Journal of Chromatography, 634 (1993) *309-315*
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 814

Peroxyoxalate chemiluminescence detection in capillary electrophoresis

Nian Wu and Carmen W. Huie*

Department of Chemistry, State University of New York at Binghamton, Binghamton, NY 13902-6000 (USA)

(First received October 15th, 1992; revised manuscript received December 14th, 1992)

ABSTRACT

The feasibility of employing peroxyoxalate chemiluminescence (PO-CL) detection in capillary electrophoresis (CE) was demonstrated using a two-step approach for the CE separation and dynamic elution (elution under pressure) of the analytes. In this approach, potential problems associated with incompatibilities between mixed aqueous-organic solvent and electrically driven separation systems were avoided by switching off the CE power supply at an appropriate time and connecting the CE capillary to a syringe pump to effect dynamic elution. The effects of dynamic flow-rate and PO-CL reagent concentration on the CL signal intensity and/or peak width were examined for the measurements of three dansylated amino acids. The average limit of detection for these analytes using this PO-CL method is about 1.2 fmol (*ca.* 85 nM) which is approximately 35-fold lower than UV absorption methods.

INTRODUCTION

Due to the small sample volume requirement in capillary electrophoresis (CE), the development of new detection methods capable of providing improvements in limits of detection (LOD) is an important area of research. A wide range of method based on well-known detection principles have been demonstrated to be quite useful for CE, including UV absorption [1–3], fluorescence [4–6], mass spectrometry [7,8], conductivity [9] and electrochemistry [10,11]. Among these methods UV absorption is the most popular because most organic analytes possess high molar absorptivities at the 210-nm region; nevertheless, UV absorption method lacks sensitivity primarily due to the short optical path-length available across the capillary column. To achieve significant gain in LOD, detection methods based on phenomena of high inherent sensitivity, such as fluorescence, should be used. For example, using a laser for fluorimetric excitation, detection of zeptomole (10^{-21} mol) quantities of analytes has been reported [5,6]. However, laser-induced fluorescence methods possess some disadvantages, such as the presence of significant background noise, e.g., Rayleigh and Raman scattering generated by the high-intensity laser, and the high price and complexity of most laser systems. These particular disadvantages could be, however, largely overcome by performing fluorescence measurements via chemical excitation, i.e., the generation of fluorescence in which the electronically excited state of the molecule is provided by a chemical reaction (chemiluminescence).

Chemiluminescence (CL) has been shown to be a highly sensitive method for detection in conventional [12,13] and microcolumn [14] high-performance liguid chromatography (HPLC). Recently the feasibility of using CL as a detection scheme in CE has been demonstrated as well using the luminol CL system for the sensitive detection of two luminol derivatives [15]. It should be noted that, however, among the most

* Corresponding author.

0021-9673/93/$06.00

common CL systems, e.g., luminol, lucigenin and the peroxyoxalate (PO)-CL reaction, the PO-CL system has been most widely used for post-column detection in HPLC [16]. The popularity of the PO-CL reaction, which is based on the oxidation of an oxalate derivative in the presence of a suitable fluorophore, is in part due to its high quantum efficiency and, perhaps more importantly, its ability to excite a wide range of different fluorophores when compared to other CL systems. Although the use of organic solvents is required in most common applications of PO-CL detection in HPLC involving reversed-phase columns due to the low solubility and instability of most oxalate derivatives in aqueous solutions, the post-column addition of PO-CL reagents to the column effluent containing the fluorophores to generate CL emission can be achieved with excellent sensitivity while maintaining good separation performance under optimized experimental conditions [17]. The involvement of mixed aqueous-organic solvent systems, however, could present some major difficulties in the use of PO-CL reaction as a detection scheme in CE due to, for examples, the influence of organic solvents on the migration behaviors of the analytes, which are strongly dependent on their mobilities in the aqueous electrophoretic buffer, and the possible effect of high electric field strength on the stability of the PO-CL reagents [18].

