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A Gas Chromatography−Flame Ionization Detection Method for Detection of Fusaproliferin in Corn

XIAORONG WU† AND J. SCOTT SMITH*,‡

Department of Biological and Agricultural Engineering, 129 Seaton Hall, and The Food Science Institute, Department of Animal Sciences & Industry, 208 Call Hall, Kansas State University, Manhattan, Kansas 66506

A sensitive and accurate detection method is of great importance in monitoring fusaproliferin levels in foods and animal feeds and evaluating its potential hazard to human and animal health. Several methods have been developed to detect fusaproliferin in cereals and cereal-related products, including thin-layer chromatography, high-performance liquid chromatography, enzyme-linked immunosorbent assay, liquid chromatography−mass spectrometry (MS), gas chromatography (GC), and GC-MS. However, these detection methods either suffer from low sensitivity, need expensive instruments, or are susceptible to interfering substances in the sample matrix. The GC−flame ionization detector method developed herein is sensitive, reliable, and easy to use for detecting fusaproliferin in corn and corn-based samples. Its detection limits were 0.04 ng for standard trimethylsilyl-fusaproliferin and about 5 ppb for fusaproliferin in corn samples. The limits of quantitation of this method were 0.15 ng fusaproliferin/injection and 20 ppb of fusaproliferin in corn samples. The recovery rates of fusaproliferin from corn samples spiked with 200, 1000, and 5000 ppb standard fusaproliferin were 109, 85.7, and 98.9% on average. The repeatability of the method was acceptable when evaluated by the Horwitz equation. Of the tested corn samples, three out of five sweet corn and the three yellow corn samples were found to have low levels of fusaproliferin (9.4−45.3 ppb). A moldy corn sample had a fusaproliferin content of 297 ppb.

KEYWORDS: Fusaproliferin; *Fusarium subglutinans*; mycotoxins

INTRODUCTION

Fusaproliferin (**Figure 1**) is a relatively recently described *Fusarium* mycotoxin. The LC_{50} of fusaproliferin was 53.4 μM to brine shrimp (*Artemia salina*), and its 50% cytotoxic concentration (CC_{50}) values were 70 μM to the Lepidopteran cell line SF-9 (*Spodoptera frugiperda*) and 55 μM to human non-neoplastic B-lymphocyte cell line IARC/LCL 171 (*1*). In a toxicity test with chicken embryos, fusaproliferin was found to be the major cause for cephalic dichotomy, macrocephaly, and limb asymmetry at levels of 1−5 mM (*2*). Another study showed that fusaproliferin can interchelate with DNA, which indicated that fusaproliferin may be a more dangerous toxin than previously thought (*3*).

Strains from seven *Fusarium* species, *Fusarium proliferatum*, *Fusarium subglutinans*, *Fusarium globosum*, *Fusarium verticillioides*, *Fusarium pseudocircinatum*, *Fusarium pseudonygamai*, and *Fusarium guttiforme* were reported to be able to produce fusaproliferin (*4*). Among these species, strains of *F. proliferatum*, *F. verticillioides*, and *F. subglutinans* are common in corn and other cereals and are associated with ear rot in corn (*5*). Thus, fusaproliferin could be a common contaminant in cereal grains and stalks. The fusaproliferin in contaminated cereals and plant stalks may easily get into foods and animal feeds and negatively affect human and animal health. Fusaproliferin contamination has been reported in moldy corn samples in Italy, South Africa (*6*−*10*), Slovakia (*11*), and in a few feed corn samples in the United States (*12*). It is important to effectively gather information about the incidence and occurrence of fusaproliferin in human foods and animal feeds and thus evaluate its possible adverse health effects on humans and animals. A sensitive, simple, and reliable fusaproliferin

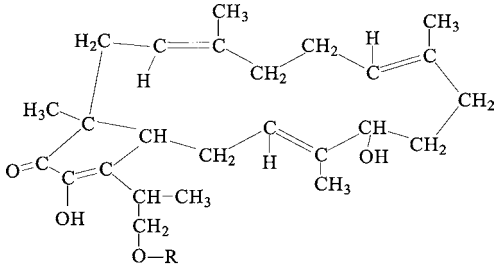

Figure 1. Chemical structure of fusaproliferin and deacylated fusaproliferin.

