis separation procedures.

The use of a single short-focal-length lens for simultaneous focusing and mixing of the two input beams inside the capillary cell significantly simplifies the optical alignment. Hence, the optical alignment is not much more difficult than that of a single-beam optical setup. The signal collection is even easier than in a conventional single-beam setup (e.g., fluorescence), since the wave-mixing signal is a laser-like beam and visible to the naked eye. By using a micro aperture-based spatial filter, the bright coherent signal spot can be easily extracted from the relatively large optical fringe pattern generated by the capillary. We have successfully used various capillary columns of commonly available sizes (e.g., from 250 μm to 50 μm I.D. so far) without any significant problems in both optical alignment and signal collection.

In a wave-mixing experiment, the input pump beam constructively interferes with the probe beam in the capillary cell to form a thermal grating, off which the "second" photon of the same pump beam is scattered to generate the signal. Thus, the polarization of the signal beam depends on the polarization planes of the input beams. When both pump and probe beams have the same polarization plane, the polarization of the signal beam is identical to the polarization of the input beams to satisfy the polarization conservation rule. When the probe beam is linearly polarized and the pump beam is circularly polarized, the signal beam has the same polarization as that of the pump beam. Taking advantage of the polarization property of the signal beam, a polarization discrimination scheme is effectively used in our detector setup to minimize the background noise. This is achieved by inserting a pair of crossed polarizers in front of the laser and the photomultiplier tube, and then a quarter-wave plate in the pump beam path. A mechanical chopper is used to modulate the probe beam so that any "modulated"

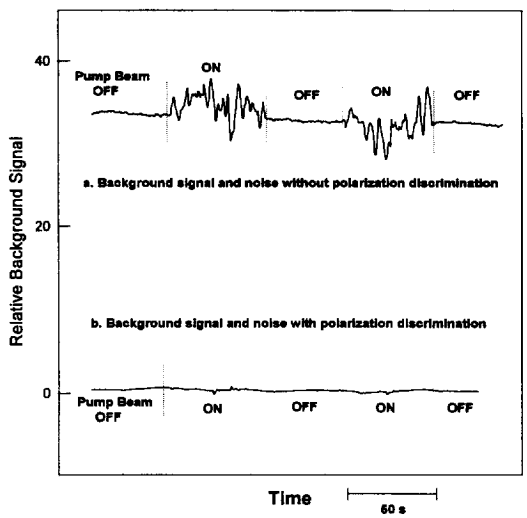

Fig. 2. Signal-to-noise ratio enhancement based on laser polarization discrimination.

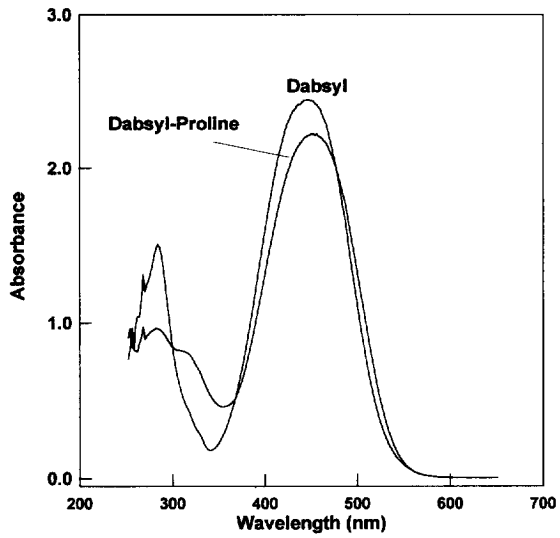

Fig. 3. Conventional non-laser UV–visible absorption spectra of $1 \cdot 10^{-4}$ *M* dabsyl chloride in acetonitrile–phosphate buffer solution and $1 \cdot 10^{-4}$ *M* dabsyl-proline in acetonitrile–phosphate buffer solution.

background noise caused by the probe beam is rejected by the polarizer placed in front of the photomultiplier tube. Fig. 2 shows the comparison of the background signal levels with and without the polarization discrimination scheme. From Fig. 2, one can observe that the background signal level without the polarization discrimination scheme is about 68 times higher than that with the polarization discrimination scheme. Hence, the overall signal-to-noise ratio (*S/N*) is significantly improved by using the polarization discrimination technique.

