

# Determination of 4(5)-Methylimidazole in Soy Sauce and Other Foods by LC-MS/MS after Solid-Phase Extraction

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**ABSTRACT:** A method for the determination of 4(5)-methylimidazole (4MeI) in naturally brewed soy sauce was developed for the first time using solid-phase extraction (SPE) and liquid chromatography–tandem mass spectrometry (LC-MS/MS). SPE on silica-based reversed-phase cartridges with heptafluorobutyric acid as an ion-pairing reagent was used for the efficient cleanup of 4MeI. A multimode ODS column was employed for the chromatographic separation. To subtract the matrix effect during LC-MS/MS analysis, a standard addition method was used. The levels of 4MeI found in naturally brewed soy sauce were extremely low (ranging from <0.002 to 0.023  $\mu\text{g/g}$ ), whereas those in soy sauces containing caramel color were generally high (ranging from 0.43 to 4.8  $\mu\text{g/g}$ ). The method proved to be useful for the analysis of 4MeI in other foods such as caramel colors, drinks, and Worcestershire sauce.

**KEYWORDS:** 4(5)-methylimidazole, soy sauce, solid-phase extraction, heptafluorobutyric acid, liquid chromatography–tandem mass spectrometry, multimode ODS column, standard addition method

## INTRODUCTION

4(5)-Methylimidazole (4MeI) is a simple nitrogen-containing heterocyclic compound (see Figure 1 for the chemical structure) that is used in the manufacture of pharmaceuticals, photographic chemicals, dyes and pigments, cleaning and agricultural chemicals, and rubber.<sup>1</sup> 4MeI is also known to be present in caramel coloring obtained from an ammonia or sulfite ammonia process (corresponding to class III or IV colors, respectively).<sup>2</sup> It is the result of a browning reaction when nitrogen compounds are present.<sup>3,4</sup> Additionally, 4MeI is present in a great many food and beverage products, even those not containing caramel color.<sup>4–11</sup>

4MeI was listed on January 7, 2011, as a chemical known to the state of California to cause cancer under Proposition 65 (Safe Drinking Water and Toxic Enforcement Act of 1986; California Health and Safety Code 25249.5 et seq.). The Proposition 65 “No Significant Risk Level” (NSRL) for 4MeI is calculated to be 16  $\mu\text{g/day}$ .<sup>1</sup> This level was based on a technical report from the National Toxicology Program (NTP).<sup>12</sup> Moreover, 4MeI is expected to be classified as a group 2B compound (*possibly carcinogenic to humans*) by the IARC Monographs.<sup>13</sup> Thus, accurate methods to determine 4MeI content in foods are needed to quantitatively determine the ingested amounts.

Several procedures have been described in the literature for the determination of 4MeI in various matrices. An ion-pair extraction method utilizing bis-2-ethylhexylphosphate has been employed for roasted coffee<sup>5</sup> and soft drinks and dark beer<sup>6</sup> and solid-phase extraction (SPE) with a strong cation exchange column for coffee, dark beer, class III caramel, and cola.<sup>4,7,8</sup> However, these extraction methods have not been applied to samples such as soy sauce containing high levels of salt and other solid content, especially nitrogen components including amino acids and low molecular weight peptides. Reversed-phase liquid chromatography (RPLC),<sup>4,7</sup> hydrophilic interaction chromatography (HILIC),<sup>8</sup> and a mixed-mode column<sup>11</sup> have also been used to quantitate 4MeI. However, these methods are not optimal for

separating 4MeI from other components of soy sauce that show similar chromatographic behavior. As well, although 4MeI has been detected in soy sauce samples supposedly containing caramel colors,<sup>9,10</sup> it is not likely to be present in high levels in naturally brewed soy sauce. Thus, an accurate quantification of 4MeI needs to employ both an efficient cleanup procedure and a method of analysis suitable for quantifying low levels of 4MeI. Liquid chromatography–mass spectrometry (LC-MS) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) both provide advantages in that 4MeI can be analyzed without derivatization.<sup>4,7,8,11</sup>

In the present study, a method for the determination of 4MeI was developed using SPE on silica-based reversed-phase cartridges with heptafluorobutyric acid as the ion-pairing reagent and LC-MS/MS with a multimode ODS column. This method was found to be suitable for the analysis of 4MeI in naturally brewed soy sauce without caramel color and other food samples.

