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# Determination of Nicotianamine in Soy Sauce and Other Plant-Based Foods by LC-MS/MS

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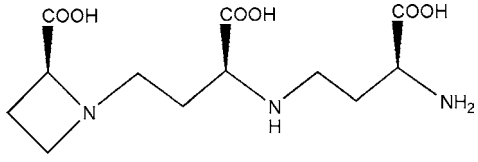
**ABSTRACT:** Nicotianamine is a nonproteinogenic amino acid, known to be an important metal chelator in plants. Recently, the antihypertensive effect of nicotianamine was discovered. In this study, a simple method to determine nicotianamine was developed using liquid chromatography−tandem mass spectrometry (LC-MS/MS) with a multimode ODS column. This method does not need derivatizing or ion-pairing reagents to retain nicotianamine, which is known for its poor retention on reversed-phase columns because of its high polarity. Moreover, this method showed a sufficient limit of detection (0.5 ng/mL), so it was found to be suitable for the analysis of nicotianamine in soy sauce and other foods, without cleanup. To subtract the matrix effect during LC-MS/MS analysis, a standard addition method was used. The levels of nicotianamine in soy sauce ranged from <0.25 to 71 μg/g. Nicotianamine was also determined in other foods, including soy milk, vegetable juice, fruit juice, and bottled tea.

**KEYWORDS:** *nicotianamine, soy sauce, liquid chromatography-tandem mass spectrometry, multimode ODS column, standard addition method*

## INTRODUCTION

The nonproteinogenic amino acid nicotianamine (Figure 1), (2*S*)-1-[(3*S*)-3-[[(3*S*)-3-amino-3-carboxypropyl]amino]-3-carboxypropyl]azetidine-2-carboxylic acid, was first identified in tobacco leaves,[1] and its structure was fully characterized.[2] Nicotianamine is widely found in higher plants,[3,4] and it was detected recently in filamentous fungi.[5] Nicotianamine chelates metal cations and is thought to be essential for the internal transport of metals in plants.[6] In graminaceous plants, nicotianamine is also known as a biosynthetic precursor of phytosiderophores of the mugineic acid family, which are secreted from the roots to solubilize iron in the soil.[6]




Nicotianamine

**Figure 1.** Chemical structure of nicotianamine.

Nicotianamine showed inhibitory activity against the angiotensin I-converting enzyme (ACE),[7] and this activity seemed not to depend solely on the effect of chelation.[8,9] Nicotianamine inhibited ACE preferentially, compared with other metal-containing enzymes,[8,9] and inhibited the contractions induced by angiotensin I in rat aorta.[10] Nicotianamine reduced blood pressure after a single oral administration in rats[7,8,11] and after a single intragastric administration in mice.[12] Nicotianamine also reduced blood pressure after long-term oral administration in rats.[11] Recently, orally administered nicotianamine improved the long-term memory function in mice.[13] Thus, it is considered that the nicotianamine present in foods has health-promoting effects, and the contents of nicotianamine in various foods of plant origin have been reported.[13−16] Previously, it was estimated that soy sauce, a condiment made of the combination of soybeans and other grains, contained about 7−8 μg/mL nicotianamine, according to the recovery rate of the ACE inhibitory activity in the purification step.[7,8] However, the real concentration value has been unclear for a long time, because nicotianamine in soy sauce is difficult to analyze. Recently, it was reported that a soy sauce sample and a soy sauce-like fermented soybean seasoning sample contained 13 and 133 μg/mL of nicotianamine, respectively,[17] which suggested that the amounts of nicotianamine in soy sauce samples vary a good deal.

The highly polar molecule of nicotianamine has poor retention on the reversed-phase columns used in high-performance liquid chromatography (HPLC) analysis. In the literature, detection of nicotianamine was achieved using amino acid analyzers,[3,4,8,11] postcolumn derivatization with *o*-phthalaldehyde for fluorometric detection after separation by cation or anion exchange,[18,19] or ion-pair reversed-phase chromatography.[12,14−16] Ion-pair reversed-phase chromatography coupled to tandem mass spectrometry,[17] hydrophilic interaction liquid chromatography coupled to mass spectrometry (HILIC-MS),[13,20] and reversed-phase chromatography coupled to quadrupole-time-of-flight mass spectrometry[21] were applied to the detection of underivatized nicotianamine. When needed, ionic adsorption−desorption using cation- or anion-exchange resin was applied to the purification of nicotianamine.[4,13−16] Moreover, *o*-phthalaldehyde was used for precolumn derivatization of nicotianamine, followed by reversed-phase chromatography for fluorometric detection.[22] Recently, for detection with

