Cheng-Huang Lin
Yi-Hsiu Chen

Department of Chemistry,
National Taiwan
Normal University,
Taipei, Taiwan

On-line identification of *trans*- and *cis*-resveratrol by nonaqueous capillary electrophoresis/ fluorescence spectroscopy at 77 K

This work presents a novel method for the accurate determining *trans*- and *cis*-resveratrol (3,5,4'-trihydroxystilbene) by nonaqueous capillary electrophoresis/fluorescence spectroscopy at 77 K. The proposed method permits not only the separation of resveratrol isomers, but also ensures that on-line spectra are readily distinguishable and unambiguously assigned. The experimental results also indicate that the effect of nonaqueous capillary electrophoresis buffer and low-temperature technique increase the detection limit by more than 150-fold.

Keywords: Nonaqueous capillary electrophoresis / 77 K fluorescence spectroscopy / *trans*- and *cis*-resveratrol
EL 4500

1 Introduction

Resveratrol (3,5,4-trihyroxystilbene), a phytoalexin found in grapes [1] and other foods [2, 3], is a wine constituent which has received considerable interest as a potential cancer chemopreventive agent [4], as well as its role in reducing heart diseases and related biological phenomena [5, 6]. The resveratrol content of grapes and commercial wines is a subject of considerable interest [3, 7, 10]. HPLC techniques are typically used for the separation of resveratrols [8–11], but GC [12, 13] and CE [7, 14–16] methods have also been reported. In particular, CE has a higher separation efficiency, greater sensitivity and requires a smaller injection volume than either HPLC or GC. However, in CE separation, the migration time and spiking methods are normally used to identify sample constituents. It is well known that migration time can cause problems because of time scale shifts (commonly known as drift), and spiking occasionally requires difficult and expensive standards, especially for identifications in complicated matrices, *e.g.*, *in vitro* and *in vivo* samples. To solve such problems, CE has also been coupled to mass spectrometry (MS) and nuclear magnetic resonance (NMR), in order to characterize analytes online [17–22]. Although both methods yield interesting results without requiring standards during analysis, CE-MS still has some problems in terms of distinguishing between isomers and CE-NMR is sometimes difficult and expensive to implement.

Correspondence: Dr. Cheng-Huang Lin, Department of Chemistry, National Taiwan Normal University, 88 Sec. 4, Tingchow Road, Taipei, Taiwan
E-mail: chenglin@cc.ntnu.edu.tw
Fax: +886-2-8931-6955

Abbreviations: CD, capillary-Dewar; **SC**, sodium cholate; **trans-resveratrol**, *trans*-3,5,4'-trihydroxystilbene

For native fluorescent species, combining CE with spectroscopic detection methods which are capable of providing spectral information on molecular analytes represents a future research trend. However, for the case of detection at ambient temperature, this method can provide limited spectral resolution due to large vibronic fluorescence bandwidths. Low-temperature fluorescence spectroscopy is much more appropriate for analyte identification than ambient temperature spectra [22–26]. The combining of CE and low-temperature fluorescence spectroscopy provides separation and identification of on-line high-resolution spectroscopy of CE-separated analyses *via* a fingerprint structure of vibrationally resolved fluorescence spectra at a low temperature [27–30]. In this study, we used a home-made "capillary-Dewar" for application to 77 K on-line spectral identification. This simple and rapid method allowed the accurate on-line identification of photo-isomerization compounds, *e.g.*, *trans*- and *cis*-resveratrol at 77 K.

2 Materials and methods

2.1 Low-temperature CE detection system

The schematic diagram of the laboratory-made CE setup is shown in Fig. 1. A high-voltage power supply (Model RR30–2R, 0–30KV, 0–2 mA, reversible; Gamma, Ormond Beach, FL, USA) was used to drive the electrophoresis. A 75 μm ID fused-silica capillary (J&W Scientific, Folsom, CA, USA) was used for the separation. The bare capillary was used after rinsing with 0.1 M NaOH for 30 min, followed by equilibration with running buffer for 10 min. All standards and electrolyte solutions were prepared with deionized water.