## MATERIALS AND METHODS

**Materials.** 4(5)-Methylimidazole and heptafluorobutyric acid were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Ammonium formate, LC-MS grade formic acid, and amino acid sequencing grade trifluoroacetic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 2-Ethylimidazole, HPLC grade acetonitrile, HPLC grade methanol, and LC-MS grade distilled water were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Caramel colors were obtained from Showa Shoji Co., Ltd. (Kumamoto, Japan). Commercial soy sauce (either naturally brewed or containing caramel color as detailed on the packaging), Worcestershire sauce,

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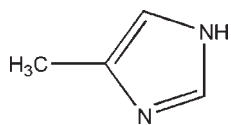


Figure 1. Chemical structure of 4-methylimidazole (4MeI).

carbonated soft drinks, dark beer, and canned coffee were bought from local shops.

A standard stock solution of 4(5)-methylimidazole (1 mg/mL) was prepared by dissolution in Milli-Q water that had undergone reverse osmosis (Millipore, Billerica, MA) and was stored at  $-20\text{ }^{\circ}\text{C}$ . A working solution (100  $\mu\text{g/mL}$ ) was prepared by dilution with 0.1% formic acid/0.1 mg/mL ammonium formate–water and then dilution to 10 and 1  $\mu\text{g/mL}$ . Calibration standards for absolute calibration curves were prepared from working solutions at levels of 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 5, 10, 20, 50, and 100 ng/mL.

**Sample Preparations.** A sample of caramel color (0.1 g) was weighed into a 10 mL volumetric flask, and water was added up to the mark. Soy sauce (2.5 g) and Worcestershire sauce (2.5 g) were made up to 10 mL in the same way. A magnetic stirrer in a beaker at room temperature was used for decarbonizing beer and carbonated soft drinks. A portion of drink (carbonated soft drinks, canned coffee, and dark beer, 5 g) was diluted with water to 10 mL. The typical volumes of solutions used for SPE were as follows: 0.8 mL for naturally brewed soy sauce, 0.2 mL for Worcestershire sauce, 0.3 mL for beer, 0.15 mL for canned coffee, and 0.012 mL for carbonated soft drinks.

If samples contained a high level of 4MeI (about  $>1\text{ }\mu\text{g/g}$ ), they were not treated by SPE but diluted (1:1000 in many cases) with 0.1% formic acid/0.1 mg/mL ammonium formate–water for direct LC-MS/MS analysis.

**SPE.** The Sep-Pak Vac 12 cc (2 g) C18 cartridge (Waters, Milford, MA) was conditioned with 12 mL of methanol, 24 mL of water, and 20 mL of 0.1% heptafluorobutyric acid/water. The loading sample was prepared using appropriate quantities of sample solution, water, and 1 mL of 1% heptafluorobutyric acid/water to 2 mL in total. When needed, 0.2 mL of 10% sodium chloride/water was used to adjust loading samples to contain from 1 to 2% of sodium chloride. The recovery efficiency of 4MeI was examined using 50, 100, and 150 ng/mL aqueous solutions. An aliquot of 1% sodium chloride/0.5% heptafluorobutyric acid/water (2 mL) was used for the blank test. After sample loading, impurities were washed out with 12 mL of 0.1% heptafluorobutyric acid/water and 8 mL of water. Retained 4MeI was eluted with 10 mL of 10% acetonitrile/water. The extract was evaporated to dryness using a rotary vacuum evaporator equipped with a water bath at  $50\text{ }^{\circ}\text{C}$ . The residues were dissolved in 900  $\mu\text{L}$  of 0.1% formic acid/0.1 mg/mL ammonium formate/water and then filtered through centrifugal filter units with a  $0.45\text{ }\mu\text{m}$  hydrophilic PTFE membrane (Millipore).

**Standard Addition Method.** To subtract the matrix effect in LC-MS/MS analysis, a standard addition method was used. (i) Ninety microliters of the sample solution was pipetted into each of five polypropylene microtest tubes. (ii) A volume of 10  $\mu\text{L}$  of each of the 4MeI standard solutions (0, 10, 20, 50, and 100 ng/mL) was pipetted into the solutions in (i). (iii) Five microliters of each sample was injected into the LC-MS/MS for 4MeI analysis. (iv) A graph of area versus concentration of added 4MeI (ng/mL) was plotted, and the native 4MeI content was calculated.