* To whom correspondence should be addressed. Tel: 785-532-1219. Fax: 785-532-5681. E-mail: jsschem@ksu.edu.
† Department of Biological and Agricultural Engineering.
‡ Department of Animal Sciences & Industry.

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detection method is needed to monitor fusaproliferin in cereals and cereal-based products in order to assess the exposure to fusaproliferin. Among the published methods for fusaproliferin detection, thin-layer chromatography (TLC) is the simplest and most inexpensive method. The disadvantages of TLC are its high detection limit (0.5−1 ppm), difficulty in verifying the separated spots, and low repeatability (*1*, *7*, *9*). The most commonly used methods for fusaproliferin detection utilize high-performance liquid chromatography (HPLC), because these methods have reasonably low detection limits (about 100 ppb) and good repeatability. The differences in detection limits among different HPLC methods may come from differences in sample preparation procedures (*1*, *6*, *7*, *9*, *13*). The accuracy and reliability of an HPLC method may be affected by interfering substances in the sample matrix. Liquid chromatography−mass spectrometry (LC-MS) methods are more sensitive than HPLC, usually have lower detection limits (1 ppb), and can verify the identities of the separated compounds (*14*). Recently, a gas chromatography−mass spectrometry (GC-MS) method for analyzing fusaproliferin and some other *Fusarium* mycotoxins was published, which had a detection limit of 50 ppb and recovery rates between 60.4 and 62.9% (*15*).

Other than these chromatographical methods, Monti and co-workers (*16*) developed an enzyme-linked immunosorbent assay method using polyclonal antibodies to detect fusaproliferin. However, the detection limit of this method is only 10 ppm (10 μg fusaproliferin/mL), which is not sensitive enough for many applications. Ritieni and co-workers (*6*) used GC to qualitatively verify the existence of fusaproliferin. However, because fusaproliferin is not volatile, it decomposes due to pyrolysis from the high temperature used in GC (*4*) and thus gives inaccurate results.

The purpose of this study was to develop a simple, sensitive, and reliable GC−flame ionization detection (FID) method to accurately detect low levels of fusaproliferin in corn and corn-based products.

MATERIALS AND METHODS

Reagents. The prepared trimethylsilyl (TMS) derivatization reagent N,O-bis(trimethylsilyl)acetamide:trimethylchlorosilane:N-trimethylsilyimidazole, 3:2:3 (formerly known as Sylon BTZ) was purchased from Supelco (Bellefonte, PA). HPLC grade pyridine, toluene, heptane, hexane, methanol, and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ). Octacosane, triacontane, and dotriacontane were purchased from Sigma Aldrich (St. Louis, MO). Dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethnane (DDD), and dichlorodiphenyldichloroethylene (DDE) were obtained from Chem-Service (West Chester, PA).

Corn Samples. Five fresh sweet corn samples were purchased from a local grocery store (Manhattan, KS). Two yellow corn samples were collected from the feed mill in the Department of Animal Sciences and Industry, Kansas State University (Manhattan, KS), and two matured corn samples (one clean and one moldy) were collected from a corn field near Manhattan, KS. All of the samples were air-dried at room temperature in a chemical hood, ground to meal, and kept in sealed plastic Ziploc bags at −20 °C until analysis. The moisture contents of the dried corn samples were tested between 6.29 and 12.08% using the conventional oven method.

Fusaproliferin Standard and Internal Standard. Fusaproliferin standard was prepared in our laboratory and verified by ^{1}H NMR, GC-MS, and LC-MS (*4*). The standard was kept as a dry powder in 5 mL vials (1.0 mg/vial) at −20 °C. Stock solutions of fusaproliferin standard (1000 and 100 ppm) were prepared by dissolving the dry film standard in methanol and storing at −20 °C.

DDT, DDD, DDE, octacosane, triacontane, and dotriacontane were kept as 100 ppm stock solutions and were tested at concentrations of 20 ppm for their appropriateness as internal standards for the determination of TMS-fusaproliferin in corn samples.

