Journal of Chromatography, 400 (1987) 169–176
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1137

APPLICATION OF BIS[4-NITRO-2-(3,6,9-TRIOXADECYLOXYCARBONYL)-PHENYL] OXALATE TO POST-COLUMN CHEMILUMINESCENCE DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

KAZUHIRO IMAI*
University of Tokyo, Branch Hospital Pharmacy, 3-28-6 Mejirodai, Bunkyo-ku, Tokyo 112 (Japan)
YORIKO MATSUNAGA
University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113 (Japan)
and
YUKIE TSUKAMOTO and ATSUHIKO NISHITANI
University of Tokyo, Branch Hospital Pharmacy, 3-28-6 Mejirodai, Bunkyo-ku, Tokyo 112 (Japan)

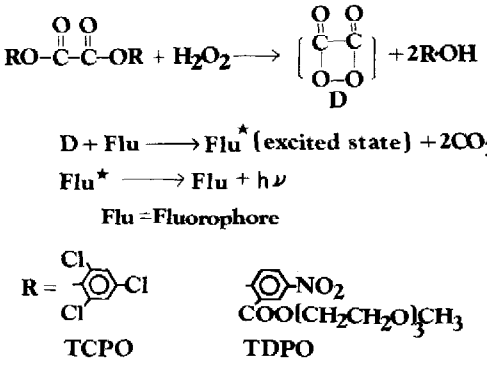
SUMMARY

Bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate (TDPO) was used to examine the peroxyoxalate chemiluminescence (CL) reaction for the detection of fluorescent compounds. Some fluorescent compounds (perylene, eosine, rhodamine, Rose Bengal, fluorescein and umbelliferone) gave higher CL intensities as the proportion of water in the reaction medium increased to *ca.* 40%, whereas dansylalanine, 8-anilinonaphthalene-1-sulphonic acid, 7-nitrobenzo-2-oxa-1,3-diazol-4-ylproline and dihydronicotinamide adenine dinucleotide gave opposite results. The effects of temperature and time on the post-column reaction in reversed-phase high-performance liquid chromatography (HPLC) were investigated. Under the optimal conditions, the detection limit for dansylamino acids was at the sub-femtomole level. The advantage of using TDPO in HPLC is its stability in the presence of hydrogen peroxide [*ca.* 10% loss of activity per 8 h *vs.* 60% per 8 h for bis(2,4,6-trichlorophenyl) oxalate].

INTRODUCTION

The peroxyoxalate chemiluminescence (CL) reaction mixture, which consists of an aryl oxalate, hydrogen peroxide and a fluorescent compound (Scheme 1), has been used as a means of detecting fluorescent compounds[1–14] and hydrogen peroxide[15] in the column eluate from high-performance liquid chromatography (HPLC). Bis(2,4,6-trichlorophenyl) oxalate (TCPO) was usually adopted as a typical oxalate, because of its stability in the organic solvents used in HPLC such as acetonitrile, acetone and ethyl acetate. However, its low solubility in these organic solvents (3, 14 and 13 m*M* in acetonitrile, acetone and ethyl acetate, respectively)[16] restricts the generation of the presumed active intermediate, 1,2-dioxetanedione, in large amounts (Scheme 1). Hence, the enhancement of the detectability of the method was limited.

0021-9673/87/$03.50



Scheme 1. Proposed mechanism for peroxyoxalate chemiluminescence reaction.

In order to supply a large amount of the intermediate, a more soluble oxalate than TCPO, bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate (TDPO) (Scheme 1) was synthesized[16]. It dissolves well in acetonitrile, acetone and ethyl acetate in the range of low to sub-molar concentrations (1, 0.8 and 0.4 *M*, respectively). It was demonstrated also that, with increasing amount of TDPO in the reaction medium, a higher intensity of CL was obtained in a static system[2].

The high solubility of TDPO in aprotic solvents should solve precipitation problems and give some information on the peroxyoxalate CL reaction in the water-rich solution. Therefore, several fluorescent compounds were selected and their CL intensities were examined in various solvent mixtures. Then, TDPO was applied to the post-column reaction detector for fluorescent compounds in an HPLC system equipped with a photon counter for monitoring the CL intensity. Also, the effect of heating of the flow-line on the signal-to-noise ratio was examined.