**Analysis of 4MeI.** The LC-MS/MS system was a Waters Acquity UPLC/Quattro micro API (Waters). Chromatographic separation was performed using a Scherzo SM-C18 column ( $3\text{ }\mu\text{m}$ ,  $150 \times 3\text{ mm}$ , Imtakt, Kyoto, Japan) with a guard cartridge SM-C18 ( $3\text{ }\mu\text{m}$ ,  $5 \times 2\text{ mm}$ ) at  $35\text{ }^{\circ}\text{C}$ . The injection volume was 5  $\mu\text{L}$ . The mobile phase consisting of solvent A, 10 mmol/L ammonium formate–water/acetonitrile (90:10), and solvent B, 150 mmol/L ammonium formate–water/ acetonitrile

(30:70) was delivered at a flow rate 0.3 mL/min. The following linear gradient was used: 0 min, 0% B; 8 min, 20% B; 10 min, 100% B; 21 min, 100% B; 21.1 min, 0% B; and 36 min, 0% B. The electrospray ionization (ESI) (positive ionization mode) conditions were as follows: capillary voltage was 0.50 kV, extractor voltage was 3.0 V, RF lens voltage was 0.0 V, source temperature was  $120\text{ }^{\circ}\text{C}$ , and desolvation temperature was  $350\text{ }^{\circ}\text{C}$ . 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 and regulated to about  $3.6 \times 10^{-3}$  mbar of the gas cell pirani pressure, and the multiplier was set to 650 V. The cone voltage and the collision energy of 4MeI were 34 V and 18 eV, respectively. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode to observe the transition of  $m/z$  83 to 56 for 4MeI quantitation.

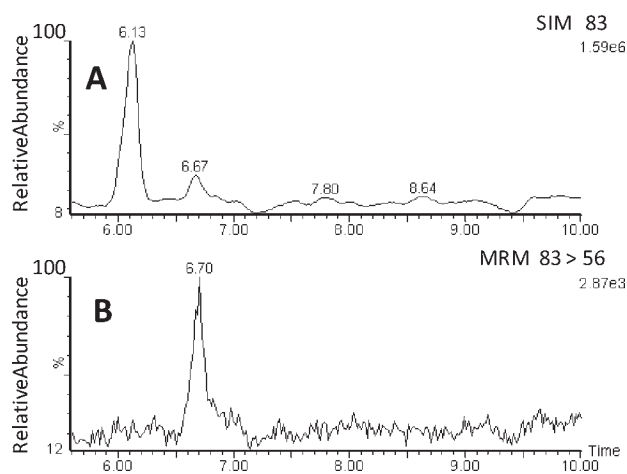
Daughter scan mode was used to obtain MS/MS spectra from the  $m/z$  83 parent ion at a collision energy of 24 eV. Selected ion monitoring (SIM) mode was used to monitor the  $m/z$  83 ion to compare chromatograms obtained by SIM with those obtained by MRM.

**Accurate Mass Analysis.** Liquid chromatography–time-of-flight mass spectrometry (LC-TOFMS) was used for accurate mass analysis. The LC-TOFMS system was an Agilent 1100 series (Agilent Technologies, Santa Clara, CA)/QSTAR Elite (AB Sciex, Foster City, CA) with an ESI source in the positive mode. The LC parameters were identical with the LC-MS/MS analysis described above, differing only in the injection volume (10  $\mu\text{L}$ ). The MS parameters were as follows: ESI spray voltage, 1200 V; capillary temperature,  $450\text{ }^{\circ}\text{C}$ . Data analysis was performed with Analyst QS 2.0 software (AB Sciex). The MS was calibrated using 2-ethylimidazole as an internal standard ( $[M + H]^+$  ion,  $m/z$  97.0760).

