Development of a Method for Determining Illudin S in Food by Gas Chromatography-Mass Spectrometry

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(Received December 15, 2005; Accepted March 6, 2006)

A method was developed for determining illudin S, a naturally occurring toxin in the mushroom *Lampteromyces japonicus* in food samples with the use of gas chromatography-mass spectrometry (GC-MS). Samples of a model food, 'Ton-jiru' (miso soup), were spiked with authentic illudin S, which was then extracted from the samples by a two-step solvent extraction with ethyl acetate and acetonitrile-*n*-hexane, followed by solid phase extraction using a normal phase Bond Elut SI cartridge. The recovery of illudin S from the samples was in the range of 61–74%. Illudin S was identified by its GC retention time and both electron and chemical ionization mass spectra. In the splitless injection mode, the calibration curves for peak area ratio of illudin S to anthracene (internal standard) were linear, in the range from 150 pg to 150 ng with correlation coefficients exceeding 0.997, and the detection limit was 36 pg per injection volume (1 μl).

Key words —— illudin S, gas chromatography-mass spectrometry, solid phase extraction, food sample

INTRODUCTION

Lampteromyces japonicus (Japanese name: Tsukiyotake) is a toxic mushroom that causes food poisoning. In 2004, 18.5% of the accidental cases of poisonous mushroom intake in Japan involved *Lampteromyces japonicus*.[1] *Lampteromyces japonicus* is similar in appearance to edible mushrooms such as *Panellus serotinus*, *Lentinus edodes*, and *Pleurotus ostreatus*, except that it has a blue-white luminescence on the pileus in the dark.

Illudin S (lampterol, Fig. 1) is a toxic sesquiterpene ingredient produced by *Lampteromyces japonicus*,[2] and is contained in all parts of the mushroom. Its content varies from 60–70 μg to 1–2 mg per gram of raw mushroom.[3,4] Poisonous symptoms, including vomiting, stomach ache, and diarrhea, appear within one hour after intake,[5] but the precise poisoning mechanism remains unclear. The LD_{50} (mouse, i.p.) of illudin S is reported to be 5 mg/kg.[2,6] The toxicity of illudin S is ascribed to its alkylating action.[7] Illudin S is also produced by a certain fungal species of the genus *Omphalotus* that is also mistakenly consumed as an edible mushroom in Europe, North America, and Australia.[8] The toxic effects of illudin family compounds have been investigated in relation to their antitumor and antiviral activity.[9–11]

Morphological observation, including verifying the blue-white luminescence on the pleat, is a primary method for identifying *Lampteromyces japonicus*. However, to clarify whether or not the processed food could induce poisoning, it is also necessary to determine the toxic ingredient, illudin S in food samples that include the mushroom as an ingredient. Some analytical methods have been reported for the determination of illudin S using gas chromatography-mass spectrometry (GC-MS),[3] liquid chromatography (LC),[4] or LC-MS.[12] These methods, however, are limited only to analysis of the fruit body a mushroom. There have been no reports of analytical methods applied to small pieces of cooked mushrooms obtained from vomit samples. We developed a forensic methodology to elucidate the cause of poisoning from the toxic mushroom *Lampteromyces japonicus* by determining the pres-

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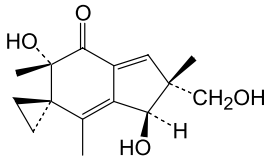

Fig. 1. Chemical Structure of Illudin S (Molecular Weight 264)

ence of illudin S through GC-MS. The analytical conditions under which GC-MS was performed were improved by adopting capillary column separation and a supplemental chemical ionization mode instead of the original GC-MS method using packed column separation.[3] We used miso soup with pork and vegetables as a sample matrix, because mushrooms are frequently consumed as ingredients in such soups in Japan. A pretreatment method was also developed to isolate and purify illudin S from the food samples.

MATERIALS AND METHODS

Reagents —— Bond Elut SI cartridges (regular type, 500 mg/3 ml) were obtained from Varian (Harbor City, CA, U.S.A.). All other chemicals used were of analytical grade. Organic solvents for the solid phase extraction (*n*-hexane, ethyl acetate) and acetonitrile were dried over anhydrous sodium sulfate. 'Ton-jiru' (miso soup with pork and vegetables) was obtained as a processed food from Nagatanien Co., Ltd. (Tokyo, Japan), and the dried content was suspended in hot water according to the preparation instructions.

