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Ultra-low-temperature non-aqueous capillary electrophoretic separation–77 K fluorescence spectroscopic detection for the on-line identification of photo-converted analytes of *trans*-resveratrol

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Abstract

We demonstrate here, for the first time, that non-aqueous capillary electrophoresis (NACE) can be interfaced with any ultra-low-temperature (ULT) separation method and 77 K fluorescence spectroscopy (FS). This novel ULT-NACE–FS system consists of a modular CE system, a dry ice bath, and instrumentation for 77 K fluorescence detection. The ULT-NACE method serves to separate structurally similar molecules by a combination of a low electrophoresis current and a high voltage at ~−70°C. When the ULT-NACE-separated analytes move into the quartz Dewar flask and traverse into the capillary detection window, liquid nitrogen was added, thus freezing the separating analyte zones, allowing the collection of 77 K fluorescence spectra for on-line spectral fingerprint identification. The first application of the ULT-NACE–FS system is described for the analysis of photo-converted analytes of *trans*-resveratrol; prospects and future applications of ULT-NACE–FS are also briefly addressed. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Non-aqueous capillary electrophoresis; Fluorescence detection; Capillary electrophoresis, ultra-low-temperature; Phenolic compounds; Resveratrol

1. Introduction

Given its high separation efficiency, short analysis time, the use of higher electric field strength without untoward effects of Joule heating, and improved detection limits, non-aqueous capillary electrophoresis (NACE) has rapidly grown in popularity over the past few years [1–6]. In addition to the above advantages, NACE provides a much lower freezing point and, as a result, separations can be readily performed at subzero temperatures. In general, when the CE separation is carried out at low temperature, plate efficiency is enhanced and the analysis time is prolonged because of the increased viscosity of the CE buffer. However, at a fixed electric field, the current also decreases with temperature, thus permitting the capillary to be shortened, speeding up the analysis. In fact, in the 1970s, gel electrophoresis in glass tubes was performed at −10°C [7]. Low-temperature (25 to −20°C) CE, where the applied voltage could be increased without loss of resolution has also been reported [8]. More recently, subzero

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separations have been described [9,10]. A modified Beckman P/ACE unit can be used at temperatures as low as −20°C [11,12]. However, no commercial instrument is currently available for use at temperatures approaching ~−70°C. For this reason, a detailed investigation on the influence of low temperature on separation efficiency has not been reported.

In CE separation, migration time and spiking methods are normally used as criteria for identifying sample constituents. However, a dependence on migration time can cause problems because of time scale shifts, and spiking occasionally requires difficult and/or expensive standards, especially for identifications of complicated matrices, e.g. in vitro and in vivo samples, or photosensitive compounds. To solve such problems, CE has been coupled to mass spectrometry (MS) and nuclear magnetic resonance (NMR) for on-line analyte characterization. Although both methods yield interesting results and do not require standards during analysis, CE–MS has drawbacks, in terms of distinguishing isomers and CE–NMR problems still exist, relative to its implementation. The combination of CE and a low-temperature (4.2 K) technique, capillary electrophoresis–fluorescence line-narrowing spectroscopy (CE–FLNS) provides frequency selection by exciting a homogeneous ensemble of molecules and has been proven to be a valuable tool for the characterization of various polycyclic aromatic hydrocarbon–DNA adducts [13–16]. On the other hand, non-line narrowing spectroscopy (NLNS), the non-selective excitation into the S_2 state with a single-frequency excitation source can be used at 77 K, leading to the excitation of all sites within the inhomogeneously broadened band [13,17]. These resulting spectra are much more characteristic for analyte identification than ambient temperature spectra.

In this work, we propose a new separation method, which we refer to as ultra-low-temperature non-aqueous capillary electrophoresis (ULT-NACE), for use in separating similar compounds, such as photo-converted molecules derived from *trans*-resveratrol, at −73°C. The combination of two analytical methods, ULT-NACE and 77 K fluorescence spectroscopy (FS), provides for the high-efficiency separation and on-line high-resolution spectroscopic identification of CE-separated analytes via the fingerprint structure of the vibrationally resolved fluorescence spectra at 77 K.