**Second SPE.** Samples prepared by SPE as described above and assessed by LC-MS/MS analysis were then used for the daughter ion scan (with LC-MS/MS) and the accurate mass analysis (with LC-TOFMS) to confirm the presence of 4MeI in the test samples. In the case of naturally brewed soy sauce, the level of 4MeI was extremely low, so a second SPE was performed as follows. Three SPE residues (prior to the dissolution) were dissolved into 1.5 mL of 0.5% trifluoroacetic acid/water. A Sep-Pak Vac 12 cc (2 g) C18 cartridge was conditioned with 12 mL of methanol, 24 mL of water, and 12 mL of 0.1% trifluoroacetic acid/water. After sample loading, impurities were washed out with 20 mL of 0.1% trifluoroacetic acid/water. Retained 4MeI was eluted with 10 mL of 0.1% trifluoroacetic acid/water. The extract was evaporated to dryness using a rotary vacuum evaporator with a water bath at  $50\text{ }^{\circ}\text{C}$ . The residue was dissolved in 450  $\mu\text{L}$  of 0.1% formic acid/0.1 mg/mL ammonium formate–water and then filtered through centrifugal filter units with a  $0.45\text{ }\mu\text{m}$  hydrophilic PTFE membrane.

## RESULTS AND DISCUSSION

**Method Validation.** For accurate determination of 4MeI at low levels, samples were subjected to SPE and LC-MS/MS analysis with the standard addition method. 4MeI is a low molecular weight, weakly basic compound. At the same time, soy sauce contains high levels of salt and other solid content, especially nitrogen components including amino acids and low molecular weight peptides. Therefore, ionic interactions as found in ion-pair extraction<sup>5,6</sup> or the ionic adsorption–desorption process as found in SPE<sup>4,7,8</sup> are difficult to apply, and to some extent a fractionation technique is needed. In fact, we first examined SPE with a strong cation exchange column (SPEC SCX 15 mg/3 mL column, Agilent Technologies) as used in the previously published methods.<sup>4,7,8</sup> However, 4MeI was recovered to only 10% of the additive amount from 10-fold diluted soy sauce, whereas it was recovered 104% from 4-fold diluted dark beer. Therefore, we employed SPE on silica-based reversed-phase

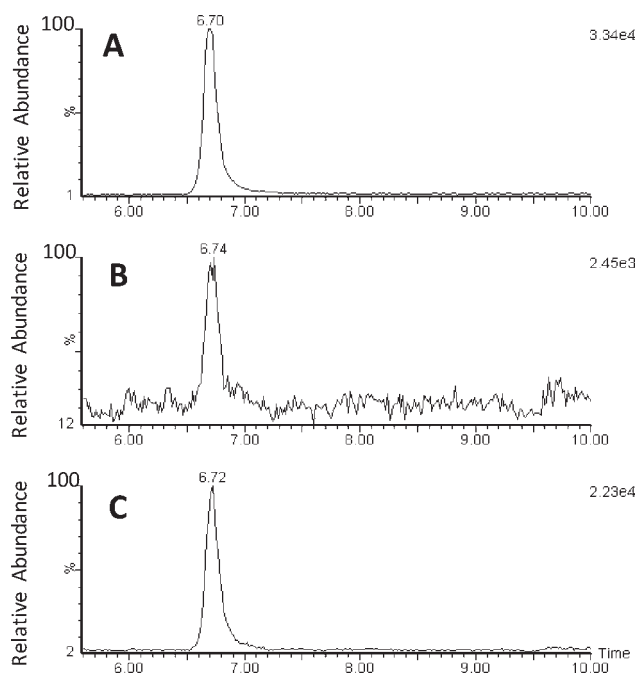


**Figure 2.** Comparison of chromatograms of 4MeI in a SPE treated sample of soy sauce A (naturally brewed, *koikuchi*): (A) selected ion monitoring (SIM) mode; (B) multiple reaction monitoring (MRM) mode.

cartridges with an ion-pairing reagent to improve the recovery of 4MeI in soy sauce.