Camilleri et al. [19] have recently demonstrated that CE separation of proteins and peptides followed by elution under pressure (dynamic elution) can be achieved with little loss of resolution, thus allowing the possible use of CE as a micropreparative technique. In this paper a similar two-step approach, involving switching off the CE power supply at an appropriate time and connecting the capillary to a syringe pump to effect dynamic flow, was demonstrated for the post-column detection of analytes in CE using the PO-CL reaction. Using dynamic elution, potential problems associated with incompatibilities between mixed aqueous-organic solvent and electrically driven separation systems are avoided. The effects of dynamic flow-rate and PO-CL reagent concentration on the CL signal intensity and/or peak width were examined for the measurements of three dansylated (Dns)-amino acids. LOD obtained using the present PO-CL method were compared to those of UV absorption method.

EXPERIMENTAL

Chemicals

Bis-(2,4,6-trichlorophenyl)oxalate (TCPO) was prepared using the procedures described by Mohan and Turro [20]. Dns-Glycine, Dns-L-arginine and Dns-L-leucine were purchased from Sigma (St. Louis, MO, USA). Hydrogen peroxide (30%) and all other chemicals were of analytical grade from Aldrich (Milwaukee, WI, USA). TCPO was dissolved in ethyl acetate and hydrogen peroxide was diluted with acetonitrile. All buffer solutions were made with distilled and deionized water.

Apparatus

CE separation was performed with a laboratory-built instrument consisting of an acrylic box designed with a safety-interlocked door to prevent operator contact with a + 30 kV high-voltage power (Glassman High Voltage, Whitehouse Station, NY, USA), which was connected to the buffer reserviors with platinum electrodes to effect CE separation. For on-line UV absorption detection, a detection window was created by burning off a small section of the polyimide coating at 40 cm from the anodic end of the electrophoretic capillary (55 cm x 75 μm I.D. x 144 μm O.D.) purchased from Polymicrotech (Phoenix, AZ, USA). Absorption of analytes which migrate pass the detection window was measured using a Spectra 100 UV-Vis detector set at 210 nm (Spectra-Physics, San Jose, CA, USA). For post-column CL detection, a post-column reactor which consisted of various fused-silica capillaries held within a Swagelock stainless-steel tee and a detection cell was constructed.

Fig. 1 shows a schematic diagram of the post-column reactor. One arm of the tee contained the electrophoretic capillary which was inserted into the reaction capillary (10 cm x 200 μm I.D. × 400 μm O.D.; Supelco, Bellefonte, PA, USA) situated at the opposite arm of the tee.