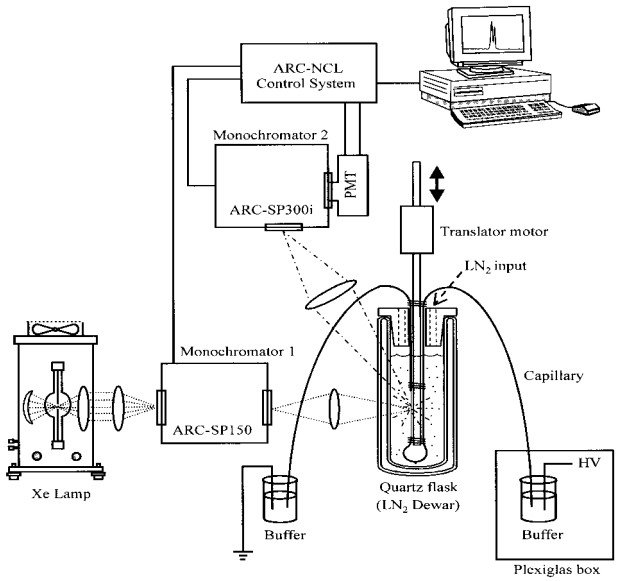

Figure 1. A schematic apparatus of the CE-fluorescence spectroscopy system used for low-temperature spectral fingerprint identification of CE-separated analytes.

Before injection, the separation buffer was filtered through a 0.45-μm syringe filter and then degassed for 5 min. The sample was hydrodynamically injected by raising reservoir 10 cm relative to the exit reservoir for 3 s, depending on the conditions. The entire reservoir at the high voltage end was enclosed in a Plexiglas box. The excitation source was selected by a monochromator (ARC, Acton Research Corporation, Massachusetts; Model SP-150, 1200 grooves/mm grating) connected with a Xe lamp (Müller Elektronik Optik, Germany; SVX/LAX 1450, 500 W) which provides an output power of over ~6 W. Fluorescence was collected at a right angle to the light source and dispersed by another monochromator (ARC Model SP-300i, 2400 grooves/mm grating) and detected by a photomultiplier tube (ARC Model P2-R928, for 190–900 nm). Electropherograms were collected at a speed of 200 s/point with a data acquisition system (ARC's Spectra-Sense NCL package), connected to a personal computer. For off-line experiments, samples were placed in quartz tubes (2 mm ID, 1.5 cm length), sealed with rubber septa (Aldrich; cat. No. Z10071-4) and brought to 77 K for low temperature fluorescence measurement. Solvent purity was checked by recording absorption and fluorescence spectra under conditions of high sensitivity.

2.2 Methods

A locally designed capillary-Dewar (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. 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. The process of separation was observed on a computer monitor. Once CE-separated analytes were shown on the screen, the HV 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 low-temperature spectroscopy. The capillary inside the CD can be moved up and down by a translator or manually to find the next CE-separated analyte. For 30-min low-temperature experiments, the consumption of liquid nitrogen was 40 ~ 60 mL. Condensation on the Dewar can easily be removed with a fan.

2.3 Reagents

trans-Resveratrol (99%) was purchased from Sigma (St. Louis, MO, USA). *cis*-Resveratrol is not commercially available, and was therefore obtained by UV photoisomerization of *trans*-resveratrol. Sodium cholate (SC; $C_{24}H_{39}O_5Na$), acetonitrile (ACN), and methanol (99.8%) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ethanol (99.8%) and ammonium acetate (CH_3COONH_4) were obtained from Riedel-de Haën (RdH Laborchemikalien, Seelze, Germany). Liquid nitrogen and nitrogen gas were supplied by Echo Chemical (Taiwan).