Prior to evaluation of the standard addition method for calculating the 4MeI content of the test samples, absolute calibration curves were prepared using 4MeI standard solutions of various concentrations (0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 5, 10, 20, 50, and 100 ng/mL,  $n = 6$ ). The evaluation of linearity was performed by using the square of the correlation coefficient ( $r^2$ ). The range showing linearity ( $r^2 = 0.9999$ ) was 0.3–20 ng/mL. 4MeI was eluted at 6.75 min within a range of 6.69–6.79 min and with a relative standard deviation (RSD) of 0.49%. The limit of detection (LOD) was 0.4 ng/mL and the limit of quantitation (LOQ) was 0.7 ng/mL based on signal-to-noise ratios (S/N) of 5 and 10, respectively. In the recent literature, the LOQs of 4MeI were 2.2  $\mu\text{g/L}$  by GC-MS after extraction and derivatization,<sup>6</sup> 0.4 ng/mL by LC-MS with SIM,<sup>7,8</sup> and 5 ppb by LC-MS/MS with MRM.<sup>11</sup> In our preliminary examination, the LOQ of 4MeI by LC-MS with SIM was 0.2 ng/mL, which was slightly better than that with MRM, but we employed MRM because of its higher selectivity than SIM (see Figure 2 for a comparison of the two methods). Figure 3 shows typical MRM chromatograms of 4MeI using LC-MS/MS. The peak widths at half-height ( $W_{0.5}$ ) of 4MeI were 0.14 min (at 10 ng/mL) and 0.15 min (at 50 and 100 ng/mL, data not shown). In the literature using LC-MS or LC-MS/MS, broader peak widths  $W_{0.5}$  of 4MeI were estimated from figures at 0.46 min,<sup>4</sup> 0.34 min,<sup>8</sup> and 0.32 min at 50 ppb.<sup>11</sup> Our chromatographic separation method (using a multimode ODS column) seemed to have an advantage for quantitation of 4MeI in complicated matrices such as soy sauce.

In real samples, such as soy sauce samples after SPE, the matrix effect (ion suppression in many cases) was often found, so an absolute calibration method was not employed. An internal standard method needs an internal standard substance, but a stable isotope-labeled derivative (a so-called surrogate) of 4MeI is not available commercially. 2-Ethylimidazole, a candidate for an internal standard for the quantitation of 4MeI, has a different extent of matrix effect from 4MeI as a result of their different retention times. Therefore, we employed a standard addition method to subtract the matrix effect. The standard addition calibration curve of each sample was used for calculating the native 4MeI content after its linearity had been checked ( $r^2 > 0.990$ ).



**Figure 3.** Representative MRM chromatograms of 4MeI using LC-MS/MS: (A) standard solution (10 ng/mL); (B) SPE treated sample of soy sauce A (naturally brewed, *koikuchi*); (C) SPE treated sample of soy sauce A spiked with 0.050  $\mu\text{g/g}$  4MeI.

The method was suitable for determining a concentration of 0.1 ng/mL of 4MeI in the standard solutions using the standard addition method. However, we defined the LOD and LOQ for each sample solution (used for injection) as 0.4 and 0.7 ng/mL on the basis of the determinations from the absolute calibration curves. For example, 0.2 g of soy sauce was loaded on the SPE column, 900  $\mu\text{L}$  of the final sample solution was obtained, 90  $\mu\text{L}$  of it was mixed with 10  $\mu\text{L}$  of each 4MeI standard solution, and the LOD and LOQ for soy sauce sample became 2.0 and 3.5 ng/g, respectively. The recovery efficiency of the method was verified by subjecting samples (soy sauce, carbonated soft drink, and canned coffee) spiked with one or three known amounts of 4MeI to SPE and LC-MS/MS analysis. 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 and RSDs are given in Table 1.

For samples containing high levels of 4MeI (about  $>1 \mu\text{g/g}$ ), direct LC-MS/MS analysis without SPE was also used. Tables 2 and 3 show the results of the method without SPE. The data suggested that our SPE method was working properly and the experimental processes used in the present study were satisfactory.

**Confirmation of the Presence of 4MeI.** Samples (caramel color A, carbonated soft drink, canned coffee, dark beer, Worcestershire sauce, and naturally brewed soy sauces A and C) were treated by SPE using the standard method and assessed by LC-MS/MS analysis and then subjected to the daughter ion scan with LC-MS/MS and accurate mass analysis with LC-TOFMS. For the naturally brewed soy sauce, a second SPE was performed. 4MeI exhibited a  $[M + H]^+$  ion at  $m/z$  83, which was used as a parent ion giving two daughter ions,  $m/z$  56 and 42 (MRM transition of  $m/z$  from 83 to 56 was used for the quantitation of 4MeI). All samples examined gave MS/MS spectra equivalent to



the standard solution. Typical MS/MS spectra are shown in Figure 4. The calculated accurate  $m/z$  value of the  $[M + H]^+$  ion for 4MeI was 83.0604, and all samples examined gave an ion with a  $m/z$  value of within  $\pm 10$  ppm. A typical mass spectrum of a naturally brewed soy sauce sample is shown in Figure 5.

