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Indirect detection of amino-substituted polycyclic aromatic hydrocarbons in cyclodextrin-modified micellar electrokinetic chromatography combined with diode laser-induced fluorometry

Takashi Kaneta, Yumiko Saito, Totaro Imasaka*

Department of Chemical Science and Technology, Faculty of Engineering, Kyushu University, Hakozaki, Fukuoka 812-8581, Japan

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Abstract

Indirect fluorescence detection in cyclodextrin (CD)-modified micellar electrokinetic chromatography (MEKC) was demonstrated by using a diode laser as an excitation source. Amino-substituted polycyclic aromatic hydrocarbons (PAHs), which have no absorption band in the deep-red region, were used as model compounds since it was difficult to separate them by conventional MEKC. In the preliminary study, the separation of amino-substituted PAHs was achieved using γ-CD as a modifier. The fluorescence intensity of the visualizing reagent, oxazine 750, increased in the presence of γ-CD due to the complex formation of oxazine 750 with γ-CD. Consequently, the peak areas of amino-substituted PAHs were observed to decrease with increasing concentration of γ-CD. Detection limits were improved due to the enhancement in efficiency and dynamic reserve by adding small amounts of γ-CD. The results obtained in this study were in good agreement with the proposed model.

Keywords: Detection, electrophoresis; Polycyclic aromatic hydrocarbons; Oxazine 750

1. Introduction

Indirect detection is a universal technique in that it is applicable to analysis of samples which inherently have no chromophore or fluorophore. This detection technique was employed in high-performance liquid chromatography (HPLC) [1,2] and capillary electrophoresis (CE) [3–5]. In indirect detection, a visualizing reagent is added to the mobile phase or the running buffer, and analytes are detected by the concomitant reduction in absorbance or fluorescence brought on by displacement of the visualizing reagent by the analyte. In HPLC, the mechanism is based on a disturbance in partitioning of the visualizing reagent between the stationary phase and the mobile phase. The mechanism is also explained by displacement of the visualizing reagent from the sample zone to maintain local charge neutrality.

Diode laser spectroscopy is useful in meeting many analytical challenges [6–8], but its main drawback is the requirement that substances must have a useful absorbance appropriate for the long emission wavelength of diode lasers. With proper choice of long-wavelength absorbing, visualization reagent, indirect detection becomes suitable for use with diode laser-induced fluorometry, thus enabling measurement of several substances. Previously, we

*Corresponding author. Tel.: +81-92-6423-563, Fax: +81-92-6325-209, E-mail: imasaka@cstf.kyushu-u.ac.jp

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reported indirect fluorescence detection of aromatic hydrocarbons in micellar electrokinetic chromatography (MEKC) using a diode laser as an excitation light source [9]. The detection mechanism was similar to that proposed for HPLC since a decrease in signal is produced by perturbation of the fluorophore–micelle complex by the sample. Signal intensity is correlated with retention factor in HPLC [10], but not in MEKC. Sensitivity is governed by the similarity of hydrophobicity between the fluorophore and the sample in MEKC.

In MEKC, a lot of additives are often required to improve resolution, such as organic modifiers and cyclodextrin. However, there is no report on the effect of additives in MEKC combined with indirect detection. In this study, the effect of cyclodextrins (CDs) on separation and sensitivity in indirect detection was studied using γ-CD-modified MEKC as a separation mode. Amino-substituted polycyclic aromatic hydrocarbons (PAHs) are used as test compounds since they yielded intense signals due to their similarity in hydrophobicity to the fluorescent visualization reagent (oxazine 750), but it was difficult to separate them by MEKC. CD-modified MEKC was often employed to separate PAHs [11–13]. However, CD should influence not only the separation but also the sensitivity and selectivity in indirect detection mode because of complex formation of CD with the visualization reagent and the sample. The effect of γ-CD on the separation, sensitivity and mechanism is discussed below.