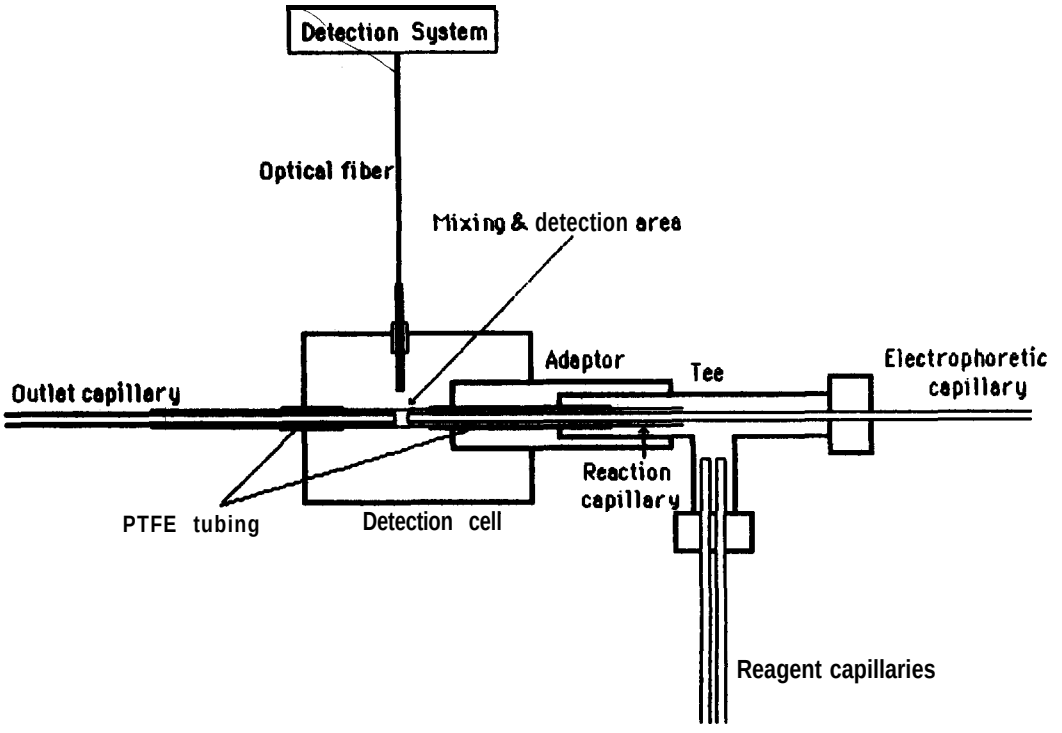

Fig. 1. Cross-sectional view of the post-column reactor.

The tee was connected to the detection cell via an adaptor and both the electrophoretic and reaction capillaries were inserted into the detection cell through the inner core of a PTFE tubing (400 μm I.D. x 1.5 mm O.D.), which served to protect the thin and fragile wall of the fused-silica capillaries. The reaction capillary extended from the tee through the entire length of the detection cell whereas the electrophoretic capillary terminated at a fixed distance near the center of the detection cell; an outlet capillary with smaller dimensions (20 cm x 100 μm I.D. × 195 μm O.D., Polymicrotech) was inserted into the opposite end of the reaction capillary to create a restricted area adjacent to the terminating end of the electrophoretic capillary to allow for mixing of the PO-CL reagents and column effluent containing analytes to occur within this post-column region. Two reagent capillaries (15 cm X75 μm I.D. x 144 μm O.D., Polymicrotech) inserted into the central arm of the tee were used to deliver the PO-CL reagents (TCPO and H_2O_2) into the mixing area through the small gaps that exist between outer surface of the electrophoretic capillary and inner surface of the reaction capillary. Dynamic elution of electrophoretic buffer and transport of the PO-CL reagents under pressure were achieved using two Sage syringe pumps (Model 341B; Orion, Boston, MA, USA).

To detect CL emission generated within the post-column mixing region, a detection window on the reaction capillary was made by burning off 2 mm length of the polyimide coating. The CL emission was collected via one end of an optical fiber bundle: 61 cm long x 1.6 mm diameter with a numerical aperture of 0.55 and an acceptence angle of 68° (Part No. 77520; Oriel, Stratford, CT, USA) situated directly above the detection window, and the other end of the fiber bundle was interfaced to the detection system. The CL emission was isolated by a 10-nm bandpass filter centered at 520 nm (Corion, Holiston, MA, USA) and was detected using a photomultiplier tube (Model 9558B; EMI, Plainview, NY, USA) operated at voltage between 700 and 800

V. The photocurrent was fed to a picoammeter (Model 7080, Oriel) and the signal was recorded on an integrator (Chromjet, Spectra-Physics).

Procedures

The CE separations were performed with a 20 m*M* sodium borate buffer (pH 8.9). Dns-amino acid stock solutions were prepared by dissolving appropriate amounts of the analytes into the buffer solutions. After making serial dilutions to obtain the desired concentrations, the sample solutions were electrokinetically injected into the CE system at the anodic end by applying 9 kV for 5 s. The electrophoretic capillary was treated by purging with 0.05 *M* NaOH for about 0.5 h and then rinsed with the run buffer for 2 h before use.

