Original Article

Isolation and determination of deoxynivalenol by reversed-phase high-pressure liquid chromatography

Abstract

Deoxynivalenol (DON) is a mycotoxin produced by food contamination. It is a pharmacologically active compound that acts on the serotonin receptor, leading to several neuroendocrine and hematological disorders. In this article we describe a simple, accurate, and sensitive method for the quantification of DON. DON was quantified using a Phenomenex® ODS analytical C18 column (150 mm × 46 mm, 5 µm) with a mobile phase composed of mixture of water-acetonitrile-methanol (5:4:1, v/v/v) at a flow rate of 1.5 ml/min and at 254 nm in an ultraviolet (UV) detector The method has been validated with isolated samples of DON and provides a tool for the control of substandard and counterfeit commercial food products.

Key words: Deoxynivalenol; *Fusarium graminearum*; HPLC; mycotoxins

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INTRODUCTION

Mycotoxins, which are by-products of fungal metabolism, can cause adverse health reactions in humans and animals consuming infected agricultural products.[1,2] Mycotoxins are usually trichothecenes, which have low molecular weight, are lipophilic in nature, and accumulate in the fat fractions of plants. They are toxic to animals.[3] Trichothecenes are a diverse range of structurally related compounds derived from 12,13-epoxytrichothec-9-ene. Two groups can be distinguished according to the chemical structure at carbon-8 (C-8): group A trichothecenes have a functional group other than a ketone at C-8 and group B trichothecenes are characterized by a ketone at C-8.[4] Deoxynivalenol (DON), also known as vomitoxin (VT) and 12,13-epoxy-3,7,15-trihydroxy-trichothec-9-en-8-one [Figure 1]), is a trichothecene mycotoxin produced by *Fusarium graminearum* and *F culmorum* and a frequent cause of toxicity after consumption of grain-based agricultural products,[5] being responsible for a variety of mycotoxicoses in animals and humans.[6]

DON is usually separated by an internal-surface reversed-phase column (octadecyl silica column) and analyzed by liquid chromatography–mass spectrometry (LC–MS), using post-derivatization with 1-anthroylnitrile.[7] Gas chromatography–mass spectroscopy (GC–MS) and GC–MSMS has been used to detect verrucarol (VER) and trichodermol (TRID) but so far there have been few reports of the use of high-pressure liquid chromatography (HPLC) and ultraviolet (UV) detector for analysis of DON. HPLC methods are more reliable and more accurate than other analytical techniques. RIA and enzyme-linked immunosorbent assay (ELISA)[8] are two methods that are most commonly used for DON analysis, but both involve multiple steps and are complicated time-consuming procedures and, besides, the results are sometimes non-reproducible. Therefore, the present study was designed to develop a methodology for estimation of DON by reversed-phase high-pressure liquid chromatography (RPHPLC) using a C-18 column and a UV detector.

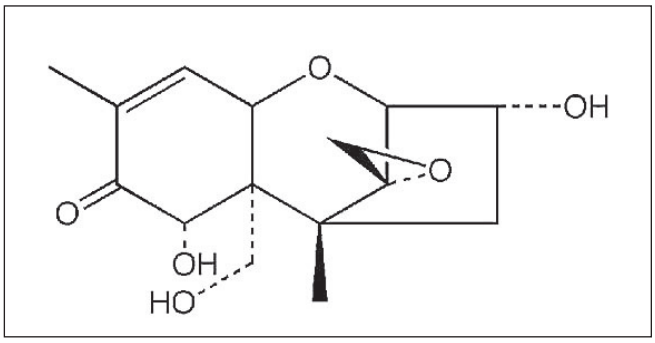

Figure 1: Structure of deoxynivalenol

MATERIALS AND METHODS

Materials

Four commercially available cereals, viz, corn, wheat, rice, and *kabli matar* (peas) were purchased from a randomly selected local vendor in Tezpur, Assam, India. Standard DON was purchased from Sigma-Aldrich (Madrid, Spain) [batch No: 303, filling code: 1364166, purity: ≥97.0% (TLC)]. The organic solvents used were HPLC grade and were purchased from Waters® (Milford, MA, USA). The other chemicals, charcoal, alumina, and silica (60–120 mesh), were obtained from Merck® (Darmstadt, Germany).

