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Development of a capillary electrophoresis-77 K luminescence detection system for online spectral identification

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We have demonstrated that capillary electrophoresis (CE) can be easily interfaced with 77 K luminescence spectroscopy (LS) for separation and online spectral identification of structurally similar analytes. This novel CE-LS apparatus consists of a regular CE system, instrumentation for LS and a specially designed capillary Dewar. When the separating molecules traverse into the cryostat detection window, liquid nitrogen is added, freezing the separating analyte zones within the capillary. At low temperature, detection limits are improved *via* signal averaging and the inherent increase in quantum yield at 77 K. We present the first application of the CE-LS system to structural isomers (2,3- and 3,4-methylenedioxymethamphetamine) and stereoisomers (*trans*- and *cis*-resveratrol). With this approach, the CE-LS interfacing provides a sensitive, accurate, rapid, simple and economic methodology for analytical chemistry.

Introduction

Capillary electrophoresis (CE) is a rapidly growing separation technique and is now a well-established and widely used analytical method in many fields, such as bioscience, pharmaceuticals, the environment, food science and forensic research. In CE, the most commonly used detection method is absorbance, which is usually shot-noise limited. Providing several orders of detection limits better than absorbance, laser-induced fluorescence (LIF) has also been used for detection. However, in both absorbance and fluorescence, analytes are usually probed briefly as they traverse detection zones located either online or in a post-column flow cell. The short temporal detection window limits the signal-to-noise (S/N) ratio. Furthermore, typically the migration time and spiking methods are used to identify sample constituents. Dependence on the migration time can cause problems if the migration timescale shifts (jitter), especially for identification in complicated matrices, *i.e. in vitro* and *in vivo* samples, and spiking requires the availability of sometimes difficult/expensive standards. To solve such problems, CE has also been coupled to mass spectrometry (MS) and nuclear magnetic resonance (NMR) for online analyte characterization. Both methods have yielded interesting results without the requirement of standards for analysis,[1–6] but CE-MS cannot distinguish between isomers and difficulties are encountered during the implementation of CE-NMR.

Of very considerable importance in future research with CE is its combination with spectroscopic detection methods that are capable of providing spectral information on molecular analytes. However, with detection at ambient temperature, this method can provide only very limited spectral resolution due to the large vibronic fluorescence bandwidths ($\sim 500\ cm^{-1}$). Low-temperature (4.2 K) fluorescence line narrowing spectroscopy (FLNS) provides frequency selection by exciting a homogeneous ensemble of molecules, and it has been proven to be a valuable tool for the characterization of various polycyclic aromatic hydrocarbon (PAH)–DNA adducts.[7–10] For nonline narrowing spectroscopy (NLNS), non-selective excitation into the S_2 state with a single frequency excitation source can be used at 77 K, leading to excitation of all sites within the inhomogeneously broadened band. The resulting spectra are much more characteristic for analyte identification than the ambient temperature spectra. The combination of two established analytical methods, CE-FLNS and CE-NLNS, provides separation and online high-resolution spectroscopic identification of CE-separated analytes *via* the fingerprint structure of vibrationally resolved fluorescence spectra at low temperature.[11–15]

This paper examines the feasibility of using a 'capillary Dewar' at 77 K to identify online spectra. This simple and rapid method can accurately identify online spectra of either structural isomers [2,3- and 3,4-methylenedioxymethamphetamine (2,3- and 3,4-MDMA)] or stereoisomers (*trans*- and *cis*-resveratrol).

Experimental

Apparatus

The schematic diagram of the regular capillary electrophoresis-luminescence spectroscopy (CE-LS) system is shown in Fig. 1. Fused silica capillary tubing (J&W Scientific, California, USA) used for CE was 75 μm id and 50 cm in length between the injection end and the detection window. The total length of the capillary was 80 cm. All standards and electrolyte solutions were prepared with deionized water. Hydrodynamic injection was obtained by raising the sample reservoir 10 cm relative to the exit reservoir for 3 s, depending on the conditions. Before injection, the separation buffer was filtered through a 0.2 μm syringe filter and then degassed for 5 min. The excitation source was selected by a monochromator (ARC, Acton Research Corporation; Model SP-150, with a 32 mm × 32 mm 1200 grooves mm^{-1} grating) connected to an Xe lamp (Muller Elektronik Optik, 500 W) which provided an output power of ~6.1 W. CE-separated analytes were probed with the light under regular conditions. Fluorescence and/or phosphorescence was collected at right angles to the light source and dispersed by another monochromator (ARC Model SP-300i, 68 mm × 68 mm 2400 grooves mm^{-1} grating). Luminescence was detected