EXPERIMENTAL

Chemicals

Distilled water for HPLC (Nakarai Chemicals, Kyoto, Japan), acetone for fluorescence spectrometry and imidazole (Merck, Darmstadt, F.R.G.), hydrogen peroxide (Mitsubishi Gas Kagaku, Tokyo, Japan), methanol, ethanol and acetonitrile for HPLC (Kanto Chemical, Tokyo, Japan), hydrochloric acid, tris(hydroxymethyl)-aminomethane (Tris) (Nakarai), nitric acid and bis[4-nitro-2-(3,6,9-trioxadecyl-oxycarbonyl)phenyl] oxalate (TDPO) (Wako, Osaka, Japan) were used. Rose Bengal, perylene, riboflavine, pyridoxine hydrochloride, dansylalanine (Dns-Ala), Dns-serine (Dns-Ser), -proline (Dns-Pro), -isoleucine (Dns-Ile), -phenylalanine (Dns-Phe) (Nakarai), rhodamine B, fluorescein (laser grade) (Kodak, Rochester, NY, U.S.A.), 8-anilinonaphthalene-1-sulphonic acid sodium salt (ANS) and umbelliferone (Tokyo Kasei Industries, Tokyo, Japan), NADH disodium salt (Kyowa Hakko, Tokyo, Japan) and 2,4,5,7-tetrabromofluorescein (eosine Y) (Nihon Rikagaku, Tokyo, Japan) were used. 7-Nitrobenzo-2-oxa-1,3-diazol-4-ylproline (NBD-Pro) was synthesized according to the published method[17].

Chemiluminescence measurement

A concentrated solution of a fluorescent compound in methanol was diluted with 0.1 *M* imidazole nitrate buffer (pH 6.1). The fluorescent compounds, perylene and NADH, which are insoluble in methanol, were dissolved in ethanol and distilled water, respectively, and diluted with the buffer.

A 40-μl volume of a fluorescent compound in acetone, 0, 200 or 400 μl of the buffer and 400, 200 or 0 μl, respectively, of acetone and 160 μl of 0.4 *M* hydrogen peroxide in acetone were pre-mixed in a borosilicate glass tube (50 × 6 mm I.D.) with a vortex mixer, then 40 μl of 16 m*M* TDPO in acetone were added with a microsyringe. The tube was immediately placed inside a Chem-Glow photometer (Aminco, Baltimore, MD, U.S.A.) and the CL intensity was measured. The time course of the intensity was recorded after adding TDPO solution, and the relative intensity, extrapolated to zero time, was compared with the intensity for 1 μ*M* Dns-Ala, which was arbitrarily taken as 100.

Fluorescence measurement

A concentrated solution of a fluorescent compound in methanol was diluted with 0.1 *M* Tris–HCl buffer (pH 8.0) or methanol. The fluorescent compounds, perylene and NADH, which are insoluble in methanol, were dissolved in ethanol and distilled water, respectively, and then diluted with the buffer.

A 300-μl volume of a fluorescent compound, 4.5, 3.0, 1.5 or 0 ml of acetone and 0, 1.5, 3.0 or 4.5 ml of 0.1 *M* Tris–HCl (pH 8.0), respectively, were mixed and the fluorescence excitation maxima and emission maxima for each compound were measured with a Hitachi 650-10s spectrophotometer at a 5-nm slit width for both monochromators. The relative fluorescence intensity was calculated relative to 1 μ*M* Dns-Ala, which was arbitrarily taken as 100.

HPLC apparatus

The HPLC–CL detection system consists of two pumps, one (LC 6A; Shimadzu, Tokyo, Japan) for the eluent and the other (LC 5A; Shimadzu) for the reagent solution, an injection valve (Rheodyne, Cotati, CA, U.S.A.) with a 20-μl loop, a TSK LS 120-T column (150 × 4.6 mm I.D.) (Toyo Soda, Tokyo, Japan), a 25-μl rotating flow mixing device where the column effluent and the reagent solution are rotated in the same direction, two inlets which enter the bottom of the vessel at opposite sides, and an outlet in the centre of the top of the vessel[17], a photon counter (LC-30-DPC 10 LC detector; JEOL, Tokyo, Japan) with a 70-μl spiral type flow cell and a recorder (Technicorder Type 3047; Yokogawa Denki, Tokyo, Japan). The first half of the flow line (stainless-steel tubing, 810 mm × 0.8 mm I.D.) after the mixer was cooled (20°C) or heated (30, 40 or 50°C) in a water-bath. The eluent for the basic experiment to obtain the optimal reaction conditions with Dns-Ser as the fluorescent compound was 0.1 *M* imidazole nitrate (pH 7.0)–acetonitrile (70:30, v/v). A similar eluent (65:35, v/v) was used for the separation of Dns-Pro, -Val, -Ile and -Phe. The flow-rate was fixed at 0.6 ml/min. The reagent solution was a 1:1 (v/v) mixture of 0.1 m*M* TDPO or 0.1 m*M* TCPO in acetonitrile–ethyl acetate (1:1, v/v) and 10 m*M* hydrogen peroxide in acetonitrile–ethyl acetate (1:1, v/v). Both solutions were mixed just prior to use. The flow-rates were 4.0, 5.0 and 6.0 ml/min. The stock solution of Dns-amino acids in 0.1 *M* imidazole nitrate (pH 7.0) was diluted with the same buffer to a concentration of 250 p*M*, and a 20-μl aliquot was subjected to HPLC analysis.