The CE separation of analytes followed by dynamic elution was accomplished by first turning on the power supply at 16.5 kV for 3.2 min. During this time, the syringe pump connected to the reagent capillaries was turned on momentarily to provide a fresh supply of PO-CL reagents into the post-column mixing region. After 3.2 min, both the reagent supply pump and high-voltage power supply were turned off. The anodic and cathodic ends of the electrophoretic capillary were then removed from buffer reserviors and attached to a syringe pump and one arm of the tee as shown in Fig. 1, respectively. Immediately afterward, delivering of the PO-CL reagents was resumed and dynamic elution of the analytes was started by turning on the two separate syringe pumps that were adjusted to the appropriate flow rates.

RESULTS AND DISCUSSION

One of the most important factors which limits detectabilities in PO-CL based HPLC systems is that without the presence of fluorescent analytes, a relatively low background CL signal can be detected when the column effluent is mixed with the PO-CL reagents [13,21]. Recent evidence suggests that this background signal may arise from reaction intermediates and/or products of the PO-CL reaction, and its intensity can be influenced by various experimental factors [17,22]. We found that the flow-rate of the PO-CL reagents (TCPO and H_2O_2) affected the background signal intensity as follows: at 2.5 μl/min or lower, the background current was found to remain relatively constant at 2.5 nA and it increased by a factor of about 2 at flow-

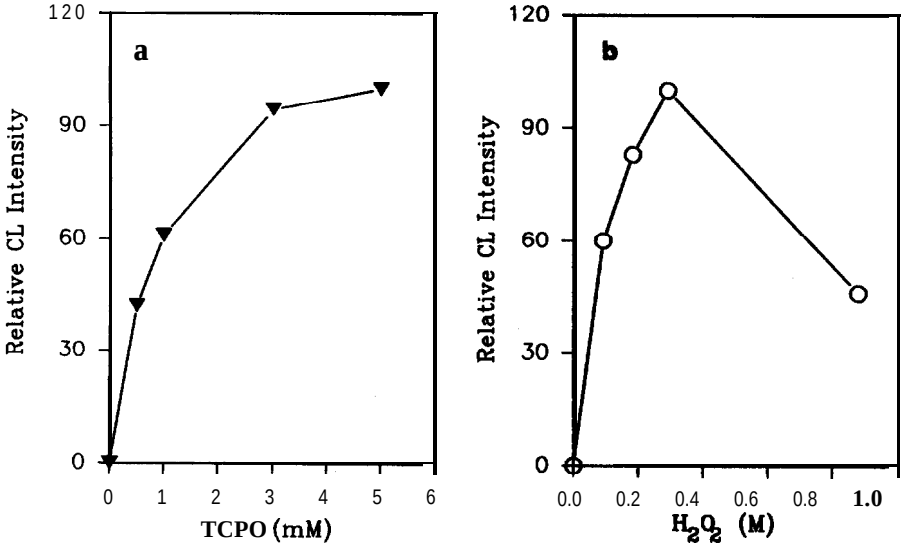

Fig. 2. Effects of (a) TCPO and (b) H_2O_2 concentrations on the relative CL intensity of 54 μM of Dns-L-leucine. The corresponding concentrations of TCPO and H_2O_2 in (a) and (b) were fixed at 5 m*M* and 0.29 M, respectively. Dynamic elution and PO-CL reagents flow-rate *were* set at 1.7 μl/min.

rates higher than 4 μl/min (dynamic flow-rate of the CE running buffer was kept at an optimum rate of 1.7 μl/min, see below). Using a flow-rate of 1.7 μl/min for both the CL reagents and running buffer, Fig. 2a and b shows that the concentrations of TCPO and H_2O_2 which produced the highest CL intensity for the post-column detection of Dns-L-leucine were found to be 5 m*M* and 0.3 *M*, respectively.

