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Journal of Chromatography A, 1053 (2004) 71–78
JOURNAL OF CHROMATOGRAPHY A
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Capillary liquid chromatographic determination of cellular flavins

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Available online 20 July 2004

Abstract

A capillary LC system was set up and optimized, in which a UV absorbance detector was used and a monolithic silica-ODS column as the separation column. Two on-line concentration techniques, namely, gradient elution mode and in-tube solid-phase ion-pair microextraction (SPIPME), were combined with the capillary LC system, which proved to be beneficial to enhance the concentration sensitivity by enabling the injection of large volumes of samples. The limits of detection at ppb levels for the flavins [riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD)] were achieved using the two techniques. For in-tube SPIPME, a monolithic silica-ODS column was employed as the extraction column, on which FAD and FMN were retained by interaction with an ion-pair reagent, tetrabutylammonium phosphate, resulting in greater than 110-fold enhancement in their concentration sensitivities relative to conventional injection method. The reproducibility and linearity of the two methods were investigated. The two methods were applied to analyze trace amounts of flavins in bacterial *Escherichia coli* cell extracts and recombinant flavoenzymes.

Keywords: Solid-phase ion-pair microextraction; Extraction methods; Flavins

1. Introduction

Flavins, which play critical roles in biochemical cycles of most organisms, represent an important class of metabolites in the cell. Among flavins, the most known is riboflavin (RF), which is phosphorylated in the cell to form two principal biological active derivatives, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN and FAD are involved as cofactor or/and substrate in many enzymatic redox processes and take part in the metabolism of other vitamins, e.g., folate and vitamin B_6 [1]. Recent mechanistic studies on neuronal nitric oxide synthase (nNOS) have demonstrated that flavins may act as redox switches by mediating electron transfer processes from nicotinamide diphosphate to heme domains that is calcium–calmodulin-dependent [2].

Owing to the biochemical importance of RF, FMN and FAD, several techniques for the determination of free RF or total flavins, after conversion of FMN and FAD into RF, have been developed, including microbiological [3], fluorimetric [4], and electrochemical [5] methods. Since RF participates in biochemical redox reactions in the form of two coenzymes, FMN and FAD, development of analytical techniques for the determination of the concentration of each flavin is helpful for the studies of the roles of these compounds in biological systems. A number of conventional high performance liquid chromatography (HPLC) methods [6–12] have been reported for the simultaneous analysis of the three flavins. More recently, capillary electrophoresis (CE) [13–16] has also been applied to separate and determine RF, FMN and FAD in different matrices because of its high resolution, rapid analysis time, and small sample requirement.

As flavins are naturally occurring fluorescent compounds [17], most of HPLC [6–11] and CE [13–16] methods for analysis of flavins use fluorescence for detection, which allows their selective detection from other compounds present in the sample matrix, especially a complicated biological sample. Compared with fluorescence detection, UV detection is more popular in LC although its sensitivity is lower.

With the trend in analytical chemistry toward miniaturization, microseparation systems have become hot research fields in separation sciences, such as capillary gas chro-

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doi:10.1016/j.chroma.2004.06.052

matography, capillary LC and CE. Capillary LC offers several advantages over conventional LC in terms of significant reduction of the consumption and disposal of organic solvents, minimization of exposure to toxic reagents because of the very small inner diameter of separation columns, low flow rates and low injection volumes.

However, few reports about the application of capillary LC were found in the literature perhaps due to two problems. One problem is the limited availability of a wide range of capillary columns. In order to meet the requirements of capillary LC for capillary columns, one type of special column, i.e., monolithic column, was developed recently, which can overcome some inherent limitations possessed by conventional commercial packed columns, such as a low permeability and a large volume of mobile phase [18–25]. The evolution of capillary columns will facilitate the faster development of capillary LC technique and its routine use. Another problem is low concentration sensitivity resulting from the smaller injection volume and narrower optical path length for UV photometric detection. In order to enhance the concentration sensitivity in capillary LC, hyphenation with sample preconcentration techniques is needed. One technique is on-column focusing, which is to dissolve analytes in a weaker solvent than the mobile phase and allows the injection of a large sample volume [26–28]. Other sample enrichment techniques are on-line or off-line solid-phase microextraction (SPME) [29,30]. On-line SPME enables continuous extraction, concentration, desorption and injection using an autosampler, which not only shortens the total analysis time but also provides better accuracy and precision relative to manual techniques, as reviewed by Kataoka [31] and Saito and Jinno [32]. On-line in-tube SPME was recently developed using an open tubular fused-silica capillary column as an extraction device instead of SPME fiber. Although the technique has been successfully hyphenated with capillary LC [33], repeating draw/eject cycles of sample solution is needed to improve extraction efficiency. Recently, Shintani et al. [34] introduced a monolithic silica-ODS column used for in-tube SPME, which demonstrated better preconcentration efficiency compared with the conventional in-tube SPME due to the merits of higher permeability and porosity possessed by a monolithic column. The method proved to be useful for concentrating and separating strongly hydrophobic standard compounds. However, it was not applied for analysis of real-world samples.

