한국가축위생학회지 제42권 제3호 (2019)
Korean J Vet Serv, 2019, 42(3), 127-133
ISSN 1225-6552, eISSN 2287-7630
https://doi.org/10.7853/kjvs.2019.42.3.127

Korean Journal of
Veterinary Service
Available online at http://kjves.org

< Original Article >

Studies on mycotoxins using LC-MS/MS for the forage produced in Incheon

Do Kyung Ra*, Jae Yeon Choi, Ju Ho Lee, Ji Hyun Nam, Jeoung Gu Lee, Sung Mo Lee

Incheon Metropolitan City Institute of Health & Environment, Incheon 22320, Korea

(Received 12 December 2018; revised 27 June 2019; accepted 2 July 2019)

Abstract

The purpose of this study was to investigate the contamination level of representative mycotoxins that have adverse effects on livestock by using LC-MS/MS method and to utilize the results as basic data for the establishment of quality control system for feed, and to provide information on production and storage. A total of nine mycotoxins, including aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, ochratoxin A, fumonisin B_1, fumonisin B_2, deoxynivalenol (DON), zearalenone (ZEN) were simultaneously analyzed in LC-MS/MS under ESI positive mode. Fumonisin B_1 and fumonisin B_2 were detected from 3 cases of 75 forage produced in Incheon area, the detection rate was 4.0%. The detection concentration was 0.01～0.02 mg/kg, which was lower than the domestic recommended limit. Fumonisins were detected in a slightly different manner from the results of mycotoxin studies reported in Korea, which is attributed to the high temperature and dry summer weather of the year. The result of LC-MS/MS method performance of 9 mycotoxins, the recovery of DON was quite low as 41.53±3.91% that is not suitable for simultaneous analysis. This is probably due to that the extract solution used in this study was not suitable for the extraction of DON, along with the characteristics of a very dry forage. For the study of mycotoxins in Incheon area forage for the first time, further investigation is needed for the safe supply of livestock products.

Key words : Forage, LC-MS/MS, Mycotoxin, Fumonisin

INTRODUCTION

Domestic consumption of livestock products is steadily increasing with economic growth, and per capita consumption has increased by 1.4 to 2 times over the past 20 years, according to data from the National Statistical Office. The domestic livestock industry has also been growing due to the continuous increase in demand, but there is an unstable situation because of the loss of productivity due to the outbreak of malignant livestock diseases, managerial instability from animal food price instability, and free trade agreement with powerful nation like Australia, Canada and New Zealand. The Government have pursued various policies to solve this problem. As part of this policy, in 1997 the government began supporting the production of raw rice hay bailing in order to establish a stable feed supply system, and the policy has continued by 2017 to produce goood quality of forages with a goal of 90% self-sufficiency rate (Ministry of Food, Agriculture, Forestry and Fisheries, 2014).

Forage can be contaminated with fungi at several stages, during which it grows as a pre-harvest crop, during which is made of after harvest, and until it is stored on a farm after purchase (Auerbach, 2003; Fink-Fremmels, 2005). It is reported that around 25% of crops were affected by the mycotoxin each year all over the world (CAST, 1989), and a high level of contamination of mycotoxins in Asian food crops was found to be 30% in aflatoxin, 69% in Fusarium, 83% in DON and 54%

*Corresponding author: Do Kyung Ra, Tel. +82-32-440-5573, Fax. +82-32-440-5582, E-mail. rara9292@korea.kr

in ZEN in 2016 (Biomin, 2016).

If the livestock feed for fungal contaminated forages such as hay, rice straw, and sailage, they once lose their palatability and their intake is reduced. If they are heavily contaminated, there is a significant nutritional loss. If forage is severely contaminated with fungus, there is also a great nutritional loss in livestock. It is known that feed contaminated with fungus generally induce a decreasing productivity of livestock by 5～10%, even when mycotoxins are not found (Schatzmayr, 2005). Feeding forages contaminated with fungus can also increase stillbirth, poor growth and respiratory diseases. Fungus produce toxins as a secondary metabolite, the representative fungus that causes nutritional and physiological reduction in livestock are aflatoxin, ochratoxin, fumonisin, deoxynivalenol, zearalenone (Scudamore and Livesey, 1998; Santi et al, 2009).

