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Analysis of the *Fusarium* Mycotoxins Fusaproliferin and Trichothecenes in Grains Using Gas Chromatography−Mass Spectrometry

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A method is described using gas chromatography−mass spectrometry (GC-MS) for the simultaneous detection of the *Fusarium* mycotoxins fusaproliferin and seven trichothecenes from grains. Sample purification of the raw extract was carried out with commercial solid phase extraction columns, and the recovery of the more polar analytes was increased by rinsing the column with acetonitrile. A significant matrix effect was found for the analysis of fusaproliferin and trichothecenes; thus, the calibrants should be prepared in a blank matrix. The response was linear in the range used. The mean recovery for fusaproliferin was 60.4 or 62.9%, depending on the spiking level. With respect to the trichothecenes, the recovery was generally higher (70.2−125.3%). The method proved to be repeatable for the analysis of fusaproliferin and trichothecenes. The limit of detection for fusaproliferin in the blank matrix mixture was 50 μg/kg, and that for trichothecenes was 5−15 μg/kg. Thirty-eight Finnish grain samples were analyzed for fusaproliferin and trichothecenes with the method developed. Fusaproliferin was not detected in any of the samples. The mean levels of deoxynivalenol, 3-acetyldeoxynivalenol, nivalenol, HT-2 toxin, and T-2 toxin in Finnish grain samples were 272, 17, 150, 40, and $<$20 μg/kg, respectively.

KEYWORDS: Mycotoxins; fusaproliferin; trichothecenes; GC-MS; *Fusarium* spp.

INTRODUCTION

Mycotoxins are secondary metabolites produced by a variety of fungi under appropriate circumstances (e.g., temperature and moisture). Some of the mycotoxins can have carcinogenic, mutagenic, or teratogenic properties, and thus the presence of mycotoxins in foods and feeds can represent a health risk for both humans and animals. In addition, mycotoxins cause large economic losses on a global scale for many commercial sectors, such as crop producers and food and animal feed processors (*1*) as well as for animal breeders. In northern temperate regions, the *Fusarium* molds are probably the most important mycotoxin-producing fungi (*2*, *3*).

Fusaproliferin is a bicyclic sesterterpene derived from five isoprenic units (*4*) (**Figure 1**). Fusaproliferin is produced by at least several isolates of *Fusarium proliferatum* and *Fusarium subglutinans* (*5*, *6*), and it has been found as a natural contaminant in different commodities in Italy, the United States, and recently also in Finland (*7*, *8*; A. Ritieni, unpublished results). However, the concentration levels of this mycotoxin in foods and feeds are still unknown in most countries. Fusaproliferin has been found to be toxic to brine shrimps (*Artemia salina* L.), the lepidopteran cell line SF-9, and the IARC/LCL171 human non-neoplastic B-lymphocyte cell line (*6*). Furthermore, fusaproliferin has been shown to have teratogenic properties to chicken embryos (*9*).

Trichothecenes are a large group of sesquiterpenoids produced mainly by different *Fusarium* strains (*1*). About 170 different trichothecenes have been identified (*10*), but the most common contaminants in cultivated and wild plants are deoxynivalenol, nivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, HT-2 toxin, and T-2 toxin (*11*). All trichothecenes contain a 12,13-epoxytrichothec-9-ene ring system (*1*), and they can be divided into four subgroups, with types A and B representing the most important members (*12*). In type A trichothecenes, there is a hydrogen atom, a hydroxyl group, or an isovaleryl group in the C8-position, whereas in the type B trichothecenes there is a ketone group in the same position (**Figure 1**). Trichothecenes are found in foodstuffs all over the world (*12*), and they can cause a wide range of symptoms, including vomiting, feed refusal, diarrhea, intestinal hemorrhage, and impairment of the immune response (*13*).