Isolates

MTCC strain 1893 *Fusarium graminearum* was obtained from the Institute of Microbial Technology (IMTECH) Chandigarh, India, and incubated as described below.

Incubation

Four conical flasks (250 ml) containing dry polished rice, corn, wheat, and peas (*kabli matar*) (100 g) were moistened with 30 ml deionized water and kept overnight in a controlled atmosphere having suitable equilibrium relative humidity (RH) (temp 25±5°C and RH 70%). The cereals were then autoclaved at 120°C for 30 min and the flasks were inoculated with 5 ml spore suspension containing approximately 10^6 macroconidia. The suspension was made from a single spore culture. The cultures were incubated (temp 20±8°C and RH 70±5%) for 3 weeks for fermentation. Five-gram samples were finely ground in a laboratory mill and thoroughly mixed before aliquots were taken for the experiment.

Extraction and cleanup

At the end of the incubation period the flasks were dried at 40±2°C in a hot-air oven for 24 h. All samples were then dry ground to the consistency of flour in a laboratory mill. Five-gram samples of ground grain were extracted with 30 ml of the extraction solvent (water:acetonitrile:methanol in a ratio of 5:4:1, v/v) in Erlenmeyer flasks, with continuous shaking for 30 min. The extraction solvent was filtered through Whatman™ No. 4 filter paper. For the cleanup process about 30 ml of each extract was placed in a 18 × 85 mm tube and filtered through a self-designed alumina–silica–activated charcoal (1:1:1) column and eluted in clear tips. The elute was evaporated to dryness on a rotary evaporator. The residue was dissolved in 1 ml of water:acetonitrile:methanol (5:4:1). Each extract was further purified by filtration through a separate Whatman™ filter (Puradisc™ 25AS, 0.45-µm pore size) and an aliquot was used for the HPLC analysis.

Instruments

A Waters® 600E system (Waters®, Milford, MA, USA) provided with a programmable UV detector was used in this study. The injector system was Rheodyne® (USA), with a maximum capacity of 20 µl and with Empower™ software. A reverse phase Phenomenex® ODS C18 column (150 mm × 46 mm, 5 µm) protected with a guard was used throughout the experiments. Chromatograms were recorded and integrated using a Waters® 745 integrator. The UV monitor was set to 254 nm, with a sensitivity of 002 or 005 absorbance units full scale. The recorder was set to 10 min (25 cm/h).

Validation studies

The rectilinear relationship between concentrations of the analytes and the UV detector response were evaluated. The concentrations used were 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 ng/ml for DON. Three different preparations of the analytical standard were analyzed in triplicate on the same day for determination of intra-day assay precision These determinations were repeated using freshly prepared standard solutions on three separate days to determine inter-day precision of analysis.

Analytical solutions and isolated sample in triplicate, freshly prepared solutions on three separate days, were used to compute the inter-day (n=6 separate determinations) and intra-day precision of the method. The stability of the analytical solutions was determined for DON at the concentrations described above for the assessment of repeatability. Analytical solutions were injected repeatedly (n=36) and RSDs % computed for the peak areas due to the respective analytes.

The optimized method for recovery was applied to the

analysis of DON content in cereal samples by spiking of DON in wheat sample (level of contamination: 1 mg DON/kg).

The performance characteristics of the method were based on the resolution between the standard DON and isolated DON and the robustness of the method as a function of small changes in the ratio of the solvent (water:acetonitrile:methanol 6:3:1 v/v) of the mobile phase, stability of analytical solutions, variation of analyst, and the effect of temperature (20–40°C) on resolution. The limits of detection and quantization for each analytes were determined as S/N of three and RSD % ≤5%, respectively.

