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On-line identification of *trans*-resveratrol in red wine using a sweeping technique combined with capillary electrophoresis/77 K fluorescence spectroscopy

The feasibility of combining the techniques of on-line concentration and capillary electrophoresis/low-temperature fluorescence spectroscopy (CE/LTFS) for the detection and identification of *trans*-resveratrol in red wine at 77 K is demonstrated for the first time. This technique, involving sweeping-micellar electrokinetic chromatography (sweeping-MEKC), was used for the initial on-line concentration and separation, after which a cryogenic molecular fluorescence experiment was performed at 77 K. In comparison with normal-MEKC mode, a ~1500-fold improvement in detection sensitivity could be obtained when the sweeping-MEKC was applied. The proposed method permits not only the separation and detection of *trans*-resveratrol from red wine extracts but also ensures that the on-line spectrum is readily distinguishable and can be unambiguously assigned at 77 K.

Keywords: Capillary electrophoresis / Low-temperature fluorescence spectroscopy / *trans*-Resveratrol / Sweeping-micellar electrokinetic chromatography DOI 10.1002/elps.200305587

1 Introduction

Several excellent descriptions of low-temperature fluorometric techniques, sometimes referred to as site-selection spectroscopy, and their applications can be found in published monographs [1–3] and reviews [4–6]. The principal advantage of the low-temperature fluorometric technique is that spectral sharpening occurs both in absorption and emission. However, using the technique of capillary electrophoresis/low-temperature fluorescence spectroscopy (CE/LTFS) either at 77 K or 4.2 K, it continues to be difficult to obtain an on-line spectrum, if the analyte is present at low concentration or is weakly fluorescent, especially for identifying such compounds in complicated matrices, *i.e.*, *in vitro* and *in vivo* samples. Jankowiak *et al*. [7] reported on a capillary electrophoresis-fluorescence line narrowing system (CE/FLNS) for the on-line structural characterization of polycylic aromatic hydrocarbons (PAHs). This procedure is based on combining CE and LTFS, and permits the separation and identification of on-line high-resolution spectroscopy of CE-separated analyses *via* a fingerprint structure of the vibrationally resolved fluorescence spectra, obtained at a low-temperature. Such a technique has been successfully applied to the analysis of DNA adducts [8–11]. Even though this method represents an excellent tool for on-line spectral identification, it is still difficult if the molecules in question are weakly fluorescent or are present at very low levels. Thus, the concentration of the samples either on-line or off-line becomes increasingly important. A series of reports on sample concentration techniques by Terabe *et al*. [12–16], as well as other groups [17–19] has appeared, concerning the so-called "stacking" and "sweeping" techniques. It is likely that such techniques have already opened new fields of investigation of molecules that are present at low levels, even in the parts per billion (ppb) range.

Resveratrol is found in grapes [20] and other foods [21, 22], and is a wine constituent that has attracted considerable interest as a potential cancer chemopreventive agent [23], as well as its role in reducing heart diseases and related biological phenomena [24, 25]. The concentrations of *trans*-resveratrol in red wines (0–6.8 ppm) from Portugal [26], grape berries (5.4–9.6 μg/100 g) [27] and red/white grape juices (~0.5/0.05 ppm) [28] have been reported. It is weakly fluorescent and is present at low levels in wines. Efforts have been made to increase the sensitivity of its detection including the use of non-aqueous CE buffer-fluorometry (LOD ~5 ppm) [29], the MEKC-UV method (LOD ~ 0.3 ppm) [30], HPLC-UV (LOD ~ 0.2 ppm) [26], and HPLC-fluorometry (LOD ~ 3 ppb) [31]. However, the use of an on-line concentration technique in CE has not yet been reported.