More sensitive and more reliable methods are needed for the analysis of mycotoxins, because several of the existing methods have problems with the recovery of the analytes or the variation of the results (*11*, *14*−*17*). These improved methods will be important in making a proper risk assessment by determining

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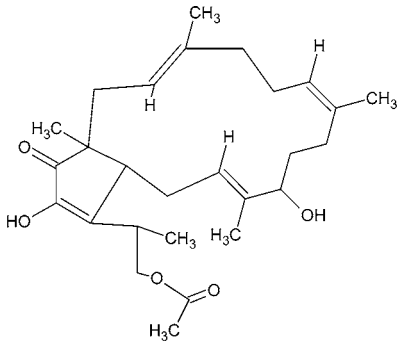

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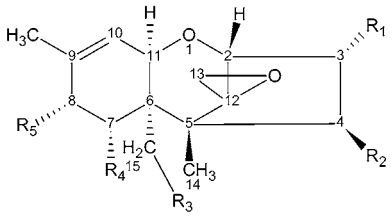

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Fusaproliferin, **1**

Neosolaniol, **2**; R_1= -OH; R_2= -OAc; R_3= -OAc; R_4= -H; R_5= -OH

Diacetoxyscirpenol, **3**; R_1= -OH; R_2= -OAc; R_3= -OAc; R_4= -H; R_5= -H

HT-2 toxin, **4**; R_1= -OH; R_2= -OH; R_3= -OAc; R_4= -H; R_5= -i-Val

T-2 toxin, **5**; R_1= -OH; R_2= -OAc; R_3= -OAc; R_4= -H; R_5= -i-Val

Deoxynivalenol, **6**; R_1= -OH; R_2= -H; R_3= -OH; R_4= -OH; R_5= -O

Nivalenol, **7**; R_1= -OH; R_2= -OH; R_3= -OH; R_4= -OH; R_5= -O

Fusarenon-X, **8**; R_1= -OH; R_2= -OAc; R_3= -OH; R_4= -OH; R_5= -O

3-acetyldeoxynivalenol, **9**; R_1= -OAc; R_2= -H; R_3= -OH; R_4= -OH; R_5= -O

Figure 1. Structures of fusaproliferin (**1**) and trichothecenes analyzed (**2**–**9**).

the levels of individual mycotoxins in different food items and estimating the average intake of these toxins. Furthermore, to be able to better assess the synergistic action of different mycotoxins, it is convenient to perform multitoxin analysis within a single run. Multitoxin analyses also have economic advantages of saving time and the use of analytical instruments.

Until now, fusaproliferin has been analyzed by using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection and/or thin-layer chromatography (TLC) (*6*−*8*, *18*−*20*). In recent years, HPLC combined with mass spectrometric detection have also been used in several studies (*21*−*23*). Only one study has mentioned the use of gas chromatography−mass spectrometry (GC-MS) for the confirmation of positive fusaproliferin results from the HPLC analysis (*7*). The extraction of the toxin has been performed with solvents of different polarities ranging from 99.5% methanol (*8*) to chloroform (*23*). The purification steps published include just filtration or liquid−liquid partitioning (*6*−*8*, *18*−*23*). This might decrease the sensitivity of the method, because the impurities still present in the sample can interfere with the detection of the analytes.

GC-MS has gained great popularity in the analysis of trichothecenes (*11*, *24*). Mass spectrometric analysis increases the sensitivity of the method considerably compared to other spectrometry-based detection techniques, because selected ion monitoring (SIM) can be used to detect only the desired ions produced by the analytes.

This study examined for the first time the quantitative analysis of fusaproliferin from grain samples using GC-MS through a modification in an existing trichothecene method. Together with fusaproliferin, seven trichothecenes (deoxynivalenol, fusarenon-X, 3-acetyldeoxynivalenol, diacetoxyscirpenol, nivalenol, HT-2, and T-2) were determined simultaneously. The developed method was applied for the analysis of Finnish grain samples from the years 2001 and 2002 and one Italian maize sample.

MATERIALS AND METHODS

Standards. Fusaproliferin was produced as by Randazzo et al. (*25*). In brief, autoclaved yellow corn kernels were inoculated with a known fusaproliferin producer, *F. proliferatum* ITEM 1494. After incubations, fusaproliferin was extracted from the matrix and purified with liquid−liquid extractions and silica column and preparative TLC. A standard solution (10 μg/mL) was prepared in methanol. Trichothecene standards deoxynivalenol, 3-acetyldeoxynivalenol, fusarenon-X, diacetoxyscirpenol, nivalenol, HT-2 toxin, T-2 toxin, and neosolaniol were purchased from Sigma (St. Louis, MO). Trichothecene standard solution mixture of deoxynivalenol, 3-acetyldeoxynivalenol, fusarenon-X, diacetoxyscirpenol, nivalenol, HT-2 toxin, T-2 toxin (1 μg/mL), and the internal standard solution (1 μg/mL neosolaniol) were prepared in acetonitrile.