A major concern in using the present approach is that after CE separation, peak broadening due to diffusion and/or other processes may occur, resulting in loss of resolution during the dynamic elution process. Fig. 3 shows the effects of flow-rate due to dynamic elution on the relative peak width (measured at the baseline) of three Dns-amino acids after CE separation. When compared to the normalized peak width of these analytes separated by CE without the influence of dynamic elution and detected by UV absorption detection method (Fig. 3: relative peak width = 1, at flow-rate = 0), it can be seen that broadening of the peak widths indeed occurred at a flow-rate of 1.2 μl/min. This broadening effects were reduced significantly as a result of an increase in the flow-rate, with the peak widths approaching those obtained without dynamic elution after about 2 μl/min; however, it should be noted that in conjunction with the decrease in peak broadening a loss in separation selectivity was found at higher flow-rates. Fig. 4 shows the effects of flow-rate due to dynamic elution on the relative CL intensity of three Dns-amino acids. It is clear that optimal CL intensity occurred at lower flow-rates (<1.5 μl/min) and started to decrease significantly, in particular for Dns-L-leucine and Dns-glycine, at flow-rates higher than cu. 1.7 μl/min. Importantly the results obtained in Figs. 3 and 4 indicated that in choosing a flow-rate for dynamic elution of the three Dns-amino acids, a compromise has to be made between separation and detection performance, and it appears that the optimum flow-rates fall in the range between cu. 1.5 and 2.0 μl/min.

Using the optimized conditions that have been determined for the flow-rates and PO-CL reagent concentrations, the separation and detection characteristics of the three Dns-amino acids after CE separation followed by dynamic elution and post column PO-CL detection are shown in Fig. 5a. For comparison purposes, Fig. 5b shows the CE separation and detection of the same Dns-amino acids without the influence of elution under pressure and using on-line UV absorption method. It can be seen that although the peak widths of the three analytes were broadened as a result of dynamic elution and post-column CL

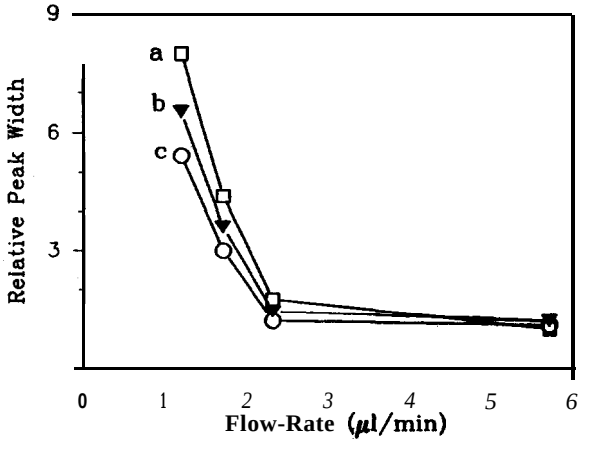

Fig. 3. Effects of flow-rate due to dynamic elution on relative peak width (at baseline) of three Dns-amino acids: (a) Dns-L-argenine = 1.24 μM; (b) Dns-L-leucine = 1.08 μ*M* and (c) Dns-glycine = 1.26 μ*M*. Flow-rate for the PO-CL reagents was 1.7 μl/min and the respective concentrations for TCPO and H_2O_2 were 4.5 m*M* and 0.29 *M*.

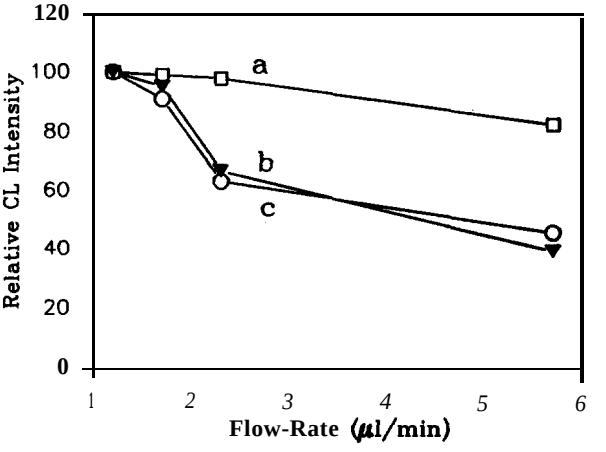

Fig. 4. Effects of flow-rate due to dynamic elution on the relative CL intensity of (a) Dns-L-argenine, (b) Dns-L-leucine and (c) Dns-glycine. Experimental conditions were as in Fig. 3.

