

## High-Performance Liquid Chromatographic Determination of Ustiloxin A in Forage Rice Silage

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**ABSTRACT.** We describe a high-performance liquid chromatographic method for determining ustiloxin A, a mycotoxin produced by *Ustilaginoidea virens*, in forage rice silage. Lyophilized silage samples were ground and extracted with water. The extracts were purified by solid-phase extraction and subjected to high-performance liquid chromatography using an octadecylsilane-bonded column. Separated ustiloxin A was detected with ultraviolet (UV) absorption at 254 nm. The limit of quantitation for ustiloxin A in silage found to be 2.5 mg/kg. The present method can be used for routine monitoring of the contamination of ustiloxin A in forage rice silage.

**KEY WORDS:** Forage Rice, HPLC, Silage, Ustiloxin A.

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The false smut balls that grow on the panicles of rice plants (Ina-kouji, in Japanese) are caused by infection by the pathogenic fungus *Ustilaginoidea virens* (Cooke) Takahashi. Suwa [7] first reported the toxicity to rabbits of the water extract from such false smut balls and Koiso *et al.* [1, 2] isolated tetrapeptide substances called ustiloxins (ustiloxin A-E; Fig. 1) from the water extract of the balls. Ustiloxins exhibit phytotoxic and mycotoxic effects through their potent inhibition of microtubule assembly [5]. Nakamura *et al.* [6] reported that the crude fraction obtained from the water extract of false smut balls or purified ustiloxin A caused liver and kidney damage in mice. Other biologically active substances in the balls, ustilaginoidins, have also been identified from ether extract of the balls [3]. These bis (naphtho- $\gamma$ -pyrone) derivatives exhibited weak cytotoxicity to human epidermoid carcinoma cells [4]. These findings indicate that false smut balls contain a number of substances that are toxic to animals.

The production of forage rice (*Oryza sativa* L.) is increasing in Japan in order to prepare greater quantities of self-supporting forage. Because forage rice is used as whole-crop silage, contamination of the rice crop with false smut balls creates concerns for feed safety. An assessment of the risk to cattle of false smut balls and the establishment of assay methods for the toxic substances in the balls are needed before forage rice can be utilized effectively.

In this study, we established a high-performance liquid chromatographic (HPLC) method for detecting ustiloxin A, a major homologue of ustiloxins, in whole-crop rice silage to evaluate the degree of false smut ball contamination of the silage.

Authentic ustiloxin A was kindly supplied by Dr. H.

Kobayashi of the University of Tokyo, Japan. Crude ustiloxin A containing fraction, used as a working standard for the determination of ustiloxin A, was prepared from air-dried false smut balls as follows. Twenty-four grams of false smut balls were extracted by 240 mL of water, and the supernatant was separated by filtration through a filter paper. A 45 mL aliquot of water extract was then applied to a Sep-Pak Vac 35cc C18 cartridge preconditioned with methanol and water. The cartridge was then washed with 100 mL of water, and the ustiloxin A-containing fraction was eluted with 100 mL of 20% (v/v) methanol. The ustiloxin A concentration of the target fraction was determined by HPLC.

Whole-crop rice silage samples were collected from fields in Hiroshima Prefecture, Japan, by using a core sampler. The degree of false smut ball contamination was estimated by counting the number of balls on each rice plant. The collected silage samples were lyophilized, finely ground in a motor-driven mill, and kept at 4°C until the time of analysis.

Five grams of ground silage sample were mixed with 100 mL of water and the mixture was then shaken for 30 min and centrifuged at 1,800 g for 10 min. The supernatant was filtered through filter paper (No. 1; Advantech, Tokyo, Japan). The precipitation was re-extracted with another 100 mL of water, and the supernatant was combined with the first extract. A 20 mL aliquot of the combined extract was mixed with 10 mL of 150 mM sodium carbonate buffer, pH 9.0, and the pH of the mixture was adjusted to 9.0 with a small amount of 0.2 N sodium hydroxide as necessary.

An Oasis MAX (6cc) cartridge (Waters, Milford, MA, U.S.A.) was wetted with 2 mL of methanol and flushed with 2 mL of water and 2 mL of 50 mM carbonate buffer, pH 9.0. Six milliliters of the extract at pH 9.0 were applied to a conditioned Oasis cartridge. Impurities were washed out first with 4 mL of 50 mM carbonate buffer, pH 9.0, then with

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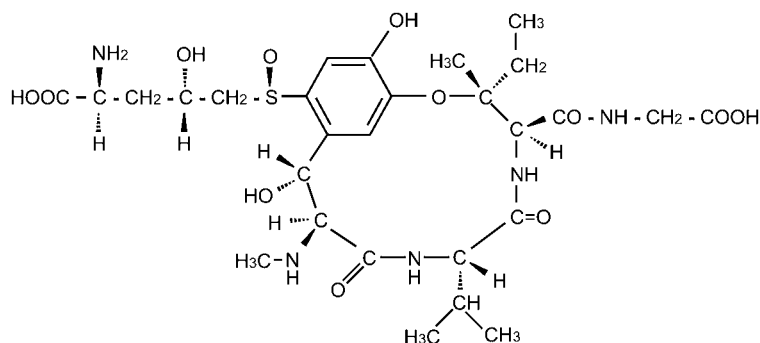


Fig. 1. Structure of ustiloxin A.

water and finally with methanol in series. Ustiloxin A was eluted with 5 mL of 50 mM ammonium acetate (pH 5.0)-acetonitrile (1:1). A 1 mL aliquot of the eluate was dried under reduced pressure and reconstituted with 0.1 mL of 20% (v/v) methanol for HPLC.

