Biosci. Biotechnol. Biochem., **71** (2), 435–441, 2007

Highly Sensitive Quantitative Analysis of Nicotianamine Using LC/ESI-TOF-MS with an Internal Standard

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Received September 12, 2006; Accepted October 17, 2006; Online Publication, February 7, 2007
[doi:10.1271/bbb.60496]

A highly sensitive quantitative method for analyzing nicotianamine (NA) by liquid chromatography/electrospray ionization time-of-flight mass spectrometry (LC/ESI-TOF-MS) is reported. Fluorenylmethoxycarbonylation of nicotianamine reduced its polarity and enabled its retention in a reversed-phase column. The adoption of N^{ε}-nicotyllysine (NL) as an internal standard ensured reliable quantification by giving a linear calibration curve drawn between the NA/NL molar ratios of standard solutions injected and the NA/NL area ratios in mass chromatograms. The high sensitivity of this analytical method allowed us to measure the amount of NA. This analytical method has applications to all research concerning NA.

Key words: 9-fluorenylmethoxycarbonyl (FMOC) derivatization; liquid chromatography/electrospray ionization time-of-flight mass spectrometry (LC/ESI-TOF-MS); nicotianamine

Nicotianamine (NA; Fig. 1A) was originally found in tobacco leaves,[1)] and has been detected in all higher plants that have been examined.[2,3)] Nicotianamine chelates many kinds of metal cations and is thought to play an important role in the internal transport of metals nutrients including iron, zinc, and copper.[4,5)] In graminaceous plants, NA is also an essential intermediate of mugineic acid family phytosiderophores (MAs), which are natural Fe chelators secreted by the roots to solubilize iron in the soil.[6–8)] Nicotianamine has recently received attention as an antihypertensive substrate.[9–12)] Nicotianamine is synthesized by the trimerization of *S*-adenosylmethionine by NA synthase (NAS).[13)] In the studies on the biosynthetic pathways of MAs, we have isolated many *NAS* genes from barley, *Arabidopsis*, rice, and maize.[14–17)]

The quantification of endogenous NA in plants is critical for elucidating the detailed function of NA in plants. In terms of its antihypertensive effect, quantifying NA in various foods is very important for determining those that are abundant in NA and that are expected to have an antihypertensive effect. Nicotianamine is usually quantified by measuring the UV absorption of its *o*-phthalaldehyde derivative that is obtained by post-labeling after high-performance liquid chromatography (HPLC),[18–20)] and its detection limit is *ca.* 10 pmol (3.0 ng) for identification and 100 pmol (30.3 ng) for quantification. However, the concentration of NA in plants examined so far is so low that the measurements are always hampered by the need for a relatively large plant sample (*e.g.*, the endogenous NA concentration in the leaves of wild-type tobacco (*Nicotiana tabacum* L. cv SR1) is $8.2 \pm 0.6\,\mu g/g$ fresh weight).[21)] Consequently, the establishment of a high-sensitivity system to measure NA has long been desired. Weber *et al.* have reported the analysis of endogeneous NA in *Arabidopsis* using liquid chromatography/electrospray ionization time-of-flight mass spectrometry (LC/ESI-TOF-MS),[22)] but their system was for a qualitative and not quantitative analysis of NA.

In this study, we developed a highly sensitive quantitative method for NA analysis using LC/ESI-TOF-MS, and demonstrated the quantification of endogenous NA in rice seed and young tobacco leaves. The adoption of an internal standard validated this quatification.

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Abbreviations: FMOC-Cl, 9-fluorenylmethoxycarbonyl chloride; HPLC, high-performance liquid chromatography; LC/ESI-TOF-MS, liquid chromatography/electrospray ionization time-of-flight mass spectrometry; MAs, mugineic acid family phytosiderophores; NA, nicotianamine; di-FMOC NA, *di*-9-fluorenylmethoxylcarbonylated NA; NL, N^{ε}-nicotyllysine; di-FMOC NL, *di*-9-fluorenylmethylcarbonylated NL