There have been many studies on fungi that is contaminated with feed and the damage to livestock in advanced countries for a long time, but there have been few studies on fungi contamination of forage in Korea until recently.

The government is required to designate a test item from examination list for each type of feed in order to secure safety and manage quality of feed. Among the fungus, aflatoxin and ochratoxin were included in the examination list (Feed inspection procedure, 2013). However, fungus is not included in the essential test item of fermentation feed, alfalfa, and hay, which are highly paid to livestock, so safety verification of feed has not been carried out enough.

In addition, the mycotoxin test method listed in the feed standard analysis method is limited to the single component analysis method using HPLC or GC, respectively.

Thus, in this study, we tried to analyze the representative mycotoxins affecting livestock through LC-MS/MS simultaneous analysis, and also tried to have a basic data for the safety of forage and establishment of quality control system based on the results.

MATERIALS AND METHODS

Materials

75 forages that were produced by two companies in Ganghwa-gun in Incheon area from June 2016 to July 2017 were used in this study. Forage source was oats, barley, and IRG. Test specimens were collected at least 150 g from each bale silage within 10 days from the date of manufacture. In order to evenly collect test specimens, the mid section of the bale silage was cut, and the samples were collected by moving the collection tube to upper and lower levels.

Standards and reagents

The mycotoxins that we tried to analyze in this study were aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, ochratoxin A, fumonisin B_1, fumonisin B_2, deoxynivalenol (DON), zearalenone (ZEN), which were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Water, acetonitril, and methanol were used as HPLC level solvents and other reagents for analysis were used as analysis grade.

Mycotoxin standards are prepared by precisely weighing 9 kinds of mycotoxins and dissolving in 50% ACN to make standard stock solution at 100 μg/mL concentration. This stock solution were kept frozen at −20°C until testing.

Each stock solutions were diluted with 0.1% formic acid (FA) in DW immediately prior to analysis. Then mixed standard solution were prepared that DON and ZEN at a concentration of 2 μg/mL and the remaining materials at 1 μg/mL. Further dilutions of the mixed standard were prepared as 0.0 μg/mL, 0.025 μg/mL, 0.05 μg/mL, 0.1 μg/mL, 0.2 μg/mL, 0.4 μg/mL (0 μg/mL, 0.05 μg/mL, 0.1 μg/mL, 0.2 μg/mL, 0.4 μg/mL, 0.8 μg/mL in case of DON and ZEN).

Sample preparation

Samples were finely cut with scissors, and 2.5 g was placed in a 50 mL PP tube. 10 mL of 50% ACN containing 0.1% FA was added, followed by shaking at

3,500 rpm for 10 minutes.

After filtration, 4 mL of the filtrate was diluted with 16 mL of DW and purified by passage through a pre-activated solid-phase cartridge column (ISOLUTE® Myco SPE colume, Biotage, Sweden). Then, 2 mL of ACN containing 0.1% FA and 4 mL of methaol were sequentially passed through the cartridge and the eluate was received in the tube. The eluate was concentrated to dryness under nitrogen at 50°C, and the residue was dissolved in 1 mL of 50% methanol containing 0.1% FA and filtered through a 0.2 μm syringe filter.

Multi-mytotoxin LC-MS/MS detection parameters

UPLC-MS/MS (Quatro Primer XE Waters, USA) equipment was used for mycotoxin analysis. The MS/MS was performed with the MRM mode using positive polarity of ESI. Column, UPLC analysis conditions, other mobile phase gradient conditions and mass spectrometer parameters are shown in Table 1 and Table 2. The individual MRM transitions and analyte-related mass spectroscopy parameters are shown in Table 3.

The method performance was evaluated by determining limit of detection (LOD), limit of quantification (LOQ), recovery and repeatability. The LOD was calculated based on the equation as 3.3* (standard deviation of the response / slope of the calibration curve), and LOQ was determined as three times LOD. Recoveries and repeatability were determined using spiked samples at levels of 0.1 mg/kg.