3 Results and discussion

3.1 The effect of nonaqueous electrolyte and low temperature

SC, a bile salt, is a natural anionic surfactant and is found in biological components. It dissolves with difficulty in ethanol or acetonitrile, and, therefore a methanol-acetonitrile mixture was used as the solvent. SC has a steroidal structure and forms a helical micelle in water. The critical micelle concentration (CMC) has reported to be 13–15 mM at 25°C [31]. Herein, the term "apparent CMC" (or CMC*) is defined as a concentration range below which the surfactant is present in organic solution as a monomer and above which all additional surfactant added to the solution forms micelles. Based on the method proposed by Cifuentes *et al*. [32], Fig. 2A shows the plots of electric current *vs.* concentration of SC under different conditions, where the current was measured at 20 kV. The concentration of SC was in the range of 1–75 mM and the nonaqueous solution was a methanol-acetonitrile mixture (7:3 v/v),

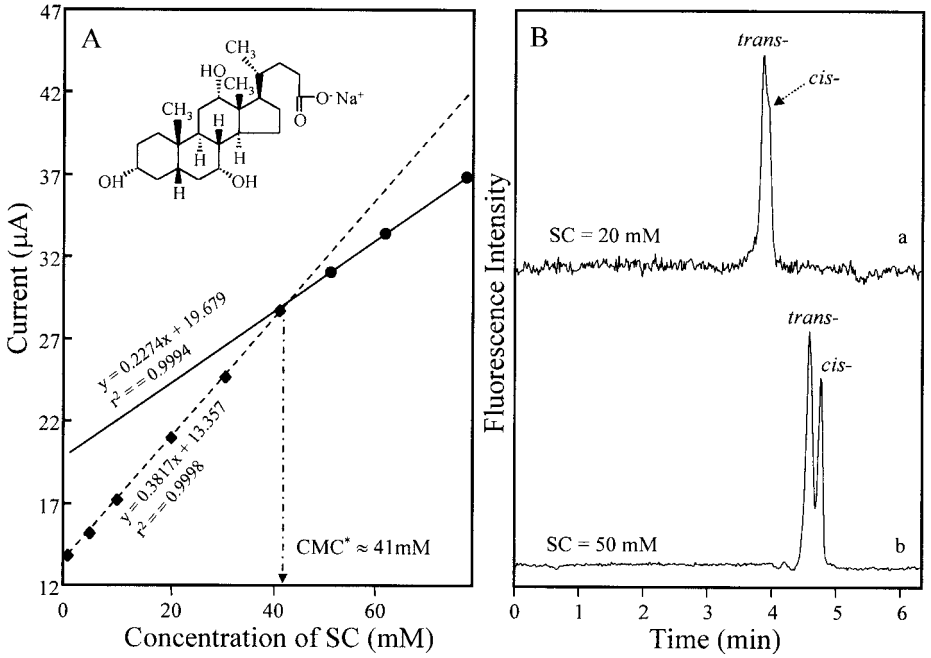

Figure 2. (A) Plots of electric current *vs*. concentration of CS under different conditions. The current was measured at 20 kV. Inset: molecular structure of SC ($C_{24}H_{39}O_5Na$). (B) CE chromatograms of 250 ppm *trans*-resveratrol. Conditions: fused-silica capillary, 75 μm ID, 30 cm to the detector; CE buffer, (a) 20 mM and (b) 50 mM of SC and 20 mM of ammonium acetate in a methanol-acetonitrile solution (7:3 v/v).

containing 20 mM ammonium acetate. The concentration at the cross point was considered to be the CMC* (~ 41 mM). To compare the effect of CMC* on the separation, 20 and 50 mM of SC were chosen for examination (Fig. 2B). The separation was not completely until the concentration of SC was beyond the value for CMC*.