Twenty microliters of reconstituted Oasis eluate were injected into an HPLC system (LC-10 System; Shimadzu, Kyoto, Japan) with a Wakosil-II 5C18 RS column (4.6 mm x 250 mm, Wako Pure Chemical, Osaka, Japan). The mobile phase, water-methanol-phosphoric acid (400:100:1), was delivered at a flow rate of 0.5 mL/min. Ustiloxin A was detected by a photodiode array detector (SPD-M10A; Shimadzu). Quantitation of ustiloxin A was performed with UV absorption at 254 nm. A calibration curve for quantitation was prepared using sequential dilutions of the crude ustiloxin A fraction.

Figure 2(a) shows a typical HPLC chromatogram of ustiloxin A working standard solution. Ustiloxin A appeared at 11.5 min under the present HPLC conditions. The peak was identified as ustiloxin A by observing the UV absorption spectrum (Fig. 2(b)), which showed absorption peaks at 207 nm, 253 nm and 290 nm [1]. Figure 3(a) depicts a chromatogram of the purified extract of forage rice silage fortified with ustiloxin A. A ustiloxin A peak in the silage extract was also identified by its UV absorption spectrum (Fig. 3(b)). Hence, observation of the specific UV absorption spectrum of ustiloxin A was effective in identifying the peak in question as ustiloxin A.

Forage rice silage samples fortified with 2.5 mg/kg or 10 mg/kg of ustiloxin A were analyzed to examine the intra- and inter-assay precision of the present method. Three replicate analyses of the samples were performed on each of three days; the results are summarized in Table 1. The recovery of ustiloxin A ranged from 89% to 96%, demonstrating the efficiency of the present extraction and purification procedure. The precision values of ustiloxin A were satisfactory at the 10 mg/kg level, while the relative standard deviation (RSD, (%)) for inter-assay experiment at 2.5 mg/kg was 19.8. Based on the present results, the limit of quantitation for ustiloxin A in forage rice silage was estimated to be 2.5 mg/kg.

The ustiloxin A concentrations of seven silage samples

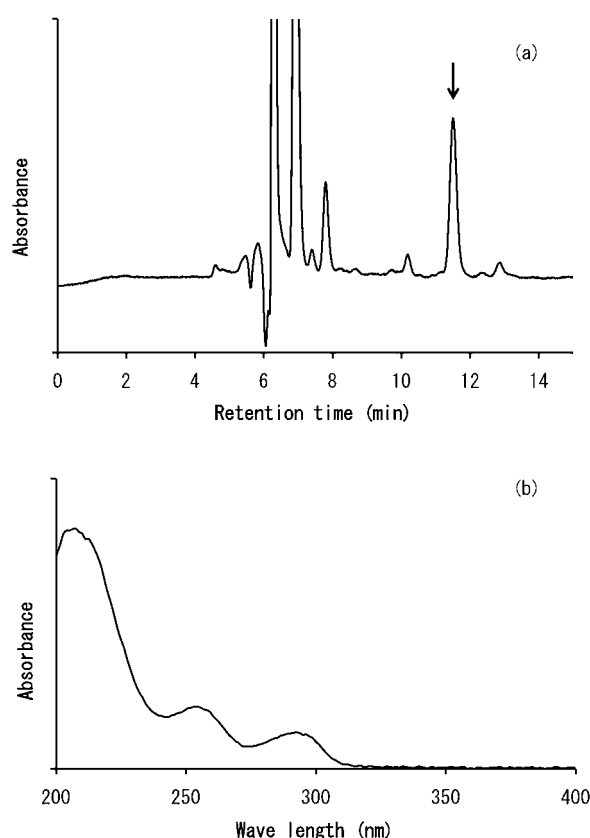


Fig. 2. Typical HPLC chromatogram (absorption at 254 nm) of a crude ustiloxin A-containing fraction prepared from false smut balls (a). The arrow indicates the peak of ustiloxin A. This peak was identified as ustiloxin A by its specific UV absorption spectrum (b).

prepared from *U. virens*-infected forage rice were determined by the present method. As shown in Table 2, ustiloxin A concentrations in silage samples heavily contaminated with false smut balls ranged from 8 to 26 mg/kg, while slightly infected samples contained less than the quantitation limit of ustiloxin A. This result indicates that the present analytical method for determining ustiloxin A in silage samples can effectively detect ustiloxin A contamina-