Fig. 3 shows the absorption spectra of dabsyl chloride and dabsyl-proline in a acetonitrile–pH 7 phosphate buffer solution. Test electropherograms indicate that there is negligible amount of unreacted dabsyl chloride after dabsyl-amino acid is extracted with diethyl ether. The effectiveness and necessity of the extraction procedure for the dabsyl-amino acids are examined. By extracting dabsyl-amino acids, one could minimize the background signal from unreacted derivatizing reagent, and hence, allow the detection of small amounts of components that are eluted early [24]. However, the electrophoregram of extracted dabsyl-amino acids show that there is negligible amount of unreacted dabsyl chloride after extraction with diethyl ether. In addition, optimum pH values for extraction of dabsyl-amino acids with diethyl ether are from 2.6 to 5.0 [25]. In order to avoid unnecessary loss of dabsyl-amino acids when a mixture of different dabsyl-amino acids is extracted at a certain pH value, we chose not to use the extraction procedure.

Fig. 4 shows an electropherogram of a mixture of four dabsyl-amino acids detected by our laser wave-

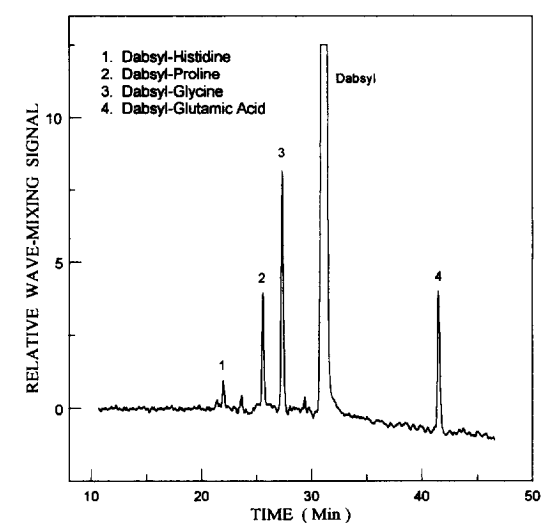

Fig. 4. Electropherogram of a mixture of four dabsyl-amino acids detected by wave-mixing on-column CE detector.

mixing detector. The amounts of dabsyl-labeled amino acids injected into the capillary tube for histidine, proline, glycine and glutamic acid are 14, 12, 11 and 7.5 fmol, respectively. Since our emphasis is in the demonstration of this new on-column wave-mixing CE detector, no attempt is made to optimize the electrophoresis separation parameters. The preliminary "injected" detection limits (at $S/N=2$) of amino acids are determined to be 7.2, 3.12, 2.0 and 1.8 fmol for dabsyl-labeled histidine, proline, glycine and glutamic acid, respectively, using a total laser power for all input beams of only 30 mW. Assuming 20-fold dilution at the detector end of the column, this corresponds to a preliminary "detected" concentration detection limit of $8.5 \cdot 10^{-8}$ M, a "detected" mass detection limit of 13 amol and an absorbance-unit detection limit of $1.35 \cdot 10^{-5}$ AU for dabsyl-glycine.

Since different optical detection methods use different analytes, lasers, wavelengths, power levels, capillary sizes, solvent systems, etc., it is difficult to directly compare the analytical sensitivity levels. Nevertheless, our detection sensitivity levels [i.e., concentration limit of detection (LOD), mass LOD, AU LOD] are comparable to those of many sensitive laser-based detection methods. While many thermo-optical methods use two-color, two-laser systems, we use a simpler one-color one-laser detection system in this study. Laser wave-mixing offers many advantages as a CE or chromatography detector since it could detect both fluorescing and non-fluorescing analytes with excellent detection sensitivity level that is approaching or equal to those of some laser-based fluorescence detectors. We have already demonstrated attomole-level detection sensitivity for strongly fluorescing analytes [20], and since the wave-mixing signal is inversely proportional to the square of analyte quantum efficiency, one could expect even a stronger signal for non-fluorescing analytes. Unlike fluorescence methods, wave-mixing detection allows virtually 100% signal collection efficiency since the signal is a laser beam that can be steered directly into a detector. Laser wave-mixing detection is applicable to both fluorescing and non-fluorescing analytes, while fluorescence detection methods can only monitor fluorescing analytes, unless one uses an indirect fluorescence detection method [26].

