Determination of acid dissociation constants of flavin analogues by capillary zone electrophoresis

Yuki Tanikami,[1] Takuma Tagami,[1] Mayu Sakamoto,[2] Yukihiro Arakawa (0000-0003-1000-5799),[3]* Hitoshi Mizuguchi (0000-0003-2396-6812),[3] Yasushi Imada (0000-0002-4844-5418),[3] and Toshio Takayanagi (0000-0002-5767-1126)[3]*

[1] Graduate School of Advanced Technology and Science, Tokushima University, 2-1 Minamijyousanjima-cho, Tokushima 770-8506, Japan

[2] Department of Chemical Science and Technology, Faculty of Engineering, Tokushima University, 2-1 Minamijyousanjima-cho, Tokushima 770-8506, Japan

[3] Graduate School of Technology, Industrial and Social Sciences, Tokushima University, 2-1 Minamijyousanjima-cho, Tokushima 770-8506, Japan

*Correspondence should be addressed to the following authors:

Toshio Takayanagi (Prof.) and Yukihiro Arakawa (Assist. Prof.)

Graduate School of Technology, Industrial and Social Sciences

Tokushima University

2-1 Minamijyousanjima-cho, Tokushima 770-8506, Japan

toshio.takayanagi@tokushima-u.ac.jp (T. T.); arakawa.yukihiro@tokushima-u.ac.jp (Y. A.)

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Abbreviations: FL, flavin; RF, riboflavin; LC, lumichrome

Abstract

Acid dissociation constants (pK_a) of 9 kinds of flavin analogues as molecular catalyst candidates were determined by CZE. Although some of the analogues are instable and degradable under the light-exposure or in alkaline aqueous solutions, the effective electrophoretic mobility of the flavin analogue of interest has been measured with the residual substance. The pK_a values of the flavin analogues were analyzed through the changes in the effective electrophoretic mobility with varying pH of the separation buffer. One or two steps pK_a values were determined by the analysis. One of the degraded species from the flavin analogues, lumichrome, was also detected in the CZE analysis, and its pK_a values were also determined. While coexisting impurities generated over the storage conditions were found in some analogues, the pK_a values of the target analogues were successfully determined with the help of the CZE separations. A pressure-assisted CZE was utilized for the determination or the estimation of the pK_a values of such analogues as possessing carboxylic acid moiety.

1 Introduction

Redox reactions are essential on organic syntheses, and molecular catalysts inducing redox reactions are intensively investigated. For example, flavin analogues contain heterocyclic isoalloxazine (flavin, FL) ring and they are one of the famous redox-active molecular catalysts [1]. In nature, the heterocyclic isoalloxazine rings are utilized as an active center of the redox enzymes, in which xenobiotic substrates are metabolized through the oxidations such as dehydrogenation and monooxygenation. Thus, FL analogues are expected to work as oxidative molecular catalysts without any active center of metal ion [2-4]. On the activity design of the molecular catalysts, acid dissociation constant (pK_a) is one of the key parameters. Since pK_a values and redox potentials are closely related to each other, experimentally determining the pK_a values of the FL analogues would be highly valuable on developing their redox catalysis. However, some of the FL analogues are not stable against light or alkaline base [5,6], which hampers the conventional methods for the pK_a determination.

Acid dissociation constants have classically been determined by potentiometric and spectrophotometric titrations. However, the homogeneous titrations are not applicable to such substances as are not pure or degradable. Spectrum shift is indispensable for

the pK_a determination by the spectrophotometric titration. On the contrary, CZE determination of acid dissociation constants is based on the changes in the effective electrophoretic mobility with protonation/deprotonation reaction under varying pH [7], and the CZE analysis is applicable to such substances as containing impurities or degradable under the measurement conditions [8-11]. Acid dissociation constants of alkaline-degradable phenolphthalein [8], labile drug compounds [9], acid-degradable tetrabromophenolphthalein ethyl ester [10], and heat-degradable bupropion [11] have been determined by utilizing the prominent characteristics of the CZE analysis.

In this study, acid dissociation constants of 9 kinds of FL analogues were determined by CZE through the measurements of their effective electrophoretic mobility. Although some of the FL analogues were degradable under alkaline conditions and/or light exposure, the acid dissociation constants were successfully determined by CZE. Aim of this study is determining the pK_a values of such difficult substances, even though they are conditional values. The target analogue was resolved from the degraded species, and changes in the effective electrophoretic mobility of the analogue gave the pK_a value(s). Some analogues contained unavoidable impurities formed under the storage conditions, the impurities were also resolved by CZE and the pK_a value(s) of the target analyte was also determined. Acid dissociation constants of the degraded species, as well as those of the impurities, were determined through the mobility change.

2 Materials and methods

2.1 Chemicals

Riboflavin and lumichrome, as well as all other reagents used for the synthesis of its analogues, were purchased from commercial supplies and used without further purification, besides *N*,3,4-trimethylaniline that was prepared according to the literature procedure [12]. Flavin analogues prepared and examined in this study are shown in Figure 1. They are categorized as monoprotic (5 analogues), diprotic (3 analogues), and triprotic (1 analogue) acids. The analogues were synthesized according to the literature previously reported, and the details are written in Supporting Information. Separation buffers of the CZE were prepared with following buffer components: 0.010 mol L^{-1} H_3PO_4 – NaOH (pH 2.38 – 3.21); 0.010 mol L^{-1} HCOOH

– NaOH (pH 3.13 – 3.53); 0.010 mol L^{-1} CH_3COOH – NaOH (pH 4.08 – 4.75); 0.010 mol L^{-1} MES – NaOH (pH 5.48 – 6.42); 0.010 mol L^{-1} HEPES – NaOH (pH 6.78 – 8.00); 0.010 mol L^{-1} TAPS – NaOH (pH 8.02 – 9.28); 0.010 mol L^{-1} CHES – NaOH (pH 8.77 – 10.36); 0.010 mol L^{-1} CAPS – NaOH (pH 9.88 – 11.27). NaOH was also used in the alkaline pH conditions (pH 11.48 – 12.38). Ionic strength of the separation buffers was adjusted at 0.010 mol L^{-1} by adding adequate amount of NaCl. Internal standards of naphthalene-1-sulfonate (1-NS^-, sodium salt) and *N*-ethylquinolinium (EtQ^+, iodate salt) were from Tokyo Chemical Industry (Tokyo, Japan). Other reagents were of analytical grade. Water used was purified by Milli-Q Gradient A10 (Merck-Millipore, Milford, MA, USA).