Conditions for TMS Derivatization of Fusaproliferin. Samples or standards containing fusaproliferin were derivatized in a mixture of 100 μL of pyridine, 100 μL of TMS derivatization reagent, 100 μL of 100 ppm dotriacontane in toluene:acetonitrile (1:1, v/v) (the final concentration was 20 ppm and served as internal standard), and 200 μL of toluene:acetonitrile (1:1) solvent in reaction vials. The vials were heated at 50, 60, 70, and 80 °C for one to several hours. The derivatized samples were injected into a GC, and the chromatograms were evaluated to determine the proper derivatization conditions. These tests were repeated four times with duplicates.

Standard Curve and Detection Limit of TMS-Fusaproliferin. A series of TMS-fusaproliferin standard solutions were prepared at levels of 0.04, 0.1, 0.2, 0.5, 2.0, 5, 20, 50, 100, 200, and 500 ppm by derivatizing at 70 °C for 1 h (the optimum observed derivatizing condition) and used to construct a standard calibration curve and determine the detection limit. A 20 ppm solution of dotriacontane was included as an internal standard in all TMS-fusaproliferin standard solutions. A linear regression equation between the peak areas of TMS-fusaproliferin and the amount of fusaproliferin in ng was established and used for quantitative analysis of fusaproliferin in samples.

Procedures for Sample Preparation and Cleanup. Ten grams of ground corn culture of *F. subglutinans* E-1583 were extracted twice with 150 mL of methanol (100 mL + 50 mL) on a wrist action shaker for 30 min each and then filtered through Whatman #4 paper. Four aliquots of 10 mL methanol extracts were used for four different preparation procedures. Two aliquots were first partitioned twice with 25 mL of heptane (15 mL + 10 mL), and then, all were evaporated to dryness on a rotary evaporator at 65 °C under a vacuum of about 300 mm Hg. The residues of these samples in the evaporating flask were first dissolved in 1 mL of methanol, followed by washing the remaining residues twice with 0.5 mL of methanol. The methanol-dissolved residues were loaded either on a Sep-Pak Florisil cartridge (6 cm^3, 1 g, Waters, Milford, MA) or a Varian Bond Elut C18 cartridge (3 cm^3, 500 mg, Harbor City, CA) after being diluted with five volumes of deionized water. These cartridges were mounted on a 12-port Visiprep vacuum manifold (Supelco) and preconditioned by passing 6 mL of methanol and 6 mL of methanol−water (1 + 5, v + v) at a flow rate of about 1 mL/min (about 20 drops/min) before sample loading. The sample-loaded cartridges were dried for 1 h with forced air under vacuum (water pump) and then washed with 6 mL of heptane, followed by elution of fusaproliferin with 3 mL of acetonitrile. The partitioned heptane, the eluate from sample loading, cartridge washing heptane, and acetonitrile eluate from each cartridge were collected separately and evaporated to dryness either by N_2 (heptane and acetonitrile eluates) or on a rotary evaporator (sample loading eluates). All residues were dissolved in 0.5 mL of toluene in 4 mL vials and evaporated to dryness with N_2 to make sure that there was no water or methanol left in the evaporated residues. Then, all of the residues were derivatized with TMS reagents in a reaction mixture that contained 20 ppm of dotriacontane as described above. Fusaproliferin and interfering substances in each of the above samples were evaluated and examined by GC. The procedure was repeated five times with duplicates.

GC-FID and GC-MS Conditions. TMS-derivatized fusaproliferin was evaluated on an HP 5890 GC fitted with an FID detector (Agilent Technologies, Palo Alto, CA) and a 30 m × 0.32 mm i.d., 0.25 μm, HP-5MS [crosslinked (5% phenyl)methylpolysiloxane] capillary column (Agilent, Kennett Square, PA). The helium flow rate was 1.0 mL/min. Major components in the treated samples (with and without cartridge cleanup) were identified by GC-MS (Agilent Technologies). As mentioned in several published papers, many factors may affect the results of quantitative GC analysis (*17*−*19*). Therefore, we also evaluated the effects of liner types, sample solvents, and syringe types on the analysis of TMS-fusaproliferin by GC-FID.