2. Experimental

2.1. Ultra-low-temperature capillary electrophoresis system

The CE set-up (Fig. 1) was fabricated in the laboratory and is similar to the one described previously [17,18]. The dry ice bath consists of a double-walled, insulated container, which can be used as a temperature controller via the use of mixtures of ice–rock salt (0 to −20°C) and dry ice–organic solvents (−15 to −100°C) [19]. The fused-silica capillary tubing (J&W Scientific, CA, USA) used for CE was 75 μm I.D. For a regular separation (without a 77 K experiment), the length of the capillary was 35 cm (10 cm from injection end to the dry ice bath, 15 cm inside the dry ice bath, and then 5 cm to the capillary detection window); for a full experiment (with 77 K fluorescence measurement), the capillary used was 90 cm (15 cm from the injection end to the dry ice bath, 15 cm inside the dry ice bath, and then 20 cm to the detection window within the Dewar flask). Hydrodynamic injection was obtained by raising the sample reservoir 5 cm relative to the exit reservoir for 3 s, depending on the conditions. Before injection, the separation buffer was filtered through a 0.45-μm syringe filter and then degassed for 5 min. The entire reservoir at the high voltage end was enclosed in a Plexiglas box. The high-voltage power supply (Model RR30-2R, 0−30 kV, 0−2 mA, reversible, Gamma, FL, USA) was used to drive the electrophoresis. The excitation source was selected by a monochromator (ARC, Acton Research Corporation; Model SP-150, 1200 grooves/mm grating) connected to a Xe lamp (Muller Elektronik Optik, SVX/LAX 1450, 500 W) which provides an output power in excess of ~6 W. Fluorescence data were collected at a right angle to the light source and dispersed by another monochromator (ARC Model SP-300i, 2400 grooves/mm grating), followed by detection by means of a photomultiplier tube (ARC Model P2-R928, for 190–900 nm). The electropherogram was collected at a speed of 200 ms/