RESULTS AND DISCUSSION

CL intensity in various organic solvents

When the amount of buffer in the reaction medium was increased from *ca.* 6% to *ca.* 40%, some fluorescent compounds, such as perylene, eosine, rhodamine, Rose Bengal, fluorescein and umbelliferone, gave higher CL intensities, whereas some others gave lower CL intensities, as shown in Fig. 1. The slopes of the CL intensity curves were similar, although slightly different, to those of the fluorescent intensity curves for perylene, esosine, rhodamine, fluorescein and umbelliferone, shown in Fig. 2. The slight differences might be ascribed to the differences in the chemical excitation yields of the test compounds[18,19] with the different solvent compositions. However, if the percentage of water was increased to *ca.* 70%, all of the fluorescent compounds tested gave a lower CL intensity. This trend was different from that observed for the fluorescence intensity (Fig. 2). It seems that side-reactions occur to a large extent in CL reactions at high water contents, and the amount of the active intermediate(s) generated is thus decreased. However, a more detailed investigation of the peroxyoxalate CL reaction in water-rich solutions is needed in order to explain this phenomenon on the basis of reaction mechanisms[20–22]. In this respect, TDPO might be a useful model, as its high solubility in aprotic solvents makes any desired experimental conditions possible without its precipitation.

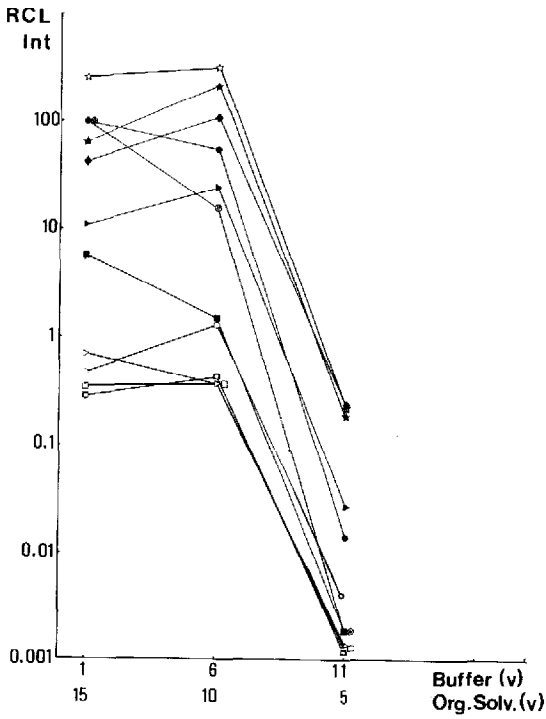


Fig. 1. Effect of solvent composition on peroxyoxalate chemiluminescence intensities with TDPO as the reagent. For experimental conditions, see text. (☆) Perylene; (★) eosine; (◆) rhodamine B; (●) Dns-Ala; (▶) Rose Bengal; (⊙) ANS; (■) NBD-Pro; (○) fluorescein; (□) umbelliferone, (▷) NADH; (□) pyridoxine.