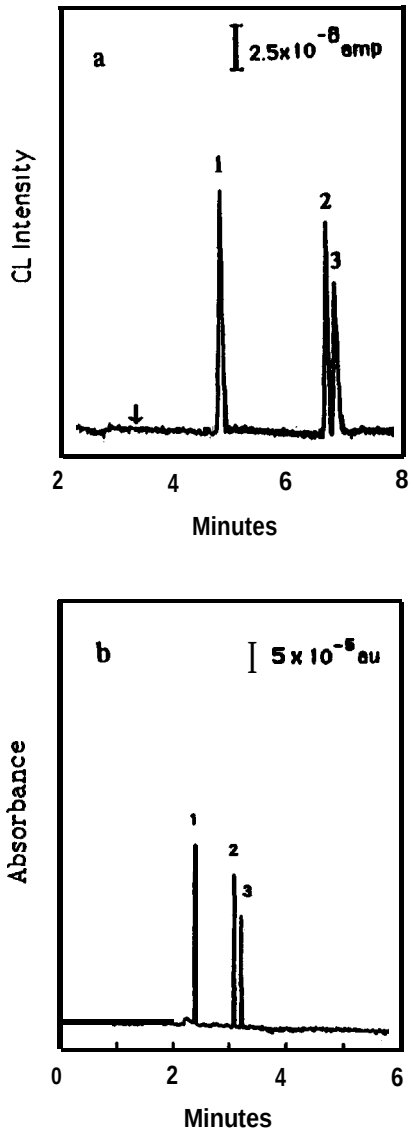

Fig. 5. (a) CE separation of (1) 1.24 μM of Dns-L-argenine; (2) 1.08 μM of Dns-L-leucine and (3) 1.26 μM of Dns-glycine followed by dynamic elution and PO-CL detection. Dynamic elution and PO-CL reagents flow-rate were set at 1.7 μl/min. Concentrations of TCPO and H_2O_2 were 4.5 mM and 0.29 M, respectively. The arrow indicates the time at which the high-voltage power supply was turned off. (b) CE separation of (1) 62 μM Dns-L-argenine; (2) 54 μM of Dns-L-leucine and (3) 63 μM of Dns-glycine and detected using on-line UV absorption method.

reaction (baseline peak widths increased by a factor of about 3 to 4 at a flow-rate of 1.7 μl/min as shown in Fig. 3), the separation of all the Dns-amino acids can still be achieved with baseline resolution. The advantage of the present method is in the improvement of LOD using the PO-CL system for sensitive detection. When compared to UV absorption detection, Table I shows an improvement factor of 1 to 2 order of magnitude in mass or concentration LOD was achieved for the measurements of the three Dns-amino acids using the present method. Average relative standard deviation (n = 3) on peak height for the three dansyl amino acids measured using the PO-CL method as shown in Fig. 5a was about 4.1%.