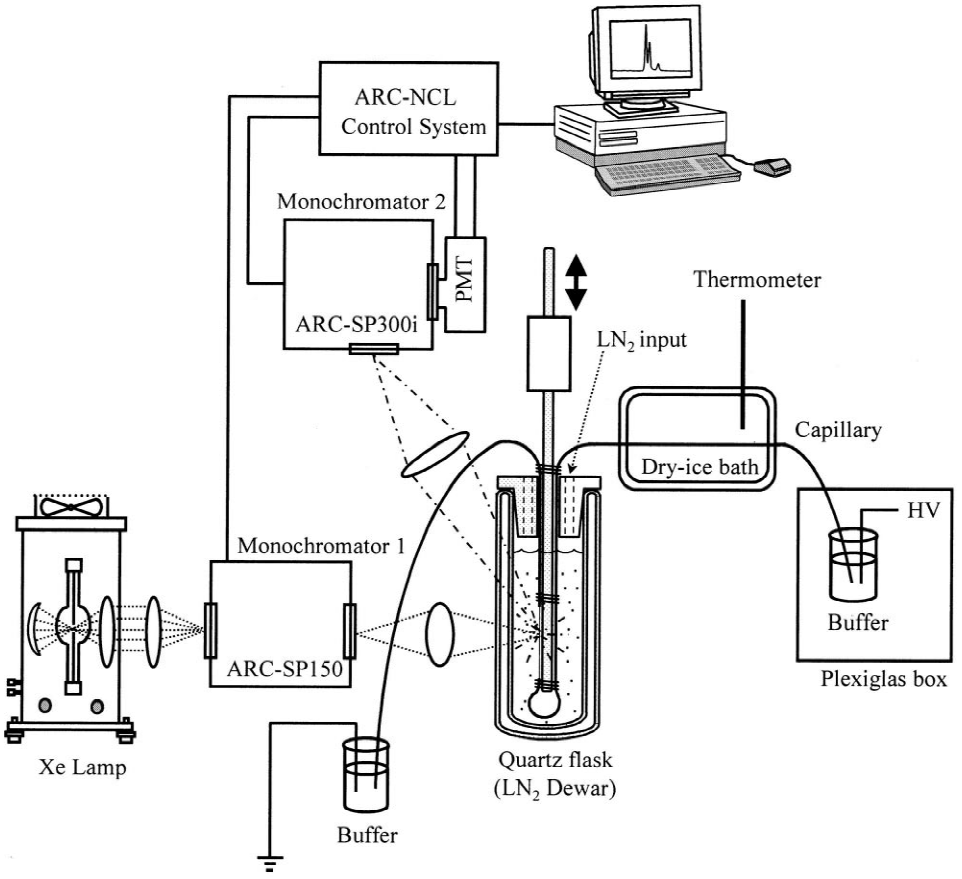

Fig. 1. A schematic apparatus for the ultra-low-temperature capillary electrophoresis–77 K fluorescence spectroscopy system.

point with a data acquisition system (ARC's Spectra-Sense NCL package), connected to a personal computer.

2.2. 77 K Fluorescence detection apparatus

A locally designed capillary Dewar flask (CD) was custom made and 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 ~10^{-5} Torr (1 Torr=133.322 Pa). The capillary was bent into a hoop, secured to a glass rod (5 mm O.D.) and positioned in the central region of the CD. The CE detection window, formed by removing the coating of the capillary was ~3 cm. The progress of the separation was observed on a computer monitor. When CE-separated analytes were shown on the screen, the high-voltage power supply was immediately turned off and liquid nitrogen poured directly into the CD. Once frozen, arbitrary detection times can be used to completely characterize the separated analytes by 77 K fluorescence spectroscopy. The capillary inside the CD can be moved up and down by a translator or manually, in order to locate the next/former CE-separated analytes. For 30-min low-temperature experiments, the consumption of liquid nitrogen was 40–60 ml. Condensation on the Dewar flask can easily be removed with a fan.

2.3. Chemicals

trans-Resveratrol (99%), *trans*- and *cis*-stilbene (95%) were purchased from Sigma (St Louis, MO, USA). Sodium cholate ($C_{24}H_{39}O_5Na$), acetonitrile (ACN) and methanol (99%) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ethanol (99.8%) and ammonium acetate (CH_3COONH_4) were obtained from Riedel-de Haen (RdH Laborchemikalien). Liquid nitrogen and nitrogen gas were supplied by Taiwan Echo Chemical Co.

3. Results and discussion

In our previously study, *trans*- and *cis*-resveratrol were successfully separated and on-line identified at 77 K [17,18]. This section describes the separation effectiveness of the photo-converted molecules by the ULT-NACE method. Fig. 2 shows a typical fluorescence electropherogram of UV-exposed and non-exposed *trans*-resveratrol ($\lambda_{ex}/\lambda_{em}$=313/365 nm) at different temperatures of the dry ice bath. In Fig 2A, the test resveratrol was obtained by irradiating a 1.1 mmol/l stock solution of *trans*-resveratrol in pure methanol for 7 min, at 366±7.8 nm selected from a Xe lamp. The CE separation occurred using a non-aqueous buffer, including 50 m*M* of SC (sodium cholate) and 20 m*M* of ammonium acetate in an acetonitrile–methanol (3:7, v/v) solution. By spiking with the *trans*-standard, the peaks a and c were found to correspond, respectively, to *trans*- and *cis*-resveratrol, as reported in our previous research [17,18]. However, in this work we found that when the temperature of the dry ice bath was decreased to −71°C, an unknown peak b gradually appeared! To determine whether peaks b and c were, in fact, generated by UV irradiation or not, we repeated the experiment using non-exposed *trans*-resveratrol under the same conditions. The results showed that neither the decomposition nor photo-reaction occurred during the ULT-NACE separation, as shown in Fig. 2B.