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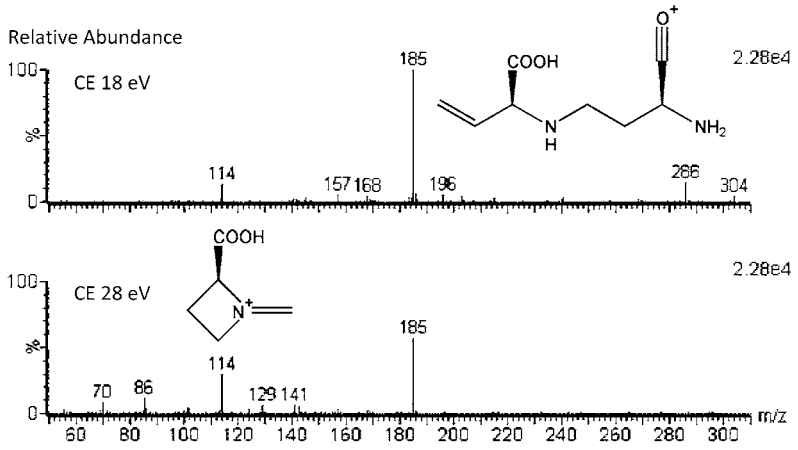


**Figure 2.** Product ion spectra of the $[M + H]^+$ ion of nicotianamine at $m/z$ 304, obtained by LC-MS/MS at collision energies (CE) of 18 and 28 eV, using standard solution (100 ng/mL). The proposed structures for the ions at $m/z$ 185 and 114 are shown.

a higher sensitivity, a derivative of nicotianamine using 9-fluorenylmethyl chloroformate was analyzed by liquid chromatography−time-of-flight mass spectrometry (LC-TOFMS),[23−25] and a derivative using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate was analyzed by liquid chromatography−mass spectrometry (LC-MS),[26,27] employing ODS columns.

However, these methods could not be applied to soy sauce easily. Soy sauce contains high levels of salt (about 150 mg/g in the typical Japanese soy sauce, *koikuchi*) and other soluble solid contents (about 160 mg/g in koikuchi) such as amino acids (about 50 mg/g in koikuchi) and organic acids (about 13 mg/g in koikuchi), which make it difficult to use ion-exchange resin for sample cleanup. The presence of other amino acids in soy sauce samples generates interference that precludes the use of amino acid analyzers for the determination of nicotianamine. Hence, to analyze nicotianamine in soy sauce samples without previous cleanup, the use of the more selective liquid chromatography−tandem mass spectrometry (LC-MS/MS), rather than LC-MS, is required. However, ion-pairing reagents are not compatible with LC-MS/MS, so they could not be applied to multiple samples. A single study[17] seemed to be the exception to this, as it described the application of an ion-pairing reagent to LC-MS/MS analysis, and the nicotianamine contents of a soy sauce sample and a soy sauce-like seasoning sample were determined, as described above; however, only a few samples were analyzed. The reported limit of detection (LOD) of HILIC-MS (2 $\mu$M = 0.6 $\mu$g/mL)[20] is insufficient for soy sauce, which should be diluted over 100 times for LC-MS or LC-MS/MS analysis because of its matrix effect. Despite the poor retention, an ODS column was used for MS detection of nicotianamine in plant samples,[21] but in the case of soy sauce, salt and other polar compounds are coeluted with nicotianamine and interfere with the determination. It is also difficult to apply derivatizing agents to soy sauce, because the derivatization efficiencies could be sensitive to the complicated soy sauce matrix, which differs depending on the samples, so suitable internal standards would have to be evaluated for the accurate determination of nicotianamine. Recently, a stable isotope-labeled nicotianamine, a strong candidate for an internal standard, was produced recombinantly,[25] but it is not yet available commercially. Because the determination of nicotianamine in soy sauce by the methodology currently available is problematic, we investigated methods that could be applicable to soy sauce and also to other foods.

In this study, a method for the determination of nicotianamine was developed using LC-MS/MS with a multimode ODS column. This method does not need derivatizing agents or ion-pairing reagents to retain nicotianamine on the column. Moreover, the sensitivity of this method is considered to be sufficient for soy sauce and is thus suitable for the analysis of nicotianamine in soy sauce and other food samples, without previous cleanup. Using this method, the levels of nicotianamine in soy sauce and other plant-based foods were determined with the aim to provide useful information from the health point of view.