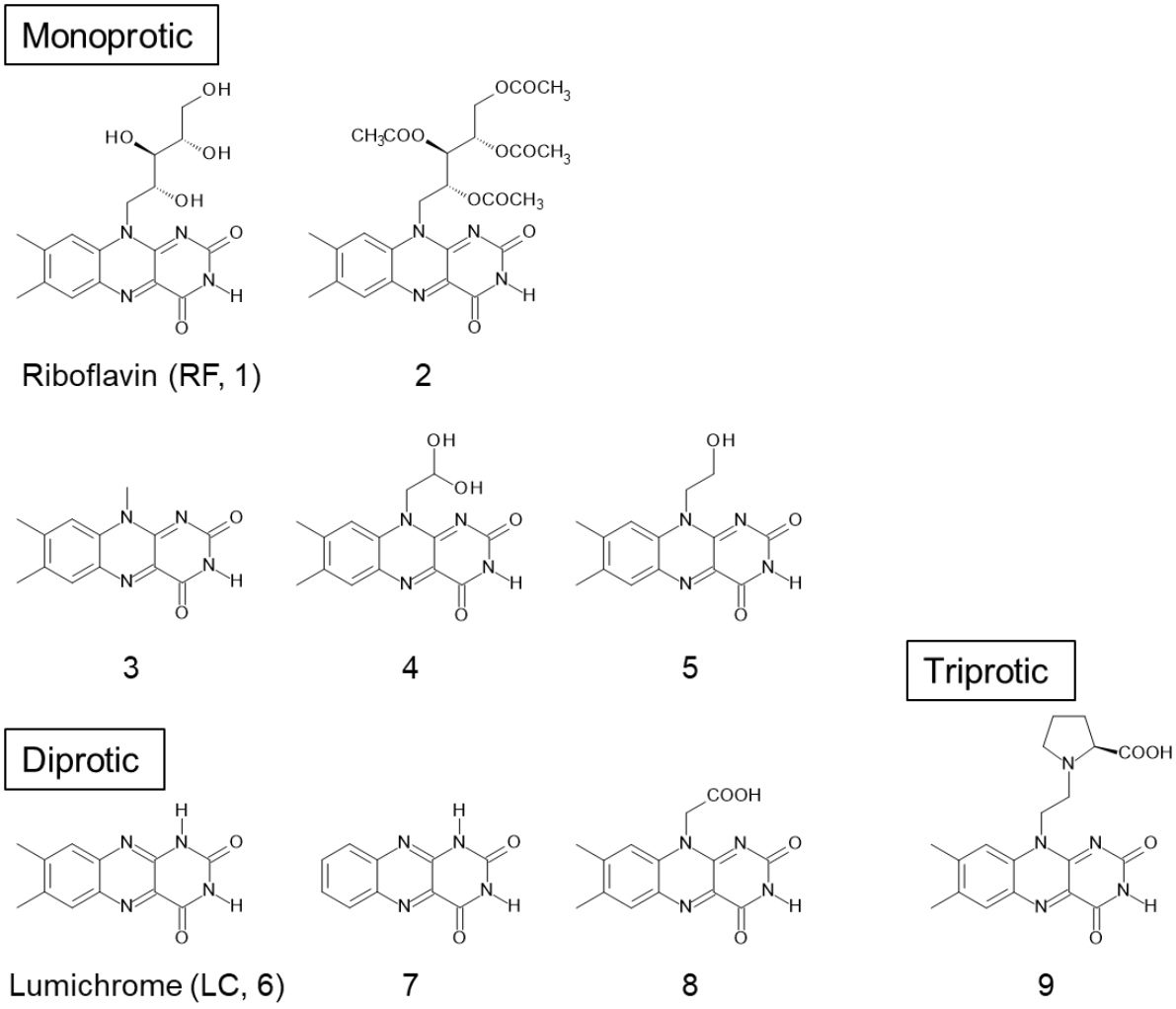

Figure 1. Structures of flavin analogues examined in this study.

2.2 Apparatus

An Agilent Technologies (Waldbronn, Germany) ^{3D}CE was used for the CZE measurements equipped with a photodiode array detector. A fused silica capillary (GL Sciences, Tokyo, Japan) was set in a cassette cartridge, and the cartridge was installed in the CZE system. Dimensions of the capillary were 64.5 cm in total length, 56 cm in the effective length from the sample injection point to the detection point, 50 μm in inner diameter and 375 μm in outer diameter. A short capillary was also used in the pressure-assisted CZE at the acidic pH conditions; the total length and the effective length of the capillary were 48.5 cm and 40 cm, respectively.

A Waters (Milford, MA, USA) ACQUITY UPLC with LCT Premire XE was used as an LC-MS system; a reversed phase column of BEH C18 (Waters, 50 mm × 2.1 mm i.d., 1.7 μm) was attached to the LC-MS system. Flow rate of the eluent was set at 1 mL min^{-1}, the MS polarity was ESI (+), and the injection volume was 20 μL. JASCO (Tokyo, Japan) PU-2080 plus and UV-2075 plus were used as a conventional RP-HPLC system with UV detection. An RP column of Unison UK-C18 (75 mm length × 4.6 mm i.d., 3 μm particle size; Imtakt, Kyoto, Japan) was attached to the system. An eluent of 70/30 (v/v) water/ethanol was used. NMR spectra were recorded using JEOL (Tokyo, Japan) JNM-ECZ-400S (^{1}H, 400 MHz). Chemical shifts are reported in ppm using TMS or the residual solvent peak as a reference. An HM-25G pH meter (TOA DKK, Tokyo, Japan) was used for the pH measurements of the separation buffers, after being calibrated daily with standard pH solutions. UV-LED light was used to expose and to degrade the FL analogues by NS375LIM (Emission maximum at 375±5 nm, 1.4 W; Nitride Semiconductors, Naruto, Japan).

2.3 Procedure

Stock solutions of FL analogues were prepared as ethanol solution at mmol L^{-1} level. The stock solution was diluted with water, and a solution of an FL analogue was prepared at the concentration of about 5×10^{-5} mol L^{-1}; it was used as a sample solution for the CZE measurements. Monoanionic 1-NS^- or monocationic EtQ^+ was added in the sample solution as an internal standard of the electrophoretic mobility. Ethanol at the concentration of 3 %(v/v) was also contained in the sample solution to monitor the electroosmotic flow (EOF). After the separation capillary being equilibrated

with a separation buffer, the sample solution was introduced into the capillary from the anodic end by applying a pressure of 50 mbar for 5 s. Both ends of the capillary were dipped in the separation buffer vials, and a DC voltage of 25 kV was applied for the CZE. During the CZE, the capillary was thermostat at 25 °C by circulating a constant-temperature air in the cassette cartridge. An analyte of the FL analogue and the internal standard were photometrically detected at 220 nm. Effective electrophoretic mobility, μ_{eff}, was calculated with the migration times of the analyte and the EOF in an ordinary manner. Acid dissociation constants of FL analogues were analyzed through the change in μ_{eff}, after standardized with the cationic or anionic internal standard.