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by a photomultiplier tube (ARC Model P2-R928, for 190–900 nm). Electropherograms were collected at a speed of 200 ms per point with a data acquisition system (ARC's SpectraSense NCL package) connected to a personal computer. The detection limit of this system was 0.1 pmol per injection for 3,4-MDMA.

Operating conditions

A locally designed capillary Dewar (CD) was custom made by Genchen Glass Co., Ltd. (Taiwan). The CD consisted of a double-walled quartz flask for introducing liquid nitrogen. The diameter of the CD was 38 mm, the inner portion was 22 mm × 120 mm in height, and it was evacuated to $\sim 10^{-5}$ Torr. The capillary was bent into a hoop, secured to a glass rod (5 mm od) and positioned in the central region of the CD. The CE detection window formed by removing the coating of the capillary was 3 cm. After separation was complete, the CE-separated analytes were rapidly frozen immediately by directly pouring liquid nitrogen into the CD. Once frozen, arbitrary detection times were used to characterize completely the separated analytes by low-temperature spectroscopy. online fluorescence and/or phosphorescence of CE-separated analytes was detected at 77 K for spectral identification. For 30 min low-temperature experiments, the consumption of liquid nitrogen was 40–60 mL. Condensation on the Dewar was easily removed with a small fan. The glass rod was attached to a translation motor. Translation of the rod and capillary in the direction of the capillary axis allowed the separated analytes to be sequentially characterized by fluorescence spectroscopy as the capillary was translated through the light excitation/detection region. The 77 K fluorescence spectra acquired during translation of the rod and capillary provided the basis for the generation of fluorescence electropherograms from a plot of the integrated fluorescence intensity as a function of time (or capillary position). Warming was achieved by an indraft of dry nitrogen gas.

Reagents

3,4-MDMA and *trans*-resveratrol were acquired from Radian International (Catalog No. M-013, 99%; 1 mg in 1 mL methanol) and Sigma (St. Louis, MO, USA), respectively. Sodium cholate (SC), acetonitrile (ACN) and methyl alcohol (99.8%) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ethyl alcohol (99.8%) and ammonium acetate (CH_3COONH_4) were purchased from Riedel-de Haen (RdH Laborchemikalien GmbH&Co. KG). Liquid nitrogen and nitrogen gas were supplied by Echo Chemical Co., Ltd. (Taiwan).

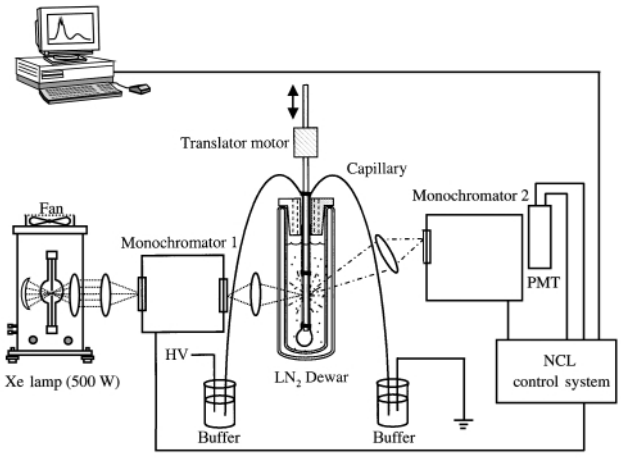

Fig. 1 Schematic diagram of the CE-LS system used for low-temperature spectral characterization of CE-separated analytes.