## MATERIALS AND METHODS

**Materials.** Nicotianamine was isolated from soybean whey, using a published method,[7,28] and its structure was confirmed by $^1$H and $^{13}$C nuclear magnetic resonance analyses (AVANCE 500) (Bruker BioSpin GmbH, Rheinstetten, Germany) and compared with the literature values.[14,29] The isolated nicotianamine exhibited a $[M + H]^+$ ion at $m/z$ 304, a $[M + Na]^+$ ion at $m/z$ 326, and a $[M + H - H_2O]^+$ ion at $m/z$ 286 by mass analysis using a Quattro micro API (Waters, Milford, MA, USA), being identical to the authentic nicotianamine described in previous papers.[7,8] The ion at $m/z$ 304 was used as a precursor ion, giving two major product ions ($m/z$ 185 and 114); the proposed structures are shown in Figure 2 by reference to the collision-induced dissociation spectra.[25,30] The nicotianamine purity (determined as 99%) was confirmed using an L-8800 amino acid analyzer (Hitachi High-Technologies Corp., Tokyo, Japan) with the biological fluid analysis setting. Ammonium acetate, LC-MS grade acetic acid, iron(II) sulfate heptahydrate, and iron(III) chloride hexahydrate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HPLC grade acetonitrile, LC-MS grade distilled water, and copper(II) acetate monohydrate were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Commercial soy sauce, vegetable juice, fruit juice, soy milk, and bottled tea were bought from local shops.

A standard stock solution of nicotianamine (1 mg/mL) was prepared by dissolution in Milli-Q water that had undergone reverse osmosis (Millipore, Billerica, MA, USA) and been stored at −20 °C. A working solution (100 $\mu$g/mL) was prepared by diluting with Milli-Q water and further diluted with 0.1% (v/v) acetic acid−water to 10 and 1 $\mu$g/mL. Calibration standards for absolute calibration curves were prepared from working solutions at levels of 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 ng/mL.

**LC-MS/MS Conditions for Column Selection.** Four types of columns other than ODS were tested to select the most suitable one for the determination of nicotianamine without derivatizing agents or

ion-pairing reagents. These columns included Unison UK-Amino as a HILIC column and Scherzo SS-C18, SM-C18, and SW-C18 as multimode ODS columns (Imtakt, Kyoto, Japan). CAPCELL PAK $C_{18}$ MG II (Shiseido, Tokyo, Japan) was used as a common ODS column for comparison. All of the columns had the same length, internal diameter, and particle size (150 mm × 3 mm i.d., 3 μm). The LC-MS/MS conditions were identical to those described below for the SW-C18, but different solvent systems and gradients were used for the other column types. For CAPCELL PAK $C_{18}$ MG II, the solvent was 0.1% (v/v) formic acid−water, isocratic. For UK-Amino, solvent A was 0.1% (v/v) formic acid−water and solvent B was 0.1% (v/v) formic acid−acetonitrile, with a gradient of 70−10% B for 0−10 min. For SS-C18, solvent A was 5 mmol/L ammonium formate−water/acetonitrile (90:10) and solvent B was 150 mmol/L ammonium formate−water/acetonitrile (30:70), with a gradient of 0−50% B for 0−6 min. For SM-C18, solvent A was 1 mmol/L ammonium formate−water and solvent B was 150 mmol/L ammonium formate−water/acetonitrile (30:70), with a gradient of 0−100% B for 0−7 min.

**Sample Preparations.** A sample of soy sauce (1 g) was weighed into a 10 mL volumetric flask, and water was added up to the mark. Vegetable juice (1 g), fruit juice (1 g), and bottled tea (5 g) were adjusted to 10 mL in the same way and then centrifuged to obtain the supernatant. Soy milk (1 g) was weighed, 1 mL of water and 60 μL of 1 mol/L HCl were added to adjust the pH to about 4.5, and then the volume was adjusted with water to 10 mL, followed by centrifugation. Sample solutions or supernatants were filtered through centrifugal filter units with a 0.45 μm hydrophilic PTFE membrane (Millipore). The filtered solutions were serially diluted with 0.1% (v/v) acetic acid−water by factors of 50 for soy sauce and tomato juice, 5 for other juices and bottled tea, and 200 for soy milk.

**Standard Addition Method.** To subtract the matrix effect in LC-MS/MS analysis, the following standard addition method was used: (i) 80 μL of 0.1% (v/v) acetic acid−water was pipetted into each of five polypropylene microtest tubes; (ii) 10 μL of five nicotianamine standard solutions (0, 20, 50, 100, 200 ng/mL) was pipetted into respective tubes in (i); (iii) 10 μL of diluted soy sauce/juice/tea/soy milk was pipetted into each tube in (i); (iv) 5 μL of sample from each tube was injected into the LC-MS/MS for nicotianamine analysis; (v) a graph of area versus added concentration of nicotianamine (ng/mL) was plotted, and the native nicotianamine content was calculated. Final dilutions of original samples for injection into the LC-MS/MS were 1:5000 for soy sauce and tomato juice, 1:500 for other juices, 1:100 for bottled tea, and 1:20000 for soy milk.