Recovery Test on Spiked Samples. Ten grams of ground, fusaproliferin negative corn samples was spiked with standard fusaproliferin at levels of 200, 1000, and 5000 ppb. The spiked samples were then extracted with 50 mL of methanol on a wrist action shaker for 30 min and then filtered through Whatman #4 paper. Methanol extracts (20 mL; equivalent to 4 g of corn sample) were used in the normal sample

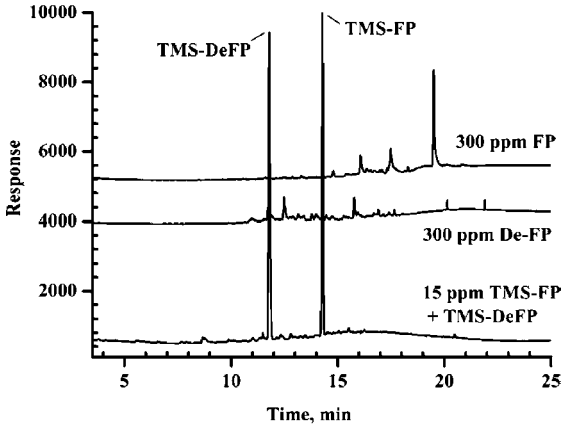

Figure 2. Irregular decomposition of fusaproliferin and deacetyl-fusaproliferin during GC process and the improved FID responses of the derivatized compounds.

preparation procedures with a C18 cartridge. The methanol extract was first evaporated to dryness on a rotary evaporator after which the residues in the evaporating flasks were dissolved three times in about 2 mL (1 + 0.5 + 0.5 mL) methanol and transferred to a 50 mL Erlenmeyer flask. The resulting 2 mL of methanol sample was then diluted with 10 mL of deionized water and loaded to a preconditioned Varian Bond Elut C18 SPE cartridge. The follow-up steps were the same as described in the sample preparation and cleanup section. The recovery tests were repeated five times. The amounts of fusaproliferin recovered were used to calculate the recovery rates (*20*, *21*). Beside recovery rates, the repeatability, specificity, and stability of the prepared samples of this method were also evaluated.

RESULTS AND DISCUSSION

Selection of TMS Derivatization Conditions. Because fusaproliferin and deacetyl-fusaproliferin are not volatile compounds, they may go through decomposition during normal GC process, affecting the determination of these compounds in a sample (*4*). According to our observation, depending on the concentration of the sample and the syringe used, 15−95% of the directly injected fusaproliferin decomposed during the GC process. Therefore, to use GC to accurately determine the fusaproliferin amount in a sample, nonvolatile fusaproliferin should first be transferred into a more volatile form. After testing various combinations of time and temperature derivatizing conditions with the TMS reagents, we found that 70 °C for 1 h was the most suitable derivatizing condition. Other derivatizing conditions (lower temperatures or shorter reaction times) resulted in incomplete derivatization, and chromatograms showed some peaks of underivatized fusaproliferin.

The GC chromatograms in **Figure 2** further demonstrate that the TMS derivatization not only overcame the problem of irregular decomposition of fusaproliferin and deacetyl-fusaproliferin during the process, it also greatly improved the FID responses of fusaproliferin and deacetyl-fusaproliferin and shortened the analysis time.

Selection of Internal Standard. Because DDT, DDD, and DDE have been used as internal standards in analysis of some trichothecenes (*22*, *23*), we evaluated these compounds and other alkanes with similar molecular weight as fusaproliferin as internal standards for detecting fusaproliferin in corn samples. Our results showed that DDT, DDD, and DDE were not suitable internal standards for the detection of TMS-fusaproliferin on the HP-5 column because of severe peak tailing and much shorter retention times. Both triacontane ($C_{30}H_{62}$) and dotriacontane ($C_{32}H_{66}$) could serve as good internal standards for TMS-fusaproliferin detection since their retention times are close to that of the TMS-fusaproliferin, and they are very stable. We chose dotriacontane ($C_{32}H_{66}$) as the internal standard because there were fewer interfering substances in the corn samples around its retention time.

