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On-line multichannel Raman spectroscopic detection system for capillary zone electrophoresis

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ABSTRACT

An on-line multichannel Raman spectroscopic detection system for capillary zone electrophoresis using a charge-coupled device as the detector is described. Resonant, near-resonant and non-resonant excitation Raman spectroscopies are employed. The 400 cm^{-1} spectral window provides adequate information to identify resolved and unresolved compounds. The use of analyte velocity reduction to allow increased data acquisition times is described. With near-resonant enhancement, the technique is shown to allow detection of 500 attomoles of methyl orange from a 5-nl injection volume, corresponding to $1 \cdot 10^{-7}\text{ M}$.

INTRODUCTION

Since its emergence in the early 1980's, capillary zone electrophoresis (CZE) has become widely accepted as a high resolution separation technique [1,2]. While ultraviolet absorption spectroscopy and fluorescence are useful, there remains a need for detection systems which provide better molecular characterization. But, the small internal diameters ($10\text{--}75\text{ }\mu\text{m}$) of electrophoresis capillaries and the consequent small sample loadings have proven to be serious problems in development of high resolution spectroscopic detectors for capillary electrophoresis. Only electrospray ionization mass spectrometric detection [3] has yet proven practical.

In an earlier communication, we demonstrated the feasibility of resonance Raman detection with a single stage monochromator and a photomultiplier detection system [4]. Although resonance Raman spectroscopy was shown to have about the same detection limits as absorption spectroscopy, the single channel detector system was unable to exploit fully the fingerprinting capability of vibrational spectra. With a multichannel detector to allow fingerprinting Raman spectroscopy may be an attractive detection system for CZE.

Since the mid-1980's the slow-scan charge-coupled device (CCD) detector has emerged as the best general purpose multichannel Raman detector [5,6]. The CCD provides low read noise, nearly-zero dark current, high responsivity, multichannel capability and large dynamic range. This detector system appears well-suited to on-line Raman spectroscopic detection for CZE.

Vo-Dinh and co-workers [7] have already reported an on-line CCD-based fluorescence detector for CZE. These authors have shown the evolution of a fluorescence spectrum at 6-s intervals during electrophoresis and have demonstrated that the CCD provides better signal-to-noise ratio than a photomultiplier tube (PMT) in on-line applications. In the present communication we demonstrate on-line CCD detection of Raman spectra over a 400-cm^{-1} interval, using resonant, near-resonant and non-resonant excitation.

EXPERIMENTAL

Capillary zone electrophoresis

A block-diagram of the experiment is shown in Fig. 1. The CZE apparatus described previously [4] was used with only minor modification. In particular, the number of collection fibers was reduced from ten to two, and the capillary/fiber holder was redesigned, as shown in Fig. 2, to provide more rigid mounting of the capillary. A $55\text{ cm} \times 75\text{ }\mu\text{m}$ I.D. capillary (Polymicro Technologies TSP/075/375) was used as separation capillary. The detection region was formed by burning off a small section of protective coating about 5 cm from the exit end of the capillary. Sample introduction was by electromigration (5–10 s, 20 kV). The usual electrophoresis working voltage was 20 kV, at a current of 65–70 μA . Electrophoresis was carried out in a 0.02 *M* Trizma buffer (Tris[hydroxymethyl]aminomethane) at pH 7.15.

Raman spectroscopic detector

The on-line multichannel Raman spectroscopic detection system is shown in Fig. 1. The excitation source was a frequency doubled Nd-YAG laser (Quantronix 416), operated at 532 nm. The laser power at the capillary was about 300 mW. A $5 \times$