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We previously demonstrated the successful on-line identification of *trans*- and *cis*-resveratrol standards by nonaqueous capillary electrophoresis/fluorescence spectroscopy at 77 K [29]. A nonaqueous CE buffer provided better fluorescence intensity (~5-fold) than an aqueous buffer for resveratrols but it was still not sufficient for measuring the on-line spectrum of minor levels of *trans*-resveratrol in red wine. To improve the sensitivity, we investigated the utility of the combination of the sweeping techniques and the low-temperature fluorometric technique in CE separation by deliberately spiking a (lysergic acid diethylamide, LSD) standard to a urine sample [32]. As a result, the diluted LSD standard could be concentrated on-line and then identified at 77 K from a urine sample. In this study, we report on the combined use of on-line sample concentration (sweeping-MEKC) and CE-LTFS at 77 K in the analysis of an actual compound (*trans*-resveratrol), which is weakly fluorescent and present at low levels (~ ppm) in red wine extracts. Several electrophoretic parameters such as the ratio of organic solvents used, SDS concentration, and the injection length required for the separation were optimized and these data are reported herein.

2 Materials and methods

2.1 Chemicals

trans-Resveratrol (99%) and SDS was purchased from Sigma (St. Louis, MO, USA). Sodium cholate ($C_{24}H_{39}O_5Na$), acetonitrile (ACN), ethyl acetate, and methanol (99.8%) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium acetate (CH_3COONH_4) was obtained from Riedel-de Haën (Seelze, Germany). Liquid nitrogen and nitrogen gas were supplied by Echo Chemical (Taiwan). The red wine was marketed by Saint-Julien in 1993 (France). Liquid nitrogen and nitrogen gas were supplied by Echo Chemical (Taiwan). All other chemicals were of analytical grade and are commercially available.

2.2 CE apparatus

A schematic diagram showing in house-fabricated CE setup is similar to that described previously [32–35]. Briefly, a high-voltage (HV) power supply was used to drive the electrophoresis and a 50 μm ID fused-silica capillary column (97 cm in length/93 cm to the detector) was used for the separation. The excitation source was selected by a monochromator connected to a Xe lamp (total power ~ 6 W). The excitation wavelength was 313 ± 8 nm; the emission was measured at 400 ± 16 nm (with 320 nm cut filter). Fluorescence data were collected at a right angle to the light source and dispersed by a second monochromator, followed by detection using a photomultiplier tube. Electropherograms were collected with a data acquisition system, connected to a personal computer. A locally designed capillary-Dewar was custom-made and consisted of a double-walled quartz flask for introducing liquid nitrogen. The capillary was bent into a hoop, secured to a glass rod and positioned in the central region of the capillary-Dewar. 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 appeared on the screen, the HV power supply was immediately turned off and liquid nitrogen poured directly into the capillary-Dewar. Once frozen, arbitrary detection times can be used to completely characterize the separated analytes by 77 K fluorescence spectroscopy. The capillary inside the capillary-Dewar can be moved up and down by a translator or manually, in order to locate the next/former CE-separated analytes.

2.3 Sweeping-MEKC

The background solution consisted of 150 mM SDS and 30 mM H_3PO_4 in a mixed methanol-water solution (25:75 v/v), pH 2.5* (conductivity, 5.3 mS/cm). The analytes (*trans*-resveratrol standard or red wine extracts) were dissolved in a 20 mM phosphate buffer resulting in a nonmicelle buffer, with 3.3 mS/cm conductivity. Under these conditions, the electroosmotic flow (EOF) was negligible. Hydrodynamic injection was achieved by raising the sample reservoir 64 cm relative to the exit reservoir for a period of 100–1200 s. By using this procedure, ~5, 14, 27, 41, 46, and 55 cm column lengths (times: 100, 300, 600, 900, 1000, and 1200 s) of solution were injected into the capillary. The total and effective lengths of the capillary were 97 cm and 93 cm, respectively. When the injection was completed, −25 kV was applied to power the CE separation. This procedure permits the SDS-anionic surfactant micelles (in the inlet reservoir) to enter the sample zone. Thus, along the capillary axis, the samples were being swept and concentrated near the junction between the sample solution and the background solution. As in the following step, the samples were separated by the MEKC mode.