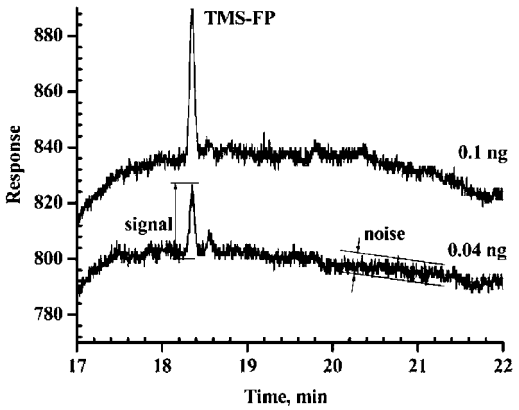

Figure 3. Detection limit of TMS-fusaproliferin by GC-FID with an HP-5MS capillary column and SGE focusliner.

Detection Limit and Standard Curve. The detection limit is of critical importance for a method, especially one used to detect toxic contaminants such as mycotoxins. Currently, several approaches, including visual inspection, signal-to-noise (S/N) ratio, and the standard deviation of the response and the slope of standard curve may be used to determine the detection limit of a method (*24*, *25*). We used the signal-to-noise ratio method to determine the detection limit of our method. That is, the lowest injected level that gives a S/N ratio of 3 was considered the detection limit. As shown in **Figure 3**, the detection limit of our method for TMS-fusaproliferin standard was 0.04 ng/injection, which corresponded to about 5 ppb of fusaproliferin in a sample.

This limit of detection was 10 times lower than that of the analytical HPLC method and the recently published GC-MS method (*15*). If the S/N ratio of 10 is taken as the limit of quantitation (LOQ) (*24*), then the LOQ of this GC method was about 0.15 ng/injection, which is equivalent to about 20 ppb of fusaproliferin in a sample. The linear regression equation between the peak area and the amount of fusaproliferin in ng was $Y = 1817.9X + 367.87$ ($R^2 = 0.9999$), where Y is the peak area of TMS-fusaproliferin and X is the amount of fusaproliferin in ng. The linear range of the standard curve was 0.1−100 ng/injection.

Procedures for Sample Preparation. Among the four different sample preparation procedures, the procedures using C18 cartridge recovered much more fusaproliferin (450−500 μg) than those using the Florisil cartridge (200−270 μg). Although heptane partition could facilitate the sample loading process and the GC chromatograms of the heptane-partitioned samples showed less well-retained impurities, we still decided to avoid the heptane partition process before rotary evaporation of the methanol extracts because up to 4.2−8.4% of the total fusaproliferin may be lost in the partition heptane. Therefore, we chose to use a C18 cartridge without the heptane partition step for sample preparation.

As shown in the chromatograms in **Figure 4**, the chosen sample preparation procedure was effective in removing both polar and nonpolar impurities, and there was essentially no detectable fusaproliferin loss during sample loading and heptane washing steps, which means that this sample preparation procedure ensures a high recovery. The main impurities removed

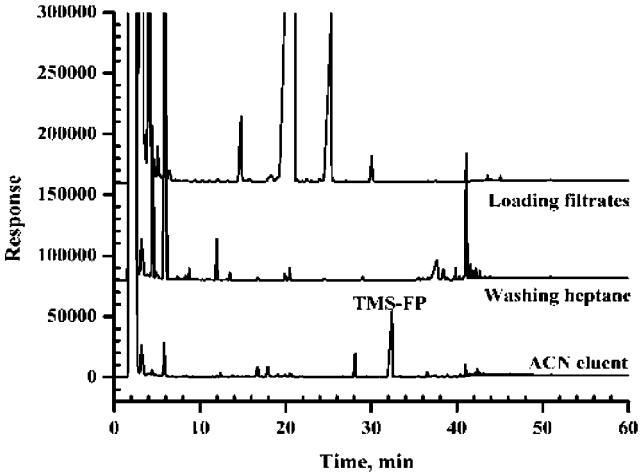

Figure 4. GC chromatograms showing impurities removed by the sample cleanup steps with the C-18 cartridge and the cleaned sample.

during the loading and heptane washing steps included large amounts of carbohydrates (D-glucitol, glucose, maltose), fatty acids (palmitic acid, oleic acid, linoleic acid), squalene, β-sitosterol, stigmasterol, and cholestenone. If these compounds had not been removed from the sample, they could have easily overloaded the capillary GC column, lengthened the retention times of later eluting peaks, and lowered the column capacity and efficiency for separation. In addition to the above possible problems, all of these compounds contain one or more groups that can be derivatized by TMS reagents, thus affecting the determination of fusaproliferin in samples.