Isolation and Purification of Illudin S from *Lampteromyces japonicus* —— Illudin S was purified from *Lampteromyces japonicus* as follows. Ten kilograms of *Lampteromyces japonicus* was extracted with ethanol, and the ethanol extract (about 4 l) was partitioned between ethyl acetate and water. The ethyl acetate fraction (33 g) was partitioned between *n*-hexane and 70% aqueous methanol. The methanol fraction (6.2 g) was concentrated, and the residue was dissolved in chloroform. The chloroform solution was passed through a Silicagel 60N column (spherical, neutral, particle size 63–210 μm, Kanto Kagaku, Tokyo, Japan). Illudin S was eluted with 5% methanol. The fraction was dried and the residue was chromatographed on a silica gel 60 F_{254} TLC plate (Merck, Darmstadt, Germany) using methanol-chloroform (1 : 9, Rf value of illudin S: 0.54). Purified illudin S (465 mg) was structurally confirmed by ^{1}H-NMR in CD_3OD [δ 0.42 (1H, m), 0.77 (1H, m), 0.95 (1H, m), 1.06 (1H, m), 1.14 (3H, s), 1.36 (3H, s), 1.68 (3H, s), 3.34 (1H, d, J = 6.4 Hz), 3.39 (1H, d, J = 11.0 Hz), 4.59 (1H, s), 6.44 (1H, s)], where the ^{1}H-NMR pattern is in agreement with that reported by Nakanishi *et al.*[2] The EI-mass spectrometric pattern of the purified illudin S is in agreement with that reported by Kawano.[3] Only one component was observed from the purified sample in the TLC and GC-MS analysis described below. The purity of illudin S was estimated at over 93% from ^{1}H-NMR data.

Pretreatment of Food Sample Using Solvent Extraction —— A 10-ml volume of miso soup, from which the pork and vegetables were removed, was spiked with 50 μl of an acetonitrile solution of illudin S (0.29–14.7 μg) in a 50-ml glass vial with a screw cap. Illudin S was extracted from the sample twice with 10 ml of ethyl acetate by shaking for 5 min at room temperature. The combined ethyl acetate extracts were dried over anhydrous sodium sulfate, filtered through No. 2 filter paper (Advantec, Tokyo, Japan), and concentrated under a stream of nitrogen at 40°C on a TurboVap LV Concentration Workstation (Zymark Co., Hopkinton, MA, U.S.A.). A 5-ml volume of acetonitrile was added to the concentrated fraction, and illudin S was extracted from the acetonitrile mixture three times with 5 ml of *n*-hexane by shaking for 5 min at room temperature, and the *n*-hexane extracts were discarded. The acetonitrile phase was completely dried under a stream of nitrogen at 40°C. The residue was dissolved with 2 ml of *n*-hexane-ethyl acetate (8 : 2) solution and subjected to the following solid phase extraction (SPE) treatment.

Solid Phase Extraction Procedure —— The SPE cartridge was attached to a VacMaster-20 sample processing station (Uniflex Co. Ltd., Tokyo, Japan), and conditioned with 2 ml of *n*-hexane-ethyl acetate (8 : 2). The pretreated miso soup sample described above was applied to the cartridge. The cartridge was washed with 5 ml of *n*-hexane-ethyl acetate (8 : 2) followed by 5 ml of *n*-hexane-ethyl acetate (6 : 4). Illudin S was eluted with 5 ml of ethyl acetate. The eluted fraction was completely dried under a stream of nitrogen at 40°C and dissolved in 100 μl of acetonitrile containing 42 ng of anthracene [internal standard (I.S.)], and subjected to GC-MS.

GC-MS —— A 1-μl volume of the purified sample was applied to the GC system, consisting of an HP 6890 series gas chromatograph combined with an HP 5973 quadrupole mass selective detector (Agilent