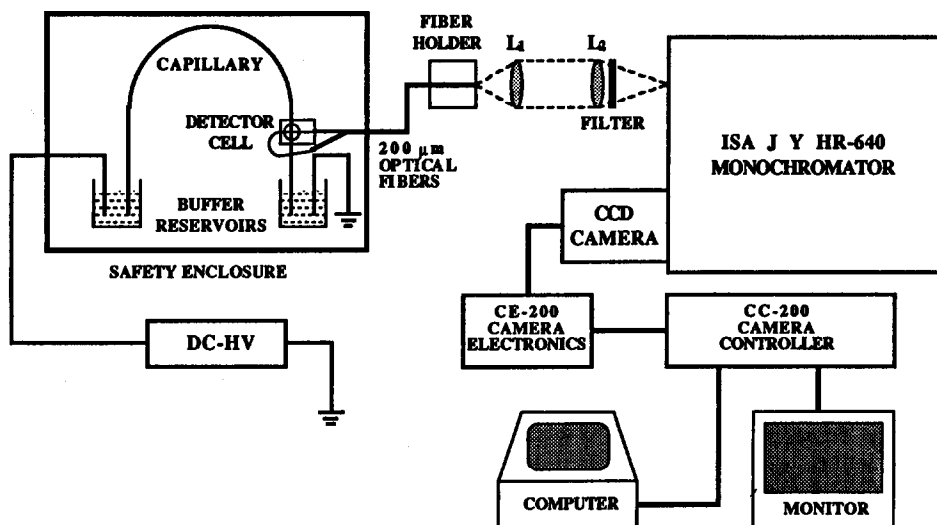


Fig. 1. Instrumentation for capillary zone electrophoresis with a CCD Raman spectroscopic detector. L_1 , $f = 50\text{ mm}$; L_2 , $f = 200\text{ mm}$; Filter, Schott OG-550.

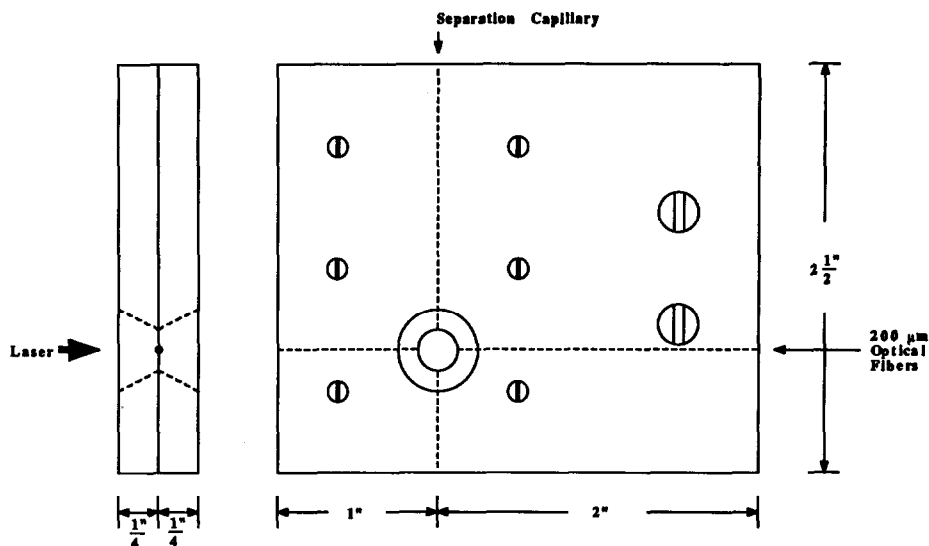


Fig. 2. Plexiglas capillary/optical fiber holder.

microscope objective (Rolyn 80.3045) focused the exciting laser beam directly on the capillary. The Raman signal was gathered by two 200- μm quartz optical fibers (General Fiber Optics, 16-200S). These fibers were placed directly against the capillary and normal to the plane of polarization of the exciting laser beam. The distal ends of the fibers were oriented parallel to the spectrograph entrance slit in a Plexiglas holder. A collimating lens, L_1 , ($f = 50$ mm) and focusing lens L_2 , ($f = 200$ mm) were used to couple the fiber output into the spectrograph. The signal collected by the fibers was filtered by a sharp cut filter (Schott OG-550) to attenuate the laser line scattering.

The Raman spectrometer consisted of a 0.64-m spectrograph (ISA Jobin Yvon HR-640) with a 1200 grooves/mm holographic grating, operated at $f/5.7$ and a cryogenically cooled charge-coupled device detector (Photometrics series 200, with PM 512 CCD chip). The laser was aligned to the capillary by centering the diffraction pattern obtained by focusing it into the capillary. The collection fiber alignment to the spectrograph and the spectrograph input optics were adjusted using intense fluorescence of Rhodamine 6G. For Raman spectroscopy the entrance slit was 300 μm , to give spectral resolution of 12 cm^{-1} . The CCD detector chip had 512 \times 512 pixels, each pixel 20 μm square. A Raman spectrum was obtained by binning the CCD vertically to form a 512 \times 1 data array. A personal computer with a locally written program controlled the detector, converted binary data from the CCD to ASCII arrays and stored spectra.