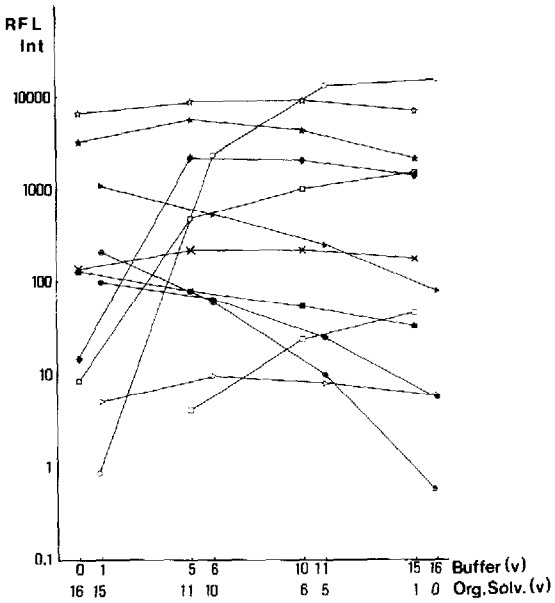


Fig. 2. Effect of solvent composition on fluorescence intensities. For experimental conditions, see text. Key as in Fig. 1.

HPLC–CL Detection of Dns-amino acids

Considering the previous results[1,2], the baseline noise seems to be caused by (1) irregularities in the pumping system and (2) flow disturbance after the mixing of the column eluate and reagent solution. The first problem can be overcome by using an air-pressure solvent-delivery system[2] or pulseless pumps, such as syringe-type pumps[4]. To solve the second problem, a rotating flow mixing device was adopted, which gave good results[17]. Also, the signal can be amplified if it can be detected at the maximum of the CL reaction. As the rate of generation of CL is dependent on the reaction temperature, control of the reaction condition by heating or cooling of the flow lines after thorough mixing could result in a high signal-to-noise ratio. Therefore, in this study, the first 35 cm of the 0.8 mm I.D. stainless-steel tubing connecting the flow cell with the mixing device was cooled or heated in a water-bath at 20, 30, 40 and 50°C.

In order to determine the optimal CL reaction time, the rate of delivery of the reagent solution was varied from 4.0, 5.0 and 6.0 ml/min. As the dead volume from the outlet of the mixing device to the flow cell was *ca.* 410 μl (810 × 8 mm I.D.), the reaction times for 4.0, 5.0 and 6.0 ml/min of reagent solution were 6.1, 4.9 and 4.1 s, respectively.

Fig. 3 shows that the peak heights decreased as the heating temperature increased at the reaction times tested, *i.e.*, heating the reaction medium accelerated the rate of the reaction and the attainment of maximal intensity was reached before the arrival of the product at the flow cell. When the dead volume of the flow line after the onset of the reaction was reduced, greater peak heights could be obtained. How-

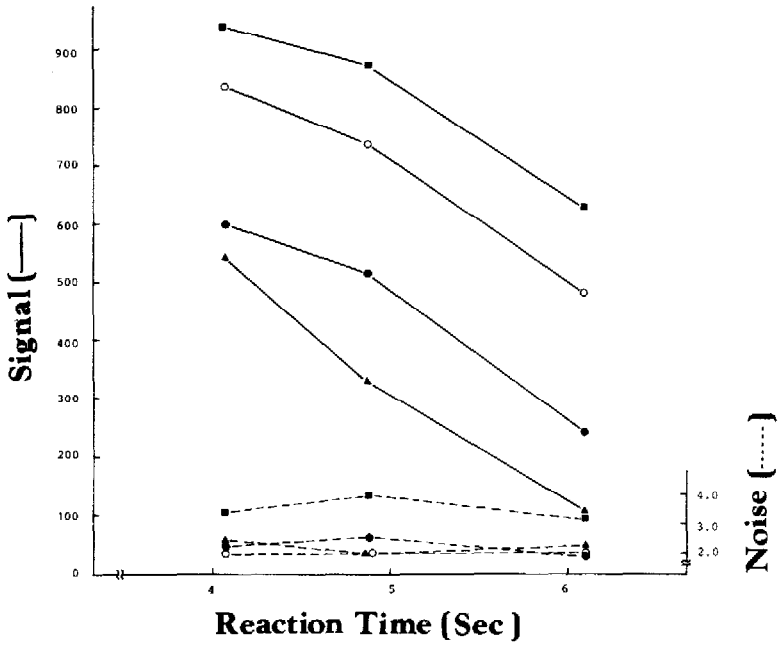


Fig. 3. Effect of reaction temperature on peak height of Dns-serine (solid lines) and noise level (broken lines). For HPLC conditions, see text. (■) 20°C; (○) 30°C; (●) 40°C; (▲) 50°C.

ever, in the present experiment, the tubing to the detector was fixed owing to space restrictions. On the other hand, as shown in Fig. 3, the noise increased as the temperature was decreased to 20°C. This means that the mixing through the mixing device may not have been thorough enough under the test conditions, and the low temperature resulted in an acceleration of the flow disturbance. A higher temperature would accelerate mixing. Hence, reduction of the dead volume of the delay tubing and the use of a high temperature, *e.g.*, 50°C, would afford a higher signal-to-noise ratio. Under the present conditions, the signal-to-noise ratio reached its maximum when the reaction time and the temperature adopted were 4.1 s (6.0 ml/min) and 30°C, respectively (Fig. 4).