In Fig. 2A, the inset shows the linear relationship of the electric currents (high voltage: 20 kV) and the temperatures of dry ice bath. Thus, separation is possible, even at temperatures as low as ~−100°C. In Fig. 2B, the inset shows the relationship of the number of theoretical plates (N) and the temperature of dry ice bath. In CE separation, the number of theoretical plates was computed from peak profiles using the formula:

$$N = 5.54\,(t/w)^2$$

where w is the full peak width at the half-maximum points. At lower temperature, the efficiency was improved, as shown in the inset. Jorgenson and Lukaces [20] derived an expression for resolution in electrophoresis as:

$$R_s = 0.177(\mu_1 - \mu_2)[V/D(\mu_{av} + \mu_{EOF})]^{1/2}$$

where R_s is the resolution. At a constant voltage, when the temperature was lower, the term $(\mu_1-\mu_2)$ will decrease, but the suppression of molecular diffusion (D) and lower value of $(\mu_{av}+\mu_{EOF})$ were more effective. As a result, the resolution was improved. In general, even though there are the other factors, such as viscosity effects, zeta potential and dielectric constant, that may affect the separation, we still can find the best temperature to improve the resolution. In this case (non-aqueous CE buffer), the best temperature for the best resolution seems to be beyond ~−70°C; whereas in most cases (aqueous CE buffer), the best temperature is around ~0°C.

These experiments also show that, when the separation is not complete, the spiking method represents a potential problem, especially for quantitative analysis. Workers in the area of isomer-separation probably must determine the optimum conditions, such as suitable CE running buffer, the ideal pH value or the addition of chiral selectors like cyclodextrins. The above experiments clearly show that the ULT-NACE method dramatically improves the resolution and efficiency.

Fig. 3A shows a typical fluorescence electropherogram of the UV-exposed *trans*-resveratrol, using a longer capillary (90 cm) at −73°C. With the ULT-CE method, the separation was improved and the unknown peak b indeed became observed by the *trans*-isomer. Although a CCD detector can record all the fluorescence information immediately along with the separation, nearly all of the detectors for CE systems currently in use obtain data at room temperature. These fluorescence spectra measured at room temperature only provide a broad fluorescence