2. Experimental

2.1. Apparatus

The experimental apparatus used in this study for CD-modified MEKC is reported elsewhere [9]. A diode laser (ILEE Laser Innovation, Urdorf, Switzerland; Model LDA1001) emitting at 660 nm (output power is less than 5 mW) is used as an excitation source. A fused-silica capillary (60 cm total length× 50 μm I.D.×375 μm O.D.) was obtained from GL Sciences (Tokyo, Japan). Fluorescence detection was carried out at a position 10 cm from the anodic end of the capillary. A photomultiplier tube (Hamamatsu photonics, Model R3896) was used to detect fluorescence. The signal from the photomultiplier tube was recorded by a strip-chart recorder (Chino, Model EB 2005). A high-voltage power supply, Model HCZE-30PN0.25 (Matsusada Precision Devices, Shiga, Japan) was used for applying the voltage.

2.2. Reagents

Tetradecyltrimethylammonium chloride (TTAC) and 1-aminopyrene were obtained from Tokyo Kasei Kogyo. Sodium dihydrogenphosphate, sodium acetate and aniline were purchased from Kishida. Oxazine 750 was supplied by Lambda Physik. The structure of oxazine 750 is shown in Fig. 1. The other reagents were obtained from Wako. All the running buffer solutions consisted of oxazine 750,

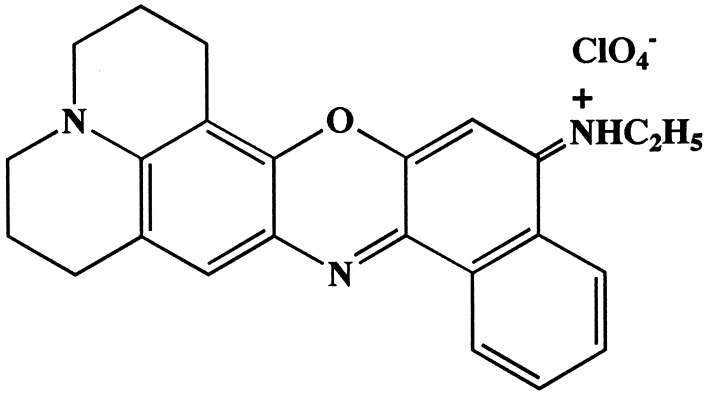

Fig. 1. Structure of oxazine 750.

TTAC and buffer components with or without γ-CD. The pH levels were adjusted to 4 and 5 by acetic acid and tris(hydroxymethyl)aminomethane (Tris) and to 6~9 by sodium dihydrogenphosphate and Tris. A mixture of aniline, 2-aminonaphthalene, 2-aminoanthracene and 1-aminopyrene was used as a sample in which the concentration of each compound was adjusted to 1.6 m*M*.

3. Results and discussion

3.1. Response mechanism

In capillary zone electrophoresis, indirect detection is explained using a displacement model, in which a sample ion displaces a similarly charged fluorophore to maintain electrical neutrality within the sample zone. A reduction in fluorescence is thus observed. The detection limit based on the displacement model is presented as [14]

$$C_{dl} = C_{flu}\frac{1}{DR \cdot TR} \quad (1)$$

where C_{dl} is the detection limit, C_{flu} is the concentration of the fluorophore, and DR and TR are dynamic reserve and transfer ratio, respectively. For MEKC, however, we previously reported that the detection limit of indirect detection is represented by the following equation as [9]

$$C_{dl} = C_{flu}\frac{3}{DR \cdot TR} \cdot \frac{F_{mc}}{F_{mc} - F_{aq}} \quad (2)$$

where F_{mc} and F_{aq} are the fluorescence intensity of a fluorophore in the micellar and aqueous phases, respectively, and the detection limit is defined as the concentration of the sample at $S/N=3$. The model of indirect detection in MEKC is based on alteration of the partitioning of a fluorophore between aqueous and micellar phases. In CD-modified MEKC, Eq. (2) is modified to account for enhancement of the fluorescence intensity in the presence of CD. A model for indirect detection in CD-modified MEKC is shown in Fig. 2. In Fig. 2, F_{comp} is the fluorescence intensity of the dye–CD complex. The fluorescence intensity of the dye molecule, which is given by F in Fig. 2, depends on its constitution. For example, the fluorescence intensity of oxazine 750, which is used in this study, is changed by complex formation with CD and partitioning into the micelle. In the present study, the order of the fluorescence intensity for oxazine 750 found was $F_{mc} >> F_{comp} > F_{aq}$. In CD-modified MEKC, CD is assumed to exist solely in the aqueous phase. Therefore, the fluorophore in the aqueous phase is equilibrated between the free and complexed forms. Then, Eq. (2) is represented as