Reagents

The running buffer, 0.02 M Trizma at pH 7.15, was prepared from Trizma base and Trizma HCl (Sigma). Epinephrine, phenylalanine and tryptophan were purchased from Sigma. Methyl orange and methyl red were purchased from Aldrich. All other reagents were of ACS reagents grade. All compounds were used as received. Type I water was used to prepare all solutions.

Procedure

Adrenochrome was prepared by reaction of epinephrine with excess triiodide in the Trizma buffer. After 2–3 min, excess triiodide was destroyed by potassium thiosulfate and the adrenochrome diluted with Trizma buffer to a final concentration of $5 \cdot 10^{-4} M$.

RESULTS AND DISCUSSION

The resonance enhanced Raman spectra of methyl orange and methyl red have been reported [8]. The base forms of methyl orange and methyl red have strongly enhanced N=N stretches around 1410 cm^{-1} . However, the absorption maxima of the basic forms of methyl orange and methyl red are 464 and 440 nm. As shown in Fig. 3, these wavelengths are about $3000\text{--}4500 \text{ cm}^{-1}$ to the blue of the frequency doubled Nd-YAG line (532 nm). Thus, our spectra are taken at an exciting wavelength closer to pre-resonance than to true resonance excitation. Nonetheless, on-line spectra can be acquired at good signal-to-noise ratio.

The required integration time is a compromise between the signal collection needs of Raman spectroscopy and the resolution requirements of capillary electrophoresis. Fig. 4 shows a time-series of on-line Raman spectra obtained during electrophoresis of $5 \cdot 10^{-4} M$ methyl orange and methyl red in Trizma buffer at pH 7.2. At high concentrations, $1 \cdot 10^{-4} M$ and higher, we could acquire spectra with acceptable signal-to-noise ratios with 1 or 2 s integration times. However, to avoid unrealistically high detection limits, we chose to increase integration times to 5 s. Although some resolution is sacrificed by the long integration time, baseline separation of this pair of compounds is still obtained, as shown in a plot of the time-evolution of the 1416 cm^{-1} signal, Fig. 5. The figure also shows the presence of impurities in the sample. The peaks at 2.7 and 3.6 min are fluorescence, not Raman

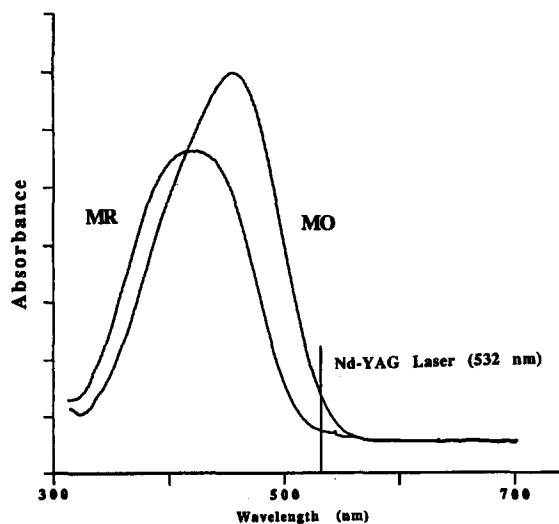


Fig. 3. Absorption spectra of methyl red (MR) and methyl orange (MO). The position of the 532 nm laser line is shown.