On the measurements of the effective electrophoretic mobility at weakly acidic pH conditions over 2.38 – 4.75, the velocity of electroosmotic flow was not fast enough, and pressure-assisted CZE [13,14] was made. A constant pressure of 30 mbar was applied to the inlet vial of the separation buffer during the electrophoresis.

2.4 Determination of acid dissociation constants of FL analogues by CZE

Three types of the acid dissociation equilibria are included in the FL analogues of monoprotic, diprotic, and triprotic acids. For monoprotic FL analogues, HA, the charge changes from 0 to −1 by the one-step acid dissociation reaction of the imide moiety. The equilibrium reaction and its acid dissociation constant are written as in Eqs. (1) and (2), respectively. Fractions of the species are related with the effective electrophoretic mobility of the analogue, μ_{eff}, and the μ_{eff} value at a particular pH condition is given in Eq. (3).

$$\mathrm{HA} \rightleftharpoons \mathrm{H^+} + \mathrm{A^-} \quad (1)$$

$$K_{\mathrm{a}} = \frac{[\mathrm{H^+}][\mathrm{A^-}]}{[\mathrm{HA}]} \quad (2)$$

$$\mu_{\mathrm{eff}} = \frac{[\mathrm{A^-}] \times \mu_{\mathrm{ep,A}}}{[\mathrm{HA}] + [\mathrm{A^-}]} = \frac{10^{-\mathrm{p}Ka} \times \mu_{\mathrm{ep,A}}}{10^{-\mathrm{pH}} + 10^{-\mathrm{p}Ka}} \quad (3)$$

In Eq. (2), $[\mathrm{H^+}]$ is conventionally used instead of $a_{\mathrm{H+}}$, and K_{a} written in Eq. (2) is exactly a mixed equilibrium constant. Value of $\mu_{\mathrm{ep,A}}$ is the electrophoretic mobility of the monoanionic FL species. The protonated FL species, HA, is neutral, and its electrophoretic mobility can be set as zero; Eq. (3) is thus given. A series of the pairs of pH and μ_{eff} value were put in Eq. (3), and the values of $\mu_{\mathrm{ep,A}}$ and pK_{a} were optimized

by a non-linear least-squares analysis [8,10]. Since the measured electrophoretic mobility may deviate under the variation of experimental conditions, the value of μ_{eff} was standardized with the electrophoretic mobility of 1-NS^-, $\mu_{eff,1\text{-}NS}$, and the standardized value of μ_{eff} / $\mu_{eff,1\text{-}NS}$ was used for the analysis. Because 1-NS^- is a monoanion over the wide pH range and its electrophoretic mobility is essentially identical under the experimental conditions, and it was chosen as an internal standard. On analyzing the pK_a value, a software of R program (Ver. 3.6.2) was used on the basis of nonlinear least-squares regression [15]. Higher ionic strength would bias the pK_a value from its thermodynamic one, pK_a^o. However, the pK_a variation is generally ~0.1 for monoprotic acid at ionic strength of 0.10 mol L^{-1}. The pK_a values are determined at ionic strength of 0.010 mol L^{-1}, and the values determined in this study would differ little from the thermodynamic ones; less than 0.1.

For diprotic FL analogues, H_2A, the charge changes from 0 to −2 by the two-steps acid dissociation reactions as in Eq. (4). The stepwise acid dissociation constants, K_{a1} and K_{a2}, are written in Eq. (5). The effective electrophoretic mobility of an FL analogue at a particular pH condition is given in Eq. (6).

$$H_2A \rightleftharpoons H^+ + HA^-, \qquad HA^- \rightleftharpoons H^+ + A^{2-} \tag{4}$$

$$K_{a1} = \frac{[H^+][HA^-]}{[H_2A]}, \qquad K_{a2} = \frac{[H^+][A^{2-}]}{[HA^-]} \tag{5}$$

$$\mu_{eff} = \frac{[HA^-]\times\mu_{ep,HA} + [A^{2-}]\times 2\mu_{ep,HA}}{[H_2A] + [HA^-] + [A^{2-}]} = \frac{10^{-pH-pK_{a1}} \times \mu_{ep,HA} + 10^{-pK_{a1}-pK_{a2}} \times 2\mu_{ep,HA}}{10^{-2pH} + 10^{-pH-pK_{a1}} + 10^{-pK_{a1}-pK_{a2}}} \tag{6}$$

Value of $\mu_{ep,HA}$ is the electrophoretic mobility of the monoanionic FL species. The electrophoretic mobility of dianionic FL species is assumed to be twice to the monoanionic FL species [8,16]. A series of the pairs of pH and μ_{eff} were put in Eq. (6), and the values of pK_{a1} and pK_{a2}, as well as $\mu_{ep,HA}$, were optimized by a non-linear least-squares analysis in a similar manner to the analysis of the one-step pK_a [16].

3 Results and discussion

3.1 Determination of an acid dissociation constant of RF

Prior to the determination of the synthesized FL analogues, an acid dissociation constant of RF was determined through the changes in the effective electrophoretic mobility in CZE. Riboflavin is a monoprotic acid, and its charge changes from 0 to −1

by the acid dissociation reaction of the imide moiety. Electropherograms of RF at several pH conditions are shown in Fig. S1 A. The migration time of RF got longer with the increase in pH, suggesting that RF is more anionic at alkaline pH conditions. Changes in the standardized electrophoretic mobility of RF are shown in Fig. S1 B. Analysis of the results with Eq. (3) gave a pK_a value of 10.29±0.06 (mean ± standard error) by a least-squares analysis. The result agrees with the reported pK_a value of 10.2 [17]. The pK_a values are summarized in Table 1.

Table 1. Acid dissociation constants of RF and its photo-degradant of LC

	pK_{a1} (this study) [a]	pK_{a2} (this study) [a]	pK_{a1} (ref.)	pK_{a2} (ref.)
RF, 1	10.29±0.06 10.24±0.03 [b]	N/A [c]	10.2 [d] 10.64 [e]	N/A
LC, 6	8.36±0.02 (degradant)	(13.54±0.10) [f]	8.2 [g]	11.4 [g]

[a] Error: standard error.
[b] Determined under the degraded conditions.
[c] Not applicable.
[d] Ref. 17.
[e] Ref. 19. Determined by spectrophotometric titration.
[f] Estimated value by extrapolation.
[g] Ref. 18.