$$C_{dl} = C_{flu}\frac{3}{DR \cdot TR} \cdot \frac{F_{mc}}{F_{mc} - F_{aq(CD)}} \quad (3)$$

where $F_{aq(CD)}$ is the fluorescence intensity in the aqueous phase containing CD, and $F_{aq(CD)}$ is given by

$$F_{aq(CD)} = \frac{K[CD]F_{comp} + F_{aq}}{K[CD] + 1} \quad (4)$$

where K is the complex formation constant between CD and the fluorophore. The complexed fluorophore should yield higher fluorescence intensity than that of the free fluorophore. In the proposed model, the fluorophore in the aqueous phase does not participate to produce a negative sample signal. Thus, the presence of CD will decrease sensitivity. However, enhancement in the fluorescence intensity was small in the presence of γ-CD. For example, the ratio of $F_{aq(CD)}$ to F_{aq} was only 1.4 even in the presence of 50 m*M* γ-CD, while F_{mc}/F_{aq} was 6.3 in 50 m*M* TTAC solution. Thus, the calculated degradation in sensitivity was only 3% using the following equation,

$$\text{Degradation (\%)} = \left(1 - \frac{F_{mc} - F_{aq}}{F_{mc} - F_{aq(CD)}}\right) \cdot 100 \quad (5)$$

Therefore, degradation in sensitivity is small for concentrations of γ-CD less than 50 m*M*.

3.2. Separation of amino-substituted PAHs

The PAHs used in this study have an amino group so that they are positively charged in neutral or acidic solutions. To improve the separation, pH was changed by titration with acetate–Tris and phosphate–Tris buffers. The MEKC separations obtained at two different pH levels, 4 and 7, are shown in Fig.