**Nicotianamine Analysis.** The LC-MS/MS system was a Waters Acquity UPLC/Quattro micro API. Chromatographic separation was performed using a 150 mm × 3 mm i.d., 3 μm, Scherzo SW-C18 column (Imtakt) at 35 °C. The injection volume was 5 μL. The mobile phase consisted of solvent A, 0.01% (v/v) acetic acid−water, and solvent B, 150 mmol/L ammonium acetate−water/acetonitrile (30:70), and it was delivered at a flow rate of 0.3 mL/min. The linear gradient used was as follows: 0−10 min, 0−100% B; 10−25 min, 100% B; 25.1−40 min, 0% B. The electrospray ionization (positive ionization mode) conditions were as follows: capillary voltage, 0.30 kV; extractor voltage, 3.0 V; RF lens voltage, 0.0 V; source temperature, 120 °C; desolvation temperature, 350 °C. The cone and desolvation gas flows were 50 and 600 L/h, respectively, and were obtained using a nitrogen source. Argon was used as the collision gas, regulated to about $3.6 \times 10^{-3}$ mbar of the gas cell Pirani pressure, and the multiplier was set to 650 V. The mass spectrometer was operated in selected reaction monitoring (SRM) mode to observe the transition of $m/z$ 304 to 185 for nicotianamine quantitation, at a cone voltage of 18 V and a collision energy of 18 eV. The transition of $m/z$ 304 to 114 was also monitored for nicotianamine confirmation at a collision energy of 28 eV. Product ion scan mode was used to obtain product ion spectra from the $m/z$ 304 precursor ion at collision energies of 18 and 28 eV.

**Effect of Coexistent Metal Cations on LC-MS/MS Analysis.** Metal salts [$FeSO_4$, $FeCl_3$, and $(CH_3COO)_2Cu$] were individually dissolved in Milli-Q water to a final concentration of 10 μmol/L. Each sample for injection into the LC-MS/MS was prepared using 71 μL of water, 9 μL of 1% (v/v) acetic acid−water, 10 μL of nicotianamine solution (50, 200, and 1000 ng/mL), and 10 μL of metal salt solution. Final concentrations were as follows: nicotianamine, 5, 20, and 100 ng/mL (0.017, 0.067, and 0.33 μmol/L); metal, 1 μmol/L; acetic acid, 0.1% (v/v).

**Nicotianamine Determination Using an Amino Acid Analyzer.** To compare the data obtained by LC-MS/MS with those obtained by amino acid analysis with a dedicated system, the nicotianamine contents in vegetable juice, fruit juice, and soy milk were determined using an L-8900 amino acid analyzer (Hitachi High-Technologies) with the biological fluid analysis setting. The LOD for the amino acid analysis was 0.3 μg/mL, based on a signal-to-noise ratio (S/N) of 3. This LOD was similar to those reported for methods using ion-pair reversed-phase chromatography followed by postcolumn derivatization with *o*-phthalaldehyde (0.6 μg/mL),[14] or using HILIC-MS (2 μM = 0.6 μg/mL).[20] Sample solutions were prepared as follows: tomato juice (2 g) and other juices (5 g) were adjusted to 10 mL with 0.1 mol/L aqueous HCl and centrifuged, and the supernatants obtained were filtered through centrifugal filter units with a 0.45 μm hydrophilic PTFE membrane, followed by a 10000 MWCO regenerated cellulose membrane (Millipore). Soy milk solution was prepared as described above; equal volumes of the filtered soy milk solution and 0.1 mol/L HCl were mixed and filtered through a 10000 MWCO membrane. Soy sauce was not evaluated by amino acid analysis, because a large amount of valine (about 3.4 mg/g in koikuchi soy sauce) was eluted just prior to nicotianamine, and the accurate and sensitive determination of the latter became impossible.

## RESULTS AND DISCUSSION

**Column Selection.** Because nicotianamine is a highly polar molecule, it has poor retention on reversed-phase columns. Ion suppression by coeluted polar compounds takes place in real samples, and quantitation becomes impossible.[26] Moreover, soy sauce contains high levels of salt, which coelutes with nicotianamine, resulting in hampered sensitivity and damage to the mass spectrometer. Therefore, we examined four types of columns other than ODS: Unison UK-Amino as a HILIC column and Scherzo SS-C18, SM-C18, and SW-C18 as multimode ODS columns, which consist of ODS, anion, and cation ligands with different ion-exchange capacities. Typical traces are shown in Figure 3. Nicotianamine was retained on the UK-Amino, but severely broad and tailing peaks were obtained, which seemed not to be suitable for quantitation. Nicotianamine was also retained on the SS-C18, but irreproducible peak areas were obtained and ion suppression was too strong to quantitate nicotianamine in real samples. Nicotianamine was retained on the SM-C18 to some extent, but peak shape was poor and retention time was irreproducible.