Chemicals. All solvents (methanol, acetonitrile, and hexane) were of HPLC grade and purchased from J. T. Baker (Deventer, Holland). The derivatization reagent BSA/TMCS/TMSI 3:2:3 (Sylon BTZ) was purchased from Supelco (Bellefonte, PA). Deionized water was purified with a Millipore Milli-Q Plus system (Millipore, Espoo, Finland). Potassium dihydrogen phosphate (KH_2PO_4) and sodium hydroxide (NaOH), used to prepare phosphate buffer, were purchased from Merck (Darmstadt, Germany) and from Eka Nobel (Bohus, Sweden), respectively.

Samples. Thirty-eight Finnish grain samples (14 wheat, 22 barley, 1 rye, and 1 oats) were collected from different parts of Finland during the years 2001 and 2002. After harvest, the grains were air-dried to a water content of <15% to avoid fungal growth during storage. Before the analysis, the samples were ground with a laboratory mill (Bamix, Mettlen, Switzerland). One ground Italian maize sample was also analyzed for the presence of fusaproliferin to approve the applicability of the method to detect fusaproliferin in naturally contaminated samples.

Sample Preparation. The method used was a modification of the method of Eskola et al. (*26*) for the analysis of trichothecenes. For validation, 25 g of a blank cereal mixture (wheat/rye/barley, 3:2:1, w/w/w) flour was spiked with fusaproliferin standard solution and the trichothecene standard mixture. Spiking was performed at two levels: 60 μg/kg trichothecenes/600 μg/kg fusaproliferin and 700 μg/kg trichothecenes/7000 μg/kg fusaproliferin. The spiked and naturally contaminated samples (25 g) were extracted with 100 mL of 84% acetonitrile for 2 h in a Swip KS-10 horizontal shaker (Edmund Bühler, Bodelshausen, Germany) at room temperature. The extracted samples were filtered through an S&S 602 $H^1/_2$ filter paper (Schleicher & Schuell, Dassal, Germany).

After filtration, 8 mL of the crude extract was purified with a Romer MycoSep 227 column (Romer Labs Inc., Union, MO). The purified extract that passed through the column (~4−5 mL) was collected, and the procedure was repeated by washing the column with 8 mL of acetonitrile to increase the recovery of the more polar compounds. Two hundred microliters of the internal standard solution was added to 8 mL of the combined fractions. The solution was evaporated to dryness under nitrogen at 50 °C with a heating evaporation unit (Pierce, Rockford, IL). The residue was transferred with 2 × 300 μL of acetonitrile to a small vial and evaporated to dryness under nitrogen at 50 °C. The derivatization reagent (50 μL) was added and the sample left for 30 min at room temperature. The derivatized sample was diluted to 250 μL with hexane and mixed thoroughly with a Vortex-Genie 2 test tube mixer (Scientific Industries Inc., Bohemia, NY). The hexane