A higher concentration of SC (100 mM) resulted in better separation although the migration time was longer. Other types of surfactants were also tested, such as SDS (sodium dodecyl sulfate) and sodium dioctylsulfosuccinate DOSS, but no separation of *trans*- and *cis*-resveratrol was detected. Although it was not easy to further optimize the separation in nonaqueous solution by controlling the EOF, pH* or ionic strength, as is commonly done in an aqueous solution, the viscosity, EOF and apparent pH values (or pH*) were investigated. Ionic molecules migrated according to their per charge ratios in aqueous solvent, whereas in nonaqueous solvents the migration and separation are influenced by the conductance, viscosity and autoprotolysis of the organic media. The viscosities in aqueous and nonaqueous CE buffer were 0.506 and 1.151 cP of that measured using an Ostwald viscometer. The EOF ($\sim 2 \times 10^{-4}$ cm^2/Vs) was measured by spiking water as the test sample with a UV detector (Jasco, Easton, MD, USA; CE-971UV). As is well known that electrophoretic mobility is directly proportional to the ε/η ratio. Methanol-acetonitrile mixtures have lower ε/η values and resulted in lower electrophoretic mobilities in this work. On the other hand, the pH* is frequently used in dealing with nonaqueous solutions due to the uncertainties in the liquid-junction potential of the glass electrode. Using a glass pH meter, the pH* of the nonaqueous CE buffer (with 50 mM SC) was found to be ~8.5. Even though in the case of the nonaqueous CE, where our knowledge concerning acid-base chemistry in organic solution is very limited, we found that with an increase in the pH* in the range of 7.5–10.5 the mobility remained relatively constant. The concentration of ammonium acetate (5–20 mM) would be expected to enhance the efficiency of separation and the use of a 20 mM solution provided best separation of *trans*- and *cis*-resveratrol.

3.2 Photostability of resveratrol

Resveratrol, a stilbene-like molecule, is extremely photosensitive and functions as glycosides in two isomeric forms in a number of plant species. Waterhouse *et al*. [8] demonstrated that *trans*-resveratrol is susceptible to UV-induced isomerization, and is converted to the *cis*-form by irradiation at 366 nm (180 μW/cm^2) and 254 nm (750 μW/cm^2) in yields of 90.6% and 10% after a 1 h exposure time, respectively. In order to investigate the photoconversion process, we measured the fluorescence spectra before and after exposure to UV-light. The test solution containing 250 ppm of pure *trans*-resveratrol was sealed in a quartz tube and irradiated for different times (0, 5, 10, and 15 min) at 366 ± 7.8 nm (~1 mW) selected from a Xe lamp. Figure 3A shows the fluorescence spectra of *trans*-resveratrol, measured after different exposure times (spectra a–d). The scan speed of the