The retention of nicotianamine on the SW-C18 was rather weak, and good peak shape, reproducible retention time, and reproducible peak area were obtained in standard solutions and real samples (Figures 3 and 4). Moreover, sensitivity was higher for this column than for the other three columns. It was presumed that the rather weak interaction between nicotianamine and SW-C18 allowed for a successful nicotianamine detection with high sensitivity, whereas a too strong interaction (although not to the extent of precluding elution) resulted in poor detection and reduced sensitivity, as observed for the other three columns. Because of the high sensitivity achieved with SW-C18, it was possible to inject highly diluted soy sauce samples into the LC-MS/MS, without the need for previous cleanup. Moreover, unlike for common ODS columns, salt was retained on the SW-C18 column separately as $Na^+$ and $Cl^-$, which were eluted after nicotianamine (3.97 and 3.80 min, respectively; the detection was performed with selected ion

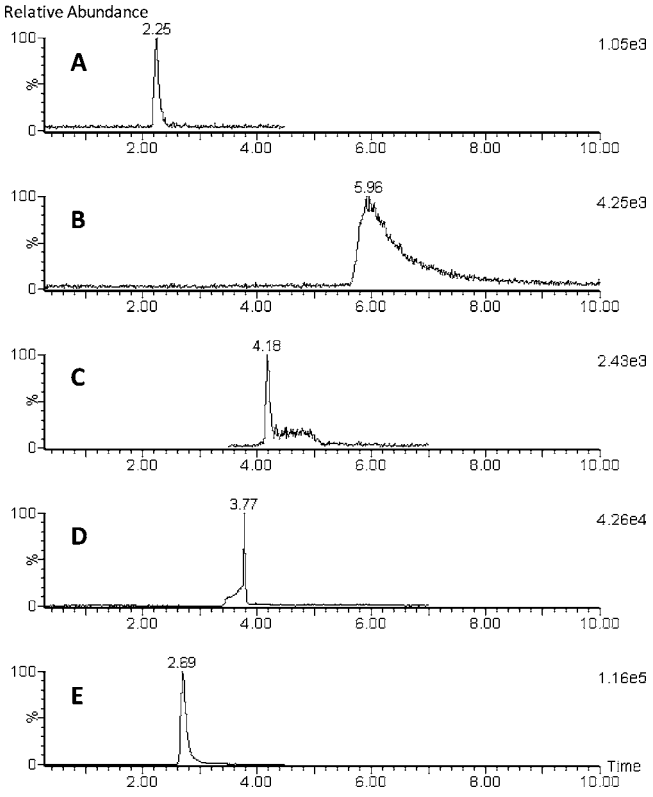


**Figure 3.** Comparison of SRM chromatograms of nicotianamine standard solutions for the transition of $m/z$ 304 to 185, using different types of columns: (A) 100 ng/mL in 0.1% (v/v) formic acid−water, using CAPCELL PAK $C_{18}$ MG II as a ODS column; (B) 1000 ng/mL in formic acid/water/acetonitrile (0.1:20:80, v/v/v), using Unison UK-Amino as a HILIC column; (C) 500 ng/mL in 0.2% (v/v) formic acid−water, using Scherzo SS-C18; (D) 1000 ng/mL in 0.2% (v/v) formic acid−water, using Scherzo SM-C18; (E) 100 ng/mL in 0.1% (v/v) acetic acid−water, using Scherzo SW-C18. Scherzo SS-C18, SM-C18, and SW-C18 are multimode ODS consisting of ODS, anion, and cation ligands with different ion-exchange capacities. Nicotianamine had poor retention on the ODS column.

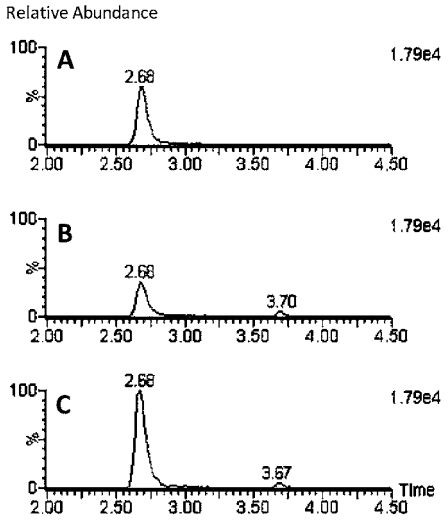


**Figure 4.** Representative SRM chromatograms of nicotianamine for the transition of m/z 304 to 185, using Scherzo SW-C18: (A) standard solution (10 ng/mL); (B) koikuchi soy sauce A (1:2000 dilution); (C) koikuchi soy sauce A spiked with 20 μg/g of nicotianamine (1:2000 dilution).

monitoring in positive electrospray ionization mode for the $m/z$ 23 ion and in negative mode for the $m/z$ 35 ion). Therefore, Scherzo SW-C18 was used for nicotianamine determination.