2.4 Extraction

A 400 μL aliquot of red wine was added to 800 μL of ethyl acetate, followed by shaking for 10 min. The upper layer was transferred to a clean tube and the organic solvent removed in a vacuum chamber. The organic phase (upper layer) was collected and evaporated to dryness. The residue was dissolved in 40 μL of sample matrix (an aqueous 20 mM H_3PO_4 solution) for the subsequent CE separation.

3 Results and discussion

3.1 On-line sample concentration

Figure 1 shows typical CE electropherograms of a *trans*-resveratrol standard separated by normal-MEKC (frame A) and sweeping-MEKC (frame B). The concentrations are 250 ppm and 250 ppb in frames A and B, respectively. Herein, in the normal-MEKC mode, the sample injection length was ~0.5 mm, whereas in the sweeping-MEKC mode the sample injection length was ~46 cm. The complete, optimal separation of *trans*-resveratrol was achieved using phosphate buffer (30 mM) containing SDS (150 mM) in a methanol-water solution (25:75 v/v). In comparison with the two electropherograms in frames A and B, a ~1500-fold improvement in detection sensitivity could be obtained. The inset in frame B shows the electropherogram corresponding to the lowest concentration of 50 ppb (S/N = 3, LOD ~ 5 ppb).

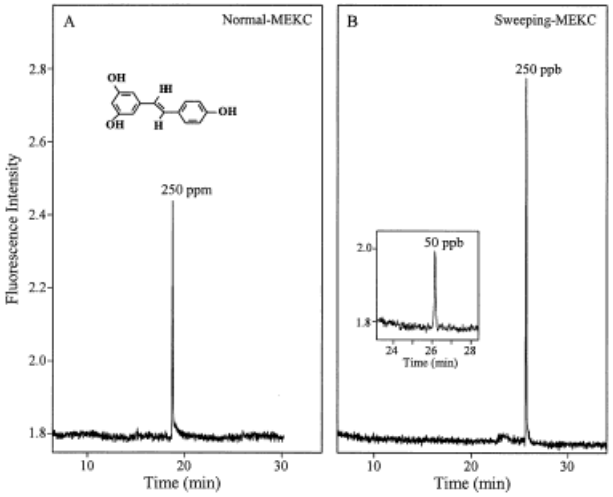

Figure 1. Electropherograms of *trans*-resveratrol obtained by (A) the normal MEKC mode (250 ppm) and (B) by the sweeping-MEKC mode (250 ppb). The inset in (B) shows the electropherogram at the lowest concentration of 50 ppb (S/N = 3, LOD ~ 5 ppb). CE conditions: (A) phosphate buffer (30 mM), SDS (150 mM) in a methanol-water solution (25:75 v/v); applied voltage, −25 kV; current, ~ −8 μA; injection length, ~0.5 mm; capillary length, 93/97 cm. (B) Injection length, ~46 cm; detection conditions: $\lambda_{ex}/\lambda_{em}$ = 313 ± 8 nm/400 ± 16 nm; UV cut filter, 320 nm; PMT, 700 V; room temperature.

Figure 2 shows the relationship between the length of the sample injection and the corresponding signal intensity in the sweeping-MEKC mode. Basically, the intensity of the signal increases linearly with the injection length. The inset shows the relationship between the length of the sample injection and plate numbers. The plate numbers decreased to 4×10^5 when the injection length exceeded ~ 50 cm. Thus, ~ 50 cm is a reasonable length for sample concentration by the sweeping mode, and, in this case, the next ~ 40 cm is necessary for the separation. For this reason, a 46 cm injection length was used in all subsequent experiments.