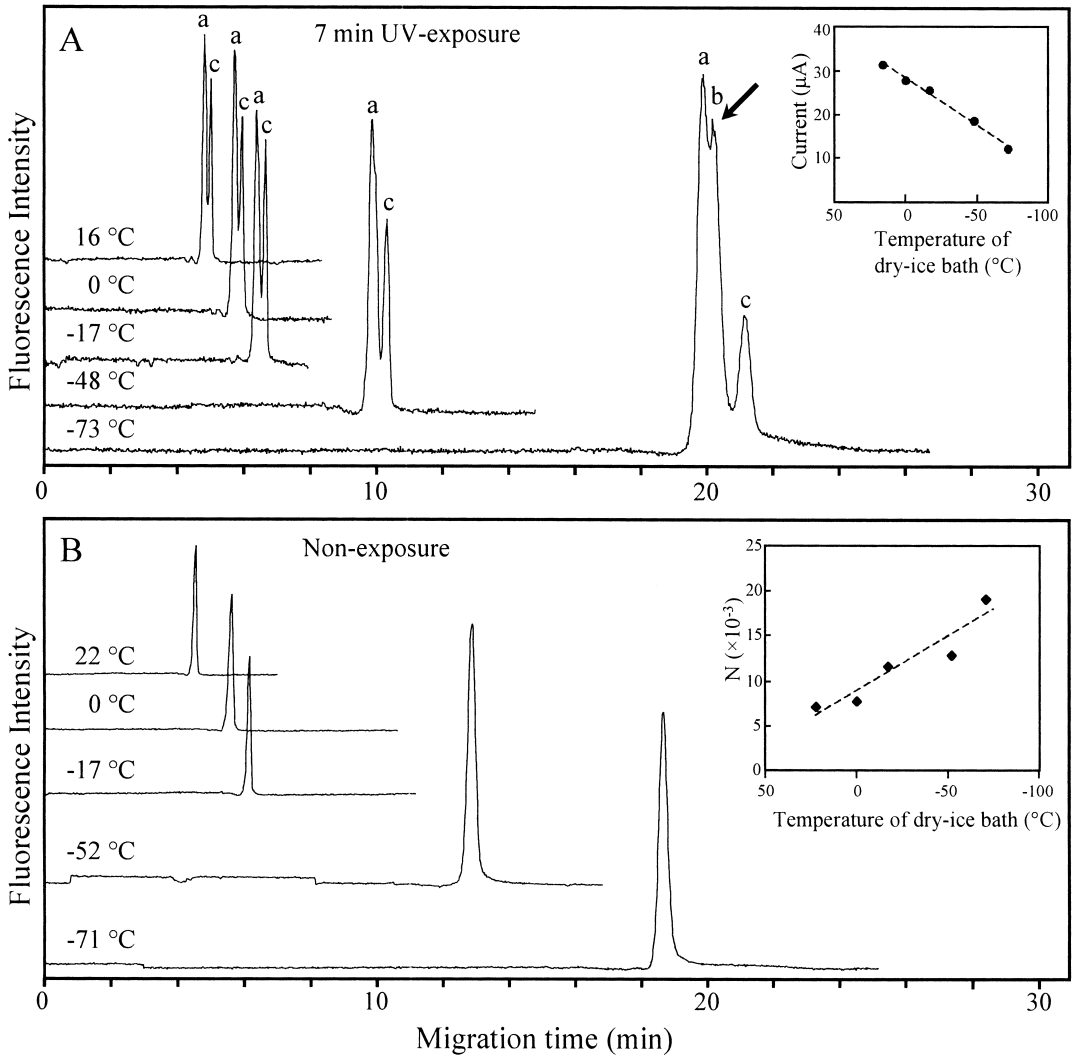

Fig. 2. (A) Typical CE fluorescence chromatograms of UV-exposed *trans*-resveratrol at different temperatures of the dry ice bath. Conditions: capillary, 35 cm (30 cm to detector, 15 cm in cooling)×75 μm I.D.; NACE buffer: 50 m*M* sodium cholate and 20 m*M* ammonium acetate in an acetonitrile–methanol (30:70, v/v) solution, applied voltage, 20 kV; currents: 32 μA/16°C, 28 μA/0°C, 26 μA/−17°C, 19 μA/−48°C, 12 μA/−71°C; λ_{ex}=313 nm, λ_{em}=365 nm. Concentration of the test sample was 250 ppm. Inset, plots of electric current versus temperature of the dry ice bath. (B) Typical CE fluorescence chromatograms of non-exposed *trans*-resveratrol at the same conditions as in (A). Inset, plots of number of theoretical plates (*N*) versus temperature of the dry ice bath.

spectrum and the identification of isomers is difficult because the isomers show similar fluorescence behavior at room temperature. While assuming that the isomerization proceeded very slowly at 77 K and no solid-phase photo-conversion occurred, our system was capable of recording the 77 K fluorescence spectra of the three analytes individually. The on-line 77 K fluorescence spectra of peaks a, b and c are shown in Fig. 3B, spectra a to c, λ_{ex}=313 nm. The (0,0) origin bands of *trans*- and *cis*-resveratrol are 360.0 (spectrum a) and 363.6 nm (spectrum c), respectively; the spectral resolution of the monochromator was 0.1 nm. Spectrum b was obtained by subtracting the background of *trans*-resveratrol. We found that the vibrational structure of the 77 K fluorescence spectrum of peak b (0-0 origin band:

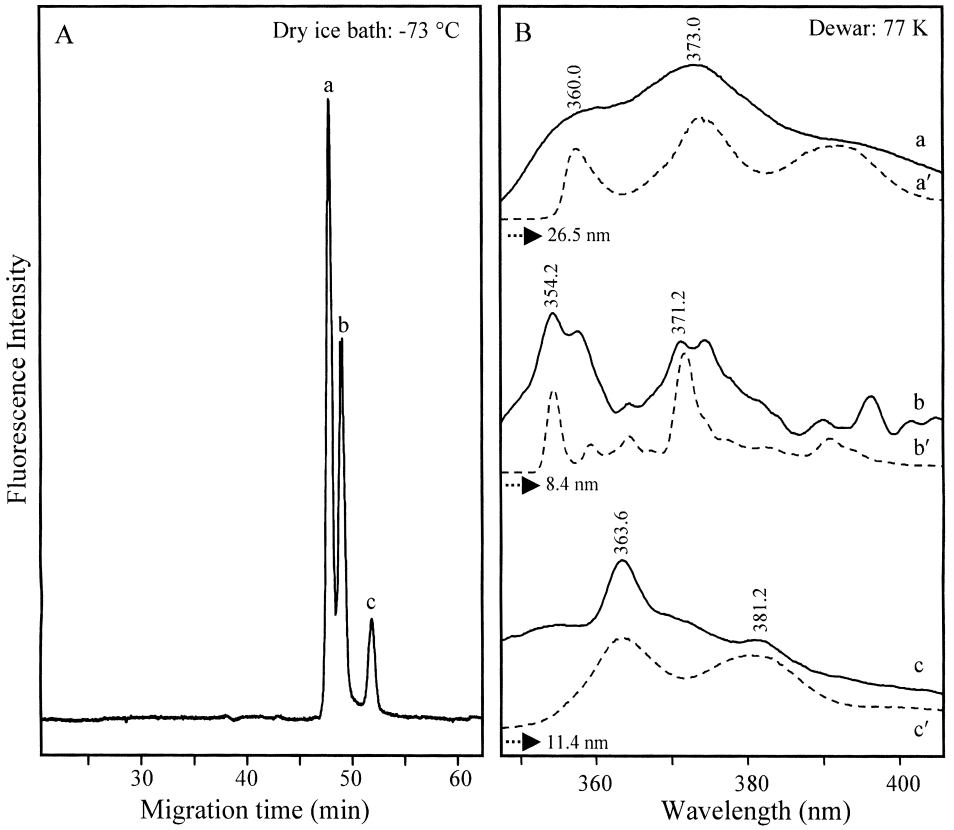

Fig. 3. (A) Typical CE fluorescence chromatogram of the UV-exposed *trans*-resveratrol (dry ice bath: −73°C). Conditions: capillary, 90 cm (50 cm to detector, 15 cm in cooling)×75 μm I.D.; NACE buffer: 50 m*M* sodium cholate and 20 m*M* ammonium acetate in the acetonitrile–methanol (3:7, v/v) solution, applied voltage, 20 kV; current: 7 μA. (B) Online 77 K fluorescence spectra (spectra a, b, and c) of the UV-exposed *trans*-resveratrol measured with different positions of capillary; $\lambda_{ex}=313$ nm. Spectra a′, b′ and c′ were the off-line 77 K fluorescence spectra of *trans*-, *cis*-stilbene and phenanthrene after being $\Delta\lambda$ red-shifted, in EtOH.