**Method Validation.** Prior to the evaluation of the standard addition method for calculating the nicotianamine content of the test samples, and to subtract the matrix effect during LC-MS/MS analysis, absolute calibration curves were prepared using nicotianamine standard solutions of various concentrations (0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 ng/mL, $n = 6$) on the transition of $m/z$ 304 to 185. The evaluation of linearity was performed using the square of the correlation coefficient ($r^2$). Concentrations in the range of 0.5−100 ng/mL showed linearity ($r^2 = 0.9996$). Nicotianamine was eluted within a time range of 2.68−2.70 min (average = 2.69 min), with a relative standard deviation (RSD) of 0.20%. The LOD was 0.5 ng/mL, and the limit of quantitation (LOQ) was 1.0 ng/mL. The LOD was based on a S/N of 10 for the transition of $m/z$ 304 to 185 and a S/N of 3 for the transition of $m/z$ 304 to 114, whereas the LOQ was based on a S/N of 15 and a S/N of 5 for the transitions of $m/z$ 304 to 185 and $m/z$ 304 to 114, respectively. The ratio of the peak areas obtained on the transition of $m/z$ 304 to 114 versus $m/z$ 304 to 185 was 0.29 with an RSD of 5.0%, and the permitted tolerance for the ratio of the peak areas was defined as 0.29 ± 0.07. In the recent literature, the LOD for nicotianamine was reported as 16 fmol (in 10 μL = 0.48 ng/mL) and the LOQ as 33 fmol (in 10 μL = 1.0 ng/mL), determined by LC-TOFMS after derivatization with 9-fluorenylmethyl chloroformate.[25] The LOD and LOQ for underivatized nicotianamine in this study were found to be comparable to the reported ones.

Nicotianamine is known to chelate metal cations under mildly acidic or higher pH values,[6] so the effect of coexistent metal cations in the solution used for the injection was evaluated. The relative peak areas of nicotianamine in the presence of metal cations versus no metal cations are shown in Table 1. The results suggested that chelation of metal cations by nicotianamine does not occur in the conditions used in this study.

**Table 1. Effect of Coexistent Metal Cations on the LC-MS/MS Analysis of Nicotianamine[a]**

| | Fe(II)[b] | | Fe(III)[b] | | Cu(II)[b] | |
|---|---|---|---|---|---|---|
| nicotianamine (μmol/L) | rel peak area (%) | RSD (%) | rel peak area (%) | RSD (%) | rel peak area (%) | RSD (%) |
| 0.017 | 104 | 6.4 | 100 | 5.8 | 97 | 3.1 |
| 0.066 | 98 | 1.8 | 98 | 2.0 | 94 | 0.6 |
| 0.33 | 97 | 3.0 | 98 | 1.2 | 97 | 2.3 |

[a]The relative peak areas of nicotianamine in the presence of metal cations versus no metal cations (taken as 100%) are shown ($n = 3$). [b]The concentration of each metal cation was 1 μmol/L.

In food samples, the matrix effect (ion enhancement in many cases) is often found, so an absolute calibration method was not used. An internal standard method needs a substance to act as internal standard, but no stable isotope-labeled derivative (surrogate) of nicotianamine is yet available on the market. $N^{\epsilon}$-nicotyl-D,L-lysine[23,24] and 2-aminobutyric acid[27] were used as internal standards, but their chemical structures seemed not to be suitable for the quantitation of nicotianamine without derivatization. Therefore, we chose a standard addition method to subtract the matrix effect. The standard addition calibration curve of each sample, obtained from the transition of $m/z$ 304

to 185, was used to calculate the native nicotianamine content after its linearity had been checked ($r^2 > 0.995$). The ratios of the peak areas for the transition of $m/z$ 304 to 114 versus $m/z$ 304 to 185, obtained from the sample solution without the addition of nicotianamine, were checked to confirm whether they were within the permitted tolerance (0.29 ± 0.07). The method was able to determine a concentration of 0.5 ng/mL of nicotianamine in the standard solutions, using the standard addition method [the concentration obtained was 0.46 ng/mL with an RSD of 4.7% ($n$ = 3)]. This result was used to establish values of 0.5 and 1.0 ng/mL for the LOD and LOQ, respectively, of each sample solution. Typical standard addition calibration curves for the test samples (soy sauce, tomato juice, and soy milk) are shown in Figure 5. All of them showed good linearity, and the different slopes were a reflection of the matrix effects of the samples (in these cases, ion enhancement).