Materials and Methods

Synthesis of the internal standard. Nicotic acid (492 mg, 4 mmol) was dissolved in 10 ml of dry C_5H_5N–CH_3CN mixture (1:5). *N*-hydroxysuccinimide (460 mg, 4 mmol) dissolved in CH_3CN (5 ml) was added to the nicotinic acid solution and thoroughly mixed. *N*,*N*′-dicyclohexylcarbodiimide (824 mg, 4 mmol) dissolved in CH_3CN (5 ml) was added to this mixture, which was kept at ambient temperature overnight and then centrifuged to remove the precipitate of *N*,*N*′-dicyclohexylurea. The supernatant was concentrated and purified by Sephadex LH-20 chromatograpy (1.5 cm i.d. × 30 cm), swelled and eluted with 1-BuOH:deionized water (6:100) in 10 ml fractions. The fractions showing a single spot on TLC (Merck GF_{254}) in EtOAc:$CHCl_3$:AcOH (20:8:1) were combined and concentrated to give crystals of an *N*-hydroxysuccinimidyl active ester (495 mg, 53% yield). The active ester of nicotic acid (93 mg, 0.42 mmol) was dissolved in DMF (1 ml) and added to a mixture of D,L-lysine-HCl (182 mg, 1 mmol) and *p*-*N*,*N*′-dimethylaminopyridine (122 mg, 1 mmol) in deionized water (5 ml). After an overnight reaction at ambient temperature, the reaction mixture was concentrated *in vacuo* and purified by Sephadex LH-20 chromatography as already described. The fractions showing a spot at R_f 0.05 on TLC in the upper phase of 1-BuOH:AcOH:deionized water (4:1:5) were collected and concentrated *in vacuo* to give a gum (*ca.* 220 mg). An aliquot (4.5 mg) of this concentrate was subjected to PEGASIL ODS-HPLC (10 mm i.d. × 30 cm, Senshu Scientific Co., Tokyo, Japan), eluted with 10% MeOH containing 0.05% AcOH at 2.0 ml/min. A peak at tR 6.5 min was collected and gave *ca.* 1.4 mg of N^{ε}-nicotyllysine (NL). HR-MS (LC/ESI-TOF-MS): obs. *m*/*z* 252.13746 (252.13482 calcd. for $[M + H]^+$), *m*/*z* 274.11678 (274.11676 calcd. for $[M + Na]^+$). ^{1}H-NMR [δ-value, D_2O contaminated with C_5H_5N]: 1.36 (5-CH_2, tt, $J = 7$, 7 Hz), 1.58 (4-CH_2, m), 1.73 and 1.83 (1H and 1H, 3-CH_2, m), 2.86 (6-CH_2, t, $J = 7$ Hz), 4.3 (2-CH, q, $J = 2.5$, 4 Hz), 7.7 (5′-H, q, $J = 6.5$, 10 Hz), 8.4 (4′-H, dt, $J = 1.7$, 8.3 Hz), 8.62 (6′-H, overlapped with C_5H_5N), 8.9 (2′-H, br.s). A JMS-T100LC AccuTOF (Jeol, Tokyo, Japan) was used for the LC/ESI-TOF-MS analysis, and a JNM-500 (Jeol, Tokyo, Japan) for the ^{1}H-NMR analysis.

Liquid chromatography-electrospray ionization time-of-flight mass spectrometry. A JSM-T100LC AccuTOF (Jeol) was used a Synergi Hydro RP column (4 µm, 80A, 150 × 2.0 mm; Phenomenex, CA, USA) in the positive ionization mode and in the mass range of *m*/*z* 200–800. The column was equilibrated with 100% eluent A (0.5% formic acid, 20% water, and 79.5% CH_3CN) for 25 min, and developed in the following conditions: 0–5 min, 100% eluent A; 5–15 min, gradient elution with increasing the concentration of eluent B (0.5% formic acid and 99.5% CH_3CN) from 0 to 100% in eluent A; and 15–35 min, 100% eluent B. The flow rate was kept at 0.2 ml/min.