It is known that RF is degradable by light-exposure to form LC [5]. When the UV light was irradiated to an aliquot of 3 mL RF solution containing 1.0×10^{-4} mol L^{-1} RF, a CZE determination of RF suggested that the residual RF gradually decreased with the irradiation time and that the residual concentration of RF was about 60 % by the 120 min irradiation. Another distinct peak-signal was detected in the electropherograms with the irradiated solution, and the signal intensity complementary increased along with the decrease in the RF concentration. Therefore, the peak signal would be attributed to a degraded substance. The photo-degraded solution was analyzed by a conventional RP-HPLC and the LC-MS. The retention time of a degradant was about 20 min in the RP-HPLC, while that of RF was about 5 min. A mass number of 243.09 was detected by LC-MS with the substance of the longer retention time; it is attributed to protonated LC.

The acid dissociation constant of RF was also determined under the degraded conditions. An aliquot of aqueous RF solution at the concentration of 5×10^{-4} mol L^{-1} was exposed under a UV lamp for 120 min, and the solution was diluted by 10 times

and used for the pK_a determination after adding 2×10^{-5} mol L^{-1} 1-NS^- as an internal standard and 3 %(v/v) ethanol as an EOF marker. Electropherograms of the degraded RF are shown in Figure 2; the residual RF (open circle) is still detected with the degraded solution. It can be noticed that additional peak indicated with the filled triangle is also detected in the electropherograms, as mentioned above. The migration time of RF, as well as that of the degradant, got longer with increasing pH of the separation buffer; both RF and the degradant become more anionic.

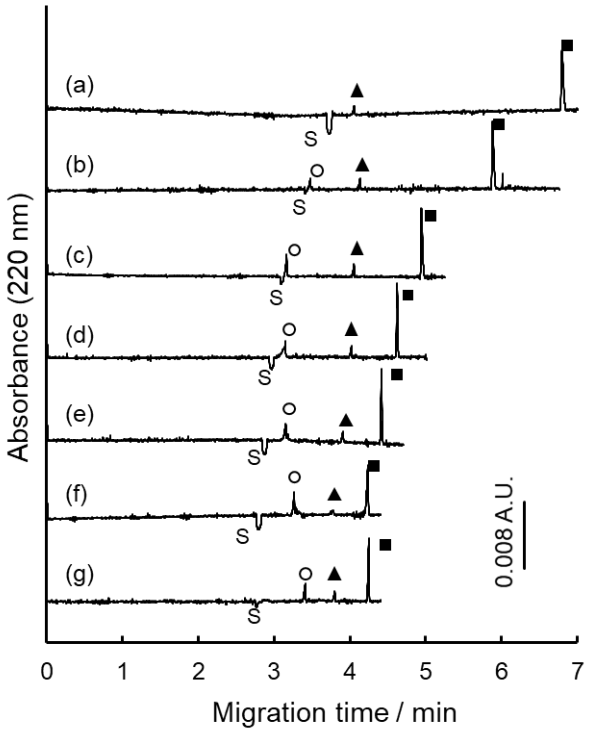

Figure 2. Electropherograms of RF at several pH conditions after UV-light exposure for 120 min. pH conditions: (a), 7.82; (b), 8.45; (c), 9.05; (d), 9.70; (e), 10.12; (f), 10.77; and (g), 11.48. Symbols: ○, RF; ▲, LC as a degradant from RF; ■, 1-NS; S, solvent (EOF). Sample solution: 5×10^{-5} mol L^{-1} RF (partly degraded) + 2×10^{-5} mol L^{-1} 1-NS^- (I.S.) + 3 %(v/v) ethanol. Separation buffers are written in the text. CZE conditions: applied voltage, 25 kV; sample injection, 50 mbar × 5 s; detection wavelength, 220 nm; capillary temperature, 25 °C.

Changes in the effective electrophoretic mobility, μ_{eff}, are shown in Figure 3 after standardized with $\mu_{eff,1\text{-}NS}$. The standardized μ_{eff} of the monoanionic degradant at pH around 11 is larger than RF, and the degradant would possess lighter molecular mass

than RF. While RF showed monoprotic profile of the mobility change, the μ_{eff} of the degradant tended to increase at the alkaline pH conditions over 12. The acid dissociation constant of RF under the degraded conditions was also determined with Eq. (3), and pK_a value of 10.24±0.03 was obtained (Table 1). The pK_a value agreed with the freshly prepared RF solution, as well as the reference value [17]. Therefore, usefulness of the CZE analysis, electrophoretic separation of the target analyte from coexisting substances [8-11], was also demonstrated in this study.

Acid dissociation constants of the degradant were also determined through the changes in the effective electrophoretic mobility. The increased μ_{eff} value at the alkaline pH conditions suggested the formation of a dianionic species. The CZE measurements, however, are limited under the pH conditions below ~12, because the higher pH conditions accompany the high salt concentrations, yielding much Joule's heat and temperature increase. Since the dianionic species is twice in its charge from the monoanionic species and its molecular mass is almost identical to the monoanionic species, μ_{eff} value of the dianionic species is supposed to be twice to the monoanionic species. This assumption was valid in phenolphthalein [8] and fluorescein derivatives [16]. Equation (6) was thus used for the determination of the two steps of the pK_a values; pK_{a1} = 8.36±0.02 and pK_{a2} = 13.54±0.10 were obtained by the least-squares analysis. It should be taken care of that the pK_{a2} value obtained in this study is the results of the extrapolated analysis. The pK_{a1} value obtained in this study agreed well with the reported value of LC (pK_{a1} = 8.2) [18], and the degradant detected in this study would be LC. Lumichrome is diprotic acid with the imide moiety and N(1)-H [18], and two steps of the mobility change would also suggest the degradant to be LC. Therefore, another usefulness of the CZE analysis is also demonstrated in this study; the degradant can directly be analyzed without any isolation from the reaction mixture. On the other hand, the pK_{a2} value obtained in this study did not agree with the reported value of 11.4 [18]. The μ_{eff} value of the degradant LC slightly changed at the pH conditions at around 11, suggesting the lack of the acid dissociation equilibrium at this pH range; the reference value determined by a spectrophotometric titration would not be correct.

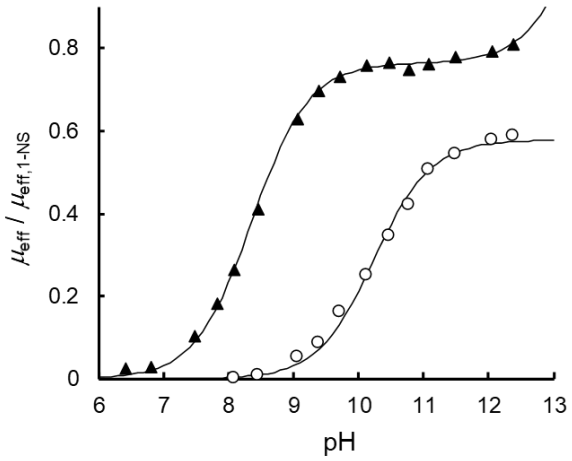

Figure 3. Changes in the effective electrophoretic mobility of RF and LC as a degradant from RF. The electrophoretic mobility was standardized with 1-NS^- as an internal standard. Symbols: ○, RF; ▲, LC as a degradant. The curves are drawn with the optimized results. The CZE conditions are the same as in Fig. 2.