Results and discussion

Application I. Structural isomers

As one of the most widely used drugs, 3,4-MDMA has been discussed in many papers.[16–21] Generally, amphetamines can be detected commercially by fluorescence polarization immunoassay (FPIA), immunochromatographic assay and thin layer chromatography (TLC). Gas chromatography-mass spectrometry (GC-MS) is doubtless one of the most popular techniques for the analysis of some amphetamines and their analogs.[18] However, GC-MS cannot distinguish between geometric isomers. This is especially important for the clinical and forensic analysis of amphetamines, since it has been shown that the enantiomers do not have identical pharmacological activity.[22] In some cases, diet pills, such as phentermine, show similar fragmentation ions to amphetamines, so that dependence on GC-MS for identification could lead to errors. In this section, we show that the CE-LS system can be used for the separation and 77 K spectral identification of amphetamines. Fig. 2 shows the excitation, fluorescence and phosphorescence spectra of 3,4-MDMA in a matrix of ethanol at 77 K (spectra a and c, full lines) and room temperature (spectra b and d, broken lines). The concentration of 2,3- and 3,4-MDMA was 100 ppm in ethanol. There is little structure to the fluorescence spectrum at room temperature, as shown in spectrum d, whereas sharp spectral bands emerge when the temperature is reduced to 77 K. Moreover, the fluorescence intensity increases two- to threefold at 77 K. We also found, for the first time, that 3,4-MDMA shows bright phosphorescence (370–540 nm) at 77 K with a maximum at $\sim$440 nm (spectrum c). 2,3-MDMA also shows similar phosphorescence, as illustrated in Fig. 3 (spectrum 1). The phosphorescence originates from the 2,3- or 3,4-dioxolane, which differs from other amphetamines, such as brolamfetamine and methylamphetamine. The inset shows a high-resolution fluorescence spectrum which reveals that the wave-

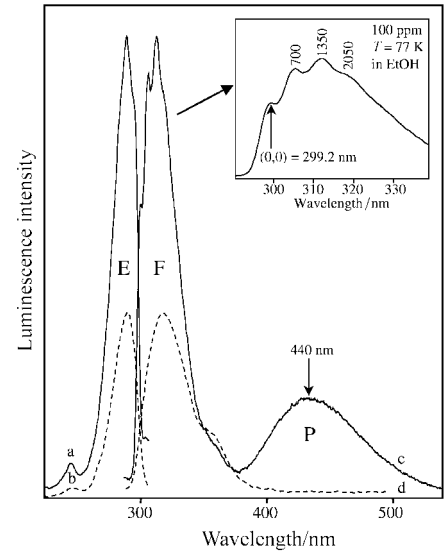

Fig. 2 Excitation, fluorescence and phosphorescence spectra of 3,4-MDMA obtained at room temperature and 77 K; λ_{ex} = 280 nm, λ_{em} = 320 nm. Inset shows a high-resolution spectrum of 3,4-MDMA in ethanol, T = 77 K. The wavenumbers 700 and 1350 correspond to the ground state (S_0) vibrational frequencies (in cm^{-1}). The sample concentration was 100 ppm.

length of the (0,0) origin band of 3,4-MDMA is observed at 299.2 ± 0.1 nm. The wavenumbers 700, 1350 and 2050 cm^{-1} correspond to the ground state (S_0) vibrational frequencies. These wavenumbers are important parameters for spectral fingerprinting.

Fig. 3(A) shows the typical fluorescence CE chromatograms of 3,4- and 2,3-MDMA isomers. The time axis is the running time for separation. The sample concentration was 2 pmol per injection. The non-aqueous CE buffer consisted of formamide–methanol solution (30%, v/v) containing 100 mM SC and 20 mM ammonium acetate. The applied voltage was 15 kV and the current was ~30 μA. By spiking with the standards, peaks 1 and 2 correspond to 3,4- and 2,3-MDMA, respectively. At room temperature, the isomers show quite similar fluorescence, precluding identification. Fig. 3(B) shows the 77 K offline spectra of 2,3-MDMA (spectrum 1) and 3,4-MDMA (spectrum 2). Spectra a and b (inset) are the 77 K fluorescence spectra of 3,4-MDMA obtained offline and online, respectively. The fluorescence detector was used online in the window after the bands were frozen. The sample concentration was 20 pmol per injection. In the cold state, a 3 cm length of detection window can be used by moving the capillary. Even though the CE buffer matrix was different from ethanol (see Fig. 3 inset), an (0,0) origin band can still be observed at 299.2 nm. Spectra c and d are the 77 K fluorescence spectra of 2,3-MDMA obtained offline and online, respectively. There is no clear (0,0) origin band, and the spectra are different from those of the 3,4-MDMA isomer. Thus, we conclude that the CE-LS system can distinguish between the positional isomers which are hard to identify at room temperature.