In conclusion we have demonstrated the feasibility of using the PO-CL system for the post-column detection of analytes after CE separation followed by elution under pressure. Clearly, more investigations are needed to optimize the various experimental factors which affect detection and separation performance using the present method. For examples, the use of deuterated running buffer may minimize the loss of resolution due to slower rate of analyte diffusion within the higher viscosity buffer solution [19]; modifications of the volume and geometry of the detection cell and connecting hardware may lead to less dispersion and/or higher degree of mixing between analytes and reagents; and also, the change in temperature, pH and the addition of catalyst and organic modifiers may affect CL efficiency and/or kinetics. Many of these factors have already been investigated in detail for the optimal determination of analytes in post-column HPLC systems involving the PO-CL reaction [12,17] and the knowledge gain in this area could be directed toward the improvements of separa-

TABLE I

COMPARISON OF CONCENTRATION AND MASS DETECTION LIMITS BETWEEN ABSORBANCE AND CHEMILUMINESCENCE DETECTION METHODS

Dns-Amino acid	LOD (S/N = 3)	
	UV absorption	PO-CL
Dns-L-Argenine	42 fmol(2.4 μM)	1.2 fmol(71 nM)
Dns-L-Leucine	32 fmol(2.3 μM)	*1.0* fmol(69 nM)
Dns-Glycine	50 fmol(3.8 μM)	1.5 fmol(l14 nM)

tion and detection performance using dynamic elution and PO-CL detection in CE.

ACKNOWLEDGEMENTS

The authors thank Dr. Richard A. Hartwick for the use of his research facilities and Spectra-Physics for financial assistance.

REFERENCES

1 Y. Walbroehl and J.W. Jorgenson, ***J. Chrotnatogr.,*** 315 (1984) 135.
2 M. Krejčí, K. Tesařík and J. Pajurek, ***J. Chromotogr.,*** 191 (1980) 17.
3 T. Tsuda and G. Nakagawa, ***J. Chromatogr., 268 (1983) 369.***
4 J.W. Jorgenson and K.D. Lukacs, ***Anal. Chem., 53*** (1981) 1298.
5 S. Wu and N.J. Dovichi, ***J. Chromatogr., 480*** (1989) 141.
6 J.V. Sweedler, J.B. Shear, H.A. Fishman, R.W. Zare and R. Scheller, ***Anal. Chem.***, 63 (1991) 496.
7 R.A. Smith, J.A. Olivares, N.T. Nguyen and H.R. Udseth, ***Anal. Chem., 60*** (1989) 436.
8 M. Moseley, L. Deterding, K. Torner and J.W. Jorgenson, ***J. Chromatogr., 480*** (1989) 197.
9 X. Huang and R.N. Zare, ***Anal. Chem.,*** 63 (1991) 2193.
10 F.E.P. Mikkers, F.M. Everaerts and Th.P.E.M. Verheggen, ***J. Chromatogr.,*** 169 (1979) 11.
11 R.A. Wallingford and A.G. Ewing, ***Anal. Chem., 59*** (1987) 1763.
12 S. Kobayashi and K. Imai, ***Anal. Chem., 52*** (1980) 424.
13 K.W. Sigvardson and J.W. Birks, ***Anal. Chem., 55*** (1983) ***432.***
14 M.L. Grayeski and A. Weber, ***Anal. Lett.***, 17 (1984) 1953.
15 R. Dadoo, L.A. Colon and R.N. Zare, ***J. High Resolut. Chromatogr., 15 (1992) 133.***
16 K. Robards and P.J. Worsfold, ***Anal. Chim. Acta, 266 (1992) 147.***
17 P.J.M. Kwakman and U.A.Th. Brinkman, ***Anal. Chim. Acta, 266 (1992) 175.***
18 R. Brina and A.J. Bard, ***J. Electroanal. Chem., 238*** (1987) ***277.***
19 P. Camilleri, G.N. Okafo and C. Southan, ***Anal. Biochem.***, 196 (1991) 178.
20 A.G. Mohan and N.J. Turro, ***J. Chem. Educ.***, 51 (1974) 528.
21 R. Weinberger, C.A. Mannan, M. Cerchio and M.L. Grayeski, ***J. Chromatogr., 288*** (1984) ***445.***
22 ***N.*** Hanaoka, H. Tanaka, A. Nakamato and M. Takado, ***Anal. Chem., 63*** (1991) 26.