#### Measurement of 4MeI in Soy Sauce and Other Foods.

Table 4 shows the amount of 4MeI analyzed in commercial soy sauce and caramel coloring. To our knowledge, this is the first quantitation of 4MeI in naturally brewed soy sauce in the absence of caramel coloring. The levels of 4MeI found in naturally brewed *koikuchi* soy sauce (the typical Japanese soy sauce) were extremely

**Table 1. Recovery Test of 4MeI Determination in Test Samples<sup>a</sup>**

sample			intraday <sup>c</sup>		interday <sup>d</sup>	
	native 4MeI ( $\mu\text{g/g}$ ) <sup>b</sup>	spike level ( $\mu\text{g/g}$ )	recovery (%)	RSD (%)	recovery (%)	RSD (%)
soy sauce A (naturally brewed, <i>koikuchi</i> )	0.0037	0.025	104.5	1.8	103.6	3.5
		0.050	97.1	2.4	98.5	2.7
		0.075	94.3	1.3	97.4	4.1
carbonated soft drink (containing caramel)	0.67	0.82	96.2	5.0	95.6	5.4
canned coffee	0.048	0.067	97.2	5.7	96.3	4.9

<sup>a</sup>Standard addition method was used in LC-MS/MS analysis with samples after SPE. <sup>b</sup>The data are shown in Table 4. <sup>c</sup>Intraday recovery test was performed on a single day ( $n = 3$ ). <sup>d</sup>Interday recovery test was performed on three different days ( $n = 6$ ).

**Table 2. Recovery Test of 4MeI Determination in Naturally Brewed Soy Sauce without SPE<sup>a</sup>**

sample			intraday <sup>c</sup>		interday <sup>d</sup>	
	native 4MeI ( $\mu\text{g/g}$ ) <sup>b</sup>	spike level ( $\mu\text{g/g}$ )	recovery (%)	RSD (%)	recovery (%)	RSD (%)
soy sauce A (naturally brewed, <i>koikuchi</i> )	0.0037	0.7	100.9	8.9	99.4	7.4
		1.0	97.5	4.9	96.0	4.2
		2.0	99.0	2.3	100.2	3.7

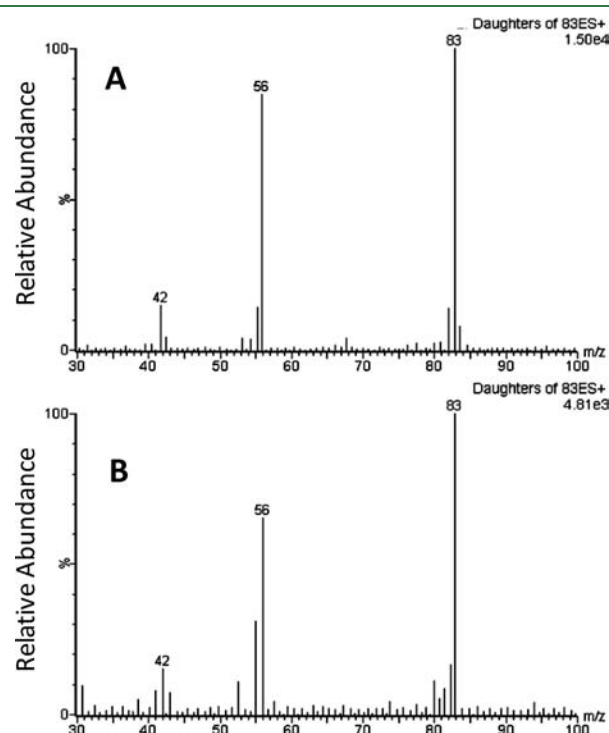
<sup>a</sup>Standard addition method was used in LC-MS/MS analysis without SPE. <sup>b</sup>The data are shown in Table 4. <sup>c</sup>Intraday recovery test was performed on a single day ( $n = 3$ ). <sup>d</sup>Interday recovery test was performed on three different days ( $n = 6$ ).