GC-FID Conditions. An RTX-65 column has been used in GC methods to detect fusaproliferin (*4*, *6*). The RTX-65 column is good for separating water insoluble, nonpolar compounds with −OH groups, but this kind of column is not as common as the 5% diphenyl-dimethyl polysiloxane columns (HP-5, Rtx-5, DB-5, etc).

Because we planned to develop a GC method that could be used by any laboratory with a GC, we chose the most commonly used HP-5 column for our method. The optimum GC conditions for separating and detecting TMS-fusaproliferin on a GC-FID with an HP-5MS capillary column included a temperature program from 180 to 235 °C at 25 °C/min, after a 10 min holding time at 235 °C, raised temperature to 260 °C at 1 °C/min with a 2 min holding time, and finally increased the oven temperature to 330 °C at 25 °C/min and held for 18 min. The helium flow rate was constant at 1.0 mL/min, and the column head pressure was 12.3 psi. The injector liner was a SGE Focusliner. The injector temperature was 250 °C, and the detector temperature was 300 °C.

As Grob reported (*19*), the construction of GC liners has great influence on the sample transfer during splitless GC process. We tested three different types of liners: double taper splitless liner (Agilent 5181-3315), single taper liner (Agilent 5181-3316), and SGE Focus liner. Our results showed that the TMS-fusaproliferin peak areas with the SGE Focus liner were about twice those with the double taper liner, which were about twice the peak areas with the single taper liner when the same amount of TMS-fusaproliferin standard was injected. The relative standard deviation of the peak areas increased in the following order: Focusliner (7.0%) < double taper liner (14%) < single taper liner (21.4%). The Focusliner, with deactivated glass wool, can enhance the sample transfer from the injector to the column and thus ensure a lower detection limit and greatly reduce the standard deviation in GC analysis. A sample injected by syringes

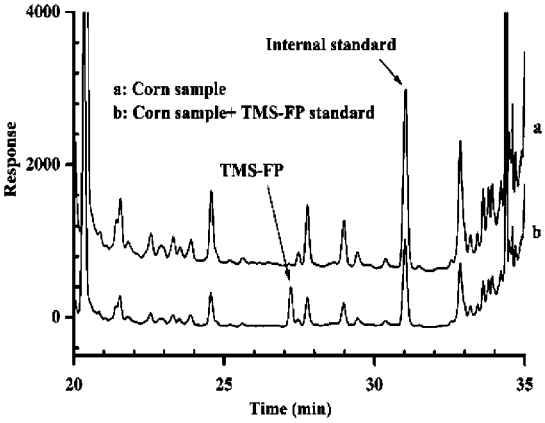

Figure 5. Verification of TMS-fusaproliferin in a sample by coinjection of TMS-fusaproliferin standard with the sample.

Table 1. Fusaproliferin Recoveries from Spiked Corn Samples

spiked level (ppb)	recovery rate ± SD (%)	RSD (%)	$0.5 \times 2^{(1-0.5\log C)}$ (%)	HORRAT
200	109.1 ± 4.41	4.04	10.19	0.40
1000	85.7 ± 1.12	1.31	8.00	0.16
5000	98.9 ± 5.87	5.87	6.34	0.95

with shorter needle lengths (43 and 50 mm) showed less heat decomposition than those injected by syringe with longer needle length (70 mm). The toluene−acetonitrile mixture (1 + 1, v + v) was a better flush solvent than the other tested solvents.

Recovery Tests. The average recoveries of fusaproliferin from spiked corn samples were 109% for 200 ppb, 85.7% for 1000 ppb, and 98.9% for 5000 ppb (**Table 1**). The high recovery rates with low standard deviation ensure accurate evaluation of fusaproliferin in the tested samples, especially at low levels.