RESULTS

Increase in the water:acetonitrile ratio resulted in an improvement in the chromatographic peak shapes. But adding small amount of methanol increased the resolution of separation of DON. The optimum ratio was water:acetonitrile:methanol of 5:4:1 (v/v/v) and the most sharp and prominent peaks were observed at this ratio. This observation may have been due to competition between the highly basic moieties of DON and the hydronium ions from the methanol and water molecules for electrostatic interaction with residual amine [Figure 2]. Further, in keeping view that instability of some silica-based columns at pH values of below 28, the method is novel that all methods are using organic solvents.

Assessment of the effect of temperature (over a range of 18–40°C) on the separation of the analyte showed that there was a gradual, albeit insignificant, decrease in retention times with increase of temperature, which did not compromise the resolution of the separation of DON. Subsequently all analyses were performed at the ambient temperature (≈20°C). The stability of analytical solutions of DON was assessed at three different concentrations (as described in the 'validation' section) and produced RSD % values (n=36 injections over 72 h) of peak areas which were typically less than 25%, 15%, and 05% at low, intermediate, and high concentrations, respectively. Thus, the solutions are sufficiently stable to justify analysis being done after preparation of fresh samples over 24-h periods. The chromatographic peak areas showed a rectilinear relationship to the concentration of the analyte within the specified ranges [Table 1] which is consistent with the expected concentrations on dilution of the innovator product of DON and isolated products. Linear regression analysis showed that the correlation coefficients (r^2) of all calibration curves were ≥0.997, with minimal variation in the slopes and intercepts [Table 2]. The performance characteristics and validation data for the method using the mobile water:acetonitrile:methanol are summarized in Table 3. The intra-day assay precision (RSD%) of peak areas for DON was limiting.

The values of recovery of DON concentrate obtained from six replicates of two samples at confidence interval at 97% probability which is accepted limit [Table 4].

DISCUSSIONS

The challenge involved in the measurement of DON in food is that the conventional methods are inaccurate.[8,9] ELISA is the established method for

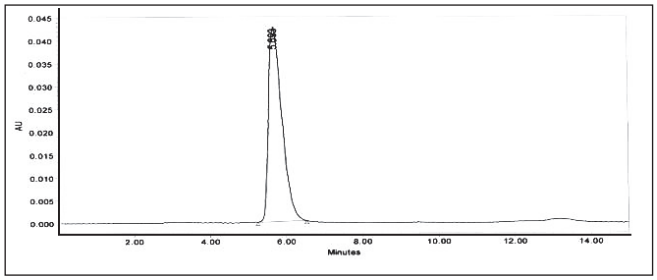

Figure 2: Chromatogram of DON toxin

Table 1: Regression analyses of calibration curves generated from the analysis of deoxynivalenol

Analyte	Range (ng/µl)	n	Slope	Intercept correlation	Coefficient (R2)
DON	10 -80	6	40.161±0.1	63.11± 4.2	0.9977 ± 0.0001

Table 2: Repeatability and sensitivity of HPLC method

	Deoxynivalenol		
	Conc.	AUC	R.S.D (%)
Inter-day precision*			
Day 1 (n= 3)	12.5ng/ml,	76321± 23.99,	1.11%
Day 2 (n=3)	25ng/ml,	221737± 26.55,	1.65%
Intra-day precision*			
Solution 1(n=3)	12.5ng/ml,	75220± 14.55,	0.561%
Solution 2(n=3)	25nµg/ml,	221737± 27.84,	0.73%
Limit of detection			
S/N (1mL)	0.04		
Limit of quantification*			
R.S.D. %≤5 (g/mL)	0.087		

*All precision data are mean± S.D. (n = 3) with R.S.D. % values in parenthesis. Precision data represents peak areas for analytes corrected for quantities of analytical standards materials used in the preparation of solutions. The concentrations in ng/ml represents the levels at which precision have been assessed. AUC: Area under Curve.