3.2 Determination of acid dissociation constants of FL analogues by CZE

3.2.1 Acid dissociation constant of FL analogue 2

Riboflavin tetraacetate (FL analogue 2) is a monoprotic acid, and its charge changes from 0 to −1 by the acid dissociation reaction of the imide moiety. Electropherograms at several pH conditions are shown in Figure S2 A, and the changes in μ_{eff} are shown in Figure S2 B after standardized with $\mu_{eff,1\text{-NS}}$. Analysis of the changes in μ_{eff} with Eq. (3) gave a pK_a value of 10.21±0.05.

An adjacent peak was detected just after the FL analogue 2. A small portion of FL analogue 2 would have hydrolyzed in the alkaline separation buffer during the CZE. When FL analogue 2 at its concentration of 1.0×10^{-4} mol L^{-1} was treated in an alkaline solution of 5×10^{-4} mol L^{-1} NaOH for 50 min, additional peak signal was detected by CZE as an adjacent peak behind to the peak signal of FL analogue 2. Thus, the newly detected signal would be attributed to the hydrolyzed species of FL analogue 2; one or more ester moieties are hydrolyzed to form alcohol group(s). Even under the alkaline-degraded conditions, the acid dissociation constant of FL analogue 2 was

successfully determined as pK_a = 10.24±0.04. The pK_a value is close to the one determined with the freshly prepared solution.

The alkaline-treated FL analogue 2 solution was analyzed by RP-HPLC and LC-MS. A certain number (~10) of peaks were detected by a conventional RP-HPLC-UV detected at 220 nm. The LC-MS chromatograms are shown in Figure S3. The mass numbers detected by the LC-MS were 545.188 (FL analogue 2 +H), 503.178, 419.157, and 377.146. The Each mass number corresponds to deacetylated substances from FL analogue 2, and the mass number 377.146 is equivalent to RF. Therefore, the newly detected peaks in Figure S2 would be attributed to the hydrolyzed substances from FL analogue 2.

3.2.2 Acid dissociation constant of FL analogue 3

Lumiflavin (FL analogue 3) is also a monoprotic acid, and its charge similarly changes from 0 to −1 by the acid dissociation reaction of the imide moiety. Electropherograms are shown in Figure S4 A, and the changes in μ_{eff} are shown in Figure S4 B after standardized with $\mu_{eff,1\text{-}NS}$. An additional peak signal indicated with filled triangle was detected with FL analogue 3; it would be attributed to an impurity formed under the storage conditions. An additional peak was also detected with the FL analogue 3 solution by a conventional RP-HPLC-UV detection; LC-MS analysis gave a substance of mass number of 243.09. The mass number is equivalent to LC, and the impurity would be LC. The migration time of FL analogue 3, as well as the additional peak signal, got longer than the EOF with the increase in pH as in the case of RF. Analysis of the changes in μ_{eff} of FL analogue 3 with Eq. (3) gave a pK_a value of 10.38±0.04. Unfortunately, the additional peak was not detected in the alkaline separation buffer, it would have been overlapped with FL analogue 3. The monoprotic behavior of the additional peak was analyzed with Eq. (3), and a pK_a value of 8.29±0.02 was obtained; the pK_a value is close to that of LC. The usefulness of the CZE analysis is also demonstrated with such substance as containing impurity, as well as the pK_a analysis of the impurity.

3.2.3 Acid dissociation constant of FL analogue 4

10-(2,2-Dihydroxyethyl)-7,8-dimethylisoalloxazine (FL analogue 4) is also a monoprotic acid, and its charge changes from 0 to −1 by the acid dissociation reaction of the imide moiety. Electropherograms are shown in Figure S5 A, and the changes in μ_{eff} are shown in Figure S5 B after standardized with $\mu_{eff,1\text{-}NS}$. A peak signal corresponding to FL analogue 4 disappeared at pH 12.05 (Figure S5 A(f)). The result suggests that FL analogue 4 has decomposed at the alkaline conditions. An additional peak signal indicated with filled triangle was also detected with FL analogue 4 as in the case of FL analogue 3. Although the migration time of the EOF became faster at alkaline pH conditions, the peak signal of FL analogue 4 much delayed from the EOF at alkaline pH conditions. Analysis of the changes in μ_{eff} with Eq. (3) gave a pK_a value of 10.22±0.08 for FL analogue 4. An acid dissociation constant of the impurity was also determined with Eq. (3), and a pK_a value of 8.40±0.02 was obtained. LC-MS analysis gave a mass number of 243.09, and the impurity was also found to be LC.

3.2.4 Acid dissociation constant of FL analogue 5

10-(2-Hydroxyethyl)-7,8-dimethylisoalloxazine (FL analogue 5) is also a monoprotic acid, and its charge changes from 0 to −1 by the acid dissociation reaction of the imide moiety. Electropherograms are shown in Figure S6 A, and the changes in μ_{eff} are shown in Figure S6 B after standardized with $\mu_{eff,1\text{-}NS}$. A peak signal corresponding to FL analogue 5 was detected. Although a peak signal of an impurity or the degradant was detected at pH around 10.5, it did not interfere with the pK_a analysis of FL analogue 5. The additional peak signal was not detected when FL analogue 5 was freshly prepared, and therefore, it would be generated under light exposure. The migration time of FL analogue 5 got longer than the EOF with the increase in pH as in the case of RF. Analysis of the changes in μ_{eff} with Eq. (3) gave a pK_a value of 10.35±0.04 for FL analogue 5.

3.2.5 Acid dissociation constants of FL analogue 7

Alloxazine (FL analogue 7) is a diprotic acid, and its charge changes from 0 to −2 by the acid dissociation reactions of the imide moiety and N(1)-H. Electropherograms are shown in Figure S7 A, and the changes in μ_{eff} are shown in Figure S7 B after

standardized with $\mu_{eff,1\text{-}NS}$. The μ_{eff} value increased in the pH range from 7 to 9, and an acid dissociation equilibrium is applied in this pH range. The μ_{eff} value further increased in the pH range over 11. This increase would be attributed to the 2nd step of the deprotonation. Two steps of the acid dissociation equilibria are analyzed with Eq. (6) in a similar manner to LC, and the pK_a values of pK_{a1} = 8.07±0.01 and pK_{a2} = 12.96±0.4 were obtained by the least-squares analysis. It should also be taken care of that the pK_{a2} value obtained in this study is the results of the extrapolated analysis, as in the case of LC. It can be noted that both pK_{a1} and pK_{a2} values of FL analogue 7 are smaller than those of LC. The result would be attributed to the lack of two methyl groups of electron donating moiety.