Table 1. Condition of LC for the analysis of mycotoxin

Description	Condition		
Column	ACQUITY UPLC BEH C_{18} 1.7 μm (2.1×100 mm)		
Column temperature	35°C		
Injection volumn	7 μl		
Flow rate	0.4 ml/min		
Mobile phase	A (0.1% FA in DW) B (0.1% FA in ACN)		
Gradient	Time (min)	A (%)	B (%)
	Initial	90	10
	3	90	10
	10	30	70
	10.1	10	90
	12	10	90
	12.1	90	10
	15	90	10

RESULTS

The results of mycotoxin detection from forage

75 specimens produced in Ganghwa-gun area from June 2016 to July 2017 were examined, and the mycotoxin was detected in 3 round bale silages produced in 2016, indicating a detection rate of 4.0%. Detected my-

Table 2. Main operating parameters of LC-MS/MS

Parameters	Value
Ionization	ESI (positive)
Capillary (kV)/Extractor (V)	4.00/3.0
Temperature (Source/Desolvation)	150°C/350°C
Gas flow (Desolvation/Cone, L/hr)	800/50
Gas (Auxiliary/Collision)	N_2/Ar

Table 3. MRM transitions and analyte-related mass spectroscopy parameters

Name of mycotoxins	Parent ion (m/z)	Product ion (m/z)		Cone (V)	Collision (V)
		Quantitative	Qualitative		
Aflatoxin B_1	313.0	284.8	240.8	50	37, 23
Aflatoxin B_2	315.0	286.8	258.7	50	30, 26
Aflatoxin G_1	329.0	242.7	282.7	40	25, 25
Aflatoxin G_2	331.0	244.8	256.8	50	30, 30
Ochratoxin A	404.0	238.7	240.7	25	22, 22
Fumonisin B_1	722.2	334.10	352.15	50	40, 40
Fumonisin B_2	706.3	336.15	318.10	50	40, 40
Deoxynivalenol	297.05	249.05	231.05	20	10, 13
Zearalenone	319.2	187.0	185.0	20	10, 23

cotoxins were fumonisin B_1 and fumonisin B_2 and mycotoxins were only detected in oat among 3 kinds of forage source. From 1 round bale silage, fumonisin B_1 was detected at the level of 0.02 mg/kg and fumonisin B_2 was at the level of 0.01 mg/kg. From other 2 round bale silages, fumonisin B_1 was detected at the level of 0.01 mg/kg each (Table 4).

Table 4. Mycotoxin contamination case of forages in Incheon area

Type of forage	No of tested	No of detected	Mycotoxin & concentration (mg/kg)
Barley	20	-	-
Oat	50	3	Fumonisin B_1 0.02 mg/kg Fumonisin B_2 0.01 mg/kg Fumonisin B_1 0.01 mg/kg Fumonisin B_1 0.01 mg/kg
IRG	5	-	-
Total	75	3	

The result of LC-MS/MS method performance

Simultaneous analysis of mycotoxins in ESI positive mode showed that nine standard chromatograms were obtained within 15 minutes. The chromatogram for each standard material is shown in Fig. 1. Based on the calibration standards for each concentration, the calibration curves showed a linearity of R2≥0.99. As a result of the recovery test using four samples, it was found that