Table 1 summarizes and compares the linearity, limit of detection (LOD) values, relative standard deviations (RSD%) of peak area and migration times, and plate num-

Table 1. LOD values, RSD values, and plate numbers for *trans*-resveratrol with nonaqueous MEKC, normal aqueous MEKC, and sweeping-MEKC

A. Nonaqueous MEKC	
Equation of the line	$y = 2582.6x + 27\,776$
Coefficient of variation	$r^2 = 0.9964$
LOD (S/N = 3)	3 ppm
Detection range	5–250 ppm
RSD	
(a) Migration time	
Intra-day (n = 2)	3.65%
Inter-day (n = 2)	3.42%
(b) peak area	
Intra-day (n = 2)	14.5%
Inter-day (n = 2)	14.4%
Plate number	3.8×10^4
B. Normal MEKC	
Equation of the line	$y = 1649.6x - 12\,892.3$
Coefficient of variation	$r^2 = 0.9703$
LOD (S/N = 3)	17 ppm
Detection range	75–250 ppm
RSD	
(a) Migration time	
Intra-day (n = 2)	6.88%
Inter-day (n = 3)	0.95%
(b) peak area	
Intra-day (n = 2)	1.46%
Inter-day (n = 3)	11.3%
Plate number	2.7×10^5
C. Sweeping-MEKC	
Equation of the line	$y = 102.38x - 5424.23$
Coefficient of variation	$r^2 = 0.9917$
LOD (S/N = 3)	5 ppb
Detection range	25–250 ppb
RSD	
(a) Migration time	
Intra-day (n = 3)	1.52%
Inter-day (n = 6)	5.81%
(b) peak area	
Intra-day (n = 3)	1.24%
Inter-day (n = 6)	8.8%
Plate number	2×10^5–8×10^5

Fluorescence emission was measured at 400 ± 16 nm (300 grooves/mm).

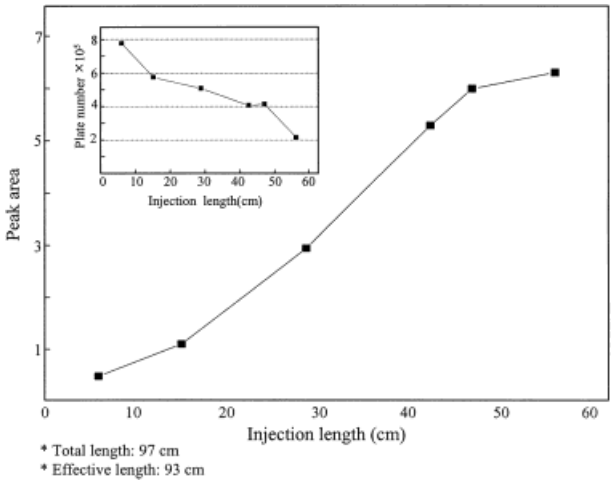

Figure 2. Relationship between sample injection length and related signal intensity, and plate numbers (inset) for sweeping-MEKC.

bers for nonaqueous MEKC (our previous study), normal aqueous MEKC and sweeping-MEKC (in this study), respectively. The linearity of these methods for *trans*-resveratrol was also fairly good. Herein, the CE buffer for nonaqueous MEKC contained 50 mM sodium cholate and 20 mM ammonium acetate in the methanol-acetonitrile solution (7:3 v/v); the applied voltage was 15 kV and the current was ~23 μA. The nonaqueous MEKC mode provided better sensitivity than normal aqueous MEKC. However, the use of an organic solvent made sweeping on-line sample concentration difficult to perform. For this reason, an aqueous CE buffer was used for on-line sample concentration in this study.