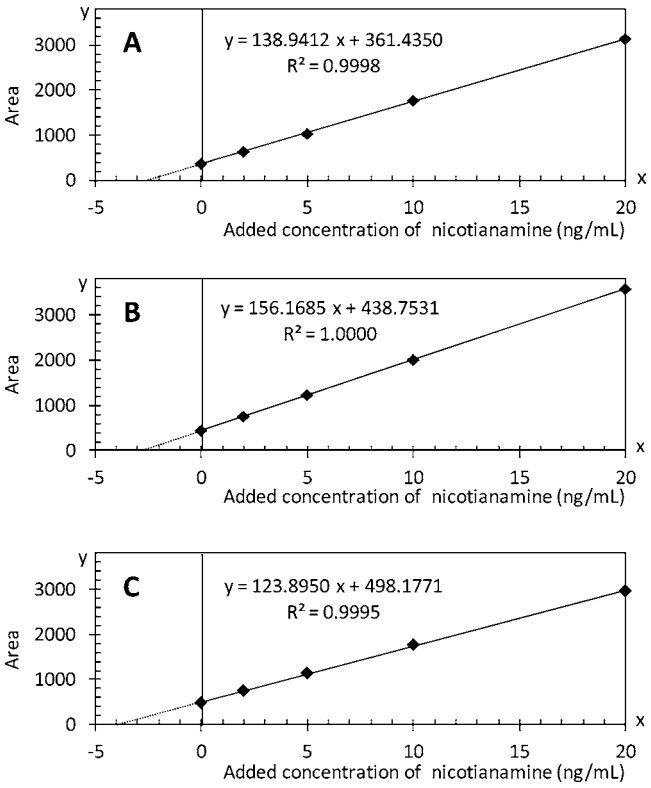


**Figure 5.** Representative standard addition calibration curves: (A) koikuchi soy sauce A; (B) tomato juice A; (C) soy milk A. The absolute value of the $x$-intercept is the concentration of nicotianamine in the sample solution.

The recovery efficiencies of the method were verified by spiking test samples with known amounts of nicotianamine (5, 10, and 20 μg/g for soy sauce A; 10 μg/g for tomato juice A; 40 μg/g for soy milk A). An intraday recovery test was performed on a single day ($n$ = 3), and an interday recovery test was performed on three different days ($n$ = 6). The mean recoveries for intraday and interday tests were in the range of 97−103 and 92−106%, with RSDs of 2.3−9.5 and 6.4−9.9%, respectively. The data suggested that the experimental procedures used in this study were satisfactory. Moreover, the values obtained by LC-MS/MS analysis were well in agreement with those obtained by amino acid analysis (Table 2), which suggested that this LC-MS/MS method was properly established.

**Measurement of Nicotianamine in Soy Sauce and Other Foods.** The amounts of nicotianamine in the commercial soy sauce, vegetable juice, fruit juice, soy milk, and bottled tea analyzed are shown in Table 3. The levels of nicotianamine found in koikuchi soy sauce (the typical Japanese soy sauce, made from roughly equal quantities of soybeans and wheat), koikuchi with reduced salt, and *usukuchi* (saltier and lighter in color than koikuchi) ranged from 6.0 to 54 μg/g. Several data were similar to previously reported values (7−8 μg/mL[7,8] and 13 μg/mL[17] for koikuchi). *Tamari*, a soy sauce made mostly from soybeans, with little or no wheat, showed a higher level of nicotianamine (71 μg/g). Nicotianamine was detected at a low level (0.31 μg/g) in *shiro* soy sauce (made mostly from wheat, with very little soybeans), and it was not detected in the soy sauce made from acid-hydrolyzed soy protein. It was reported that soybeans contain high levels of nicotianamine (340 μg/g dry weight[8] and 32.9−44.9 mg/100 g dry weight[16]), whereas in wheat, nicotianamine was not detected.[8] Soy protein is separated from water-soluble components, including nicotianamine, during the production process, so the levels of nicotianamine in soy sauce may reflect the contents of nicotianamine in the raw materials.

Table 3 shows the levels of nicotianamine in other foods of plant origin. Several data were similar to previously reported values (31 mg/kg fresh weight for tomato fruit[13] and 0.090 mg/g fresh weight for soy milk[14]). It was possible to determine the low levels of nicotianamine in samples such as bottled tea (e.g., 0.18 μg/g in bottled oolong tea). Samples were analyzed with good repeatability; RSDs were in the range of 1.2−12.5% ($n$ = 3 or 7).