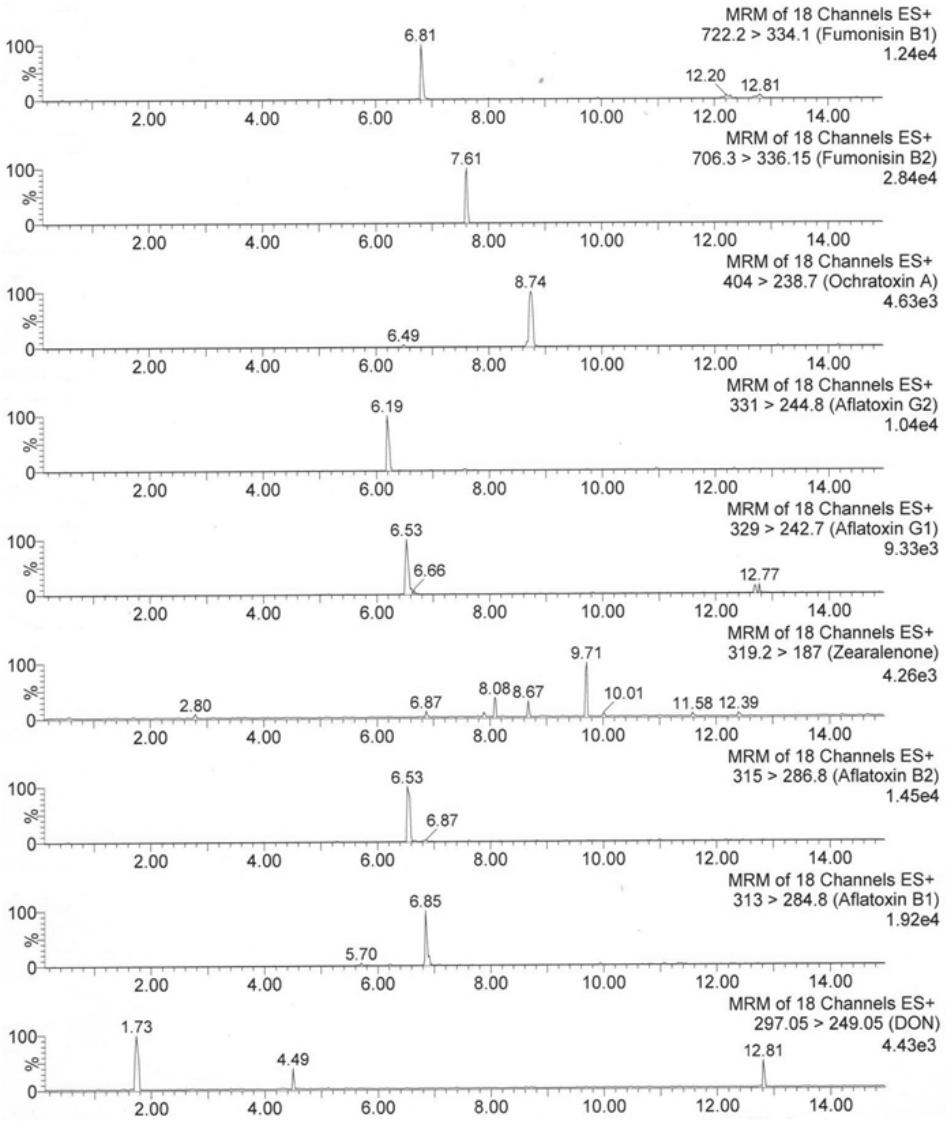

Fig. 1. The chromatogram of mycotoxin standard materials at 0.1 mg/kg (DON, ZEN: 0.2 mg/kg).

Table 5. LOD, LOQ and recovery rate of mycotoxins in forage

	Aflatoxin B_1	Aflatoxin B_2	Ochratoxin A	Aflatoxin G_1	Aflatoxin G_2	Deoxynivalenol	Fumonisin B_1	Fumonisin B_2	Zearalenone
Recovery (%, n=4)	78.05	76.20	81.88	97.98	83.83	41.53	93.65	98.68	70.50
±SD (%)	±1.79	±2.64	±1.26	±4.15	±3.90	±3.91	±4.16	±3.19	±3.35
LOD (mg/kg)	0.013	0.010	0.016	0.008	0.024	0.108	0.011	0.016	0.263
LOQ (mg/kg)	0.042	0.031	0.468	0.025	0.072	0.325	0.034	0.047	0.079

all the materials except DON had a recovery rate of 70% or more (Table 5).

DISCUSSION

Mycotoxins are toxins produced by fungi during the growth, storage and distribution of agricultural products and are stable to heat, so they are not degraded even after cooking and processing. So, people or animals who consume food or feed contaminated with mycotoxins can experience various disorders and, there has been a growing interest in the world because it is related to carcinogens such as liver cancer and esophageal cancer especially.

In 2002, the WTO published chronic contagious food contaminants, with mycotoxins as the first, phytotoxins in the second, and unbalanced diet in the third.

The fungus grows mainly in agricultural products, but when animals consume food contaminated with mycotoxins, they accumulate in the body and cause acute and chronic disorders in the animals. In addition, human can be secondly contaminated when they eat animals such as livestock products or aquatic products, so the standards for the use of animal feed as well as food have been set around the world (Kim and Jang, 1999).

At least 77 countries are involved in the regulation of mycotoxin among international trade by defining acceptable mycotoxin and concentrations in certain feeds (CAST, 2003), Korea also regulates aflatoxins (B_1, B_2, G_1, G_2) and ochratoxin A as the major mycotoxin to be controlled according to the type of feed (Control of livestock and fish feed act).