3.2 Optimized conditions for the separation of *trans*-resveratrol in red wine

Figures 3A and B show CE electropherograms of red wine extracts at different SDS concentrations and various ratios of methanol-water for CE separation by sweeping-MEKC mode, respectively. In all of these experiments, the sample injection length was 46 cm. In frame A, the CE buffers were methanol-water solutions (25:75 v/v), containing 30 mM H_3PO_4 and different concentrations of SDS (electropherograms a and b: 50 and 200 mM). The arrows show the peak corresponding to *trans*-resveratrol by the spiking method (data not shown). Neither 50 nor 200 mM concentrations of SDS provide efficient separation capability for *trans*-resveratrol from red wine extracts. An excellent separation for this analyte was achieved only at 150 mM of SDS (Fig. 4). In order to investigate the effects of organic solvent, we selected 150 mM of SDS as the test concentration and under exactly the same experimental

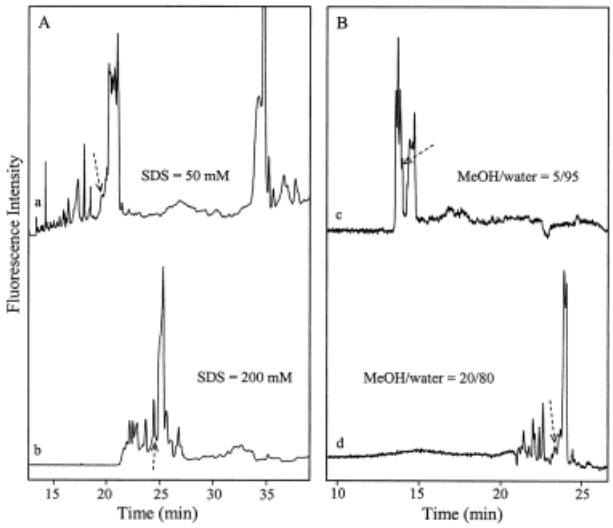

Figure 3. CE electropherograms of red wine extracts at different concentrations of SDS and various ratios of methanol-water by sweeping-MEKC mode. CE conditions: (A) 30 mM H_3PO_4 in methanol-water solutions (25:75 v/v) with different SDS concentrations (electropherograms a and b: 50 and 200 mM). (B) 30 mM H_3PO_4 and 150 mM SDS in different ratios of methanol-water solution (electropherograms c and d: 5/95 and 20/80 v/v). The arrows correspond *trans*-resveratrol peak by the spiking method (data not shown). Applied voltage, −25 kV; current, ~ −8 μA; injection length, 46 cm.

conditions, methanol-water solutions (electropherograms c and d: 5/95 and 20/80 v/v) were investigated. The findings show that these ratios did not lead to the isolating *trans*-resveratrol. The complete, optimal separation of *trans*-resveratrol from the red wine extract can be achieved with a phosphate buffer (30 mM) containing SDS (150 mM) in a methanol-water solution (25/75 v/v).

3.3 On-line sample concentration-CE/fluorescence spectral identification at 77 K

Figure 4 shows a typical CE electropherogram of a red wine extract by applying the sweeping-MEKC mode. The peak marked by an arrow is *trans*-resveratrol by the spiking method, the concentration of which was calculated to be 3.4 ppm. The inset spectra (solid line and dashed line) show the on-line 77 K spectra of a *trans*-resveratrol (10 ppm) standard and the CE-separated analyte (arrow) from the red wine extract, respectively. In this experiment, a 2400 groove/mm grating was used to acquire a higher spectral resolution (0.3 nm). Although higher spectral resolution always leads to poorer sensitivity, the fluorescence intensity increased by over ~30-fold for *trans*-resveratrol when the temperature was decreased to 77 K.