Under the optimal conditions described above (4.1 s reaction at 30°C), 5 fmol of each dansylated amino acid were separated and detected, as depicted in Fig. 5. The sensitivity was several times greater than that reported previously[1] and almost the same as that in another study[2], although the HPLC conditions and oxalate used (TCPO in the former two instance) were different from those in the present experiments.

An advantage of the use of TDPO in the post-column reaction in HPLC is that it is stable for 5 h in the combined mixture of hydrogen peroxide in acetone and ethyl acetate at room temperature (23°C), in contrast to TCPO, which showed a *ca.* 40% decrease of intensity in 5 h on storage under the same conditions (Table I). The triethylene glycol moiety in the ester component could enhance the stability of the oxalate in the presence of hydrogen peroxide for some unknown reason. A single-

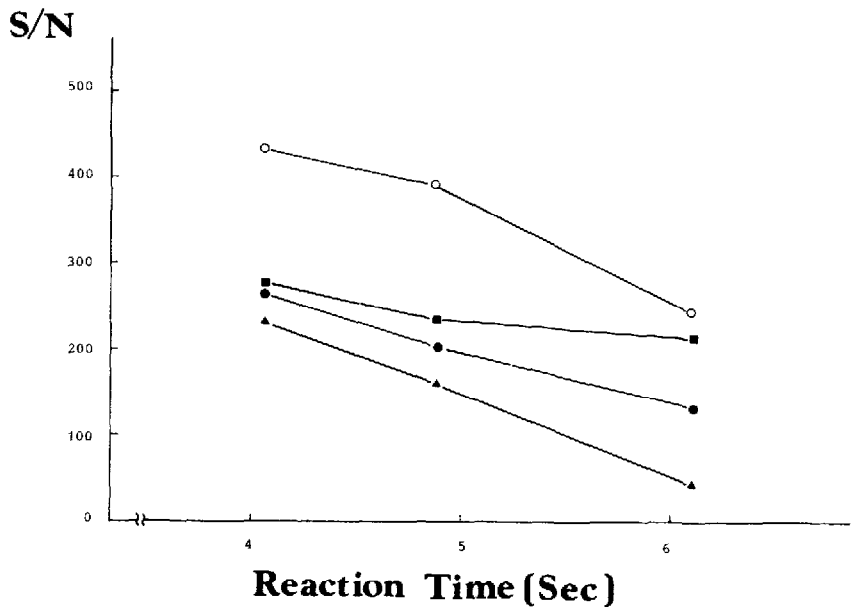


Fig. 4. Effect of reaction temperature on signal-to-noise ratio for Dns-serine. For HPLC conditions, see text. Key as in Fig. 3.

pump delivery system for the reagent solution, as adopted in this study, would be of advantage in routine analysis.

In our previous work, recrystallisation of imidazole in diethyl ether lowered the background[14], but in the present experiments imidazole was used as received, as

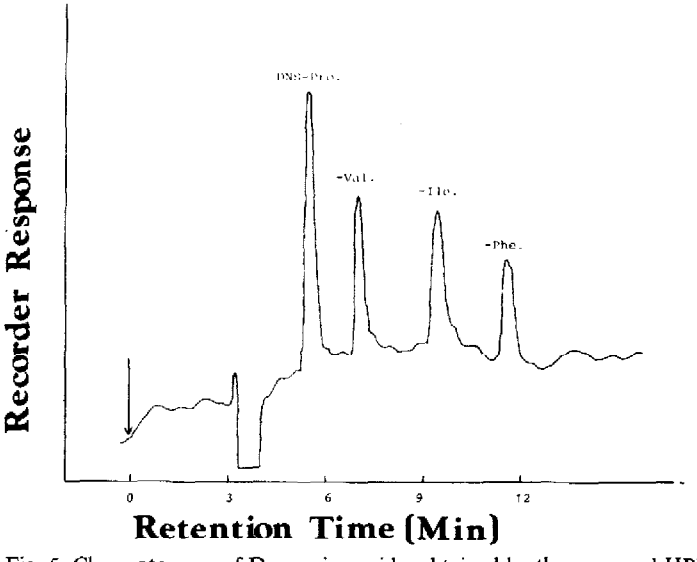


Fig. 5. Chromatogram of Dns-amino acids, obtained by the proposed HPLC CL detection system. A 5-fmol amount of each Dns-amino acid was injected and detected. For HPLC conditions, see text.