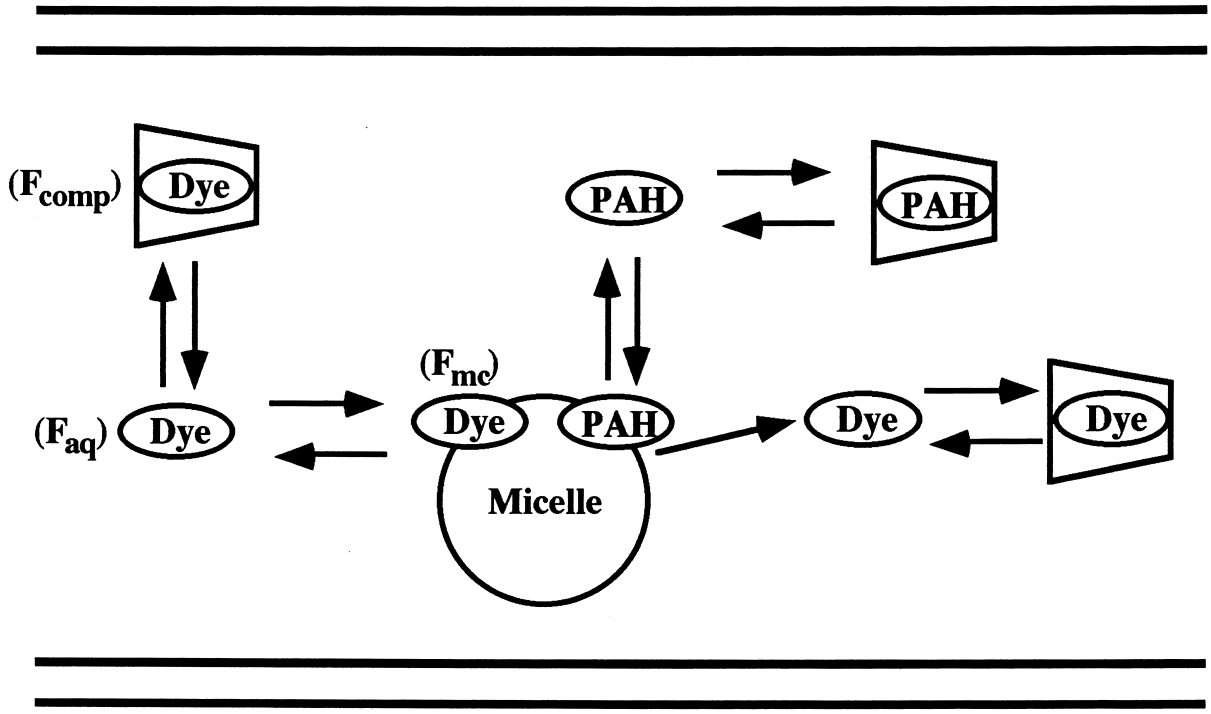

Fig. 2. A model of indirect detection in CD-modified MEKC.

3. As noted in Fig. 3, the separation was not improved by changing the pH. In Fig. 3A, 2-aminonaphthalene was distinguishable from the blank peak, which is assigned to oxazine 750. This is attributed to the change in the electrophoretic mobility of 2-aminonaphthalene by protonation of the amino group. However, the separation between 2-aminoanthracene and 1-aminopyrene was not achieved even when the pH was lowered from 9 to 4. Furthermore, the peak intensity of aniline was smaller at pH 4 than that at pH 7. Poor sensitivity for aniline at pH 4 is easily explained by taking protonation of aniline into account. The acid dissociation constant of anilinium ion is $2.2 \cdot 10^{-5}$ *M* so that greater than 80% of aniline is cationic at pH 4. In this study, a cationic surfactant, TTAC, was used as a micellar phase. Therefore, protonated aniline, which has a positive charge, does not penetrate into the micellar phase because of electrical repulsion. Consequently, aniline gives smaller signal at lower pH.

As shown in Fig. 3, the separation between 2-aminoanthracene and 1-aminopyrene requires improvement. Thus, γ-CD, which is size-specific for anthracene, was selected as a modifier. The effect of γ-CD on the separation of amino-substituted PAHs is shown in Fig. 4. Also, the apparent electrophoretic mobilities calculated using the following equation are given in Table 1.

$$\mu = \mu_{obs} - \mu_{eo} = \frac{L}{Et_m} - \frac{L}{Et_{eo}} \quad (6)$$

where μ is the electrophoretic mobility, μ_{obs} is the apparent electrophoretic mobility (which can be calculated from the migration time, t_m), μ_{eo} is the electroosmotic mobility (calculated from the migration time of the electroosmotic flow, t_{eo}), and L and E are the effective length of the capillary and the electric field strength, respectively. By adding 5 m*M* γ-CD, 2-aminonaphthalene was separated completely from the blank, but 2-aminoanthracene was not separated from 1-aminopyrene. The separation between 2-aminoanthracene and 1-aminopyrene was achieved by adding 10 m*M* γ-CD. As noted in Table