Derivatization with FMOC-Cl. Each aqueous substrate solution (2.5 µl) was finely mixed with 2.5 µl of 50 mM EDTA•2Na and 5 µl of sodium borate (1 M, pH 8.0). Ten µl of 25 mM 9-fluorenyl methoxycarboxyl chloride (FMOC-Cl; dissolved in CH_3CN) was added with immediate vortexing. The sample was allowed to react at 30 °C for 60 min. Ten µl of 50 mM 3-amino-1-propanol was added to the sample with vortexing to consume excess FMOC-Cl. An aliquot (5 µl) of each reaction mixture (30 µl) was injected for the LC/ESI-TOF-MS analysis.

Reproducibility of the measurement by adopting an internal standard. A solution (25 µl) of authentic NA (1 nmol) and NL (1 nmol) was prepared. An aliquot (2.5 µl) of the solution was FMOC derivatized and subjected to the LC/ESI-TOF-MS analysis (triplicate). The peak area of the mass chromatograms for the $[M + H]^+$ ions of di-FMOC NA, that for the $[M + H]^+$ ions of di-FMOC NL, and their peak area ratio (NA/NL) were calculated.

Calibration curve with an internal standard. Standard solutions (25 µl) containing 0.25 nmol, 0.5 nmol, 1.0 nmol, 2 nmol, and 4 nmol of NA and 1 nmol of NL were prepared. An aliquot (2.5 µl) of each solution was FMOC derivatized and subjected to the LC/ESI-TOF-MS analysis (triplicate). The peak area ratios of the mass chromatograms for the $[M + H]^+$ ions of di-FMOC NA and di-FMOC NL were adopted for the calibration curve.

Validating the quantification. Solutions (25 µl) of authentic NA (1 nmol) and NL (1 nmol), endogenous NA in rice seed and NL (1 nmol), and endogenous NA supplemented with authentic NA (1 nmol) and NL (1 nmol) were prepared. An aliquot (2.5 µl) of each solution was FMOC derivatized and subjected to the LC/ESI-TOF-MS analysis (triplicate). The peak area ratios were determined as described in *Calibration curve with an internal standard*.

Quantification of NA in a rice grain. One grain of dry, mature, unpolished rice seed (*Oryza sativa* L. cv Tsukinohikari; about 20 mg) was finely homogenized with a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan), weighed, and suspended in 400 µl of deionized water. The sample was then centrifuged at 15000 × *g* for 10 min and the supernatant was collected. This extraction was conducted three times, and all the supernatants were collected in one tube. After adding 1 nmol of NL (1 µl of a 1 mM solution) to the supernatant, the sample was concentrated to approximately 300 µl in Micro Vac (Tomy Seiko, Tokyo), passed through a 10000 NMWL Filter Unit (Millipore, MS, USA), further concentrated

to dryness. The dried sample was dissolved in 25 µl of deionized water. Three concentrated extracts were prepared from three independent grains. An aliquot (2.5 µl) of each concentrated extract was FMOC derivatized and subjected to LC/ESI-TOF-MS analysis. The quantification was carried out once for each grain sample.

Comparison of the NA content of a plant sample determined by LC/ESI-TOF-MS and by HPLC. Approximately 1 g of dry, mature, unpolished rice seed (approximately 50 grains) was finely homogenized with a Multi-Beads Shocker (Yasui Kikai). Three lots of 20 mg of the homogenate (approximately the same weight as one rice grain) were prepared and used for the LC/ESI-TOF-MS analysis. The extract from each lot was FMOC derivatized and analyzed once. The remaining homogenate was used for the HPLC analysis as described previously.[18–20] One extract was prepared from all the remaining homogenate, and the measurement was executed three times.

One g of fresh young tobacco leaves (*Nicotiana tabacum* L. cv SR) was finely homogenized with a mortar and a pestle. The homogenate was suspended in 20 ml of deionized water. The sample was then centrifuged at 15000 × *g* for 10 min and the supernatant was collected. This extraction was conducted three times, and all the supernatants were collected in one tube. Three lots of an aliquot (120 µl; corresponding to 2 mg of the homogenate) of the supernatant were, after adding 1 nmol of NL, was concentrated to approximately 300 µl in Micro Vac (Tomy Seiko), passed through 10000 NMWL Filter Unit (Millipore), further concentrated to dryness. Each dried sample was dissolved in 25 µl of deionized water (1/10 the concentration in case of the rice seed). The three concentrated extracts were subjected to the LC/ESI-TOF-MS analysis, and the remaining extract was used for the HPLC analysis as just described.