The aim of this study was to develop a method for analysis of RF, FMN and FAD in a complicated sample matrix by a capillary LC with UV absorbance detection. During the method development, a monolithic silica-ODS column was used as the separation column. Two on-line concentration methods, namely, gradient elution mode and in-tube solid phase ion-pair microextraction (SPIPME) using a monolithic silica-ODS column as an extraction medium, were used to enhance concentration sensitivity. The applicability of the methods was demonstrated by assessment of flavins content from several types of samples including the *Escherichia coli* cell extracts, the recombinant nNOS full-length flavoenzyme and its reductase domain (FAD/FMN).

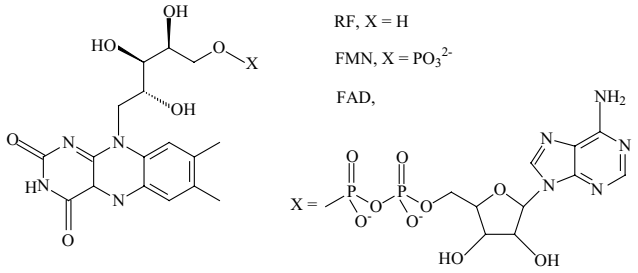

Fig. 1. Structures of RF, FMN and FAD.

2. Experimental

2.1. Reagents

RF, FMN and FAD were purchased from Wako (Osaka, Japan), Sigma (St. Louis, MO, USA) and TCI Chemicals (Tokyo, Japan), respectively. Their structures are shown in Fig. 1. FMN and FAD stock solutions were prepared as 2 mg/mL in methanol–water (50:50, v/v). RF stock solutions were prepared as 1 mg/mL in deionized water. The stock solutions were stored at 4 °C prior to use. Methanol and tetrabutylammonium phosphate (TBAP) were of HPLC grade and purchased from Wako. Water used for mobile phase and sample preparations was obtained from a Milli-Q water purification system (Millipore, MA, USA).

2.2. Apparatus and procedure

The capillary LC system was set up in our laboratory, which consists of a micro-flow pump (GL Sciences MP681, Tokyo, Japan), a microbore HPLC injection valve (Valco VICI, Switzerland) and a UV detector (Jasco CE 970, Tokyo, Japan) equipped with a capillary flow-cell holder. Data were acquired by a data acquisition board (National Instrument, USA) at a sampling rate of 50 Hz and a program written in Labview 6.0. Cross-capillary UV illumination was used for UV detection. The inner and outer diameters of the capillary are 100 and 360 μm, respectively. A monolithic silica-ODS column (250 mm × 0.2 mm i.d.) was obtained from GL Sciences. The monolithic silica-ODS columns (150 mm × 0.2 mm i.d. and 500 mm × 0.2 mm i.d.) were prepared according to the previously described method [23]. Gradient elution was performed with methanol–30 mM phosphate buffer (pH 3.0) at a flow rate of 2 μL/min. The gradient started at 5% methanol, which was maintained isocratically for the first 1 min, thereafter the methanol concentration was raised linearly to 95% in 15 min and kept at 95% for 10 min. In in-tube SPIPME experiments, isocratic elution was performed with 4.5 mM TBAP in methanol–30 mM phosphate buffer (pH 3.0) (30/70, v/v) as the mobile phase at

a flow rate of 2 μL/min. Detection wavelength was set at 265 nm.

2.3. In-tube SPIPME

A monolithic silica-ODS column (150 mm × 0.2 mm i.d.) was used as the in-tube SPIPME device and mounted in the position of the sample loop of the six-port valve injector. A syringe pump (KDS200, New Hope, USA) was used to control the injection volume and flow rate of the sample solution. The monolithic column was washed and conditioned first by methanol (80 μL), then 4.5 mM TBAP aqueous solution (80 μL) at a flow rate of 5 μL/min. The extraction of flavins into the monolithic column was performed by the injection of the sample at a flow rate of 5 μL/min with the six-port valve in the LOAD position. Then the six-port valve was switched to the INJECT position and the extracted flavins were directly desorbed from the monolithic column by mobile phase flow, transported to the capillary LC column, and then detected by UV detector.

2.4. Preparation of Escherichia coli cell extracts

The *E. coli* BL21 was cultured in 100 mL of 2 × YT medium at 37 °C by shaking. The 2 × YT medium (100 mL) contains 1.6 g tryton, 1.0 g yeast extract, and 0.5 g NaCl. Growth was monitored by measuring optical density at 600 nm. As the number of bacteria increased with the duration of culture, the growth was discontinued when the cell concentration reached about 2×10^8 living cells/mL. Then 10 mL of culture medium was withdrawn and centrifuged at 4 °C and 3200 rpm for 30 min. The supernatant medium was removed and the residual *E. coli* cells in the tube were washed with 10 mM phosphate buffer (pH 7.0) to prevent the contamination of 2 × YT medium and stored at −80 °C until extraction.