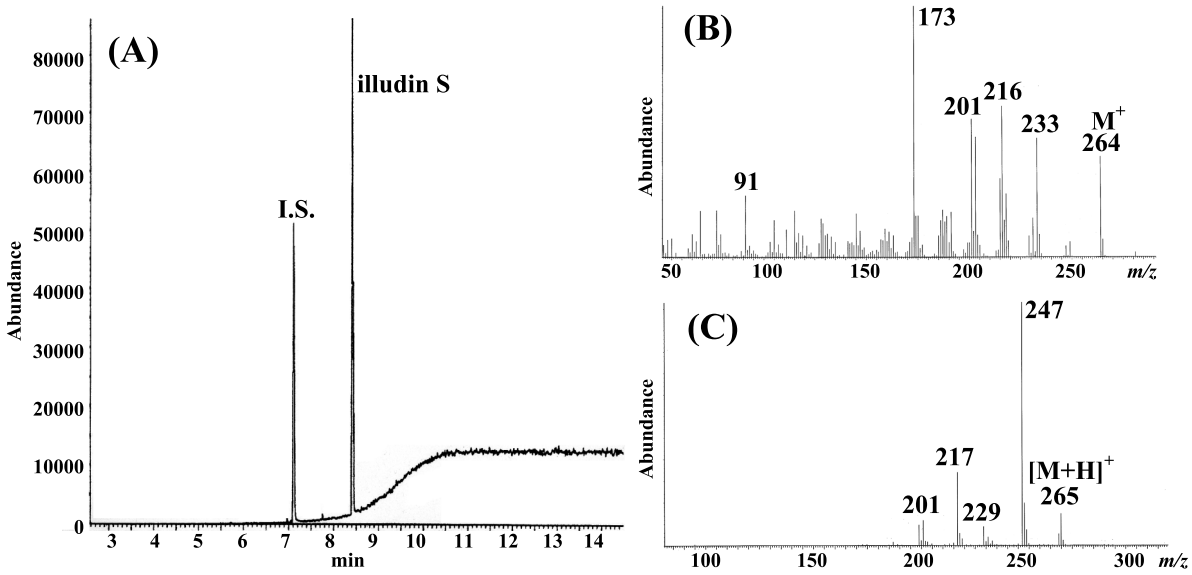

Fig. 2. Total Ion Chromatogram (A), EI Mass Spectrum (B), and Positive CI Mass Spectrum (C) of Standard Illudin S

The volume injected on the total ion chromatogram was 1 μl containing 7.4 ng of illudin S and 0.84 ng of anthracene (I.S.) in acetonitrile. Detailed GC-MS conditions are described in the Experimental section. Peaks corresponding to illudin S and I.S. are shown on the total ion chromatogram.

Technologies, Inc., Palo Alto, CA, U.S.A.). The capillary column was DB-5MS (30 m × 0.25 mm i.d., 0.25 μm in thickness; J&W Scientific, Folsom, CA, U.S.A.). The carrier gas (helium) flow rate was adjusted to 1.0 ml/min, and the splitless injection mode was used. The injection port and transfer line were maintained at 200 and 250°C, respectively. Electron ionization (EI, ionization energy 69.9 eV, ionization current 34.6 μA) and positive chemical ionization (CI, isobutane as reaction gas, ionization energy 149.7 eV, ionization current 149.6 μA) were used as the ionization mode. The ion source was adjusted to 230 and 250°C for EI and positive CI mode, respectively. The oven temperature was controlled by a program [initially at 120°C (1 min hold), ramped to 290°C at 20°C per min (5 min hold)]. The acquisition mass range was 50–550 for the EI mode or 60–550 for the positive CI mode, and the scan rate was 0.8 scans per sec. Acquisition was started 2.5 min after sample injection. The extracted ion chromatograms for the EI mode were obtained at *m/z* 173 and 264 for illudin S, and *m/z* 178 for the I.S., respectively. The analyte concentrations were calculated from the peak area ratios of the analytes to the I.S., using a standard calibration curve, prepared by plotting the peak area ratios against designated concentrations of the standard illudin S (0.15–147 μg/ml in acetonitrile, triplicate). For a standard calibration curve for the food sample matrix, both illudin S and I.S. were spiked into a fraction which was prepared in advance from dried miso soup by a two-step solvent extraction followed by SPE pretreatment of the miso soup, in turn followed by GC-MS analysis.

RESULTS AND DISCUSSION

Calibration Curve of Illudin S by GC-MS

The total ion chromatogram and EI and positive CI mass spectra of illudin S are shown in Fig. 2. The retention index of illudin S against straight-chain hydrocarbon was 2095. In the EI mass spectrum, the *m/z* 173 peak was found to be the base peak, and an *m/z* 264 peak, corresponding to the molecular ion M^+, was also observed. In the positive CI mass spectrum, a peak at *m/z* 265 corresponding to the protonated molecular ion $[M + H]^+$ was found, and the des-hydroxyl molecular ion ($[M - OH]^+$, *m/z* 247) was also observed as a base peak. Calibration curves for the peak area ratio of illudin S to I.S. were linear in the range of 150 pg to 147 ng, with correlation coefficients exceeding 0.997 (Fig. 3). The repeatability [relative standard deviation (R.S.D.), $n = 3$] of the determination of 740 pg and 14.7 ng of illudin S per injection volume (1 μl) on extracted ion chromatograms (*m/z* 173, 264) was 7.1, 8.8 and 2.6, 3.5%, respectively. The limit of detection (LOD) for illudin S was 36 pg per injection volume (S/N = 3