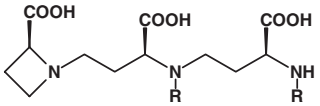
Results

General aspects

Since NA (Fig. 1A) is not retained in a reversed-phase column, NA was FMOC derivatized to form di-FMOC derivative (di-FMOC NA), which was less polar and could be retained in the column. The di-FMOC NA was eluted at tR 16.9 min under the HPLC conditions described in the Materials and Methods, and showed the ions of m/z 748 ($[M + H]^+$) and 770 ($[M + Na]^+$) at the ratio of 100:5. The LC/ESI-TOF-MS analysis developed in this study enabled us to quantify as little as 1 pmol (0.3 ng) of NA, while HPLC required 100 pmol (30.0 ng) to clearly detect and quantify NA as its o-phthalaldehyde derivative even though it could detect 10 pmol (3.0 ng) of the NA derivative.[18–20]

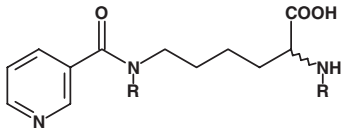
It is preferable to use an internal standard whose chemical property is similar to that of NA, and NA labeled with a stable isotope could be an ideal choice.

A. Nicotianamine and its FMOC derivative



R = H: Nicotianamine (NA), R = FMOC: di-FMOC NA

B. N^{ε}-nicotyl-D,L-lysine and its FMOC derivative



R = H: N^{ε}-nicotyl-D,L-lysine (NL), R = FMOC: di-FMOC NL

Fig. 1. The Chemical Structures of Nicotianamine (NA), N^{ε}-Nicotyl-D,L-lysine (NL), and Their FMOC Derivatives.

However, the preparation of the stable isotope-labeled NA is too laborious, so we prepared N^{ε}-nicotyllisine (NL, Fig. 1B). N^{ε}-nicotyllysine was expected to behave similarly to NA on HPLC when they were FMOC derivatized, because both of their FMOC derivatives still carry a basic amino group and carboxyl group(s). Furthermore, the difference between their molecular weights (MW) is in a reasonable range (MW 251 for NL and MW 303 for NA). Followed by FMOC derivatization, NL was detected at tR 17.9 min as a di-FMOC derivative showing the ion of m/z 696 ($[M + H]^+$).

In this study, we selected the $[M + H]^+$ ions of di-FMOC NA (m/z 748) and the $[M + H]^+$ ions of di-FMOC NL (m/z 696) for the quantification of NA. The mass chromatograms of the ions at m/z 748 and 696 were shown in Fig. 2A. A sufficient supply of FMOC-Cl for derivatization was confirmed by detecting the FMOC derivative of 3-amino-1-propanol, a quencher, as the ion of m/z 298 ($[M + H]^+$) at tR 15.9 min.

Reproducibility of the measurement by adopting an internal standard

We first examined the effect of the internal standard on the reliability of the measurement. A solution of authentic NA and NL were FMOC derivatized and subjected to the LC/ESI-TOF-MS analysis. The peak areas of mass chromatograms for the $[M + H]^+$ ions of di-FMOC NA varied somewhat in each measurement as often occurs in LC/ESI-MS. A similar problem happened to the peak areas of mass chromatograms for the $[M + H]^+$ ions of di-FMOC NL (Table 1). However, the ratios of peak areas of the $[M + H]^+$ ions of NA and NL (NA/NL ratios) were similar in each measurement and reproducible (Table 1). We therefore adopted the NA/NL ratios to evaluate the quantification of NA.