In conventional laser- or non-laser-based absorption methods, absorbance is determined based on a small difference between two large light intensities. In wave-mixing detection, absorbance is determined based on a large absolute positive signal measured against a virtually dark background. Since the signal is visible to the naked eye even at trace-concentration levels, the signal-to-noise ratio is excellent even when using very low laser power levels. The wave-mixing optical alignment is not much more complicated than a single-beam optical method, and the optical alignment remains stable once it is set up. Furthermore, unlike many absorbance-based laser methods, only a single laser is needed to produce all the input beams in this one-color one-laser wave-mixing detector.

As shown in Eq. (1), the wave-mixing signal has a cubic dependence on the total laser intensity. This nonlinear dependency can be used advantageously to obtain excellent detection sensitivity, since an order of magnitude increase in laser intensity results in three orders of magnitude increase in signal strength. The background noise has just a linear dependence, and hence, signal-to-noise ratio enhancement could be very significant when the laser intensity is increased. Background noise sources include scattering of E_1 and E_2 input beams from the optical components. Since the input beam E_2 propagates away from the direction of the signal beam E_3 and the photodetector, it is filtered and trapped effectively. This E_2 scattering noise is further minimized by the use of the polarization discrimination arrangement. The scattering noise from the input beam E_1 is effectively minimized by optically modulating the input beam E_2, as shown in Fig. 1. Background scattering from E_1 is further minimized by the use of a beam trap and a spatial filter. Since the nonlinear signal beam E_3 is a coherent laser-like beam, it can be effectively filtered and extracted from the incoherent background optical noise. Taking advantage of the coherent properties of the signal beam, we have already demonstrated excellent wave-mixing S/N even when using pulsed laser energy levels in the nJ range and continuous-wave laser intensity levels in the mW range.

Quadratic dependence of signal on concentration could be viewed advantageously or disadvantageously depending on whether the analyte absorption coefficient or concentration is increasing or decreas-

ing. Regardless, any disadvantages encountered at low absorptivity is more than compensated for by the brute strength of the "coherent" signal beam, its cubic dependence on laser intensity, and its optical collection efficiency (virtually 100%). Quantitative measurement (e.g., peak strength vs. concentration) is still straightforward in this "nonlinear" method, since the log–log signal vs. concentration plot has a slope value of two instead of the usual slope value of one as in conventional methods. One of the useful features of this quadratic dependence on concentration, especially in electropherograms and chromatograms, is its ability to yield inherently narrower CE or LC peaks (i.e., squared Gaussian), and hence, better separation resolution, as compared to conventional linear (Gaussian) CE or LC peaks.

Since only a short absorbance path length (e.g., less than 0.1 mm) is necessary in a wave-mixing detector to obtain excellent detection sensitivity, this detection technique is especially suitable for capillary on-column detection. Using a lens with an appropriate focal length, one could focus the input beams to fit them all inside small capillary tubes commonly used. Since the laser probe volume is sufficiently small to interact mostly the fluid inside the capillary tube and not much with the glass walls, light scattering off the capillary wall can be minimized. Hence, wave-mixing detection method has many potential applications as an ultrasensitive short-path-length on-column "absorbance"-based detector for many popular CE or LC systems.

4. Conclusion

We have demonstrated forward-scattering four-wave mixing as a sensitive "absorbance" detector for a CE system. Advantages of wave-mixing detection over conventional laser-based detection methods include excellent optical signal collection efficiency, generation of signal in the form of a coherent laser-like beam, signal measurement against a virtually dark background, excellent detection sensitivity for measuring absorbance values, effective use of very short analyte path length for absorbance measurements, ability to detect both fluorescing and non-fluorescing analytes, relatively simple one-color one-laser optical setup, low power or energy requirements for continuous-wave or pulsed lasers and convenient on-column detection for many CE and chromatography systems.

Acknowledgments

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