Thus, the method developed in this study made it possible to determine the content of the highly polar nicotianamine in soy sauce, which contains high levels of salt and other soluble solid contents, without the need of derivatizing agents or ion-pairing reagents. Moreover, this method is expected to be applicable for the analysis of the nicotianamine content of various food

**Table 2. Comparison of Nicotianamine Contents Obtained by Two Different Methods**

| | LC-MS/MS[a] | | | amino acid analyzer[b] | | |
|---|---|---|---|---|---|---|
| sample | nicotianamine (μg/g) | RSD (%) | $n$ | nicotianamine (μg/g) | RSD (%) | $n$ |
| tomato juice A | 14 | 4.3 | 7 | 14 | 1.2 | 3 |
| carrot juice | 7.1 | 6.2 | 3 | 7.7 | 1.7 | 3 |
| apple juice A | 1.1 | 1.6 | 3 | 1.3 | 3.8 | 3 |
| grape juice A | 3.6 | 6.3 | 3 | 4.4 | 3.7 | 3 |
| pineapple juice A | 3.5 | 7.9 | 3 | 3.4 | 0.9 | 3 |
| peach juice | 9.5 | 10.1 | 3 | 11 | 3.5 | 3 |
| soy milk A | 79 | 5.0 | 7 | 78 | 1.0 | 3 |

[a]Method developed in this study. [b]L-8900 amino acid analyzer (Hitachi High-Technologies) with the biological fluid analysis setting.

**Table 3. Nicotianamine in Food Samples**

| sample | | nicotianamine (μg/g) | RSD (%) | n |
|---|---|---|---|---|
| koikuchi soy sauce | A | 13 | 5.4 | 7 |
| | B | 6.0 | 8.1 | 3 |
| | C | 19 | 3.4 | 3 |
| | D | 30 | 2.3 | 3 |
| | E | 18 | 4.2 | 3 |
| | F | 25 | 5.5 | 3 |
| | G | 22 | 10.7 | 3 |
| | H | 18 | 7.9 | 3 |
| | I | 48 | 5.1 | 3 |
| | J | 54 | 5.3 | 3 |
| koikuchi soy sauce with reduced salt | A | 17 | 6.9 | 3 |
| | B | 23 | 2.8 | 3 |
| | C | 25 | 8.4 | 3 |
| usukuchi soy sauce | A | 17 | 5.6 | 3 |
| | B | 13 | 8.9 | 3 |
| | C | 8.9 | 9.1 | 3 |
| | D | 17 | 7.9 | 3 |
| | E | 10 | 2.3 | 3 |
| tamari soy sauce | | 71 | 9.4 | 3 |
| shiro soy sauce | | 0.31 | 10.3 | 3 |
| soy sauce made from acid-hydrolyzed soy protein | | <0.25 | - | 3 |
| tomato juice | A | 14 | 4.3 | 7 |
| | B | 18 | 2.3 | 3 |
| | C | 14 | 5.4 | 3 |
| carrot juice | | 7.1 | 6.2 | 3 |
| apple juice | A | 1.1 | 1.6 | 3 |
| | B | 1.2 | 8.5 | 3 |
| grape juice | A | 3.6 | 6.3 | 3 |
| | B | 4.6 | 8.3 | 3 |
| pineapple juice | A | 3.5 | 7.9 | 3 |
| | B | 4.3 | 7.7 | 3 |
| grapefruit juice | | 2.4 | 8.1 | 3 |
| pink grapefruit juice | | 1.9 | 6.8 | 3 |
| orange juice | | 0.40 | 12.5 | 3 |
| Satsuma mandarin juice | | 0.59 | 5.1 | 3 |
| peach juice | | 9.5 | 10.1 | 3 |
| soy milk | A | 79 | 5.0 | 7 |
| | B | 84 | 3.0 | 3 |
| | C | 77 | 5.3 | 3 |
| bottled green tea | | 0.25 | 5.3 | 3 |
| bottled oolong tea | | 0.18 | 1.2 | 3 |
| bottled black tea | | 0.69 | 4.9 | 3 |

samples, without cleanup, because of the high sensitivity achieved by using a multimode ODS column. The daily intake of soy sauce is estimated to be 17.5 g in Japan,[31] so the amount of nicotianamine from soy sauce is insufficient to produce antihypertensive effects by itself. However, other foods, such as tomato juice or soy milk, also contain high levels of nicotianamine. Thus, the determination of nicotianamine in various food samples will elucidate the contribution of nicotianamine in the total diet to the antihypertensive and other health-promoting effects.

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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS USED

ACE, angiotensin I-converting enzyme; LC-MS/MS, liquid chromatography−tandem mass spectrometry; SRM, selected reaction monitoring; LOD, limit of detection; LOQ, limit of quantitation; RSD, relative standard deviation; S/N, signal-to-noise ratio.

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