Calibration curve with an internal standard

We next investigated the relationship between the molar ratios (NA/NL) and the peak area ratios (NA/

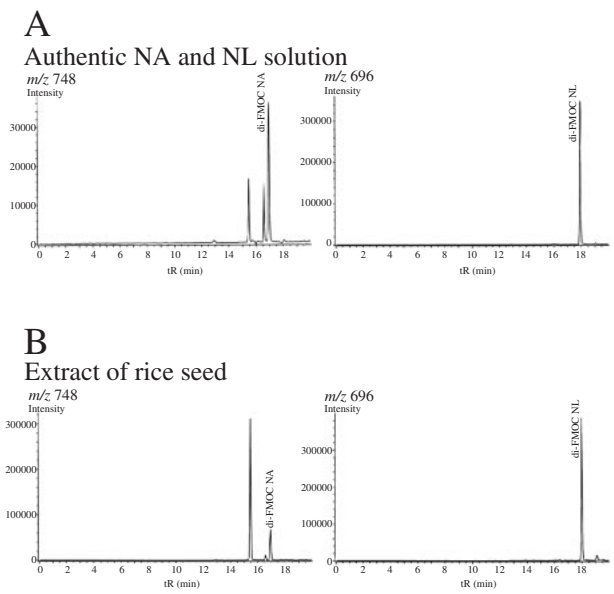


Fig. 2. Mass Chromatograms of Ions at m/z 748 and 696.

A, The peak of the $[M + H]^+$ ion of di-FMOC NA (m/z 748) was detected at 16.9 min, and that of di-FMOC NL (m/z 696) was detected at 17.9 min. The other peaks apparent in both the mass chromatograms were irrelevant to di-FMOC NA and di-FMOC NL. B, The peak of the $[M + H]^+$ ion of di-FMOC NA (m/z 748) was detected at 16.9 min and di-FMOC NL (m/z 696) at 17.9 min.

NL). Five standard solutions (molar ratios of 0.25, 0.5, 1, 2, and 4) were prepared. An aliquot (5 µl) of each solution was FMOC derivatized and subjected to the LC/ESI-TOF-MS analysis. Linearity was apparent between the molar ratios and the peak area ratios (Fig. 3), and the formula for the regression line was $y = 0.0977x + 0.0125$ (x is the molar ratio and y is the peak area ratio; $R^2 = 0.99$). We therefore adopted the regression line for quantification of NA.

Validating the quantification

Generally, EDTA is not used for FMOC derivatization.[23,24] However, when a concentrated extract of rice seed was subjected to the derivatizing reaction without EDTA, the derivatization efficiency of NA in the extract was much lower than that of authentic NA in deionized water. Since NL in the concentrated extract was derivatized almost quantitatively, it was thought that this inefficient derivatization occured specifically to NA. We circumvent the problem by adding sufficient amounts of EDTA to FMOC deivatizing solution (NA/NL ratio without EDTA, 0.06 (n = 3); with EDTA, 0.19 (n = 3)).

In order to validate the quantification, solutions (25 µl) of authentic NA (1 nmol) and NL (1 nmol), of endogenous NA in rice seed and NL (1 nmol), and of endogenous NA supplemented with authentic NA (1 nmol) and NL (1 nmol) were prepared. The solution of endogenous NA in rice seed was adjusted to be a similar concentration as that of the authentic NA solution based on the result of a preliminary experiment. An aliquot was FMOC derivatized and subjected to the LC/ESI-TOF-MS analysis. The increase in peak area ratio derived from the addition of authentic NA approximated the peak area ratio calculated when equimolar authentic NA was derivatized and analyzed (Fig. 4). This indicated that the derivatizing efficiency of NA in the extracts of rice seed was similar to that in deionized water. We concluded that the regression line depicted in Fig. 3 could be applied to quantify NA in a plant extract.

Table 1. Drift of Peak Areas and Peak Area Ratios

Solution	Di-FMOC NA peak area (intensity·sec)	Di-FMOC NL peak area (intensity·sec)	NA/NL ratio
1	444254	2978483	0.149
2	306893	1991742	0.154
3	378588	2571624	0.147

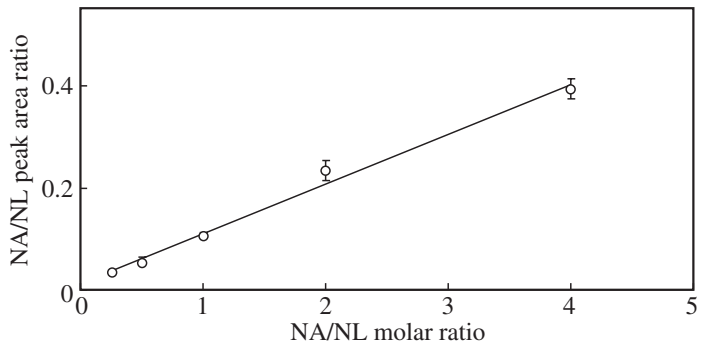


Fig. 3. Calibration Curve with an Internal Standard.