Repeatability. The precision of the results from a method can be evaluated by the relative standard deviation (RSD or coefficient of variance, CV). The smaller the RSD value is, the more precise the results are. Usually, RSD increases as the levels of analytes decrease. The well-known Horwitz curve ($\%RSD = 2^{1-0.5\log C}$ or $\%RSD = 2C^{-0.1505}$) is used to evaluate the precision of results in mycotoxin analyses (*26*−*28*). If the RSD (%) of results from different laboratories falls below the calculated value from the above Horwitz equation, the precision of the results is considered acceptable and under statistical control. For intralaboratory data, the acceptable RSD (%) should be half of the value calculated from the Horwitz equation (*29*). The so-called Horwitz ratio (HORRAT), HORRAT = RSD (found)/RSD (calculated from Horwitz equation), is also used to evaluate the acceptability of analytical data (*28*, *30*). A HORRAT of <1 for intralaboratory data (<2 for interlaboratory data) indicates that the precision of data is acceptable, which also means that the analytical method is acceptable. The recovery data listed in **Table 1** showed that our recoveries at the three spiked levels were acceptable in precision.

Specificity. Specificity is the ability of a method to accurately and specifically detect an analyte in the presence of other interference substances in the sample matrix. In our study, we used both the standard addition methods and the GC-MS to validate the specificity of our method in detecting TMS-fusaproliferin. In the standard addition method, we verified the TMS-fusaproliferin peak by comparing the GC chromatogram of a sample with that of the sample plus 0.5 ng of standard TMS-fusaproliferin, which is clearly shown in **Figure 5**. The MS spectrum of TMS-fusaproliferin detected in the spiked corn

Table 2. Fusaproliferin Levels in Tested Corn Samples[a]

sample	SC1	SC2	SC3	SC4	SC5	YC1	YC2	YC3	MC
fusaproliferin (ppb)	38.8	28.4			9.2	41	32.6	45.3	297

[a] Note: SC, sweet corn; YC, yellow corn; and MC, moldy corn.

sample was essentially the same as that of the standard TMS-fusaproliferin, which had a parent ion at *m*/*z* 588 as well as several other major fragments at *m*/*z* 498, 456, 423, 296, and 224. The GC-MS spectrum further confirmed that the marked peak in **Figure 5** was TMS-fusaproliferin.

Stability of the Prepared Sample. The stability of the final sample solution is also critical for a GC method because many laboratories run their samples overnight by using autosamplers. Therefore, the final sample solutions should be stable at least for the normal duration of analysis or even better and be stable long enough for later verification. During our study, our final derivatized samples were kept at room temperature, and we found there were no significant differences between results on day one and those obtained 1 week later. Therefore, our sample solutions were stable for at least 1 week at room temperature.

Fusaproliferin Levels in Tested Corn Samples. Among the nine tested corn samples, no fusaproliferin was detected in two of the five sweet corn samples, and the other three sweet corn samples were detected with 9.4, 28.4, and 38.8 ppb fusaproliferin (**Table 2**). All of the four matured yellow corn samples were found fusaproliferin positive with 32.6 to 45.3 ppb in the normal corn, and 297 ppb was found in the moldy corn sample. These results indicate that our GC-FID method is suitable for detecting low levels of fusaproliferin in corn samples.

The described GC-FID method is sensitive and reliable for detecting low levels of fusaproliferin in corn. The method includes sample preparation procedures with average fusaproliferin recoveries of 109, 85.7, and 98.9% when samples are spiked with 200, 1000, and 5000 ppb of fusaproliferin standard. The repeatability of this method was acceptable when evaluated with the Horwitz equation. The limit of detection and LOQ for TMS-fusaproliferin standard by GC-FID with SGE Focusliner were 0.04 and 0.15 ng per injection, which correspond to 5 and 20 ppb of fusaproliferin in corn samples. The linear regression equation of the standard curve had a R^2 of 0.9999 and a linear range from 0.1 to 100 ng. The final derivatized sample solutions had good stability. When this method was applied to detect fusaproliferin in corn samples, we found that it was a useful method for detecting low levels of fusaproliferin in corn samples; most normal corn samples in this study were contaminated with low levels (<50 ppb) of fusaproliferin.

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