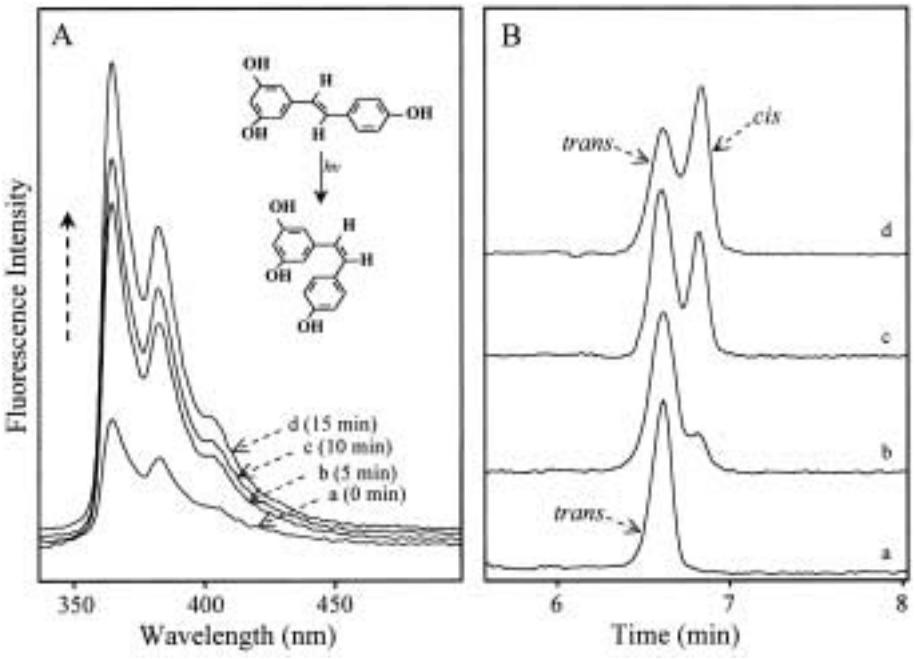

Figure 3. (A) Fluorescence spectra a–d of the test *trans*-resveratrols measured with different exposure times (0, 5, 10, and 15 min); λ_{ex} = 313 nm, room temperature. The fluorescence intensity was increased with longer exposure times. Inset: molecular structure of *trans*- and *cis*-resveratrols. (B) CE chromatograms a–d of the test resveratrols indicated in (A). CE buffer, 50 mM of SC and 20 mM of ammonium acetate in methanol-acetonitrile solution (7:3 v/v).

monochromator was 0.2 nm/200 ms and the excitation wavelength was 313 nm. This figure reveals that an increase in exposure times caused a rapid growth in the total fluorescence intensity. This suggests that the fluorescence spectra were, in fact, mixed spectra of *trans*- and *cis*-resveratrol. It should be noted that the measurement of an actual spectrum for a single isomer is extremely difficult when the isomerization caused by UV light is so fast. Therefore, online identification is ideal for the compounds such as this, when standards are not easily acquired.

Figure 3B shows the typical fluorescence CE chromatograms of the test resveratrols after different exposure times (electropherograms a–d). Based on CE electropherogram a, no *cis*-resveratrol was present before the irradiation. However, for a 5-min exposure time (electropherogram b), the fluorescence intensity of the *cis*- was observed from behind the *trans*-isomer. Furthermore, after the passage of 10 and 15 min of exposure time, the fluorescence intensity of *cis*-isomer became greater than that of *trans*-resveratrol. The *cis*-resveratrol increased in intensity at a rate of ~8% per min at room temperature. This situation complicated efforts to measure the fluorescence spectrum of either *trans*- or *cis*-resveratrol, which is undergoing photoconversion during the measurement. Although a CCD camera can be used to record the fluorescence immediately, nearly all of the detectors of CE systems currently in use obtain data at room temperature. These fluorescence spectra only provide a broad band of fluorescence spectra and the identification of isomers is difficult, because the isomers have similar fluorescence behaviors at room temperature. While assuming that the isomerization of *trans*- and *cis*-isomers proceeded extremely slow at 77 K and no solid-phase photoconversion occurred, our system was capable of measuring the 77 K fluorescence spectra online for the two isomers, as described below.

In Fig. 4A, CE electropherogram a was acquired using an aqueous buffer which contained 50 mM SC and 20 mM ammonium acetate in water-acetonitrile (7:3 v/v), whereas electropherogram b was obtained using a nonaqueous buffer instead of 70% water to methanol. The nonaqueous CE buffer provided a larger theoretical plate number than the an aqueous buffer by a factor of about 1.2-fold. According to our results, at least a ~2.7-fold enhancement in efficiency was obtained when using a lower sample concentration and a higher applied voltage (50 ppm of SC and 25 kV applied voltage). This strategy also provided an increase in fluorescence intensity by a factor 5 as compared to an aqueous buffer. The effect of low temperature was also investigated. Many organic and inorganic compounds are used as matrices for obtaining highly-resolved (quasiline) spectra. Frozen normal alkanes typically represent a common and useful type of matrix for aromatic molecules. Our results indicated that *trans*-resveratrol has a markedly enhanced fluorescence at 77 K in either CE buffer or methanol. This property is similar to that of *trans*-stilbene which is only weakly fluorescent (Φ_F = 0.05) at room temperature, but strongly fluorescent (Φ_F = 0.75) at 77 K [33]. Figure 4B, spectra a and b, show off-line 77 K and room temperature fluorescence spectra of *trans*-resveratrol in a methanol matrix