3.2.6 Acid dissociation constants of FL analogue 8 at weakly alkaline pH region

7,8-Dimethyl-10-carboxymethylisoalloxazine (FL analogue 8) is a diprotic acid, and its charge changes from 0 to −2 by the acid dissociation equilibria of a carboxylic acid moiety and an imide moiety. The carboxylic acid moiety would dissociate at weakly acidic pH region, and the imide moiety at weakly alkaline pH region. Electropherograms in the neutral to weakly alkaline pH range are shown in Figure S8, and the changes in μ_{eff} are shown in Figure 4 A after standardized with $\mu_{eff,1\text{-}NS}$. A peak signal attributed to FL analogue 8, as well as an impurity, is detected in the electropherograms. According to the presence of the carboxylate moiety, FL analogue 8 is anionic in the pH range between 7.9 and 12.0. On the other hand, detection of FL analogue 8 was difficult at acidic pH conditions below 4.5, because the electroosmotic flow was too slow at the acidic pH conditions to detect the anionic species. In Figure 4 A, the μ_{eff} value of FL analogue 8 increased in the pH range at around 11, suggesting that the charge changes from −1 to −2 in the pH range: the 2nd step of the deprotonation. The acid dissociation equilibrium of K_{a2} is expressed as in Eq. (7) with its acid dissociation constant (8). The effective electrophoretic mobility is contributed from both charged species of HA^- and A^{2-}, and it is written as in Eq. (9).

$$HA^- \rightleftharpoons H^+ + A^{2-} \quad (7)$$

$$K_{a2} = \frac{[H^+][A^{2-}]}{[HA^-]} \quad (8)$$

$$\mu_{eff} = \frac{[HA^-] \times \mu_{ep,HA} + [A^{2-}] \times \mu_{ep,A}}{[HA^-] + [A^{2-}]} = \frac{10^{-pH} \times \mu_{ep,HA} + 10^{-pK_{a2}} \times \mu_{ep,A}}{10^{-pH} + 10^{-pK_{a2}}} \quad (9)$$

Values of $\mu_{ep,HA}$ and $\mu_{ep,A}$ are the electrophoretic mobility of the monoanionic and dianionic species, respectively. A series of the pairs of pH and μ_{eff} were put in Eq. (9), and the values of $\mu_{ep,HA}$, $\mu_{ep,A}$ and pK_{a2} were optimized by a non-linear least-squares analysis in a similar manner after the standardization with 1-NS^- as an internal standard. A pK_{a2} value of 10.84±0.02 was obtained by the analysis. Optimized values of standardized $\mu_{ep,HA}$ and $\mu_{ep,A}$ were 0.72 and 1.32, respectively. The standardized $\mu_{ep,A}$ value is 1.83 times to that of $\mu_{ep,HA}$ value, and the twice charge of A^{2-} against HA^- would be appropriate. The effective electrophoretic mobility of the impurity showed two-steps increase (Figure 4 A), and the increase was also analyzed as two-steps acid dissociation equilibrium, as written in Eq. (6); acid dissociation constants of pK_{a1} = 8.45±0.02 and pK_{a2} = 12.91±0.09 were determined by the analysis. An LC-MS analysis gave a mass number of 243.09, and the impurity was also LC.

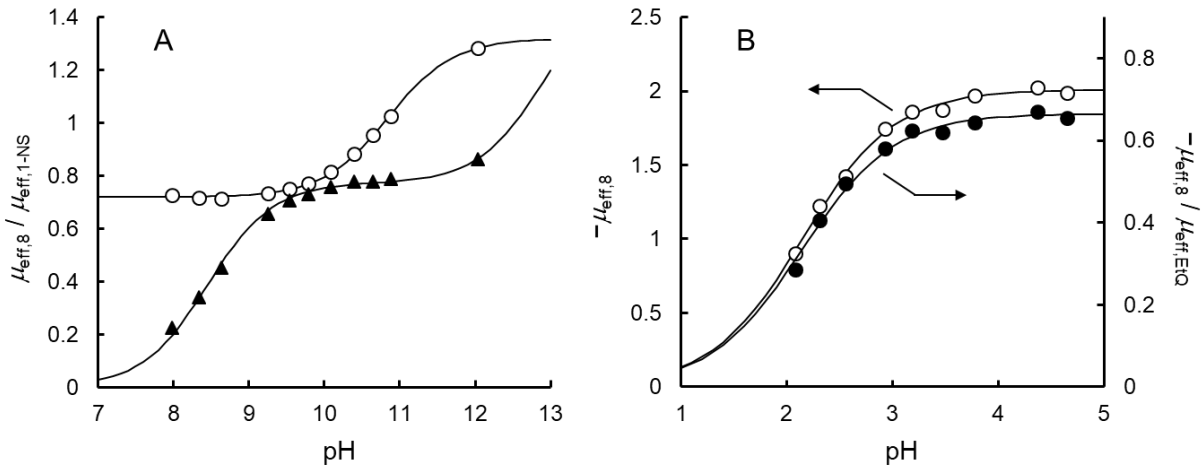

Figure 4. Changes in the effective electrophoretic mobility of FL analogue 8. (A) At weakly alkaline conditions. The μ_{eff} values are standardized with $\mu_{eff,1\text{-}NS}$. ○, FL analogue 8; ▲, impurity. (B) At weakly acidic conditions. ○, the μ_{eff} values are directly used for the analysis; ●, the μ_{eff} values are used for the analysis after standardized with $\mu_{eff,EtQ}$. The curves are drawn with the optimized results. CZE conditions: (A), applied voltage, 25 kV; sample injection, 50 mbar × 5 s; detection wavelength, 220 nm; capillary temperature, 25 °C; (B), pressure-assisted CZE under 25 kV applied voltage and 30 mbar pressure assist, other conditions are as in (A).

3.2.7 Acid dissociation constants of FL analogue 9 at weakly alkaline pH region

2-(7,8-Dimethyl-10-isoalloxazyl)ethyl-L-proline (FL analogue 9) is a triprotic acid, and its charge changes from +1 to −2 by the three-steps acid dissociation reactions of a carboxylic acid moiety, a protonated pyrrolidine moiety, and an imide moiety.

Electropherograms are shown in Figure S9, and the changes in μ_{eff} are shown in Figure 5 A after standardized with $\mu_{eff,1\text{-}NS}$. The migration time of FL analogue 9 got longer than the EOF with the increase in pH. Two-steps increase in the μ_{eff} is read out, and the acid dissociation constants of pK_{a2} (protonated pyrrolidine moiety) and pK_{a3} (imide moiety) have been analyzed by Eq. (6) through the μ_{eff} values; pK_{a2} = 8.23±0.04 and pK_{a3} = 10.93±0.06 were determined. The pK_a values for the FL analogues determined by the CZE are summarized in Table 2.