Flavins extraction procedure was similar to the method described previously with minor modification [35,36]. Briefly, 2 mL of ice-cooled methanol was added to the tube containing the cells and incubated at room temperature for 10 min, then placed at −20 °C for 30 min. Then the solution was centrifuged at 4 °C and 3000 rpm for 25 min. The supernatants were transferred to a 15 mL plastic tube. Chloroform (1.6 mL) and deionized water (640 μL) were added to the methanol solution and mixed thoroughly for about 30 s to remove phospholipids liberated from cell membrane. After 10 min, the solution was centrifuged at 4 °C and 3000 rpm for 25 min. Then the upper layer solution was withdrawn and centrifugally filtered through a Millipore M_r 5000 cutoff filter at 4 °C to remove proteins and other debris. The filtrate was evaporated using a micro centrifugal vacuum concentrator MV-100 (Tomy Medical, Japan) at room temperature. Prior to analysis, the dried sample was reconstituted in 500 μL of deionized water.

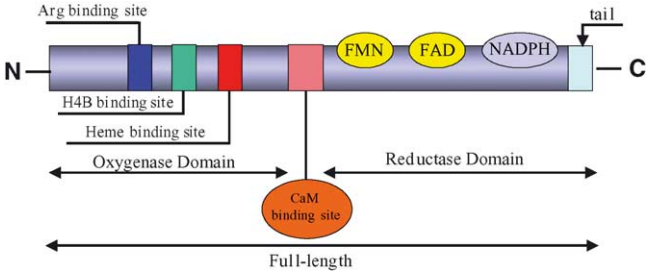

Fig. 2. Structure of nNOS full-length flavoenzyme.

2.5. Flavoenzyme sample preparation

The recombinant nNOS full-length flavoenzyme and its reductase domain (FAD/FMN) were harvested from *E. coli* BL21 cells, and then purified [37]. Fig. 2 depicts the structure of the nNOS full-length flavoenzyme, which is composed of an N-terminal oxygenase domain, a C-terminal reductase domain and a functional peptide calmodulin (CaM). The flavoenzyme solutions (0.5 mL) were heated in a water bath at 100 °C for 5 min, thereby the noncovalently binding FAD and FMN would be released from the protein. The solutions were placed in an ice bath for about 5 min to allow for protein precipitation, then centrifuged for 10 min at 15,000 rpm and the supernatants containing free FAD and FMN were collected and diluted with water by 10-fold for capillary LC analysis.

3. Results and discussion

3.1. Optimization of the capillary LC system

The laboratory-assembled capillary LC system was first optimized. In our capillary LC system, a 5 cm capillary was employed between the outlet of the column and the detection window and capillary flow cell was used. When a capillary with narrower inner diameter was used, although the band broadening originating from the detection cell and connecting capillaries was less, the short optical path length resulted in a substantial loss of concentration sensitivity. Six alkylbenzenes (methylbenzene, ethylbenzene, propylbenzene, butylbenzene, pentylbenzene and hexylbenzene) were used as samples to optimize the inner diameter of the connecting capillary between the column and the detector. Acetonitrile (80%, v/v) at a flow rate of 2 μL/min was used as the mobile phase and the volume of sample loop was 0.16 μL. When a capillary with 100 μm inner diameter was used between the column and the detector, the concentration sensitivity and the resolution were better. Hence, all further experiments were conducted with a capillary (100 μm i.d.) between the column and the detector.

The reproducibility of the capillary LC system in isocratic and gradient elution modes were investigated. In isocratic elution mode, when acetonitrile–water (80:20, v/v) at a flow

rate of 2 μL/min was used as the mobile phase, the relative standard deviations (R.S.D.s) of retention time, peak area and peak height of the six alkylbenzens were all less than 3% (n = 4). In gradient elution mode, when the gradient started at acetonitrile–water (60:40) and the acetonitrile concentration was raised linearly to (100:0) in 15 min and kept at 100% for 5 min at a flow rate of 2 μL/min, the R.S.D.s of retention time, peak area and peak height of the six alkylbenzens were all less than 5% (n = 4).

3.2. Gradient elution

In order to enhance the concentration sensitivity in capillary LC, hyphenation of sample preconcentration techniques is needed. Gradient elution is one of the most popular on-line concentration techniques in LC, which allows the injection of a large sample volume without considerable effect on band broadening. At first, the type of organic solvents, gradient program and the pH of the phosphate buffer were optimized. The three flavins contain acidic groups. As shown in Fig. 1, RF contains a weakly acidic lactam group on the isoalloxazine tricyclic ring (pK_a = 10.2) [38] and ribose polyol group. FMN and FAD contain additional phosphate groups. The pH of the mobile phase affected the dissociation of acidic groups, resulting in affecting the hydrophobicity of the flavins. When the pH of the phosphate buffer was in the range from 3.0 to 5.0, the three flavins were baseline separated. The elution followed the hydrophobicity order, FAD < FMN < RF. When the pH of the phosphate buffer was 7, FMN and FAD coeluted. Phosphate buffer (30 mM, pH 3.0) and methanol were used as mobile phases to optimize the gradient program. When the gradient started at 5% (v/v) of methanol, which was maintained isocratically for the first 1 min, thereafter the methanol concentration was raised linearly to 95% in 15 min and kept at 95% for 5 min, the three flavins obtained better separation.