The formula representing the regression line was $y = 0.977x + 0.0125$; (x is the molar ratio, y is the peak area ratio; $R^2 = 0.99$).

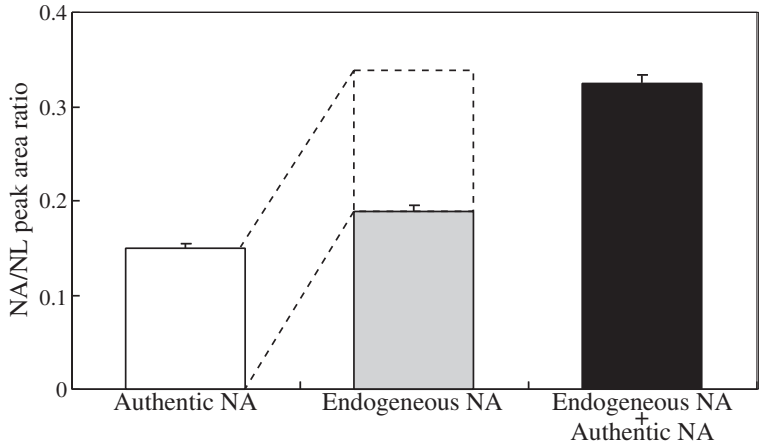


Fig. 4. Validating the Quantification.
Authentic NA and NL, endogenous NA in rice grains and NL, and endogenous NA supplemented with authentic NA and NL were prepared, and aliquots were subjected to the LC/ESI-TOF-MS analysis.

Quantification of NA in a rice grain

Using the regression line in Fig. 3, we measured NA in small plant using our analytical method. Concentrated extracts from one rice grain were FMOC derivatized and subjected to the LC/ESI-TOF-MS analysis. The mass chromatograms of ions at m/z 748 and 696 were shown in Fig. 2B. The NA content in one rice grain was calculated to be $21.2 \pm 0.9\,\mu g/g$ dry weight.

Comparison of the NA contents of a plant sample determined by LC/ESI-TOF-MS and by HPLC

Using the same plant sample, the NA content calculated by the LC/ESI-TOF-MS analysis was compared with the result from the HPLC analysis.[18–20] Since large amounts of a plant sample would be needed for the HPLC analysis because of its lower sensitivity, approximately 1 g of dry, mature, unpolished rice seed (approximately 50 grains) was homogenized finely. This homogenate was used for both the LC/ESI-TOF-MS analysis and the HPLC analysis. The NA content in rice seed was calculated to be $20.7 \pm 2.1\,\mu g/g$ dry weight in the LC/ESI-TOF-MS analysis and $15.3 \pm 0.3\,\mu g/g$ dry weight in the HPLC analysis. The value of the calculated NA content was slightly higher in the LC/ESI-TOF-MS analysis than in the HPLC analysis.

Using another plant sample, we also executed a comparison of the NA contents determined by the LC/ESI-TOF-MS analysis and by the HPLC analysis. One g of fresh young tobacco leaves was homogenized finely. This homogenate was used for both the LC/ESI-TOF-MS analysis and the HPLC analysis. The LC/ESI-TOF-MS analysis gave the mass chromatograms of ions at m/z 748 and 696 similar to those of the rice grain extracts (data not shown). The NA content in tobacco leaves was calculated to be $48.4 \pm 4.8\,\mu g/g$ fresh weight in the LC/ESI-TOF-MS analysis and $41.9 \pm 0.4\,\mu g/g$ fresh weight in the HPLC analysis. As in the case of the rice seed, the value of the NA content in the tobacco leaves calculated in the LC/ESI-TOF-MS analysis was slightly higher than that in the HPLC analysis.