An acid dissociation constant of FL analogue 9 was also determined by spectrophotometric titration at alkaline pH region. Changes in the absorbance at 356 nm was used for the analysis. One step of the absorbance change was observed with a clear isosbestic point at 368 nm, and a pK_a value of 10.74±0.02 was determined. The pK_a value corresponds to the pK_{a3} value at the imide moiety determined by the CZE. However, the spectrum slightly changed in the pH range from 6.7 to 9.4 where the pK_{a2} value was determined by the CZE analysis. The absence of the spectrum shift by the acid dissociation equilibrium would be because of the pyrrolidine moiety being shielded from the resonance skeleton. Therefore, CZE analysis is applicable to such substance without any spectrum shift.

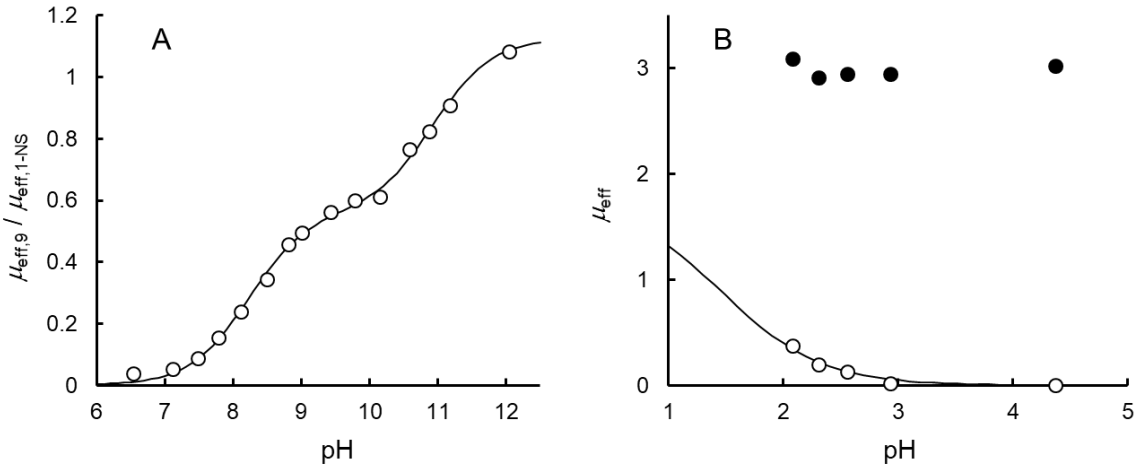

Figure 5. Changes in the effective electrophoretic mobility of FL analogue 9. (A) At weakly alkaline conditions. ○, FL analogue 9 standardized with $\mu_{eff,1\text{-}NS}$. (B) At weakly acidic conditions. ○, FL analogue 9; ●, EtQ^+. The curves are drawn with the optimized results. CZE conditions: (A), applied voltage, 25 kV; sample injection, 50 mbar × 5 s; detection wavelength, 220 nm; capillary temperature, 25 °C; (B), pressure-assisted CZE under 25 kV applied voltage and 30 mbar pressure assist, other conditions are as in (A).

457 **Table 2**. Acid dissociation constants of FL analogues determined in this study

	pK_{a1} [a]	pK_{a2} [a]	pK_{a1} [a]	Remark
Analogue 2	10.21±0.05 10.24±0.04 [b]	N/A [c]	N/A [c]	Degraded at alkaline conditions: hydrolysis of ester moiety.
Analogue 3	10.38±0.04	N/A	N/A	Degraded at storage conditions: LC was formed.
Analogue 4	10.22±0.08	N/A	N/A	Degraded at alkaline conditions: LC was formed.
Analogue 5	10.35±0.04	N/A	N/A	Degraded at storage conditions with light exposure: LC was formed.
Analogue 7	8.07±0.01	(12.96±0.4) [d]	N/A	Impurity of LC was contained.
Analogue 8	2.15±0.02 [e]	10.84±0.02	N/A	
Analogue 9	(1.46±0.03) [d],[e]	8.23±0.04 (−) [f]	10.93±0.06 (10.74±0.02) [f]	

458 [a] Error: standard error.
459 [b] Degraded at alkaline conditions.
460 [c] Not applicable.
461 [d] Estimated value by extrapolation.
462 [e] Determined by pressure-assisted CZE; no standardization of μ_{eff}.
463 [f] Determined by spectrophotometric titration.
464

465 3.3 Determination of acid dissociation constants of FL analogues 466 by pressure-assisted CZE

467 Acid dissociation constants of the carboxylic acid moiety on FL analogues 8 and 9
468 were difficult to analyze by CZE, because their peak signals were not detected at acidic
469 pH conditions due to the slow EOF. Therefore, pressure-assisted CZE [13,14] was
470 examined to compensate the slow EOF and to detect the analytes even at the acidic
471 pH conditions. An assisting air-pressure of 30 mbar was applied to the inlet vial during
472 the electrophoresis. The migration time of the EOF got faster and the analytes of
473 interest can be detected by the pressure-assisted CZE.

474 3.3.1 Acid dissociation constant of FL analogue 8 at acidic pH region

475 As noted in the previous section, FL analogue 8 is electrically neutral at acidic pH
476 conditions and its charge changes from 0 to −1 by the acid dissociation reaction of the
477 carboxylic acid moiety. Pressure-assisted electropherograms are shown in Figure 6.
478 FL analogue 8 migrated slower than the EOF, and it is also anionic in this pH range.
479 The peak widths became wider by applying the pressure compared with the normal

CZE, because the analyte in the plug zone is dispersed by the parabolic flow in the directions of both the tube axis and the tube diameter. However, the effective electrophoretic mobility of the internal standard, EtQ^+, was affected little by the pressure, and therefore, the effective electrophoretic mobility of the analyte would be measured properly, and it would be used for the pK_a analysis. Changes in μ_{eff} of FL analogue 8 are shown in Figure 4 B; both directly measured μ_{eff} values and standardized μ_{eff} values with EtQ^+ were used for the analysis. Since anionic internal standard of 1-NS^- was not detected even by the pressure-assisted CE, cationic EtQ^+ was used for the standardization. Fortunately, protonated species of FL analogue 8 is electrically neutral, its electrophoretic mobility is zero and Eq. (3) was used for the analysis on the basis of an acid dissociation equilibrium (1). A pK_{a1} value of 2.15±0.02 was obtained with the direct analysis of μ_{eff} values, whereas a pK_{a1} value of 2.14±0.03 was obtained after the standardization with EtQ^+. Therefore, the standardization worked well.