**Table 3. Comparison of the Quantification of 4MeI with and without SPE<sup>a</sup>**

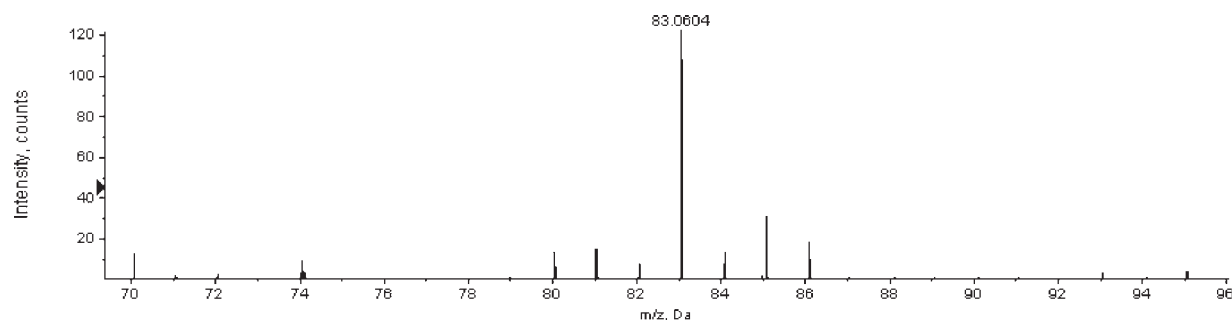
sample	without SPE		with SPE	
	4MeI ( $\mu\text{g/g}$ )	RSD (%)	4MeI ( $\mu\text{g/g}$ )	RSD (%)
caramel color A (sulfite ammonia process)	128	1.1 <sup>b</sup>	121	5.8 <sup>c</sup>
soy sauce I (acid-hydrolyzed, containing caramel)	1.6	0.4 <sup>b</sup>	1.6	2.6 <sup>d</sup>
soy sauce J (Chinese dark soy sauce, containing caramel)	5.1	5.4 <sup>b</sup>	4.8	6.3 <sup>d</sup>

<sup>a</sup>Standard addition method was used in LC-MS/MS analysis after SPE or without SPE (with SPE, the same data as shown in Table 4). <sup>b</sup> $n = 3$ . <sup>c</sup> $n = 6$ . <sup>d</sup> $n = 4$ .

low, ranging from 0.0035 to 0.0085  $\mu\text{g/g}$ . 4MeI was not detected in *usukuchi* (light color soy sauce). *Tamari*, a soy sauce that is darker in appearance than *koikuchi*, showed a slightly higher level of 4MeI. Thus, formation of 4MeI by the Maillard reaction in naturally brewed soy sauce may occur at low levels. From our analysis, most soy sauce samples containing caramel color (likely class III or IV) contained high levels of 4MeI ranging from 0.43 to 4.8  $\mu\text{g/g}$ . It is likely that given our results, plain caramel may be used in soy sauce G as a low level of 4MeI (0.013  $\mu\text{g/g}$ ) was detected. Previous studies have indicated that soy sauce contains high levels of 4MeI in the range of 2.83 mg/kg<sup>9</sup> and 0.35–0.55 ppm.<sup>10</sup> These levels were thought to be derived from the caramel colors incorporated into the samples. In caramel originating from a sulfite ammonia process, a high level of 4MeI was detected similar to the values reported for caramel from an ammonia



**Figure 4.** MS/MS spectra of 4MeI using LC-MS/MS: (A) standard solution (10 ng/mL); (B) sample solution prepared from soy sauce A (naturally brewed, *koikuchi*) by SPE followed by a second SPE treatment and assessed by LC-MS/MS analysis. This sample was also used for the analysis shown in Figure 5.



**Figure 5.** Accurate mass analysis for 4MeI in soy sauce A (naturally brewed, *koikuchi*) using LC-TOFMS. The sample was prepared by SPE followed by a second SPE treatment and assessed by LC-MS/MS analysis. The same sample was used for the analysis shown in Figure 4B.