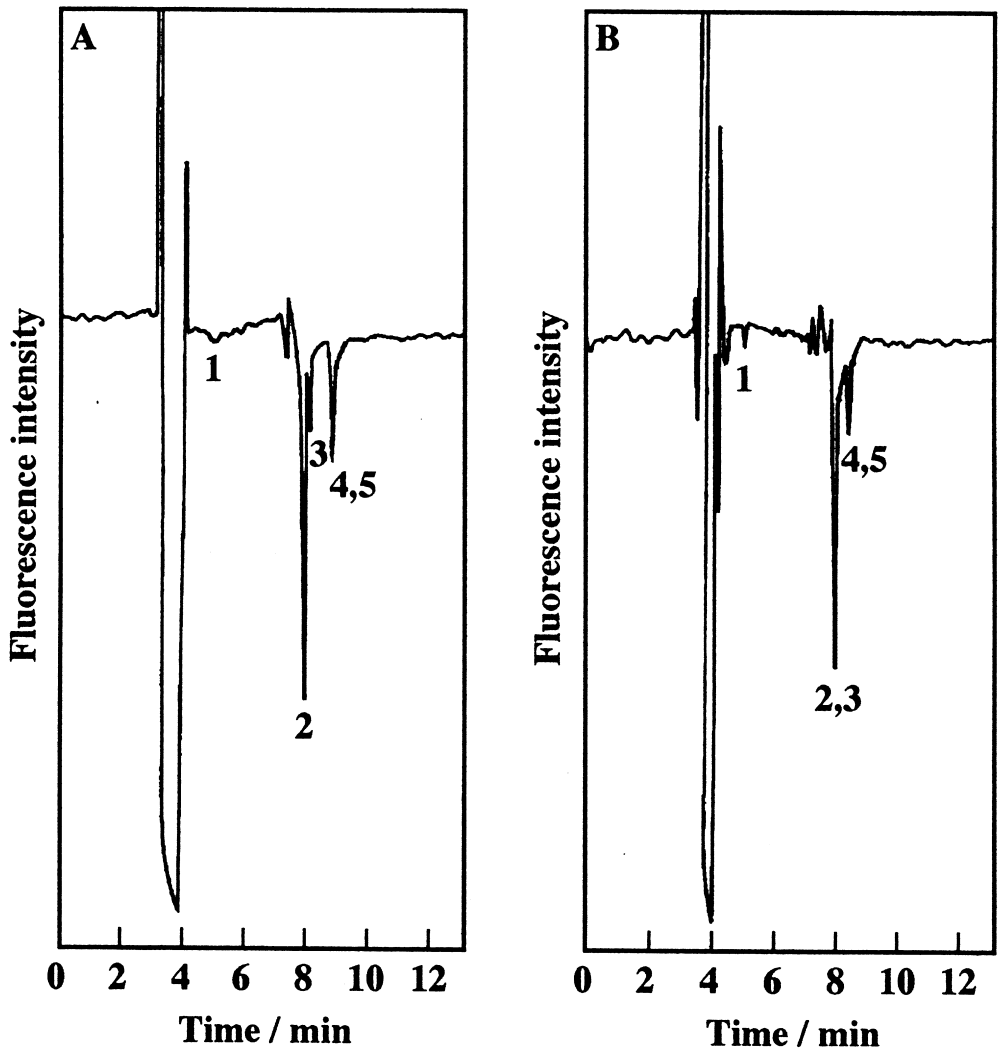

Fig. 3. Effect of pH on the separation of amino-substituted PAHs. (1) Aniline, (2) blank, (3) 2-aminonaphthalene, (4) 2-aminoanthracene, (5) 1-aminopyrene. (A) pH 4 (50 m*M* TTAC, 10 m*M* acetic acid, 1 m*M* Tris and 20 μ*M* oxazine 750), (B) pH 7 (50 m*M* TTAC, 10 m*M* NaH_2PO_4, 1 m*M* Tris and 20 μ*M* oxazine 750); applied voltage, 20 kV.

1, the electrophoretic mobilities were decreased with increasing concentration of γ-CD. The reduction in electrophoretic mobility is explained by two factors; one factor is the increase in the viscosity of the running buffer and the another is the reduction in distribution coefficients caused by complex formation between the γ-CD and the samples. At a concentration less than 10 m*M*, a decrease in the electrophoretic mobilities of aniline and 2-aminopyrene might result from increasing viscosity. On the other hand, the electrophoretic mobilities of 2-aminoanthracene and 1-aminopyrene are reduced by complex formation with γ-CD. However, at a concentration greater than 10 m*M*, the electrophoretic mobility of aniline is decreased by complex formation since changes in the electrophoretic mobility are not observed for 2-aminopyrene at the range of 5–15 m*M*. Thus, it is predicted that 2-aminopyrene would