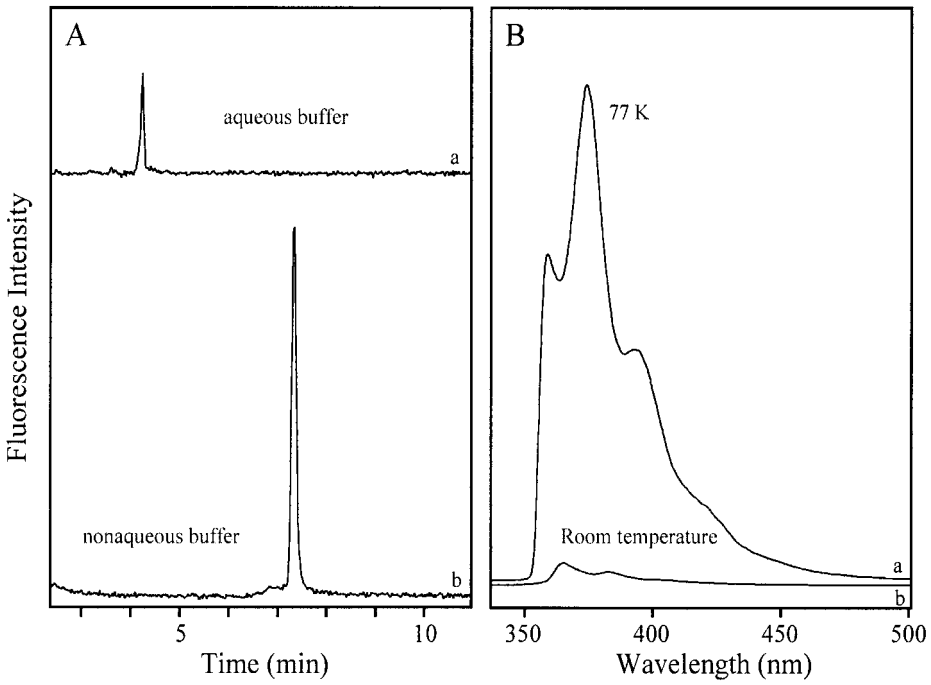

Figure 4. (A) Effect of aqueous and nonaqueous buffer on the separation of 250 ppm *trans*-resveratrol. CE conditions were the same as described in Fig. 3B. Applied voltage, 15 kV; current, a: ~62 μA, b: ~23 μA. (B) Effect of low temperature: spectrum a, 77 K; spectrum b, room temperature, in methanol, λ_{ex} = 313 nm. The fluorescence intensity was increased 30-fold when the temperature was cooled to 77 K.