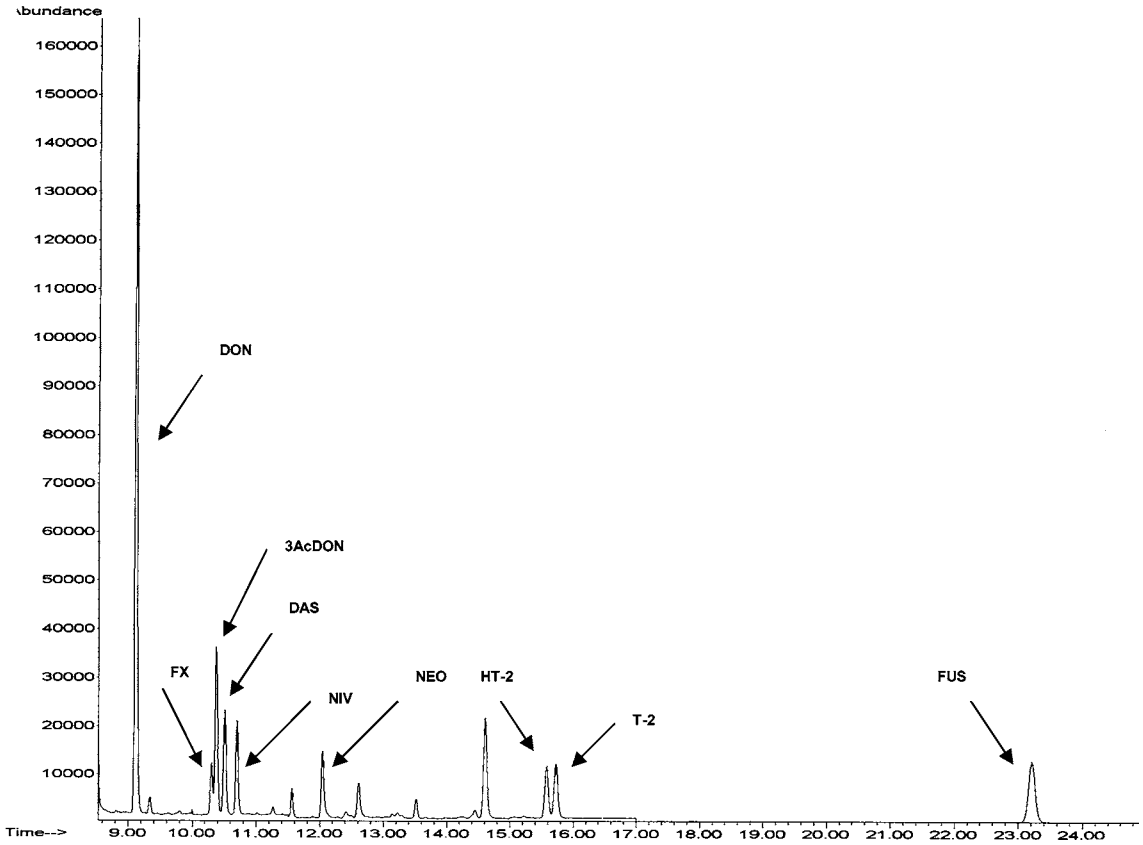

Figure 2. Total ion chromatogram of a spiked blank matrix mixture (fusaproliferin, 3000 μg/kg; trichothecenes, 300 μg/kg).

was washed with 1 mL of phosphate buffer (60 mM, pH 7) and mixed until the upper layer was clear. The hexane layer was transferred to an autosampler vial for the chromatographic analysis.

For the calibration curve, a blank matrix mixture was extracted and purified in the same way as for the samples. However, the column was flushed only once, and to 4 mL of the purified extract was added 200 μL of the internal standard solution with appropriate amounts of trichothecene standard mixture and fusaproliferin standard solution, corresponding to 10, 20, 50 100 300, 600, and 1000 μg/kg of trichothecenes and 100, 200, 500, 1000, 3000, 6000, and 10000 μg/kg for fusaproliferin.

GC-MS Analysis. Fusaproliferin was analyzed together with seven trichothecenes in the same analytical run using a Hewlett-Packard 5890 GC and a Hewlett-Packard 5971A MS (Hewlett-Packard, Palo Alto, CA). The capillary column used was a 30 $\times$ 0.25 mm i.d., 0.25 μm DB-5MS (J&W Scientific, Folsom, CA). The injection port temperature was 250 °C with injection in the splitless mode. The injection volume was 3 μL for the lower spiking level and 1 μL for the higher level. The difference in the injection volumes is due to the expansion of the solvent vapors in the injector liner. In trace analyses, large sample volumes are favored, but this may also lead to sample loss through the septum purge line (*27*). With low sample concentrations this loss is smaller than with high sample concentrations. By using different sample volumes and internal standard, the effect of this phenomenon could be decreased. The hold time of the injector was 2 min. Helium was used as carrier gas. The initial GC temperature was 80 °C, and the temperature was increased to 245 °C at 60 °C/min. After a 3 min hold time, the temperature was increased to 260 °C at 3 °C/min and finally to 270 °C at 10 °C/min and then held for 7 min. Selected ion monitoring (SIM) was used for the detection of the analytes. The ions monitored were *m*/*z* 589 and 456 (fusaproliferin), *m*/*z* 235 and 422 (deoxynivalenol), *m*/*z* 480 (fusarenon-X), *m*/*z* 392 and 377 (3-acetyldeoxynivalenol), *m*/*z* 378 (diacetoxyscirpenol), *m*/*z* 379 and 482 (nivalenol), *m*/*z* 347 and 466 (HT-2), *m*/*z* 350 and 436 (T-2), and *m*/*z* 290 (neosolaniol).