According to the range of the harmful substances in the feed and the acceptance criteria from Standards for feed (MAFRA, 2017), 50 ppb of aflatoxin (B_1+B_2+G_1+G_2) and 250 ppb of ochratoxin A are set as the minimum acceptable limits for single vegetable ingredient. Also, 4 mycotoxins such as deoxynivalenol (vomitoxin), zearalenone, fumonisin (B_1+B_2) and T-2 / HT-2 were designated as mycotoxin recommended control to manage the quality of the feed efficiently and safely.

Studies on the fungi and mycotoxin contamination of domestic forage are at an early stage, and very little is reported. As a result of regional survey on mycotoxin contamination of rice straw wrapping silage in Korea, 48% in Gyeonggi, 33% in Gangwon, 40% in Chungcheong, 50% in Yeongnam, and 57% in Honam were contaminated with mycotoxins. Of these, 44% were contaminated with only one mycotoxin, and 15% were contaminated with two types of mycotoxin (Sung, 2013). It was reported that the detected mycotoxins were zearalenone, deoxynivalenol and ochratoxin A, and the concentration of each mycotoxin was 38～847 ug/kg, 59～1914 ug/kg, and 1.2～5.8 ug/kg, respectively.

In this study, the detection rate of mycotoxins was 4.0%, which is lower than other study. The reason for this is that first of all, the test was conducted for silage within 10 days from the date of production so the silage was not exposed to environmental problems such as storage problems or mold contamination after opening, and the silage type was also different from other study.

Fumonosin detected in this study, is a mycotoxin mainly produced in corn by *Fusarium* sp., the most recently discovered of major mycotoxin. It has been found in almost all parts of the world and has been reported to occur in hot and dry year rather than in cool season (Nelson, 1993).

According to the weather data from 1973 to August 2017 of Korea Meteorological Administration, the summer of 2016 showed the highest daily maximum temperature (more than 33℃) and the highest daylight hours, and

the lowest precipitation. The sailage used in the test also had a very low moisture content of less than 6%. So fumonisin, which is rarely reported in other domestic silage mycotoxin research literature, may have been detected due to the influence of summer climate condition. In 2009, Sung et al. reported that harmful fungi secret mycotoxins such as *Fusarium* sp. and *Penicillium* sp. were detected in the study of risk analysis through genetic analysis of contaminated fungi in feed straw in Korea. This result also seem to support the detection possibility of fusmonisin.

The recommendation for fumonisin management in domestic single ingreient feed is 60 mg/kg at present, so the contamination level of 0.01～0.02 mg/kg detected in this study is very low.

However, fumonisin is classified as group 2B according to IARC (nternal Agency for Research on Cancer) because of the possibility of carcinogenesis in humans when overdosed. Fumonisin toxicity have been also reported such as hepatotoxicity in most of animals and pulmonary edema in pigs, it is necessary to pay attention to the silage that is fed to ruminants.

On the other hand, the sample treatment method used for the simultaneous analysis of mycotoxin in this study is a convenient method to extract mycotoxins at a time from the forage in a short time using a solid-state cartridge column. Mycotoxin detection is also the method that can detect all nine targets within 15 minutes under ESI positive mode using LC-MS/MS, but there is a limit in which the instrument can not use two analysis modes at a time. So the recovery rate of DON was quite low as 41.53±3.91%, which is not suitable for simultaneous analysis.

Sung et al. (2013) reported the recovery rate of DON from grains was 79.05% analyzed by ESI (−), and 10 mycotoxins except DON were analyzed by ESI (+) by HPLC-MS/MS. Based on this report, it is necessary to use a device capable of switch mode in order to perform simultaneous analysis of mycotoxins by LC-MS/MS. In addition, it was difficult to expect a high recovery rate due to the characteristics of dry forage. To overcome this, it is considered that various studies on extraction solvent application and selection of various immunoaffinity columns are necessary.

CONCLUSION

In order to increase the production of domestic self-supporting forage, it is important not only to increase the yield of the forage but also to supply good quality forage that does not cause pollution or deterioration during harvesting, storage and paying period.