**Table 4.** Amount of 4MeI in Food Samples in the Presence or Absence of Caramel Color<sup>a</sup>

sample	4MeI ( $\mu\text{g/g}$ )	RSD (%)	<i>n</i>
caramel color A (sulfite ammonia process)	121	5.8	6
caramel color B (plain)	<0.06		2
carbonated soft drink (containing caramel)	0.67	8.9	6
canned coffee	0.048	6.4	6
dark beer	0.017	6.4	6
Worcestershire sauce (containing caramel)	0.027	4.0	6
soy sauce A (naturally brewed, <i>koikuchi</i> )	0.0037	13.1	6
soy sauce B (naturally brewed, <i>koikuchi</i> )	0.0035	12.6	6
soy sauce C (naturally brewed, <i>koikuchi</i> )	0.0085	3.6	2
soy sauce D (naturally brewed, <i>koikuchi</i> )	0.0051	3.9	2
soy sauce E (naturally brewed, <i>usukuchi</i> )	<0.002		2
soy sauce F (naturally brewed, <i>tamari</i> )	0.023	5.0	2
soy sauce G (acid-hydrolyzed, containing caramel)	0.013	4.7	2
soy sauce H (acid-hydrolyzed, containing caramel)	0.43	4.0	2
soy sauce I (acid-hydrolyzed, containing caramel)	1.6	2.6	4
soy sauce J (Chinese dark soy sauce, containing caramel)	4.8	6.3	4

<sup>a</sup> Standard addition method was used in LC-MS/MS analysis after SPE.

process (73.3–187.8  $\mu\text{g/g}$ <sup>7</sup>). As expected, 4MeI was not detected in plain caramel.

Table 4 also shows the 4MeI levels found in other foods. They were similar to previously reported values as follows: cola type soft drinks, 0.30–0.36  $\mu\text{g/mL}$ ,<sup>4</sup> 188–613  $\mu\text{g/L}$ ,<sup>6</sup> 0.33–0.74 mg/kg,<sup>9</sup> 0.17–0.70 ppm,<sup>10</sup> and 280–793 ppb;<sup>11</sup> and dark beer, 3–424  $\mu\text{g/L}$ <sup>6</sup> and 1.58–28.03 ng/mL.<sup>7</sup> Roasted coffee beans contained 4MeI at levels of 0.307–1.241 mg/kg,<sup>5</sup> 0.39–2.05  $\mu\text{g/g}$ ,<sup>7</sup> and 0.35–1.45  $\mu\text{g/g}$ .<sup>8</sup> These values correspond to 0.020–0.097  $\mu\text{g/mL}$  in brewed coffee obtained by preparing 10 g of ground coffee beans and 150 mL of hot water under the assumption that the extraction efficiency is 100%. The 4MeI value for Worcestershire sauce was much lower (0.027  $\mu\text{g/g}$ ) than the previously reported values (1.0–3.4 ppm).<sup>10</sup> This is thought to be due to the class of caramel colors incorporated into the food and not from loss of sample because in this study the recovery of 4MeI from the Worcestershire sauce sample was 99.8% at a spiked level of 0.10  $\mu\text{g/g}$  (*n* = 1, data not shown).

Samples were analyzed with good repeatability; RSDs were in the range of 2.6–8.9% for common analyte concentrations and

in the range of 12.6–13.1% for the extremely low concentrations normally found in naturally brewed soy sauce (Table 4).

Thus, the newly developed method made it possible to quantify extremely low concentrations of 4MeI in naturally brewed soy sauce that does not contain caramel color. Although reports in the literature support the idea that soy sauce contains a high level of 4MeI, this is believed to be derived from the caramel color incorporated into the food. Moreover, this method is expected to be applicable for analysis of the 4MeI content of various food samples because of the efficiency of SPE on silica-based reversed-phase cartridges with heptafluorobutyric acid as an ion-pairing reagent and the good chromatographic separation achieved by using a multimode ODS column.

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## ABBREVIATIONS USED

4MeI, 4(5)-methylimidazole; LC-MS, liquid chromatography–mass spectrometry; SPE, solid-phase extraction; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LC-TOFMS, liquid chromatography–time-of-flight mass spectrometry; ESI, electrospray ionization; LOD, limit of detection; LOQ, limit of quantitation; RSD, relative standard deviation.

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