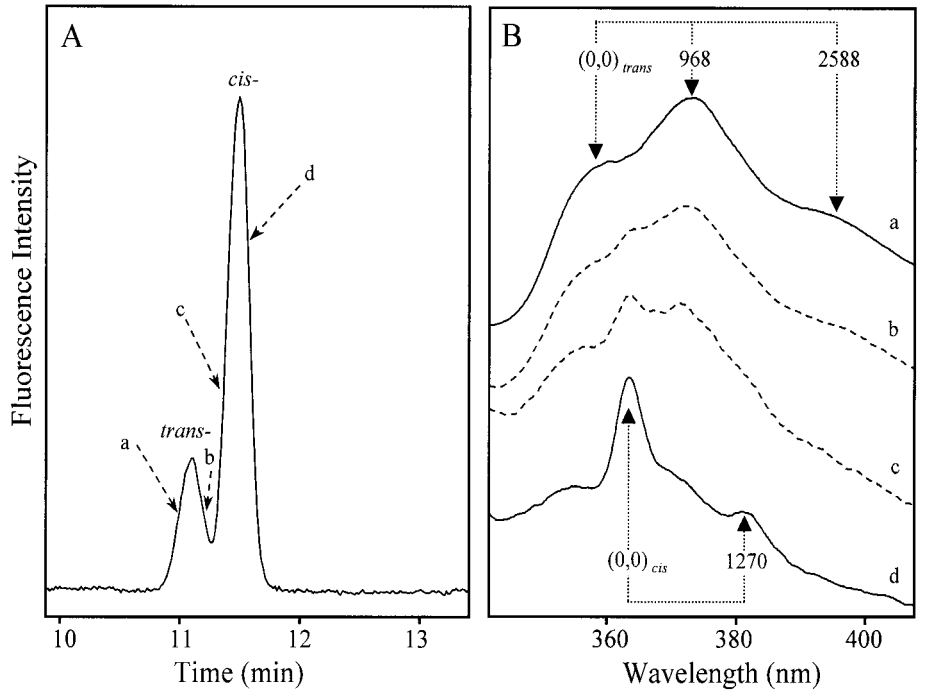

Figure 5. (A) CE-separated chromatogram of the test resveratrol after a 22-min exposure time. Conditions: fused-silica capillary (75 μm ID), 80 cm total length, detection window ~ 40 cm from the injection end; CE buffer, 50 mM SC and 20 mM ammonium acetate in methanol-acetonitrile solution (7:3 v/v). (B) On-line 77 K fluorescence spectra (spectra a–d) for the CE-separated eluate (position a–d, in frame A), λ_{ex} = 313 nm.

with the same scale. The fluorescence intensity increased over ~30 fold when the temperature was cooled to 77 K. The (0,0) origin band of *trans*-resveratrol was observed at 359.0 nm in methanol which was blue-shifted by 1.0 nm, compared the nonaqueous CE buffer, too.

3.3 On-line spectral identification

Figure 5A shows the CE-separated chromatogram of the test resveratrols after a 22-min exposure time. Positions a–d on the capillary detection window indicate the differ-

ent CE-separated spots. Measuring the 77 K fluorescence spectra from different positions allows the determination of the population of the two isomers inside the capillary, as shown in Fig. 5B (spectra a–d, corresponding to positions a–d; λ_{ex} = 313 nm). It should be noted that the physical distance between the two peaks was 1.1 cm. Spectra a and d were assigned to *trans*- and *cis*-resveratrol, respectively. The observed wavelength of the (0,0) origin band of *trans*- and *cis*-resveratrol was 360.0 and 363.6 nm, respectively. The other bands are labeled with their ground-state vibrational frequencies, in cm^{-1}. These numbers are important parameters for spectral fingerprinting. It is obvious that spectra b and c (dash lines) were spectra of mixtures of *trans*- and *cis*-resveratrol in different ratios. Thus, applying a CE method and low-temperature technique was, in fact, useful in resolving the room-temperature photoisomerization of analytes.

4 Concluding remarks

This work demonstrates that *trans*- and *cis*-resveratrol are readily distinguishable and can be unambiguously assigned. A CE method has also been successfully used to perform separations and on-line spectral identification of *trans*- and *cis*-resveratrol, *via* low-temperature fluorescence spectroscopy at 77 K. The nonaqueous CE buffer and 77 K fluorescence detection method increased detection limit by 150-fold. We believe that combining CE and low-temperature fluorescence spectroscopy will be useful for the identification of native fluorescent molecules where the individual isomers must be identified. In addition, it provides a simple, fast, and economic technology for people who are interested in CE and Shpol'kii spectroscopy at 77 K and has potential for use in the identification of *in vitro* and *in vivo* samples which contain photosensitive compounds.

The authors would like to thank the National Science Council of Taiwan for financial support of this research under Contract No. NSC-89-2113-M-003-016.

Received January 14, 2001

5 References

[1] Creasy, L. L., Coffee, J. J., *J. Am. Soc. Hortic. Sci*. 1988, *113*, 230–234.

[2] Jeandet, P., Bessis, R., Gantheron, B., *Am. J. Enol. Vitic*. 1991, *42*, 41–46.

[3] Siemann, E. H., Creasy, L. L., *Am. J. Enol. Vitic*. 1992, *43*, 49–52.

[4] Mgbonyeby, O. P., *Int. J. Oncol.* 1998, *12*, 865–869.

[5] Sporn, M. B., Newton, D. L., *Fed. Proc.* 1979, *38*, 2528–2534.

[6] Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W. W., Fong, H. H. S., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C., Pezzuto, J. M., *Science* 1997, *275*, 218–220.

[7] Gu, X., Creasy, L. L., Kester, A., Zeece, M. G., *J. Agric. Food Chem.* 1999, *47*, 3223–3227.