Validation. The following validation parameters were determined for the method used: selectivity, repeatability, limit of detection (LOD), limit of quantification (LOQ), recovery percent, and linearity. Eight sets of samples were analyzed, each having six replicates ($p = 8$, $n = 6$).

RESULTS AND DISCUSSION

The total ion chromatogram of a standard prepared in blank matrix mixture is presented in **Figure 2** showing the retention times of the analytes.

Selectivity. Selectivity is the effect of the background, that is, the sample matrix, on the method. The difference in the slopes of the calibration curves with and without matrix are due to the matrix effect, which is common in gas chromatographic trace analysis (*28*). The differences in the responses are caused by the adsorption of the analytes by the active sites in the GC injector and the first part of the capillary column (*29*). When matrix components are present, they compete with the analytes for these active sites, and the responses of the analytes are higher. In this study, the differences between the slopes of the calibration curves with and without blank matrix mixture were found to be statistically significant for each of the analytes (two-sided *t* test). Pettersson (*29*) also reported a normal 10−30% matrix enhancement in trichothecene analysis techniques. Especially with the MS detector and trimethylsilyl derivatives, this effect was considerable. Due to the matrix effect, the calibrants in the method described should be prepared in blank matrix, as recommended also by Pettersson (*29*).

Linearity. The method was linear for trichothecenes in the range of 10−1000 μg/kg and for fusaproliferin in the range of 100−10 000 μg/kg. The acceptable linearity of each point of the calibration curve for fusaproliferin was tested with the method of van Trijp and Roos (*30*) on each day ($p = 8$) of the validation process. A tolerance of 100 $\pm$ 10% was accepted for the separate calibration points for good linearity. On this

Table 1. Comparison of the Original (a) (*26*) and Improved (b) Methods for the Recovery-Corrected Concentrations (Micrograms per Kilogram) of the Analytes in Naturally Contaminated Samples

sample	method	deoxy-nivalenol	fusarenon-X	3-acetyldeoxy-nivalenol	diacetoxy-scirpenol	nivalenol	HT-2	T-2	fusapro-liferin
1	a	905	nd	52	nd	800	nd	nd	nd
	b	987	nd	61	nd	884	nd	nd	nd
2	a	52	nd	nd	nd	53	nd	nd	nd
	b	59	nd	nd	nd	68	nd	nd	nd
3	a	172	nd	19	nd	96	63	<20	nd
	b	152	nd	15	nd	89	82	<20	nd
4	a	108	nd	nd	nd	896	70	50	nd
	b	122	nd	nd	nd	1120	67	57	nd