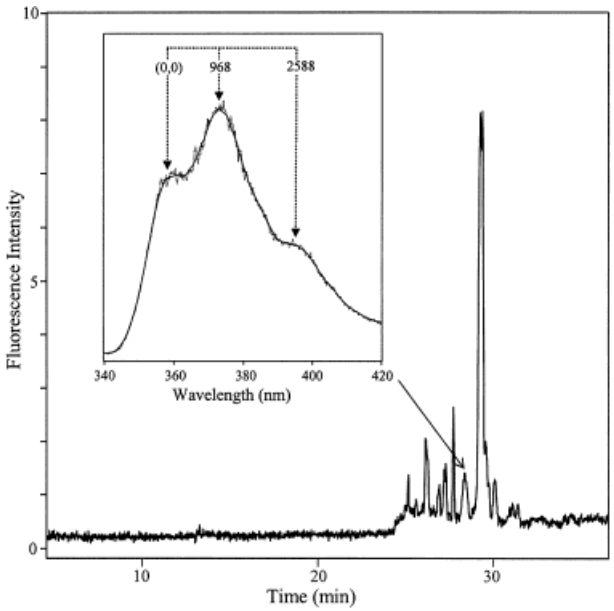

Figure 4. CE electropherogram of a red wine extract. CE conditions: phosphate buffer (30 mM), SDS (150 mM) in a methanol-water solution (25:75 v/v). Applied voltage, −25 kV; current, ~ −8 μA; injection length, 46 cm. Inset, the on-line 77 K fluorescence spectra for a 10 ppm *trans*-resveratrol standard (solid line) and the CE-separated analtye (dashed line) obtained using the sweeping-MEKC mode.

This property is similar to that of *trans*-stiblene which is only weakly fluorescent (Φ_F = 0.05) at room temperature but strongly fluorescent (Φ_F = 0.75) at 77 K. Hence, once the analyte was frozen, a 77 K fluorescence spectrum can be easily recorded. The 77 K fluorescence spectrum of the arrow marked peak was super imposable with the spectrum of the *trans*-resveratrol standard. Thus, we conclude that the *trans*-resveratrol in red wine could be absolutely identified using this approach. The observed wavelength of the (0,0) origin band of *trans*-resveratrol in this aqueous CE buffer (a mixed methanol-water solution; 25:75 v/v) was 360 nm, whereas its (0,0) origin band was observed at 359 nm when it was dissolved in methanol at 77 K. A red-shift of 1 nm was observed. The ground-state vibrational frequencies for the other bands are labeled in cm^{-1}. These numbers are important parameters for spectral fingerprinting. Although a CCD detector can be used to record the fluorescence spectra of these peaks immediately, nearly all of the detectors currently used in CE systems are designed to obtain data at room temperature. In most cases, at room temperature, these regular fluorescence spectra only provide a broad fluorescence spectrum, leading to difficulties in fingerprint identification, especially for the structurally similar compounds.

Although a number of reports of the analysis of *trans*-resveratrol have appeared, including MEKC-UV [34], HPLC-UV [31], and HPLC-fluorometry [35], all of these methods only provide broad band spectra for spectral identification. In this study, the proposed sweeping-MEKC method, the initial on-line concentration and separation at high sensitivity (LOD ~5 ppb)-low spectral resolution (400 ± 16 nm) was achieved by a 300 grooves/mm grating, after which, a cryogenic molecular fluorescence experiment was performed at 77 K using a 2400 grooves/mm grating with high spectral resolution. This proposed method may solve problems encountered for analytes that have a weak fluorescence intensity, broader fluorescence behavior at room temperature, or are present at low levels.

4 Concluding remarks

This work demonstrates that *trans*-resveratrol in red wine is readily distinguishable and can be unambiguously assigned through on-line sample concentration, thus improving the sensitivity and on-line 77 K fluorescence spectral identification (fingerprinting). The complete, optimal separation of *trans*-resveratrol from a red wine extract could be achieved with phosphate buffer (30 mM) containing SDS (150 mM) in a methanol-water solution (25:75 v/v) and a 1500-fold improvement in detection sensitivity was obtained compared with the normal-MEKC method. Although all of the separation conditions and the results of excitation by a Xe lamp are discussed, it is definitely clear that the use of a combination of a sweeping technique and a laser, especially a UV laser, can further improve the analysis of *trans*-resveratrol in wine.

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