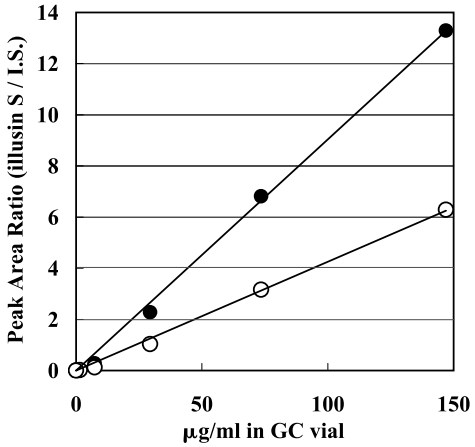

Fig. 3. Calibration Curves for Peak Area Ratio of Illudin S to I.S. on Extracted Ion Chromatograms

Closed circles (●) and open circles (○) indicate *m/z* 173 and 264 (M^+) peaks, respectively.

on extracted ion chromatogram of *m/z* 173), and the limit of quantification (LOQ) for illudin S was 120 pg per injection volume (S/N = 10 on extracted ion chromatogram of *m/z* 173). Standard illudin S in methanol solution stored at –20°C was stable for at least 3 months.

The illudin S peak was slightly split into two neighboring peaks. The mass spectrometric patterns of the two split peaks were almost identical, and the GC-MS quantification of illudin S was possible irrespective of the peak splitting. In GC-MS analysis in the split injection mode, the illudin S peak was not split. The reason for the peak splitting remains unclear at this point, but we consider that illudin S is converted to a diastereomeric mixture in the injection port.

Solvent Extraction from Food Samples

We used ethyl acetate as the first solvent for the extraction of illudin S from the food samples, to remove solid and water-soluble impurities. The recovery rate of illudin S was over 80% for aqueous samples within the pH range of 5–9. The second extraction, using *n*-hexane-acetonitrile, was intended to remove fat fractions from the samples. Typically, food samples contain large amounts of long-chain fatty acid esters, such as oleic esters, which interfere with the GC-MS analysis of illudin S. Most of the oily contaminants, however, were removed by the *n*-hexane extractions, and a nearly quantitative recovery (> 98%) was achieved.

Solid Phase Extraction from Food Samples

It was difficult to determine illudin S prepared by the two-step solvent extraction from food samples containing high levels of oily contaminants. We used the normal-phase silica SPE cartridge for the purification of illudin S to remove hydrophobic contaminants that are not removed from the two-step solvent extraction. Illudin S was retained to the Bond Elut SI under the solvent conditions of ethyl acetate-*n*-hexane (6 : 4) even in the presence of the miso soup matrix. The eluted solvent fraction (ethyl acetate) containing illudin S could be readily to dryness. The recovery of standard illudin S in the SPE treatment was over 96%. Reversed-phase SPE was not adopted, because contaminated hydrophobic compounds remaining in the two-step solvent extraction fraction interfered with the GC-MS analysis of illudin S, and a time-consuming step was required to dry the aqueous eluate completely before subjecting it to GC-MS (data not shown).

Analysis of Illudin S-Spiked Food Samples

To confirm the efficacy of the established GC-MS method with the pretreatment, the miso soup sample was analyzed. A 10 ml food sample was spiked with illudin S (0.29–14.7 μg) and subjected to GC-MS after the pretreatment procedures. As shown in Fig. 4, on the total ion chromatogram and the extracted ion chromatograms of the base peak (*m/z* 173) and the molecular ion peak (*m/z* 264), the illudin S peak was clearly observed with a retention time of 8.4 min. The peak was split into two closely overlapping peaks, like the standard illudin S sample (Fig. 2). Figure 4D shows the EI mass spectrum of the peak with a retention time of 8.4 min (Fig. 4A), which is nearly superimposable onto the spectrometric pattern of standard illudin S (Fig. 2B). Table 1 shows the recovery values for illudin S from the miso soup sample. More than 61% of the illudin S was recovered in the analyte concentration range from 0.029 to 1.47 μg/ml. The LOQ and LOD for illudin S was 37 and 11 ng per ml of miso soup sample, respectively.