Next, the injection volume was optimized. Fig. 3 shows the chromatograms of flavins at different injection volumes. The concentration sensitivity increased with the increasing of the injection volume. The resolution decreased slowly with the increasing of the injection volume. The resolutions of FAD and FMN, FMN and RF are 1.94, 2.35 (0.16 μL), 1.86, 2.31 (1 μL) and 1.73, 2.25 (5 μL), respectively. Hence, a 1 μL sample loop was used for investigation of the reproducibility, linearity and sensitivity for the determination of the three flavins, as shown in Table 1. For the three flavins, the reproducibility of the retention time, peak area and peak height and linearity of peak area response against concentration were found satisfactory and limits of detection (LODs) were in the low ppb levels.

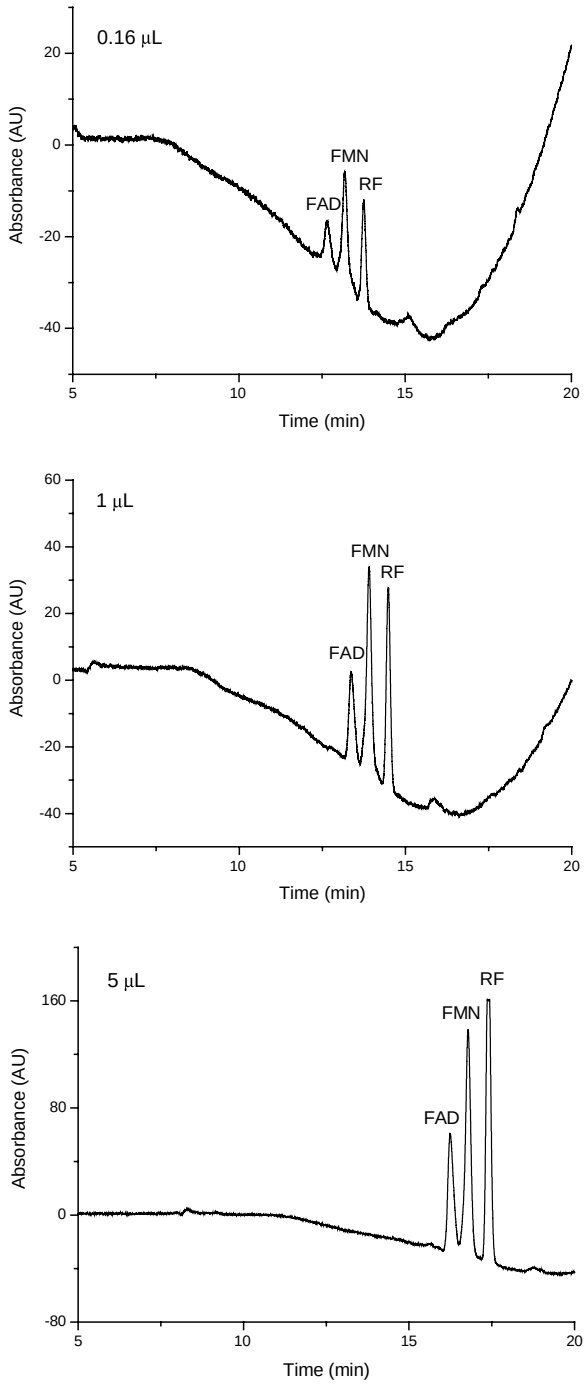

Fig. 3. Chromatograms of flavins at different injection volumes in gradient elution mode. *Conditions:* gradient elution was performed with methanol–30 mM phosphate buffer (pH 3.0) at a flow rate of 2 μL/min. The gradient started at 5% (v/v) of methanol, which was maintained isocratically for the first 1 min, thereafter the methanol concentration was raised linearly to 95% in 15 min and kept at 95% for 5 min. Detection wavelength was set at 265 nm. The concentration of each analyte was 1 μg/mL except for FMN (2 μg/mL).