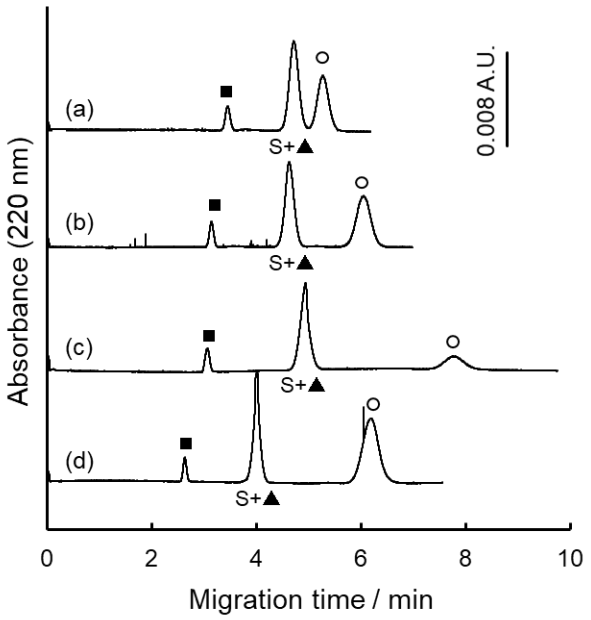

Figure 6. Electropherograms of FL analogue 8 at acidic pH conditions by the pressure-assisted CZE. pH conditions: (a), 2.08; (b), 2.56; (c), 3.47; (d), 4.65. Symbols: ○, FL analogue 8; ▲, LC as a degradant; ■, EtQ^+; S, solvent (EOF). Sample solution: 6×10^{-5} mol L^{-1} FL analogue 8 + 3×10^{-5} mol L^{-1} EtQ^+ (I.S.) + 3 %(v/v) ethanol. Separation buffers are written in the text. CZE conditions: applied voltage, 25 kV; assist pressure, 30 mbar; sample injection,

50 mbar × 5 s; detection wavelength, 220 nm;
capillary temperature, 25 °C.

3.3.2 Acid dissociation constants of FL analogue 9 at acidic pH region

An acid dissociation constant of FL analogue 9 was also examined by the pressure-assisted CZE. Its charge changes from +1 to 0 by the acid dissociation reaction of the carboxylic acid moiety, and the dissociated species is zwitterion. Electropherograms are shown in Figure 7; broad peaks are also detected as in the case of FL analogue 8. It can be seen from Figure 7 that FL analogue 9 migrated faster than the EOF at acidic pH conditions and it is cationic. Although only a broad peak is detected the EOF position at pH 2.56 (b), the broad peak was found to be composed of two overlapped peaks of FL analogue 9 and the EOF when detected at 254 nm. Changes in the electrophoretic mobility are shown in Figure 5 B. FL analogue 9 becomes more cationic at acidic conditions. Different from FL analogue 8, the electrophoretic mobility of the protonated species of H_3A^+ is not zero, and the value is difficult to measure. The acid dissociation equilibrium and its acid dissociation constant are written as in Eq. (10) and (11), respectively. The effective electrophoretic mobility is contributed from the positively charged species, H_3A^+, and it is given in Eq. (12).

$$H_3A^+ \rightleftharpoons H^+ + H_2A \quad (10)$$

$$K_a = \frac{[H^+][H_2A]}{[H_3A^+]} \quad (11)$$

$$\mu_{eff} = \frac{[H_3A^+] \times \mu_{ep,H3A}}{[H_3A^+] + [H_2A]} = \frac{10^{-pH} \times \mu_{ep,H3A}}{10^{-pH} + 10^{-pK_a}} \quad (12)$$

Value of $\mu_{ep,H3A}$ is the electrophoretic mobility of the monocationic species. Another problem is that CZE measurements at pH conditions below 2 is also difficult owing to the high salt concentrations and the generated Joule's heat. To estimate the acid dissociation constant of the carboxylic acid moiety of FL analogue 9, the electrophoretic mobility of the cationic species of +1 charged one was assumed to be equal to that of the anionic species of −1 charged one. An acid dissociation constant of 1.46±0.03 was estimated under such assumption.

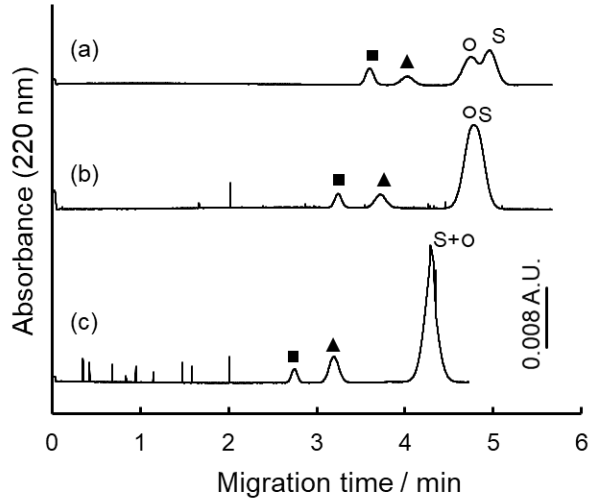

Figure 7. Electropherograms of FL analogue 9 at acidic pH conditions by pressure-assisted CZE. pH conditions: (a), 2.08; (b), 2.56; (c), 4.37. Symbols: ○, FL analogue 9; ▲, impurity; ■, EtQ^+; S, solvent (EOF). Sample solution: 6×10^{-5} mol L^{-1} FL analogue 9 + 3×10^{-5} mol L^{-1} EtQ^+ (I.S.) + 3 %(v/v) ethanol. Separation buffers are written in the text. CZE conditions: applied voltage, 25 kV; assist pressure, 30 mbar; sample injection, 50 mbar × 5 s; detection wavelength, 220 nm; capillary temperature, 25 °C.

4 Concluding remarks

In this study, acid dissociation constants were determined by CZE for a series of molecular catalyst candidates of FL analogues. Changes in the μ_{eff} by the protonation or deprotonation were used for the CZE analysis. The CZE analysis was proved to be useful for the separation of the target analyte from the coexisting substances including degraded species and impurities. The CZE analysis is also applicable to such substances as without spectrum shift. Pressure-assisted CZE was also utilized for the measurements of the effective electrophoretic mobility at weakly acidic pH conditions, where the electroosmotic flow is slow.

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Conflict of interest

The authors have declared no conflict of interest.

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Supporting information

Supporting information file: Syntheses of FL analogues 2, 3, 4, 5, 7, 8, and 9 are described. Typical electropherograms and the changes in the effective mobility are shown in Figures S1, S2, S4, S5, S6, and S7 for FL analogues 1, 2, 3, 4, 5, and 7, respectively. Typical electropherograms are shown in Figures S8 and S9 for FL analogues 8 and 9, respectively. LC-MS chromatograms for alkaline-treated FL analogues 2 are shown in Figure S3.