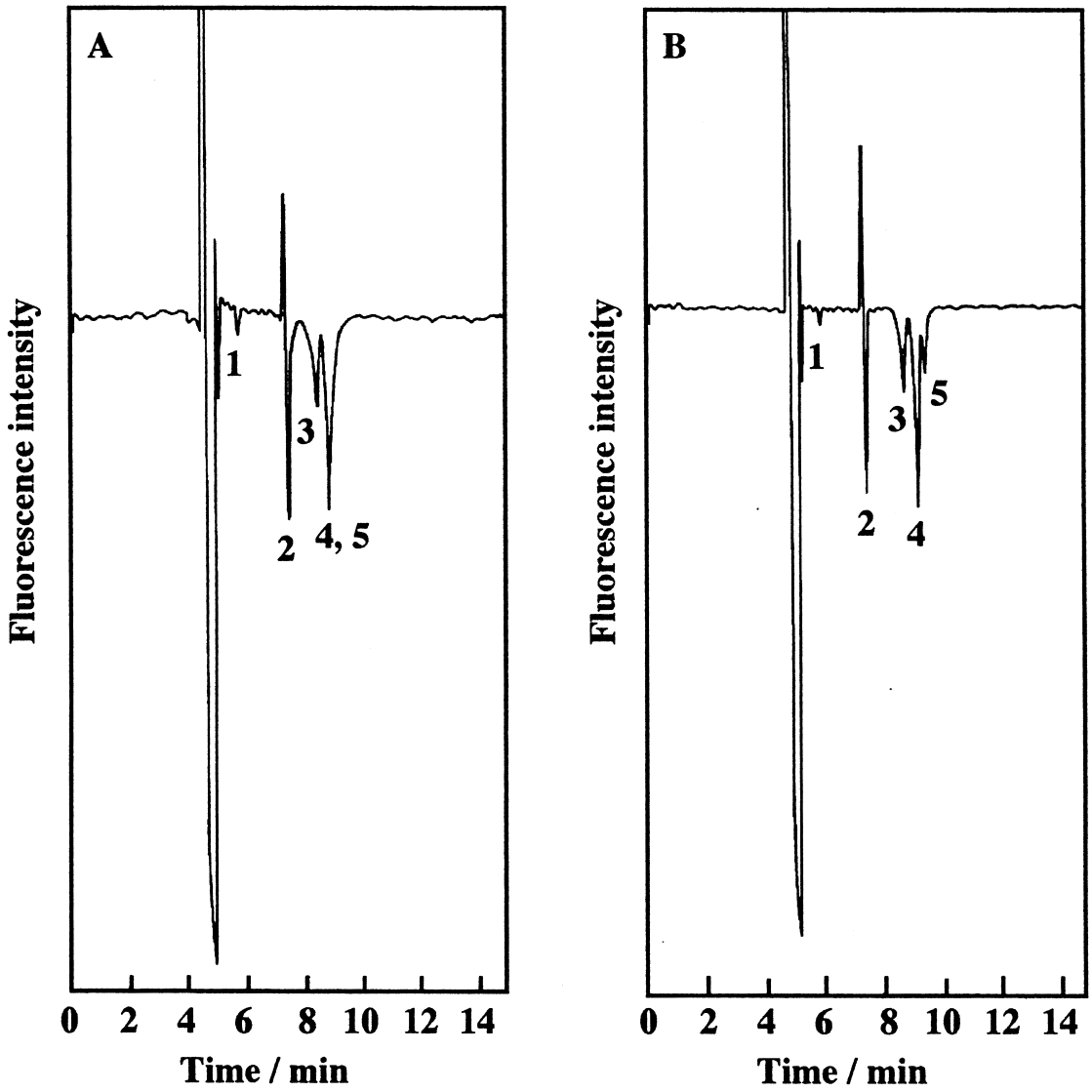

Fig. 4. Effect of γ-CD concentration on the separation of amino-substituted PAHs. (1) Aniline, (2) blank, (3) 2-aminonaphthalene, (4) 2-aminoanthracene, (5) 1-aminopyrene. (A) 5 m*M* γ-CD; (B) 10 m*M* γ-CD; buffer, 10 m*M* NaH_2PO_4, 1 m*M* Tris containing 50 m*M* TTAC and 20 μ*M* oxazine 750; applied voltage, 20 kV.

have the smallest complex formation constant with γ-CD among the amino-substituted PAHs used in this study.