3.3. In-tube SPIPME

A monolithic silica-ODS column (150 mm × 0.2 mm i.d.) was used as the in-tube SPME device. At first, the influence of the pH of the mobile phase methanol–30 mM phosphate (30/70, v/v) at a flow rate of 2 μL/min on the separation of the three flavins was investigated while keeping the injection volume at 0.16 μL. When the pH of the phosphate buffer was in the range from 3.0 to 5.0, the three flavins were baseline separated. The retention factors of the three flavins decreased

Table 1
Reproducibility, linearity and sensitivity for FMN, FAD and RF in gradient elution mode (the injection volume was 1 μL)

Name	R.S.D. (n = 3) (%)			Calibration line		LOD (S/N = 3)	
	Retention time	Peak area	Peak height	$y = ax + b$	r^2	ng/mL	nM
FAD	1.6	0.70	0.53	$y = 1.09 \times 10^4 x + 9.93 \times 10^2$	0.9995	28	34
FMN	1.5	2.5	2.0	$y = 8.57 \times 10^3 x + 2.59 \times 10^3$	0.9996	28	59
RF	2.9	1.1	0.70	$y = 2.08 \times 10^4 x + 1.36 \times 10^3$	0.9997	12	32

y: peak area; x: sample concentration (μg/mL); r: correlation coefficient.

Table 2
Sensitivity enhancement factor in terms of peak height (SEF_{height}), LOD and plate number

		FAD	FMN	RF
SEF_{height} [a]		113	142	123
Plate number ($\times 10^3$)	Conventional injection (0.16 μL)	5.92	4.65	4.16
	In-tube SPIPME (100 μL)	9.97	7.79	6.88
LOD (in-tube SPIPME)	ng/mL	2.1	1.4	0.38
	nM	2.5	2.9	1.0

[a] $SEF_{height} = \dfrac{\text{Peak height with in} - \text{tube SPIPME}}{\text{Peak height with conventional injection}} \text{Dilution factor.}$

with the increase in the pH of the phosphate buffer. The retention factors of FAD, FMN and RF were 0.28, 0.47 and 0.77, respectively, when methanol–30 mM phosphate (pH 3.0) (30/70, v/v) was used as the mobile phase. The phosphate buffer (pH 3.0) was used for further experiments. When the extraction monolithic column was conditioned by methanol, then water and 10 μL of flavin standards solutions dissolved in water were injected, the concentration efficiencies of FAD and FMN were very poor. This is perhaps due to the ionization of FAD and FMN in water, resulting in their weak retention onto the extraction column, since the pK_a values of FMN and FAD are 1.3 and 6.5 from their phosphates groups [14]. This point can be attested by the smaller retention factors of FAD and FMN. But it is possible to enhance the retention factors of FAD and FMN by their interactions with an ion-pair reagent. Therefore, the use of an ion-pair reagent was investigated.

TBAP was tested as an ion-pair reagent. Firstly, the concentration of TBAP in the mobile phase was optimized. Addition of TBAP in the concentration range from 3.0 to 6.0 mM in methanol–30 mM phosphate buffer (pH 3.0) (30/70, v/v) provided the desired increase in the retention factors of FAD and FMN due to their attractions by the positive charge on the column. These flavins are negatively charged at pH 3.0. The elution order (RF, FMN, FAD) was reversed in comparison with that using a mobile phase devoid of TBAP. When 4.5 mM TBAP was added to methanol–30 mM phosphate buffer (pH 3.0) (30/70, v/v), the retention factors of FAD, FMN and RF were 2.2, 1.5 and 0.68, respectively. It should be noted that the retention factor of RF decreased when TBAP was added to the mobile phase, compared with that when the mobile phase devoid of TBAP was used. This is due to the decrease of the hydrophobic adsorption of RF onto the monolithic silica-ODS column. When TBAP was added to the mobile phase, the ion-pair reagent was equilibrated between the stationary and mobile phases, resulting in reducing the retention of RF molecule, which was neutral at pH 3.0 according to its pK_a value (10.2) [38]. TBAP (4.5 mM) was used for subsequent experiments.

Next, the pH of the mobile phase and the concentration of methanol were optimized since varying pH and the concentration of methanol allow a considerable control over both retention and resolution. When the pH of the mobile phase was in the range from 3.0 to 7.0, the three flavins were baseline separated. The pH 3.0 was used for further investigation. The mobile phase with the concentration of methanol ranging from 30 to 35% provided better retention and resolution for the three flavins. When the concentration of methanol was 25%, the separation took about 40 min, which was about three fold longer than that using a mobile phase with 30% methanol. When the concentration of methanol was 40%, FAD and FMN were partially separated since the adsorption of TBAP to the stationary phase was reduced, leading to the reduction in the retention of FAD and FMN. Based on the results, a mobile phase with 30% methanol was used for subsequent experiments.

Table 3
Reproducibility and linearity for FAD, FMN and RF when hyphenating in-tube SPIPME with capillary LC (the injection volume was 10 μL)

Name	R.S.D. (n = 3) (%)			Calibration line	
	Retention time	Peak area	Peak height	$y = ax + b$	r^2
FAD	1.6	1.2	2.0	$y = 4.75 \times 10^4 x - 6.01 \times 10^3$	0.9999
FMN	0.90	2.2	3.0	$y = 4.40 \times 10^4 x + 6.01 \times 10^3$	0.9998
RF	0.69	0.86	1.1	$y = 1.06 \times 10^5 x + 1.17 \times 10^4$	0.9996

y: peak area; x: sample concentration (μg/mL); r: correlation coefficient.