Discussion

We have described a new analytical method for quantifying NA using LC/ESI-TOF-MS. Nicotianamine was stably retained on a reversed phase column when derivatized with FMOC-Cl. Much higher sensitivity was achieved for detecting NA in this analytical method than in the HPLC analysis.[21] This analytical method was capable of quantifying NA (Fig. 3 and 4), and we could determine sensitively the content of NA in a minute plant sample (*e.g.*, one grain of unpolished rice seed; $21.2 \pm 0.9\,\mu g/g$ dry weight). The values of NA content calculated in the LC/ESI-TOF-MS analysis and the HPLC analysis were almost the same.

As mentioned in the Results, for resolving the inefficient derivatization relevant specifically to NA, we added EDTA to the FMOC deivatizing solution. Our hypothesis was as follows: NA is a strong chelator of many kinds of transition metal cations in the neutral and alkaline pH range, at which FMOC derivatization is generally performed (pH 8.0 in this study). Consequently, NA would chelate metal cations derived from the plant sample at pH 8.0 in the reaction mixture of the concentrated extract, and that the formation of NA-metal complex should make itself inert to the derivatization. The restoration of reaction efficiency by adding EDTA suggested that NA chelated the metal cations in the extracts and the formation of the NA-metal complex hampered derivatizing NA. Although one study quantified NA using FMOC derivatization[22] and another measured other natural metal chelator MAs (derivatives of NA) through derivatization with FMOC-Cl,[25] neither study mentioned ways to improve the reaction efficiency. Therefore, the precise quantification of NA or MAs might not have been accomplished in those studies.

The NA content of each plant sample obtained by the LC/ESI-TOF-MS analysis differed slightly from that of the same sample by the HPLC analysis, the former method giving a slightly higher value. The adoption of the internal standard in the LC/ESI-TOF-MS analysis should reasonably compensate for the loss during the purification process, while such compensation did not work in the HPLC analysis without an internal standard. This could be one of the reasons why the LC/ESI-TOF-MS analysis gave a higher value than the HPLC analysis for the NA content of the same sample.

We should refer to the analysis of NA in tobacco leaves, for which we had to pay careful attention to the amount of FMOC-Cl used for drivatization. In the initial analysis of the tobacco leaves (20 mg), the amount of FMOC-Cl, which was sufficient for 20 mg of the rice seed, was insufficient, because the FMOC derivative of 3-amino-1-propanol was not detected and the derivatization efficiency of NA in the extract was much lower than those of authentic NA in deionized water. This insufficiency of FMOC-Cl would have been due to large amounts of consumers like other amines and amino acid, in tobacco leaves. When the extract was diluted to 10 times the volume and the diluted extract was used for the LC/ESI-TOF-MS analysis, the FMOC derivative of 3-amino-1-propanol was detected, and the derivatization efficiency of NA was restored. We concluded that a sufficient supply of FMOC-Cl was essential for the derivatization and smaller aliquot of the sample should be applied at the onset of analyzing a novel sample. Since this LC/ESI-TOF-MS analytical method allowed us to measure the content of NA in a plant sample very abundant in contaminats such as tobacco leaves, we concluded that this method could be applied to various plant samples.

For scientists studying the metal nutrition of graminaceous plants, the quantification of MAs is of major interest in addition to the quantification of NA. In this study, FMOC-derivatized deoxymugineic acid was detected in an extract of rice seed (data not shown). This suggested that our analytical method for NA might be applicable for the highly sensitive quantification of MAs. We plan to investigate whether this method can be used to quantify several kinds of MAs in detail.

High sensitivity is not the only advantage of our analytical method. While the HPLC analysis identifies NA based solely on its retention time, the LC/ESI-TOF-MS analysis uses both its ion peak and retention time, which identifies NA more reliably. In addition, identification using its ion peak reduces the necessity for the strict separation of NA from other substrates, which drastically reduces the analysis time. This analytical method could contribute greatly to clarifying the role of NA in higher plants, as well as to research efforts investigating NA as an antihypertensive substrate.

Acknowledgment

We thank Dr. Shinji Nagata, Department of Applied Biological Chemistry, The University of Tokyo, for his technical advices in the LS/ESI-TOF-MS analysis and measuring HR-MS.

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