The content of illudin S in raw mushroom is reported to be from 60–70 μg to 1–2 mg per gram.[3,4] When mistakenly harvested *Lampteromyces japonicus* is cooked, illudin S elutes into a liquid fraction from the fruit body during boiling and salt curing.[3,4] It is reported that only 15% of the total illudin S in the raw fruit body was recovered in the boiled fruit body,[3] and 77% of the total illudin S was recovered in a boiled water extract.[4] Illudin S

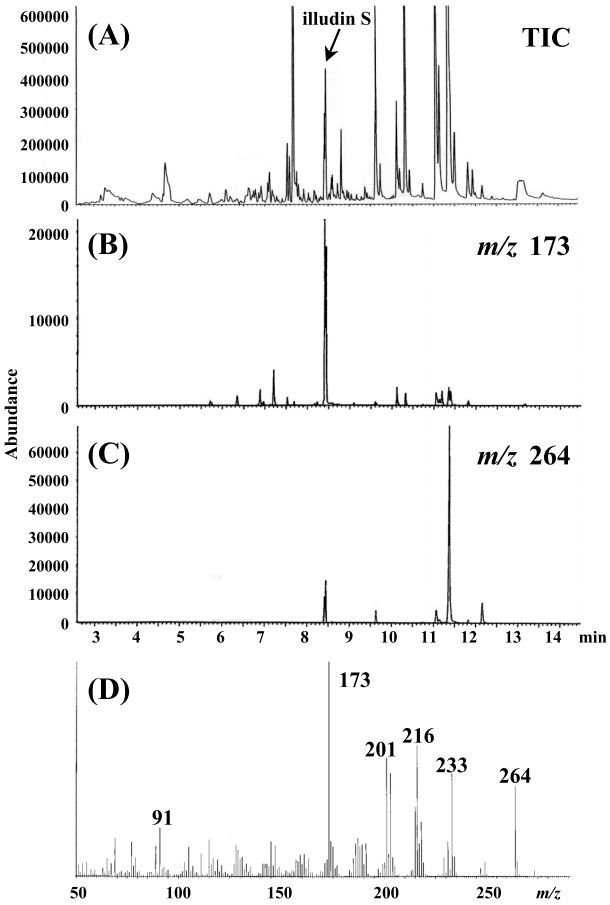

Fig. 4. Total Ion Chromatogram (A), Extracted ion Chromatograms [*m/z* 173 (B), 264 (C)], and Mass Spectrum (D, Retention Time 8.4 min) of a Miso Soup Sample

Miso soup (10 ml) spiked with 1.47 μg of illudin S was pretreated by the two-step solvent extraction procedure, followed by SPE. Detailed conditions are described in the Experimental section. The peak corresponding to illudin S is shown on the chromatogram.

Table 1. Recovery Yields of Illudin S from Miso Soup

Prepared Concentration	Recovery Yield[a] (%)	
(μg/ml)	intraday	interday
0.029	73.6 ± 2.3	71.2 ± 1.0
0.15	62.5 ± 2.9	60.7 ± 3.2
1.5	69.2 ± 8.0	66.9 ± 8.0

Quantitation of illudin S was performed based on the extracted ion chromatogram at *m/z* 173. *a*) Value represents the average of five determinations ± standard deviation.

is stable during heating at 100°C for 10 min.[4] In Japanese cooking using mushrooms, the fruit body is usually dipped into soup at a weight ratio of 0.1–10%. Therefore, 10 ml of the finished soup sample would contain 0.01–1 g raw mushroom. If *Lampteromyces japonicus* is mistakenly cooked, 0.6 μg–2 mg of illudin S is anticipated to be included in 10 ml of a soup sample. This level of illudin S in such a food sample is higher than the LOD value established in this paper to permit a valid analysis to be carried out. When 100 ml of such processed soup is consumed, the content is diluted by approximately 10-fold by the gastric contents, and 0.06–200 μg of illudin S is likely to be included in 10 ml of a vomit sample. It is also possible to determine illudin S in such a vomit sample, unless the mushroom portion in the soup is so small that the level of illudin S is extremely low.

We also attempted this pretreatment procedure followed by GC-MS to determine illudin S from raw *Lampteromyces japonicus*. This procedure used a methanol extraction fraction in which illudin S was recovered from raw *Lampteromyces japonicus*, evaporated, and then dissolved in water. Its content was 0.42 mg of illudin S per gram of raw mushroom by GC-MS determination. Illudin S was detected from real mushroom samples stored at –20°C for 3 months, and the detection level was not significantly lowered.

The proposed GC-MS method combined with the pretreatment should provide for the adequate determination of illudin S in cases of poisoning induced by the intake of a *Lampteromyces japonicus* mushroom, and the overall procedure requires less than 1.5 hr.

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