3.3. Sensitivity

As described above, sensitivity in CD-modified MEKC is affected by CD concentration. Thus, parameters, which are considered to influence the sensitivity, were determined by changing the concentration of γ-CD. The parameters, dynamic reserve, theoretical plate number, detection limit and peak area are listed in Table 2. As described in Section 3.1, peak area is decreased by increasing γ-CD concentration. As shown in Table 2, peak areas of amino-substituted PAHs except for aniline

Table 1
Apparent electrophoretic mobilities of samples

	Mobility (10^{-4} cm^2 s^{-1} V^{-1})			
	γ-CD concentration (m*M*)			
	0	5	10	15
Aniline	1.9	1.4	1.1	1.1
2-Aminonaphthalene	3.5	2.8	2.6	2.6
2-Aminoanthracene	3.9	3.0	2.7	2.8
1-Aminopyrene	3.9	3.0	2.8	3.0

were indeed decreased by increasing the concentration of γ-CD. The peak area for aniline does not change even in the presence of γ-CD up to a concentration of 10 m*M* since the size of γ-CD is too large to include aniline. A large DR is needed to enhance sensitivity as predicted by Eq. (3). In Table 2, dynamic reserve was remarkably improved by adding 5 to 10 m*M* γ-CD. The value of dynamic reserve at 10 m*M* CD (155) is larger than that obtained by MEKC in the previous report (86). Thus, the detection limit of aniline at 5 and 10 m*M* γ-CD is somewhat better than that without γ-CD. The dynamic reserve in the case of 15 m*M* of γ-CD is smaller than that for 10 m*M* γ-CD. It is speculated that the decreasing dynamic reserve at 15 m*M* might be caused by adsorption of γ-CD onto the capillary wall. In the present study, a cationic surfactant, TTAB, was used so that the capillary wall is coated by TTAB. Thus, it is possible that γ-CD adsorbs onto the capillary wall by interaction with TTAB. On the other hand, theoretical plate number was increased by adding small amounts of γ-CD (5 and 10 m*M*) though it was degraded at higher γ-CD concentration (15 m*M*). Peak broadening reduces sensitivity since the detection limit is defined as the concentration at $S/N=3$. Thus, enhancement in sensitivity at the concentration of 5 m*M* is explained by an increase in the dynamic reserve and the theoretical plate number. The range of the detection limits for these compounds (33 to 680 μ*M*) was lower than that previously reported (420–1600 μ*M*). This implies that oxazine 750 is excluded more efficiently from the micelle by amino-substituted PAHs.

4. Conclusions

Indirect detection of amino-substituted PAHs is

Table 2
Parameters calculated from chromatograms obtained at different CD concentrations

	CD concentration (m*M*)			
	0	5	10	15
Dynamic reserve	80	151	155	97
Theoretical plate number				
Aniline	8600	25 000	14 000	9400
2-Aminonaphthalene	–	14 000	15 000	14 800
2-Aminoanthracene	–	–	20 000	9200
1-Aminopyrene	–	–	17 000	5300
Detection limit (μ*M*)				
Aniline	910	450	680	1100
2-Aminonaphthalene	–	74	78	170
2-Aminoanthracene	–	–	33	63
1-Aminopyrene	–	–	100	250
Relative peak area[a]				
Aniline	1	1	1	0.7
2-Aminonaphthalene	–	5.3	4.3	4.0
2-Aminoanthracene	–	–	12	9.7
1-Aminopyrene	–	–	4.0	3.3

[a] Peak area was normalized by the area of aniline obtained without γ-CD.

accomplished by CD-modified MEKC combined with diode laser-induced fluorescence detection. The separation of amino-substituted PAHs was achieved at a γ-CD concentration greater than 10 m*M*. Sensitivity was also affected by adding γ-CD, since fluorescence intensity was changed by complex formation of oxazine 750 with γ-CD. Peak areas decreased with increasing γ-CD concentration because of the enhancement of fluorescence intensity by the complex formation. Degradation of sensitivity was not observed even in the presence of γ-CD since both efficiency and dynamic reserve were enhanced by addition of γ-CD. Thus, CD-modified MEKC combined with indirect detection is preferential for applying diode laser-induced fluorescence detection to the determination of several compounds having no fluorophore by using the present technique.

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