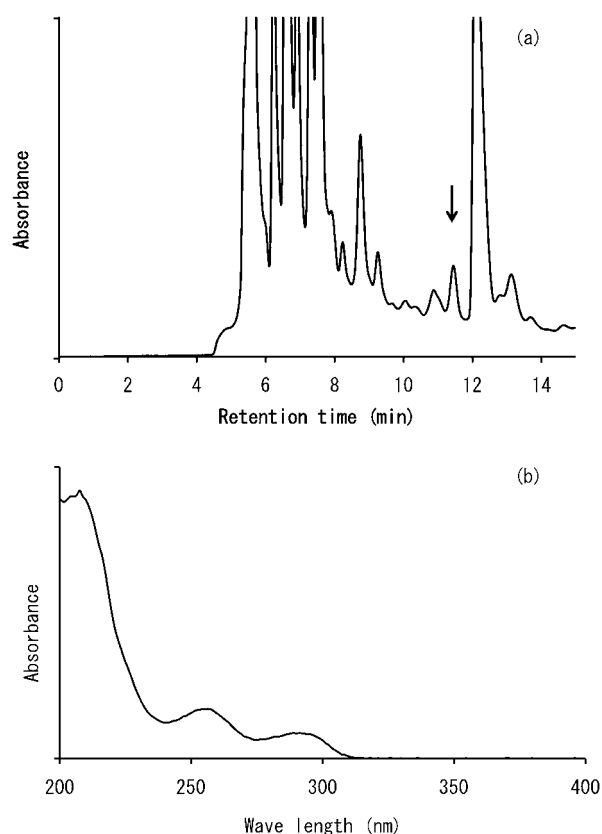


Fig. 3. Typical HPLC chromatogram (absorption at 254 nm) of the extract of the forage rice silage fortified with ustiloxin A (a). The arrow indicates the peak of ustiloxin A. This peak was identified as ustiloxin A by its specific UV absorption spectrum (b).

tion in forage rice silage without counting the number of smut balls.

To the best of our knowledge, this is the first reported method for determining ustiloxin A in forage rice silage. The present method may be useful for detecting contamination by false smut balls in forage rice silage.

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Table 1. Precision of the determination of ustiloxin A in forage rice silage by the present HPLC method

Spiked level 10 mg/kg		Spiked level 2.5 mg/kg	
Recovery (%)	96	Recovery (%)	89
RSD* (%)		RSD (%)	
inter-assay	10.4	inter-assay	19.8
intra-assay	6.61	intra-assay	11.9

\* Relative standard deviation.

Table 2. Ustiloxin A concentrations in the forage rice silage samples prepared from *Ustilaginoidea virens*-infected forage rice

No.	Degree of infection	Ustiloxin A concentration (mg/kg)
1	High	7.7
2	High	14
3	High	26
4	Low	< 2.5
5	Low	< 2.5
6	Low	< 2.5
7	Low	< 2.5

## REFERENCES

1. Koiso, Y., Li, Y., Iwasaki, S., Hanaoka, K., Kobayashi, T., Sonoda, R., Fujita, Y., Yaegashi, H. and Sato, Z. 1994. Ustiloxins, antimitotic cyclic peptides from false smut balls on rice panicles caused by *Ustilaginoidea virens*. *J. Antibiot.* **47**: 765–773.
2. Koiso, Y., Natori, M., Iwasaki, S., Sato, S., Sonoda, R., Fujita, Y., Yaegashi, H. and Sato, Z. 1992. Ustiloxin: a phytotoxin and a mycotoxin from false smut balls on rice panicles. *Tetrahedron Lett.* **33**: 4157–4160.
3. Koyama, K. and Natori, S. 1988. Further characterization of seven bis(naphtho- $\gamma$ -pyrone) congeners of ustilaginoigins, coloring matters of *Claviceps virens* (*Ustilaginoidea virens*). *Chem. Pharm. Bull.* **36**: 146–152.
4. Koyama, K., Ominato, K., Natori, S., Tashiro, T. and Tsuruo, T. 1988. Cytotoxicity and antitumor activities of fungal bis(naphtho- $\gamma$ -pyrone) derivatives. *J. Pharmacodyn.* **11**: 630–635.
5. Luduena, R.F., Roach M.C., Prasad, V., Banerjee, M., Koiso, Y., Li, Y. and Iwasaki, S. 1994. Interaction of ustiloxin A with bovine brain tubulin. *Biochem. Pharmacol.* **47**: 1593–1599.
6. Nakamura, K., Izumiyama, N., Ohtsubo, K., Koiso, Y., Iwasaki, S., Sonoda, R., Fujita, Y., Yaegashi, H. and Sato, Z. 1994. "Lupinosis"-like lesions in mice caused by ustiloxin, produced by *Ustilaginoidea virens*: a morphological study. *Nat. Toxins* **2**: 22–28.
7. Suwa, M. 1915. Experimental *Ustilaginoidea* toxicosis. *Igaku Chuo Zasshi* **13**: 661–686 (in Japanese).