Table 3: Precision of RP-HPLC analysis of deoxynivalenol substances in isolated sample

	Deoxynivalenol
Inter-day precision*	
Mean ±S.D. (*n* = 3) (% w/w)	80.12% ± 0.66
R.S.D. %	0.70%
Intra-day precision*	
Mean ±S.D. (*n* = 3) (% w/w)	82.63% ± 0.58
R.S.D. %	0.83%

*All precision data are mean± S.D. (n = 3) with R.S.D. % values in parenthesis. Precision data represents peak areas for analytes corrected for quantities of analytical standards materials used in the preparation of solutions.

Table 4: Recoveries of deoxynivalenol for wheat and rice determined by HPLC method

Actual spiking deoxynivalenol		0.1 mg/kg	1 mg/kg
Concentration			
Analyst I*	Wheat	87.90 ± 4.50	89.67± 4.55
	Rice	86.11 ± 6.11	93.57± 6.09
Analyst 2	Wheat	86.82 ± 6.11*	87.99.± 5.90*
	Rice	86.04 ± 5.22*	94.02± 5.92**

*All precision data are mean %± S.D. (*n* = 6) Significantly different (**P*<0.05, ***P* <0.01) from sham operated control group.

measurement of DON. DON is readily oxidized to the respective quinines, which are substances containing the highly basic qunoids moiety. This interacts with residual silanols on silica-based columns.[10,11]

Pascale[7] was the first to determine T_2 toxin (which are similar to DON in chemical structure) by liquid chromatography, with fluorescence detection derivatization with 1-anthroylnitrile. Though, after derivatization the molecules became stabilized for HPLC analysis, the accuracy and sensitivity of this method were questionable.

Determinations of DON by ELISA methods are rapidly using but RPHPLC by using UV detector rapid and simple methods are unavailable. Acetyl ($CH_3C=O$) is responsible for the toxicity of DON: 3-acetyl-DON is more prevalent in Europe and 15 acetyl-DON is more prevalent in North America.[9,12] These toxins are often present at levels of 10%–20% in DON, and differ only with an acetyl group. The present investigation shows that using of CH_3OH in mobile phases provides a competing ion to reduce analyte–silanol interactions. The validation of the repeatability (inter- and intra-day precision) of the proposed HPLC method using the isolated product [Table 3] yielded RSD % values typically less than 2%. It is obvious that the difficulties in its synthesis, the absence of regulatory standards, and toxin counterfeiting have resulted in the marketing of products.

Compared with the other reported methods for estimation of DON, this method is simple and quick. A previous study showed that for isolation and increases maximum absorbance in UV range DON was passes through immunoaffinity column,[13] whereas in the present study DON was separated using alumina-silica-activated charcoal (1:1:1), which is more economic and easier.

Previously, DON was isolated by Sephadex™ LH20 column[14] and other methods, viz, solid phase extraction, liquid extraction, ion-exchange columns, immunoaffinity columns, etc.[15] The new procedure described here for isolation of DON has several advantages: it is economical, simple, and less time consuming than the previous methods for large-scale separation of DON.

HPLC methods are sensitive and more accurate compared with LC–MS and GC–MS and most manufacturers prefer the HPLC methods.[16] Besides, LC–MS and GC–MS are expensive techniques, and not all manufacturers are able to bear the costs involved. The present investigation presents an alternative to LC–MS for analysis for DON. The method has a novel approach, using an immunoaffinity column for isolation of DON.

CONCLUSIONS

DON is a potential toxin found in food grains. HPLC methods can be useful techniques for quality control and monitoring of this contamination. In this article we have described an accurate, sensitive, and easy technique to estimate DON in a food sample. The present method validation and repeatability compiles with standard. Therefore, the present investigation has importance for alternates of ELISA, LC-MS analysis for DON.

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