The forage is paid neatly, but most of it is stored and paid. Forage type and storage environment affect the quality of the forage in a complex relationship with microorganisms. Fungi can occur during the growth of the forage crop, during the harvest and after the harvest, if only oxygen, water and temperature are adequate. Fungal contamination of the forage increases the loss of palatability, reduction of intake, nutritional loss as well as the incidence of a diseases. Some fungi produce mycotoxins that are toxic to livestock and ultimately reduce the productivity of livestock. Several factors such as temperature and humidity affect the growth of fungi and the production of toxins. It is possible that the forage will be contaminated with mycotoxin because any fungus actually grows in any suitable growing condition, whether during the growing season, packaging or storage.

Investigations on forage mycotoxin contamination have not yet been conducted enough in Korea. In order to precisely understand the contamination status of mycotoxins and to take preventive measures, various studies will be needed depending on the region, species, and climate factors.

In addition, methods to prevent mycotoxin contamination during storage and transport before feeding to livestock should be further developed, which is essential for safe livestock supply.

REFERENCES

Auerbach H. 2003. Mould growth and mycotoxin contamination of silage: sources, types and solution. pp. 247-265. In: Nyons TP, Jacques KA (ed.). Nutritional biotechnology in the feed and food industries. Nottingham Univ. Press, United Kingdom.

Biomin. 2016. Mycotoxin survey 2016 third quarter (July-September). https://nutricionanimal.info/wp-content/uploads/2016/11/Mycotoxin-Survey-Presentation-Q3-2016-1.pdf

CAST (Council for Agricultural Science & Technology). 1989.

Mycotoxins: economics and health risks. Task Force Report No.116. Ames, Iowa.

CAST (Council for Agricultural Science & Technology). 2003. Mycotoxins: lisks in plant, animal and human systems. Task Force Report No. 139. Ames, Iowa.

D'Mello JPF, Placinta CM, Macdonald AMC. 1999. Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. Anim Feed Sci Tech 80: 183-205.

Fink-Gremmels J. 2005. Mycotoxin in forages. pp. 249-268. In: Diaz DE (ed.). The mycotoxin blue book. Nottingham Univ. Press, United Kingdom.

Kim CJ, Jang JH. 1999. Food microbiology. Soohaksa, Seoul.

Ministry of Agriculture, Food and Rural Affairs. 2014. Forage supply policy direction. pp. 4-23

Ministry of Agriculture, Food and Rural Affairs. 2016. Control of livestock and fish feed act (notofication No.14481).

Ministry of Agriculture, Food and Rural Affairs. 2017. Acceptance criteria from Standards for feed (notofication No. 2017-28).

National institute of agricultural sciences, Rural development administration. 2016. Understanding and prevention of mycotoxins

National institute of food and drug safety evaluation. 2016. The method to analyze mycotoxins in food in 2016. pp. 61-63.

Nelson CE. 1993. Strategies of mold control in dairy feeds. J Dairy Sci 76: 898-902.

Nho WG, Yeo JM, Kim WY, Lee JH, Seo S, Kim MK, Seo GS. 2012. Taxonomy and Identification of fungi isolated from round bale silage. Journal of Practical Agriculture and Fisheries Research 14: 61-80.

Santi DU, Sung HG, Lee CH, Lee SY, Kim SW, Cho KJ, Ha JK. 2009. Comparative study on the aflatoxin B1 degradation ability of rumen fluid from Holstein steers and Korean native goats. L Vet Sci 10: 29-34.

Schatzmayr G. 2005. Effective solution to control mycotoxins. Hoard's West. February(25): W42-W43.

Scudamore KA, Livesey CT. 1998. Occurrence and significance of mycotoxins in forage crops and silage; a review. J Sci Food Agric 77: 1-17.

Sung HG. 2013. The studies on real condition of mycotoxin contamination in the fields before harvest and by the storage of rice straw using as roughage in Korea. J Kor Grassl Forage Sci 33(1): 21-29.

Sung HG, Kim SG, Lee JK. 2009. The presence of mycotoxigenic fungi in rice straw during the earlier spring of Korea. New paradigm for forage production in the East Asian region. Proceeding of the 3rd Korea-China-Japan joint symposium on Grassland Agriculture and Livestock Production. pp. 48-49.

Sung HG, Lee JK, Seo S. 2011. Studies on fungal contamination and mycotoxins of rice straw round bale silage. J Kor Grassl Forage Sci 31: 451-462.

Sung HG, Lee JK, Seo S, Lim DC, Kim JD. 2010. Mold growth and mycotoxin contamination of forages. J Kor Grassl Forage Sci 30: 77-88.