[8] Trela, B. C., Waterhouse, A. L., *J. Agric. Food Chem.* 1996, *44*, 1253–1257.

[9] Vastano, B. C., Chen, Y., Zhu, N., Ho, C., Zhou, Z., Rosen, R. T., *J. Agric. Food Chem.* 2000, *48*, 253–256.

[10] Celotti, E., Ferrarini, R., Zironi, R., Conte, L. S., *J. Chromatogr. A* 1996, *730*, 47–52.

[11] Juan, M. E., Lamuela-Raventos, R. M., Torre-Boronat, M. C., Planas, J. M., *Anal. Chem.* 1999, *71*, 747–750.

[12] Blache, D., Rustan, I., Durand, P., Lesgards, G., Loreau, N., *J. Chromatogr. B* 1997, *702*, 103–110.

[13] Barlass, R., Miller, M., Douglas, T. J., *Am. J. Enol. Vitic.* 1987, *38*, 65–68.

[14] Berzas Nevado, J. J., Contento Salcedo, A. M., Castaneda Penalvo, G., *Analyst* 1999, *124*, 61–66.

[15] Cartoni, G., Coccioli, F., Jasionowska, R., *J. Chromatogr. A* 1995, *709*, 209–214.

[16] Gu, X., Chu, Q., O'Dwyer, M., Zeece, M., *J. Chromatogr. A* 2000, *881*, 471–481.

[17] Lu, W., Poon, G. K., Carmichael, P. L., Cole, R. B., *Anal. Chem.* 1996, *68*, 668–674.

[18] Qzaki, H., Itou, N., Terabe, S., Takada, T., Sakairi, M., Koizumi, H., *J. Chromatogr. A* 1995, *716*, 69–79.

[19] Cai, J., Henion, J., *J. Anal. Toxicol.* 1996, *20*, 27–37.

[20] Locke, S. J., Thibault, P., *Anal. Chem.* 1994, *66*, 3436–3446.

[21] Cai, J., Henion, J., *J. Chromatogr. A* 1995, *703*, 667–692.

[22] Olson, D. L., Lacey, M. E., Sweedler, J. V., *Anal. Chem.* 1998, *70*, 645–650.

[23] Jankowiak, R., Zamzow, D., Ding, W., Small, G. J., *Anal. Chem.* 1996, *68*, 2549–2553.

[24] Zamzow, D., Lin, C.-H., Small, G. J., Jankowiak, R., *J. Chromatogr. A* 1997, *781*, 73–80.

[25] Shu, M., Ariese, F., Small, G. J., Jankowiak, R., Hewer, A., Phillips, D. H., *Carcinogenesis* 1995, *16*, 2561–2569.

[26] Todorowic, R., Ariese, F., Devanesan, P., Jankowiak, R., Small, G. J., Rogan, E. G., Cavalieri, E. L., *Chem. Res. Toxicol.* 1997, *10*, 941–947.

[27] Jankowiak, R., Roberts, K. P., Small, G. J., *Electrophoresis* 2000, *21*, 1251–1266.

[28] Roberts, K. P., Lin, C.-H., Singhal, M., Casale, G. P., Small, G. J., Jankowiak, R., *Electrophoresis* 2000, *21*, 799–806.

[29] Roberts, K. P., Lin, C.-H., Jankowiak, R., Small, G. J., *J. Chromatogr. A* 1999, *853*, 159–170.

[30] Jankowiak, R., Lin, C.-H., Zamzow, D., Roberts, K. P., Li, K.-M., Small, G., *J. Chem. Res. Toxicol.* 1999, *12*, 768–777.

[31] Nishi, H., *J. Chromatogr. A* 1996, *735*, 57–76.

[32] Cifuentes, A., Bernal, J. L., Diez-Masa, J. C., *Anal. Chem.* 1997, *69*, 4271–4274.

[33] Sharafy, S., Muszkat, K. A., *J. Am. Chem. Soc.* 1971, *93*, 4119–4125.