Application II. Stereoisomers

Resveratrol, a wine constituent, is a phytoalexin found in grapes and other foods. Recently, resveratrol has attracted interest as a cancer chemopreventive agent, as well as an agent that can reduce heart disease and other biological phenomena.[23–26] This section describes an online method to identify the stereoisomers of *trans*- and *cis*-resveratrol at 77 K by fluorescence spectroscopy. The test resveratrol was obtained by irradiating a 1.1 mmol L^{-1} stock 100% methanol solution of *trans*-resveratrol for ~20 min at 366 ± 7.8 nm selected from an Xe lamp. Fig. 4(A) shows the typical fluorescence CE chromatograms of the test resveratrol. The CE separation was performed using a non-aqueous buffer, containing 50 mM of SC and 20 mM of ammonium acetate in ACN–methanol solution (30:70, v/v). The applied voltage and current were 15 kV and ~30 μA, respectively. The non-aqueous buffer provided approximately fivefold enhanced fluorescence compared to aqueous buffer (data not shown). We found that, at room temperature, *cis*-resveratrol showed an increase in fluorescence intensity at a rate of ~8% min^{-1}. This situation causes problems in the measurement of the fluorescence spectrum of *cis*-resveratrol, which is photoconverted during the measurement. Although a charge-coupled device (CCD) detector can record the fluorescence immediately, nearly all of the detectors used in CE systems obtain data at room temperature. These provide a broad fluorescence spectrum and cannot be used to identify isomers because they have similar fluorescence behavior at room temperature. By assuming that the isomerization of *trans*- and *cis*-isomers proceeds very slowly at 77 K and that no solid phase photoconversion occurs, we believe that our system can measure the 77 K fluorescence of the two isomers separately. The measured 77 K fluorescence spectra of peaks 1 and 2 are shown in Fig. 4(B) (spectra a and b, λ_{ex} = 313 nm). Spectrum a was obtained from the left shoulder of peak 1 and this spectrum is completely identical to the offline *trans*-resveratrol reference standard in the same CE buffer at 77 K (data not shown). Spectrum b, from the right shoulder of peak 2, is the actual 77 K spectrum of *cis*-resveratrol. The distance from peak 1 to peak 2 is 1.1 cm. The capillary detection window can easily be moved up and down in order to record the online spectra. The observed wavelengths of the (0,0) origin band of *trans*- and *cis*-resveratrol are 360.0 and 354.6 nm, respectively. The other bands are labeled with their ground state vibrational frequencies (in cm^{-1}). These wavenumbers are also important parameters for spectral fingerprinting. Also investigated herein was the effect of low temperature. The fluorescence intensity increased over ~30-fold when the temperature was cooled to 77 K, demonstrating the benefit of obtaining the low-temperature spectra with high sensitivity and spectral resolution (data not shown). Thus, the application of the CE method and low-temperature technique (77 K) resolves the problems encountered with the room temperature photoisomerization of the analytes.