~354.2 nm) was different from that of the resveratrol isomers. The photochemical reaction for stilbene is known (Fig. 4, part I) [21]. Because resveratrol is a stilbene-like molecule, is extremely photosensitive and exists as glycosides in two isomeric forms in a number of plant species, we supposed that resveratrol also underwent a similar photo-conversion, as shown in Fig. 4 (part II). Treda and Waterhouse reported that *trans*-resveratrol is susceptible to UV-induced isomerization, and is converted to the *cis*-form by irradiation at 366 nm (180 $\mu W/cm^2$) and 254 nm (750 $\mu W/cm^2$) in yields of 90.6 and 10% after a 1-h exposure time, respectively [22]. However, in this study we found that another compound was present, which could be 2,4,6-trihydrophenanthrene. Both 2,4,6-trihydrophenanthrene and *cis*-resveratrol are not commercially available. For the assignment of these photo-converted analytes, we measured and compared the 77 K fluorescence spectra of *trans*-, *cis*-stilbene and phenanthrene. Red-shift is usually observed by substitution of hydrogen with a hydroxyl group. We assume that, as the skeleton molecule of resveratrol, the 77 K fluorescence spectrum of stilbene should be similar to resveratrol. The origin band of *trans*-stilbene was observed at 333.5 nm, in EtOH at 77 K ($\lambda_{ex}=313$ nm). With this in mind, the spectrum was moved (red-shifted) for 26.5 nm so as to match the *trans*-resveratrol on screen, shown as spectrum a′ (dash line). They were found to have similar vibrational structures at 77 K, after neglecting the solvent effects. Using the same approach, *cis*-isomers also

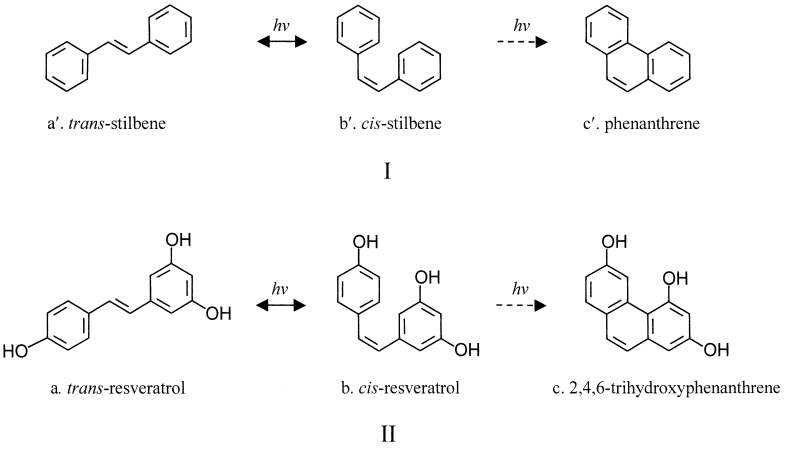

Fig. 4. Photochemical reaction schemes for *trans*-stilbene (I) and *trans*-resveratrol (II).

show similar vibrational structures, as shown in spectra c and c′. Based on these similarities, we assigned peak b (spectrum b) to 2,4,6-trihydroxyphenanthrene because of the similarity of the 77 K fluorescence spectrum of phenanthrene (spectrum b′, dashed line). This assumption is supported by observations reported by Baderschneider and Winterhalter, who isolated several stilbene derivatives, including 2,4,6-trihydroxyphenanthrene from wine [23]. This relationship explains the photo-conversion of *trans*-resveratrol to *cis*-resveratrol and 2,4,6-trihydroxyphenanthrene, either in the laboratory or naturally by exposure to sunlight.

4. Conclusions

We demonstrated, for the first time, that the ULT-CE technique can be interfaced with NACE and 77 K fluorescence spectroscopy for the on-line detection and spectroscopic identification of molecular analytes. The proposed ULT-NACE–FS system allows for the excellent separation of the photo-converted analytes of *trans*-resveratrol and their presence can be unambiguously assigned. Although the applications in this work were for native fluorescent compounds, it is clear that the ULT-NACE technique can be applied to any type of CE separation because the dry ice bath is separate from the detection system. The concept that we have developed here is a simple, economic and sensitive methodology. We believe that the ULT-NACE technique will be very useful in the future in the separation of closed molecules which are separated with difficulty at room temperature. The combination of ULT-NACE and 77 K low-temperature fluorescence spectroscopy will be valuable for the identification of native fluorescent molecules where individual isomers must be identified.

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