The capillary LC system was hyphenated with in-tube SPIPME. TBAP (4.5 mM) in water was substituted for water as the solution for pre-conditioning the extraction monolithic column. When methanol–30 mM phosphate (pH 3.0) (30/70, v/v) devoid of TBAP was used as the mobile phase and 10 μL of flavins solutions were injected, the three flavins were concentrated well, as shown in Fig. 4A. But their separation became poor. After the extraction column was pre-conditioned with 4.5 mM TBAP, the elution order of the three flavins was reversed due to the effect of TBAP in the extraction column. Based on the results, 4.5 mM TBAP in methanol–30 mM phosphate (pH 3.0) (30/70, v/v) was used as the mobile phase for further investigation of the effect of injection volume. The peak height had linear relationship with the injection volume in the range from 5 to 100 μL. Greater than a 110-fold enhancement in the concentration sensitivity for flavins relative to the conventional injection method was achieved, as shown in Table 2. The LODs for FAD, FMN and RF (S/N = 3) were in the low ppb levels. Moreover, the separation efficiency was improved compared with the conventional injection as reflected by plate number (Table 2). Panels B and C of Fig. 4 depict the chromatograms of flavins comparing in-tube SPIPME and conventional injection. With a conventional injection at a volume of 1 μL, the peaks broadened seriously due to excess injection volume, as shown in Fig. 4B. When in-tube SPIPME was used at an injection volume of 100 μL, the flavins exhibited excellent separation and peak shape compared with 1 μL conventional injection, as shown in Fig. 4C.

An injection volume of 10 μL was used to investigate the reproducibility and linearity for the determination of three flavins by hyphenating in-tube SPIPME with the capillary LC system. Table 3 presents the reproducibility data obtained for the retention time, peak area and peak height, the calibration lines (peak area versus concentration in μg/mL) and the linearity of the calibration lines.

3.4. Sample analysis

To test the validity of the two developed capillary-LC methods, applications to evaluate flavins concentration in the *E. coli* cell extracts and characterize the recombinant nNOS full-length flavoenzyme and its reductase domain (FAD/FMN) were carried out under the optimum conditions. Peaks of FAD and FMN monitored in the samples were identified by their retention times in comparison with external standards and by standard addition method.

Panels A and B of Fig. 5 present chromatograms of *E. coli* cell extracts analyzed by the gradient elution mode and in-tube SPIPME capillary LC, respectively. The concentrations of FAD and FMN in the *E. coli* cell extracts were found to be 0.72 ± 0.04 μg/mL (0.87 ± 0.05 μM), 0.68 ± 0.06 μg/mL (1.41 ± 0.13 μM), respectively, by triplicate analysis of the cell extracts using the gradient elution mode. RF was not detected in the *E. coli* cell extracts. Similar experiments were performed to evaluate the flavins content in the cell extracts