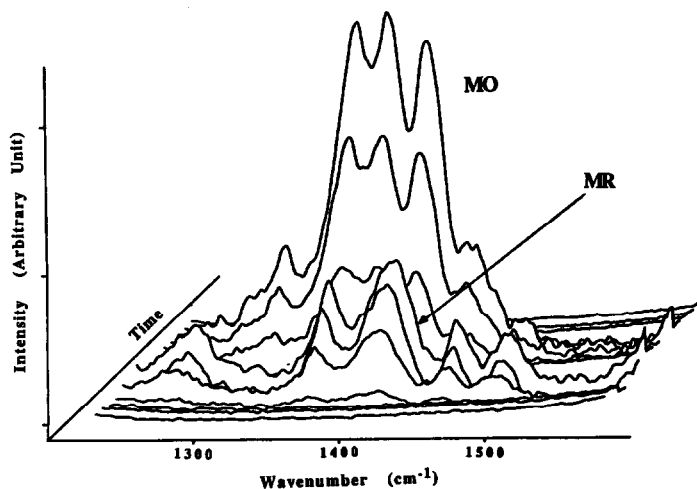


Fig. 4. Time series Raman spectra of $5 \cdot 10^{-4}$ M methyl orange (MO) and methyl red (MR) in 0.02 M Trizma buffer, pH 7.2. CCD integration time of 5 s for each spectrum. Sample injection: 20 kV, 5 s; CZE at 20 kV; laser power: 250 mW.

scatter. At both peaks a high background was observed, but there were no resolved Raman bands.

Despite the theoretical loss of sensitivity from the use of pre-resonance excitation, the detection limits ($S/N = 3$) for 5 s integration time, $6 \cdot 10^{-7}$ M, compare favorably with both absorbance detection limits and our earlier single-channel resonance Raman results [4]. That detection limits are unchanged despite the use of a less-favorable excitation wavelength can be attributed to two factors. First, the incident laser power has been increased from 40 mW to 250 mW. Second, the CCD has

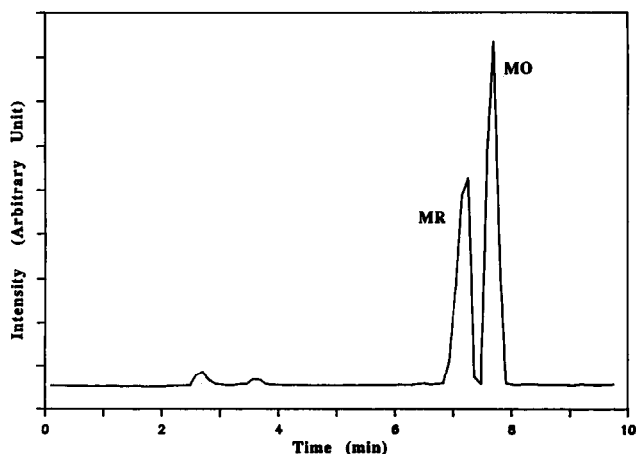


Fig. 5. Raman-detected (1416 cm^{-1}) electrophoretogram of $5 \cdot 10^{-4}$ M methyl red (MR) and methyl orange (MO) in 0.02 M Trizma buffer. Sample injection: 20 kV, 5 s; CZE at 20 kV; laser power: 250 mW.

a higher quantum efficiency and is inherently quieter than the PMT employed in our earlier studies.

By increasing the integration time to 10–40 s, we can further decrease the detection limits without increasing laser power or changing excitation wavelength. This long time scale is incompatible with sample residence time in the field of view of the detector, of course. To circumvent this problem, we reduce the driving DC voltage from 20 kV to 7 kV when the sample zone reaches the detection window. Fig. 6 compares the Raman spectra at the equivalent points in the electrophoretogram for a series of integration times. This strategy allows reduction of detection limits to 4, 2 and $1 \cdot 10^{-7} M$ for 10, 20 and 40 s integration times. With 5 nl injection volume and 40 s integration time, the limit of detection (LOD) for this system is 500 attomoles ($5 \cdot 10^{-16}$ moles).

If the width of a CZE band is strictly limited by diffusion, then there is some loss of resolution associated with reduction of the driving voltage [9]. In practice, bands are rarely as narrow as predicted from diffusion coefficients, so that the resolution loss from the increased residence time is negligible. A greater concern is the possible destruction of delicate compounds by prolonged laser irradiation. While we can not detect any decomposition of these azo compounds, driving voltage reduction is not a risk-free technique.

The most common chromatographic derivatization strategy is to attach a chromophore or fluorophore to a molecule. Typically, the accessible electronic transitions of the derivative will be localized on the added chromophore. Consequently, such a derivative will have a resonance Raman spectrum which is more characteristic of the derivatizing reagent than of the target molecule. Raman detection of such derivatives is not especially informative.