TABLE I

STABILITY OF OXALATES IN THE PRESENCE OF HYDROGEN PEROXIDE

HPLC conditions as in Fig. 3.

Oxalate	*Relative peak height*							
	1 h	*2 h*	*3 h*	*4 h*	*5 h*	*6 h*	*7 h*	*8 h*
TDPO	100.0	100.0	100.0	100.0	99.0	95.7	93.8	91.1
TCPO	100.0	85.9	78.8	70.1	60.6	55.6	50.5	44.7

the background CL can be easily subtracted by the photon counting system. However, the impurities in the imidazole nitrate buffer greatly increased the background level. Therefore, we changed to 0.1 m*M* oxalate in this experiment. In this respect, the desirec enhancement of detectability by the use of TDPO in the reaction detector was not achieved. Purification of imidazole, hydrogen peroxide and nitric acid would be required to lower the baseline level and enhance the detectability.

ACKNOWLEDGEMENTS

The authors express their thanks to Misses Y. Sekine and K. Natori for their technical assistance.

REFERENCES

1 K. Kobayashi and K. Imai, *Anal. Chem.*, 52 (1980) 424.
2 K. Kobayashi, J. Sekino, K. Honda and K. Imai, *Anal. Biochem.*, 112 (1981) 99.
3 G. Mellbin, *J. Liq. Chromatogr.*, 6 (1983) 1603.
4 K. W. Sigvardson and J. W. Birks, *Anal. Chem.*, 55 (1983) 432.
5 K. Miyaguchi, K. Honda and K. Imai, *J. Chromatogr.*, 303 (1984) 173.
6 K. Miyaguchi, K. Honda and K. Imai, *J. Chromatogr.*, 316 (1984) 501.
7 G. Mellbin and B. E. F. Smith, *J. Chromatogr.*, 312 (1984) 203.
8 G. J. de Jong, N. Lammers, F. J. Spruit, U. A. Th. Brinkman and R. W. Frei, *Chromatographia*, 18 (1984) 129.
9 T. Koziol, M. L. Grayeski and R. Weinberger, *J. Chromatogr.*, 317 (1984) 355.
10 K. W. Sigvardson, J. M. Kennish and J. W. Birks, *Anal. Chem.*, 56 (1984) 1096.
11 M. L. Grayeski and A. J. Weber, *Anal. Lett.*, 17 (1984) 1539.
12 K. W. Sigvardson and J. W. Birks, *J. Chromatogr.*, 316 (1984) 507.
13 R. Weinberger, C. A. Mannan, M. Cerchio and M. L. Grayeski, *J. Chromatogr.*, 288 (1984) 445.
14 K. Miyaguchi, K. Honda, T. Toyo'oka and K. Imai, *J. Chromatogr.*, 352 (1986) 255.
15 K. Honda, K. Miyaguchi, H. Nishino, H. Tanaka, T. Yao and K. Imai, *Anal. Biochem.*, 153 (1986) 50.
16 K. Imai, H. Nawa, M. Tanaka and H. Ogata, *Analyst (London)*, 111 (1986) 209.
17 T. Toyo'oka, Y. Watanabe and K. Imai, *Anal. Chim. Acta*, 149 (1983) 305.
18 K. W. Sigvardson, J. M. Kennish and J. W. Birks, *Anal. Chem.*, 56 (1984) 1096.
19 K. Honda, K. Miyaguchi and K. Imai, *Anal. Chim. Acta*, 177 (1985) 111.
20 C. L. Catherall, T. F. Palmer and R. B. Cundall, *J. Chem. Soc., Farady Trans. 2*, 80 (1984) 823.
21 C. L. Catherall, T. F. Palmer and R. B. Cundall, *J. Chem. Soc., Farady Trans. 2*, 80 (1984) 837.
22 F. J. Alvarez, N. J. Parekh, B. Matuszewski, R. S. Givens, T. Higuchi and R. L. Schowen, *J. Am. Chem. Soc.*, 108 (1986) 6435.