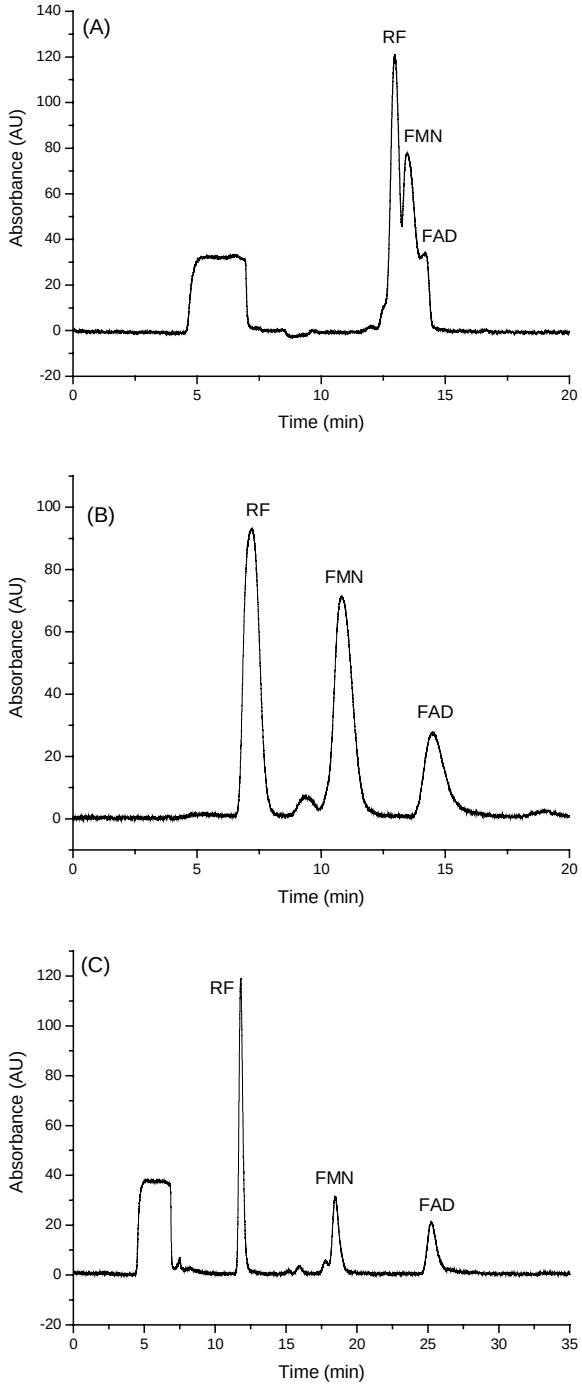

Fig. 4. Chromatograms of flavins comparing in-tube SPIPME and conventional injection. *Conditions:* flow rate: 2 μL/min, wavelength: 265 nm. (A) The injection volume was 10 μL with in-tube SPIPME. The mobile phase was methanol–30 mM phosphate buffer (pH 3.0) (30/70, v/v). The concentration of each analyte was 1 μg/mL except for FMN (2 μg/mL). (B) The injection volume was 1 μL with conventional injection. The mobile phase was 4.5 mM TBAP in methanol–30 mM phosphate buffer (pH 3.0) (30/70, v/v). The concentration of each analyte was 10 μg/mL except for FMN (20 μg/mL). (C) The injection volume was 100 μL with in-tube SPIPME. The concentration of each analyte was 0.1 μg/mL. Other conditions as in (B).

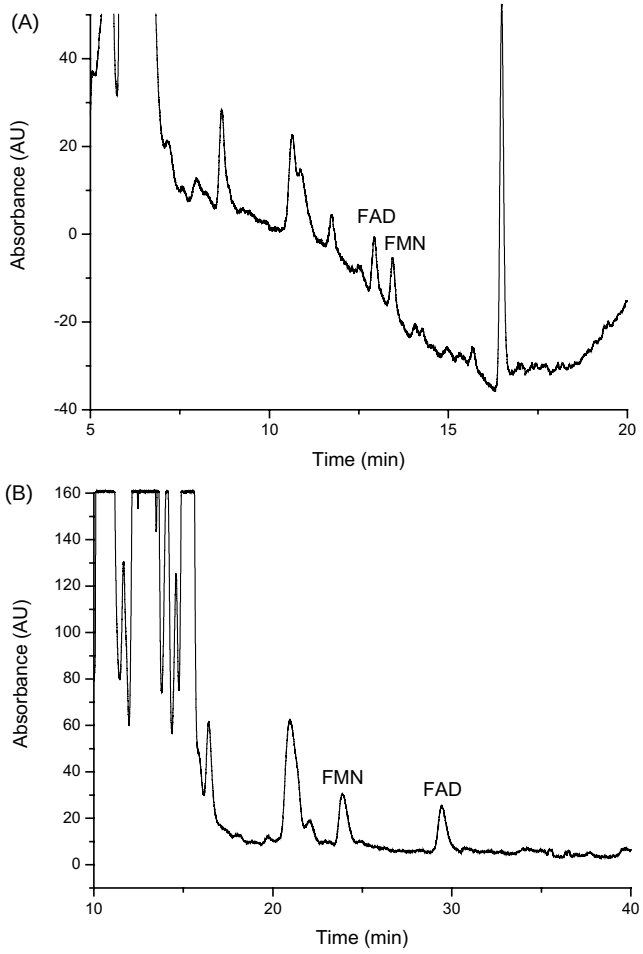

Fig. 5. Chromatograms of the *E. coli* cell extracts analyzed by the capillary LC system. (A) Gradient elution mode. The injection volume was 1 μL. Other conditions are the same as in Fig. 3. (B) In-tube SPIPME. The separation column was monolithic silica-ODS column (500 mm × 0.2 mm i.d.). The injection volume was 10 μL. Other conditions as in Fig. 4C.