Among the simplest exceptions to this generalization are derivatives which are formed by oxidation of molecules. Although oxidation may be followed by rearrangement of the molecule, or by uptake or expulsion of small groups such as hydroxyl ions,

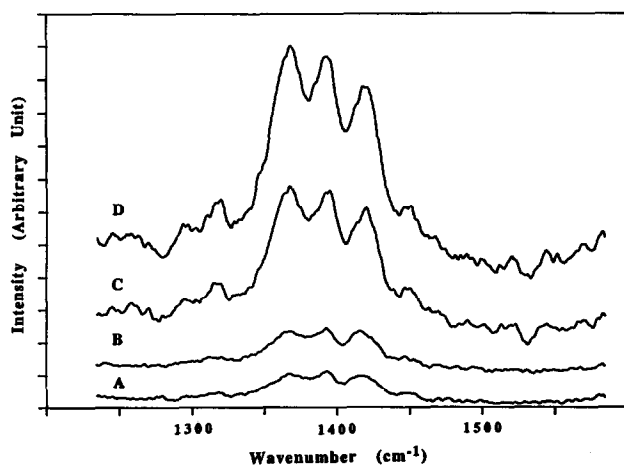


Fig. 6. On-line Raman spectra at different integration times for $5 \cdot 10^{-5} M$ methyl orange in 0.02 *M* Trizma buffer at pH 7.2. (A) 5 s; (B) 10 s; (C) 20 s; (D) 40 s. Sample injection: 20 kV, 5 s; laser power: 250 mW.

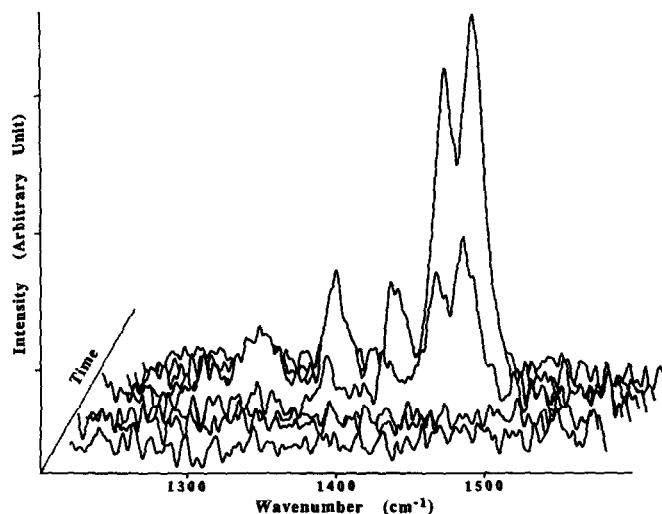


Fig. 7. Time series Raman spectra of $5 \cdot 10^{-4} M$ adrenochrome in $0.02 M$ Trizma buffer, pH 7.1. CCD integration time of 20 s for each spectrum. Sample injection: 20 kV; 5 s; CZE at 20 kV; laser power 220 mW.

the resulting final species will often reflect the structure of the starting material quite faithfully. In such cases the specificity of vibrational spectroscopy can be usefully employed. As a test of this strategy, we have chosen to oxidize epinephrine to the red-orange adrenochrome ($\lambda_{\max} = 490 \text{ nm}$). Adrenochrome has relatively strong $C=N^+$ or $C=N$ stretches at 1474 and 1454 cm^{-1} [10]. Fig. 7 shows a time-series of on-line resonance Raman spectra of $5 \cdot 10^{-4} M$ adrenochrome in Trizma buffer at pH 7.1. However, adrenochrome is not completely stable over time or under the high power density of our experiment. Although the samples were filtered prior to introduction into the column, we observed flashes of scattered laser light, characteristic of suspended particles, with a longer migration time than adrenochrome itself. The light scattering is probably due to polymerization of adrenochrome to water-insoluble melanins [11]. Even so, with reduced running voltage and 20 s integration, the detection limits for adrenochrome is about $10^{-5} M$. This value is about 10-fold higher than can be obtained with larger volumes in static samples.