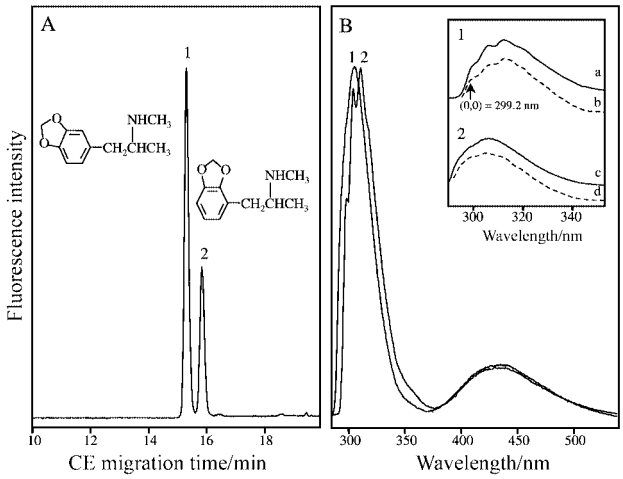

Fig. 3 (A) Typical CE fluorescence chromatograms of 3,4-MDMA (peak 1) and 2,3-MDMA (peak 2) at room temperature. Conditions: capillary, 80 cm (40 cm to detector) × 75 μm id; buffer, 100 mM sodium cholate, 20 mM ammonium acetate in formamide–methanol solution (30:70, v/v); applied voltage, 15 kV; current, ~30 μA; λ_{ex} = 280 nm, λ_{em} = 320 nm; sample concentration, 2 pmol per injection. (B) offline 77 K spectra of 3,4-MDMA (spectrum 2) and 2,3-MDMA (spectrum 1). Inset shows the online 77 K fluorescence spectra for the CE-separated peaks and standards of 3,4-MDMA (offline, spectrum a; online, spectrum b) and 2,3-MDMA (offline, spectrum c; online, spectrum d). Sample concentration, 20 pmol per injection.

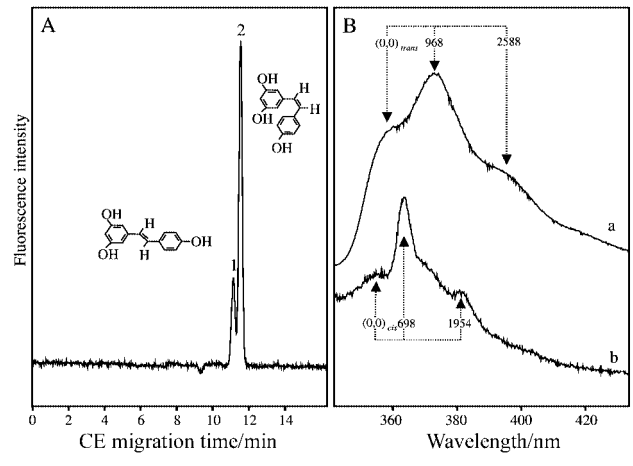

Fig. 4 (A) Typical chromatogram of *trans*- and *cis*-resveratrol for 20 min irradiation at room temperature. Conditions: 50 mM of sodium cholate and 20 mM of ammonium acetate in ACN–methanol solution (30:70, v/v); applied voltage, 20 kV; current, ~32 μA. Concentration of the test sample was 20 pmol per injection. (B) online fluorescence spectra for the CE-separated peaks of *trans*- and *cis*-resveratrol; T = 77 K, λ_{ex} = 313 nm.

Conclusions

The use of a CE method for the separation and online spectral identification of closely related analytes, *via* low-temperature fluorescence spectroscopy at 77 K, has been demonstrated for 2,3/3,4-MDMA and *trans*/*cis*-resveratrol. Application of the CE method and low-temperature technique resolves the problems encountered with the room temperature photoisomerization of the analytes. The observed variations in intensity of *trans*-resveratrol show that a non-aqueous buffer and 77 K fluorescence detection improve the detection limit at least ~150-fold. Successful separation of these isomers was accomplished using a non-aqueous buffer. Although the applications in this work were for fluorescent molecules, this technique also appears to be potentially useful for non-fluorescent molecules. For example, this method has been applied to DNA adducts in which DB[*a*,*l*]PDE-14-N^{6}dA (dibenzo[*a*,*l*[pyrene diolepoxide-14-N^6-deoxyadenosine) adducts were identified.[14] Our recent research also shows that K^+- and Ag^+-9-anthracene-dibenzo-19-crown-6-ester have the same fluorescence spectra at room temperature. However, they show different fluorescence spectra at 77 K. The idea that we have developed here is a simple, fast and economic methodology for people who are interested in CE and Shpol'skii spectroscopy at 77 K. We believe that the combination of CE and low-temperature luminescence spectroscopy will be useful in the future in stereoisomeric studies and in the investigation of other fluorescent molecules where isomers need to be identified.

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