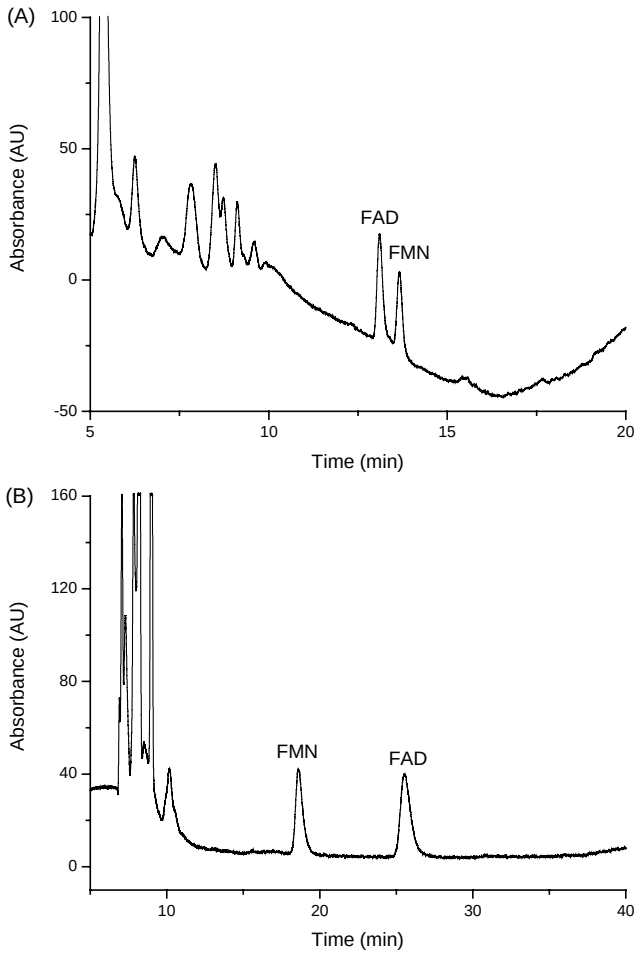

Fig. 6. Chromatograms of the recombinant nNOS full-length flavoenzyme analyzed by the capillary LC system. (A) Gradient elution mode. The injection volume was 1 μL. Other conditions as in Fig. 3. (B) In-tube SPIPME. The injection volume was 10 μL. Other conditions as in Fig. 4C.

by in-tube SPIPME. FAD was separated baseline from other substances in the cell extracts. Unfortunately, FMN was not separated completely from other substances contained in the cell extracts, resulting in the difficulty in the accurate quantitation of the content of FMN. To evaluate FMN content in the cell extracts accurately, a longer monolithic silica-ODS column (500 mm × 0.2 mm i.d.) was employed to analyze the cell extracts. As shown in Fig. 5B, FMN was separated baseline from other substances in the cell extracts. The contents of FAD and FMN in the cell extracts were 0.73 ± 0.05 μg/mL (0.89 ± 0.07 μM), 0.70 ± 0.07 μg/mL (1.46 ± 0.10 μM), respectively, which are in good agreement with the results obtained by the gradient elution mode. Higher concentration of FMN relative to FAD were measured for the *E. coli* cell extracts.

Panels A and B of Fig. 6 depict chromatograms of the recombinant nNOS full-length flavoenzyme analyzed by the gradient elution mode and in-tube SPIPME capillary LC, respectively. The chromatograms of the recombinant nNOS reductase domain (FAD/FMN) are similar to those of the recombinant nNOS full-length flavoenzyme. Triplicate measurements of flavin concentrations in the recombinant nNOS full-length flavoenzyme and its reductase domain (FAD/FMN) separately by the gradient elution mode resulted in FAD/FMN ratios of 0.90 and 0.96 in the recombinant nNOS full-length flavoenzyme and its reductase domain (FAD/FMN), respectively, which are in good agreement with theoretical expectation of 1/1 molar ratio of FAD/FMN for the flavoenzymes. Similar experiments were performed to characterize the proteins by in-tube SPIPME, revealing FAD/FMN ratios of 0.90 and 0.98 in the recombinant nNOS full-length flavoenzyme and its reductase domain (FAD/FMN), respectively, which were in agreement with the results obtained by the gradient elution mode.

4. Conclusions

Two capillary LC methods were developed for analysis of cellular flavins, where a monolithic silica-ODS column

was used as the separation column, which possesses the advantages of high permeability and reduction of the consumption of mobile phase. Two on-line concentration techniques, namely, gradient elution mode and in tube SPIPME, proved to be useful for enhancing the concentration sensitivity, giving LODs at ppb level for the three flavins. In gradient elution mode, the resolution still decreased with the increase of the injection volume slowly although it allowed the injection of a large sample volume without considerable adverse effect on band broadening. When the capillary LC system was hyphenated with in-tube SPIPME, the technique not only enhanced the concentration sensitivity of flavins, but also improved the separation efficiency. The successful application of the two methods in analyzing the flavin contents in the *E. coli* cell extracts and the recombinant nNOS full-length flavoenzyme and its reductase domain (FAD/FMN) demonstrated the potential of capillary LC in metabolome analyses. The new capillary LC-based strategies for analysis of other low-concentrations metabolites in a cell will be explored.

Acknowledgements

L.J. is grateful to the Japan Society for the Promotion of Science (JSPS) for supporting her postdoctoral studies. The author would like to acknowledge GL Sciences for donating the monolithic capillary column. Appreciation is offered to Ms. J. Fu and Professor Iyanagi in the Department of Life Science for donating the *E. coli* cells and the recombinant flavoenzyme samples. Special thanks are given to Dr. H. Hisamoto and Dr. N. Matsubara for their helpful in-laboratory assistance. This research was supported in part by a JSPS Fellowship (no. 02366) and Grant-in-Aid for Scientific Research (no. 15350049) from the Ministry of Education, Culture, Sports Science and Technology, Japan.

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