Although it is insensitive, non-resonance Raman is not entirely unfeasible. Fig. 8 shows a time series of on-line non-resonance Raman spectra taken during electrophoretic separation of $0.05 M$ of phenylalanine (PHE) and tryptophan (TRP) in Trizma buffer at pH 7.1. This mixture is incompletely resolved under these conditions and a non-selective detector, such as a refractive index detector, shows only a single broad peak. However, in the Raman spectrum it is easy to identify the phenylalanine, 1006 cm^{-1} and tryptophan 1013 cm^{-1} bands. Although it is just barely observable above the background noise, the 880 cm^{-1} band tracks tryptophan migration only. The phenylalanine 1033 cm^{-1} band tracks the migration of that molecule through the detector region with somewhat better signal-to-noise ratio.

Allowed in-plane aromatic ring modes are typically strong Raman bands [12]. Phenylalanine and tryptophan represent rather favorable test cases for non-resonance Raman. Comparable results would be expected for other water-soluble aromatic

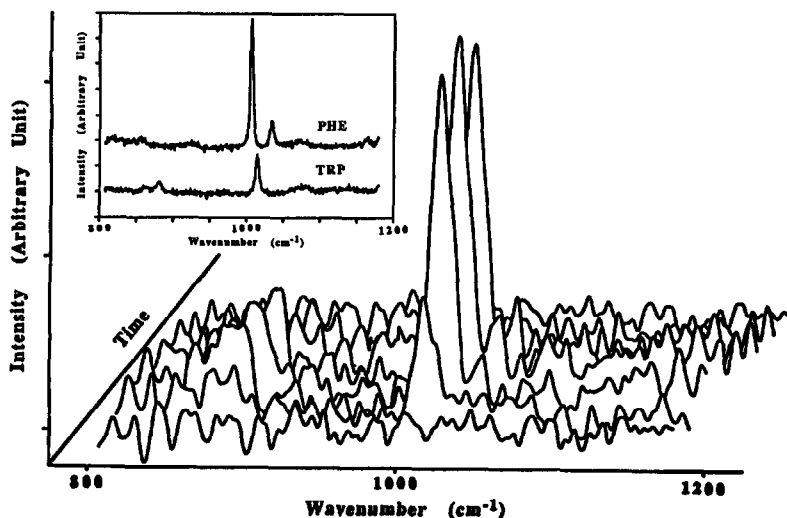


Fig. 8. Time series Raman spectra of 0.05 *M* phenylalanine and tryptophan in 0.02 *M* Trizma buffer, pH 7.2. CCD integration time of 10 s for each spectrum. Sample injection: 20 kV, 10 s; CZE at 20 kV; laser power 200 mW. Non-resonant Raman spectra of phenylalanine (PHE) and tryptophan (TRP) in Trizma buffer, pH 7.2, is shown in inset.

molecules. Better sensitivity could be obtained by increasing laser power to several watts and by multipassing the laser beam through the sample. However, it is unlikely that detection of molecules at concentrations much below 10^{-3} *M* is feasible if the exciting wavelength is far from resonance.

CONCLUSION

The feasibility of on-line multichannel Raman spectroscopic detection for CZE has been demonstrated. However, resonance or pre-resonance enhancement is required to achieve satisfactory detection limits. To be more generally useful, excitation in the ultraviolet or near-ultraviolet is probably necessary. Because scattering intensity increases with the fourth power of frequency, required ultraviolet laser powers are quite low. The familiar He–Cd 325 nm line may prove useful at power levels below 20 mW, and the recently developed neon ion lasers may provide adequate power at various wavelengths between 332 and 370 nm. In general, the use of low resolution ($6\text{--}12\text{ cm}^{-1}$) is adequate. A wider spectral window is desirable, and could be achieved by the use of shorter focal length monochromators and coarser gratings. Our CCD is a 512×512 pixel device designed for imaging. Low noise chips optimized for spectroscopy (1152×298 pixels) are now becoming available and should also contribute to improved performance of a Raman detector.

The fiber optic collection system is quite flexible. Other fibers could be added to provide simultaneous absorbance detection slightly upstream of the Raman detector. An absorbance/Raman detector would allow optimization of the reduced velocity technique for Raman spectrum acquisition.

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