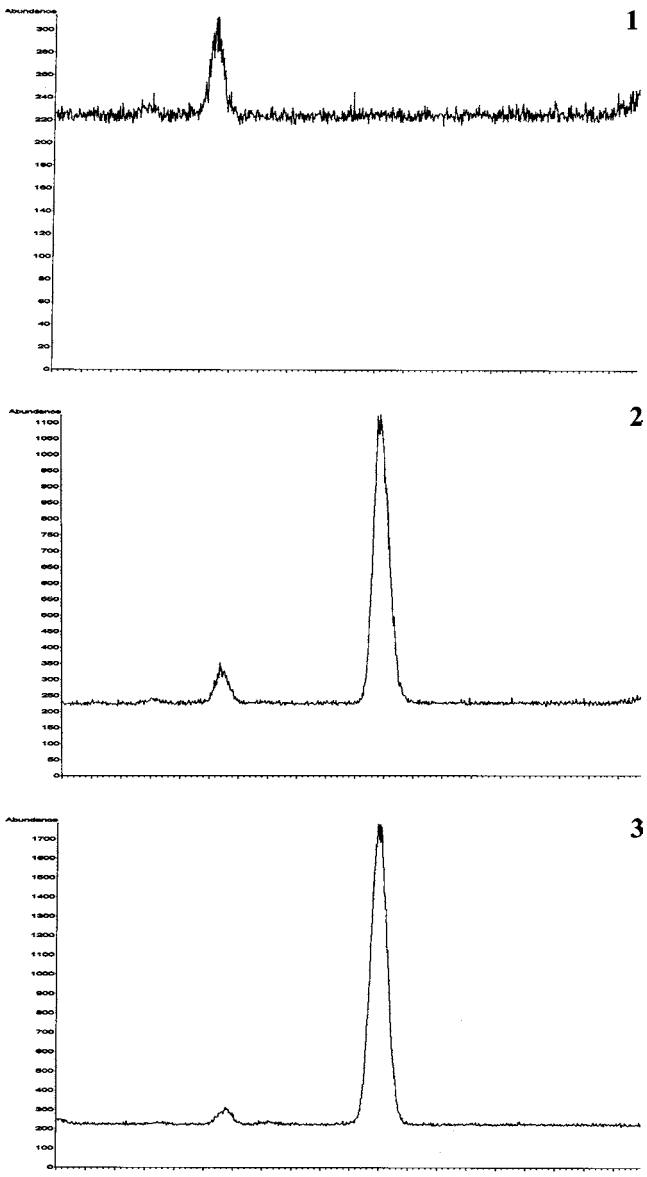

Figure 3. Extracted ion chromatograms of fusaproliferin (*m/z* 589) of a blank matrix mixture (1), a blank matrix mixture assisted standard at LOQ level (100 μg/kg) (2), and a positive Italian maize sample (3).

basis, the method can be considered as being linear for the analysis of fusaproliferin. The linearity of the method for the trichothecenes was determined earlier during our in-house validation (data not shown).

The slopes of the calibration curves for different analytes with blank matrix mixture were reproducible throughout the validation process ($p = 8$). The coefficient of variation (CV%) of the slopes for different analytes varied between 10.1% for deoxynivalenol and 21.2% for fusaproliferin. The variation in the slope values is due to the contamination of the MS ion source during the validation process.

Repeatability and Recovery. The mean recovery of fusaproliferin was 62.9% at the lower (600 μg/kg) spiking level and 60.4% at the higher (7000 μg/kg) spiking level, with coefficients of variations of 14.2 and 12.6%, respectively. The recoveries of the trichothecenes at the lower (60 μg/kg) spiking level varied between 81.7% for nivalenol and 125.3% for deoxynivalenol. At the higher (700 μg/kg) spiking level the recoveries varied between 70.2% for nivalenol and 113.2% for T-2 toxin. For trichothecenes, the coefficient of variations of the mean recoveries ranged from 4.7% for deoxynivalenol at the higher spiking level to 17.4% for nivalenol at the lower spiking level. The recoveries of the more polar compounds, fusaproliferin and nivalenol, were increased significantly by rinsing the MycoSep 227 column with acetonitrile (data not shown), but they remained lower when compared to those of the other analytes. Krska (*31*) also reported the adsorption of nivalenol into the purification column. The recovery of fusaproliferin was, however, about the same (50−60%) as in the existing methods, although many attempts with different solvents, solvent mixtures, and procedures have been made to improve the recovery (*32*; A. Ritieni, unpublished results). The reason for the relatively poor recovery of fusaproliferin might be due to the strong interactions of the analyte with the sample matrix. For this reason, more effective extraction methods [e.g., accelerated solvent extraction (ASE)] should be tried to improve the recovery of fusaproliferin.

The high recoveries (>100%) for some trichothecenes are attributable to the differences in the preparation of samples and calibrants. When the purification column was rinsed with acetonitrile and the eluates of the two elutions combined, the actual amount of sample matrix for further sample preparation was 1−2 g. With the calibrants, instead, the amount was exactly 1 g. This can, however, be compensated for by correcting the results for the recovery.

The CV% for each of the analytes highlighted the good repeatability of the method. A so-called Horwitz equation ($RSD_R = 2C^{-0.1505}$) is often used to quantify the relationship between the RSD_R (interlaboratory relative standard deviation) and analyte concentration in mycotoxin analysis (*33*). Some researchers have suggested that when applying the equation to within laboratory studies, as in our study, the goal value should be two-thirds of the RSD_R predicted from the Horwitz equation. The Horwitz equation is very useful in evaluating analytical methods (*34*) by calculating the Horwitz ratio [HORRAT = RSD_R(found)/RSD_R(predicted)] (*35*). A HORRAT value of <2

indicates that the method is acceptable, precise, and clearly under statistical control (*34*). The HORRAT values [RSD_R (predicted) = 2/3 of the RSD_R obtained from the Horwitz equation] for the analytes in this study were between 0.33 for deoxynivalenol and 1.58 for fusaproliferin, showing that the method can be considered as being acceptable for each of the analytes.

LOD and LOQ. The LOD and LOQ for the trichothecenes were determined previously during the in-house validations (LOQ = 10 μg/kg for deoxynivalenol, 3-acetyldeoxynivalenol, fusarenon-X, and diacetoxyscirpenol; = 20 μg/kg for HT-2 and T-2 toxins; = 30 μg/kg for nivalenol). For fusaproliferin, these parameters were calculated from the extracted ion chromatograms of a standard prepared in the blank matrix mixture (LOD = 3 × S/N ratio; LOQ = 2 × LOD). The calculated values were 28 and 56 μg/kg for LOD and LOQ, respectively. For practical reasons, the lowest calibrant analyzed was, however, 100 μg/kg for fusaproliferin, and it was used as the LOQ instead of the theoretical value. Correspondingly, the LOD for fusaproliferin was 50 μg/kg. The LOD values in the matrix (maize) of the previously published methods have ranged from 50 μg/kg analyzed with LC-MS (*22*) to 2.5 mg/kg with HPLC using UV detection (*7*).

Fusaproliferin in Natural Samples. To ensure the usefulness of the method for the detection of fusaproliferin in naturally contaminated grains, a positive maize sample from Italy was analyzed with the method developed. A sample earlier found to be positive for fusaproliferin (A. Ritieni, unpublished results)-with HPLC-UV was also found to be positive with the GC-MS analysis. The extracted ion chromatograms of fusaproliferin (*m*/*z* 589) for a blank matrix mixture, a standard prepared in blank matrix mixture at LOQ level (100 μg/kg), and a positive Italian sample are presented in **Figure 3**.

The 38 samples from Finland were all negative for fusaproliferin as well as for fusarenon-X and diacetoxyscirpenol. Trichothecene ranges in the samples varied from 0 to 4300 μg/kg for deoxynivalenol (mean = 272 μg/kg), from 0 to 1390 μg/kg for nivalenol (mean = 150 μg/kg), from 0 to 100 μg/kg for 3-acetyldeoxynivalenol (mean = 17 μg/kg), from 0 to 320 μg/kg for HT-2 toxin (mean = 40 μg/kg), and from 0 to 92 μg/kg for T-2 toxin (mean < 20 μg/kg). The detailed results of the trichothecene concentrations will be presented in a subsequent paper. In that paper will also be presented the contaminating *Fusarium* species determined from the samples. Neither *F. subglutinans* nor *F. proliferatum* was detected, which could explain the absence of fusaproliferin, although it is possible that also other species of *Fusarium* can produce fusaproliferin. The most prevalent species in grain samples in Finland during the recent years have been *F. avenaceum*, *F. arthrosporioides*, and *F. sporotrichioides* (*36*). Probably these species are not capable of producing fusaproliferin, at least in the growth conditions typical of Finland. Further studies are needed to investigate the toxin production of common Finnish *Fusarium* species under different climatic conditions.

The comparison of the original method developed for trichothecenes only (*26*) and the improved method for the analysis of fusaproliferin and trichothecenes was carried out by analyzing four naturally contaminated (trichothecenes) samples. With the improved method, the concentrations of the analytes were in most of the cases slightly higher than those obtained with the original method. However, the differences were not statistically significant (paired *t* test), which means that, concerning the analysis of trichothecenes, the two methods are well in agreement (**Table 1**).

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