Work done (Final)

Major research Project

TITLE: "Serological and molecular investigation of deoxynivalenol from humans and cattle from several districts of Karnataka".

File no: 42-465/2013 SR

Introduction:

Deoxynivalenol is a widespread mycotoxin that poses serious health hazards like gastrointestinal problems and immunosuppression to humans and animals. The potential health effects of DON as well as toxicological mechanisms have not been reviewed from India. The toxin is mainly produced by *Fusarium graminearum* and *Fusarium culmorum* and belongs to the group of type B trichothecenes. Humans are chronically exposed to DON through ingested food, in particular, cereal-based products. Regulatory limits for food and feed were established by many authorities, and a recommended tolerable daily intake for humans was defined as 1 μg kg−1 bodyweight per day. The same has not been documented from India. Therefore the present research work has been completed with kind support of UGC.

Work done as per objectives

Analysis done as follows:

I) Purchasing of the following:

Instrument:

Deep Freezer -20^{oc}

Chemicals and reagents: Standard of mycotoxin Deoxynivalenol was purchased from Sigma (India). Acetonitral and water HPLC garde was purchased from Himedia, With related miscellaneous chemicals and Glass wares.

II) IHuman Urine samples analysis

Since DON is very concentrated, in the first morning urine, the same was obtained from each participating individual from different regions of the southern districts of Karnataka and stored at "20 %C until analysis. A total of 30 samples were collected using 50 mL sterile urinary Vaccutainer. The participants were not subjected to any diet restrictions before sampling. Therefore not much inconvenience or health risk was involved for the participants. Furthermore, all the participants were asked to indicate their sex as M for male, F for female, C for children and their respective age. All samples were frozen within 6 hrs after collection (Rodriguez-Carrasco *et al*., 2014).

The study was approved by the University of Mysore Human Ethical Committee, Mysuru, India.

Sample preparation

The collected urine sample were kept outside to obtain room temperature. A simple "dilute and shoot" approach was used for the analysis of urine samples. The 1 ml of urine samples centrifuged for 3 min at 5,600×g from this Five hundred microliters of the supernatant was pipetted and mixed with the same amount of dilution solvent (ACN/ water, 10:90). After appropriate mixing, 20µl of the samples were injected into the LC-MS/MS system.

LC–MS conditions

The UHPLC system consisted of an Acquity UPLC. The analytical column used was a Waters Acquity UPLC® HSS T3 2.1 × 100 mm, 1.8 µm column kept at 40 °C preceded by a Waters Acquity UPLC BEH C18 VanGuard, 1.7 µm, 2.1 mm × 5 mm precolumn. Two mobile phases were used : Mobile phase A was water, mobile phase B was Acetonitral with 0.2 % Formic acid. For the analysis in negative mode.

III) Cattle Urine samples analysis

20 cattle urine samples were collected from farmers cattle raring house/ field (Mysore, District India) and all were kept frozen at −20 °C until analysis.

Sample preparation

The collected urine sample were kept outside for thawing. 1 ml of urine samples centrifuged for 2 min at 6,600×g from this Five hundred microliters of the supernatant was pipetted and mixed with the same amount of dilution solvent (ACN/ water, 10:90). After appropriate mixing, 20µl of the samples were injected into the LC-MS/MS system.

LC–MS conditions

The UHPLC system consisted of an Acquity UPLC. The analytical column used was a Waters Acquity UPLC® HSS T3 2.1 × 100 mm, 1.8 µm column kept at 40 °C preceded by a Waters Acquity UPLC BEH C18 VanGuard, 1.7 µm, 2.1 mm × 5 mm precolumn. Two mobile phases were used : Mobile phase A was water, mobile phase B was Acetonitral with 0.2 % Formic acid. For the analysis in negative mode.

Results:

The standard Deoxynivalenol was processed for the LCMS the Deoxynivalenol conjugated with the water and formic acid molecule. The molecular weight of Deoxynivalenol was 296 and shown peak at (DON+2H_2O) and (DON + Formic Acid). This show the fragmentation pattern with the peak at m/z 331 and m/z 341 with combining to water and formic acid.

Table 1: MS parameters of the DON standard.

Analytic	Retention time (min)	Precursor ion	Product ions (m/z)
DON Standard	1.36	297 (M+H)	341 331

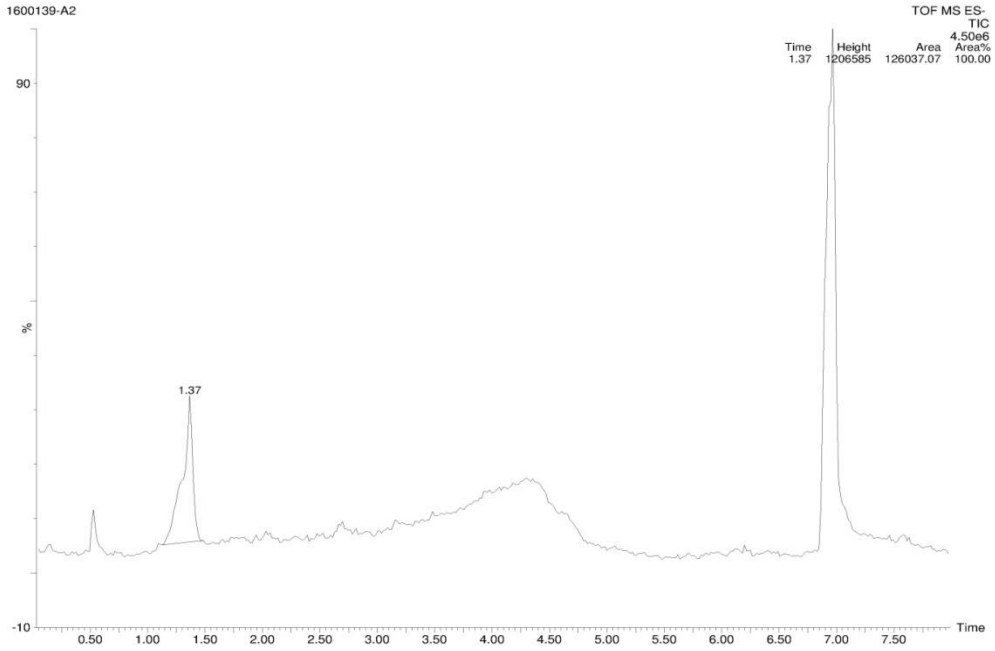

Fig: 1: LC-MS Chromatogram obtained for Deoxynivalenol standard, Retention time 1.37 min.

Table 2: MS parameters recorded for the Human Urine sample.

Analyte	Retention time (min)	Precursor ion	Product ions (m/z)
Human urine	2.4	297 (M+H)	331

The urine samples were analyzed in the above procedure. In Human urine sample the deoxynivalenol was conjugated with 2 molecules of Water (M+46) and the peak was found in 331m/z. (M+35).

Table 2.2: MS parameters recorded for the cattle urine sample.

Analyte	Retention time (min)	Precursor ion	Product ions (m/z)
Cattle	1.7	297 (M+H)	341

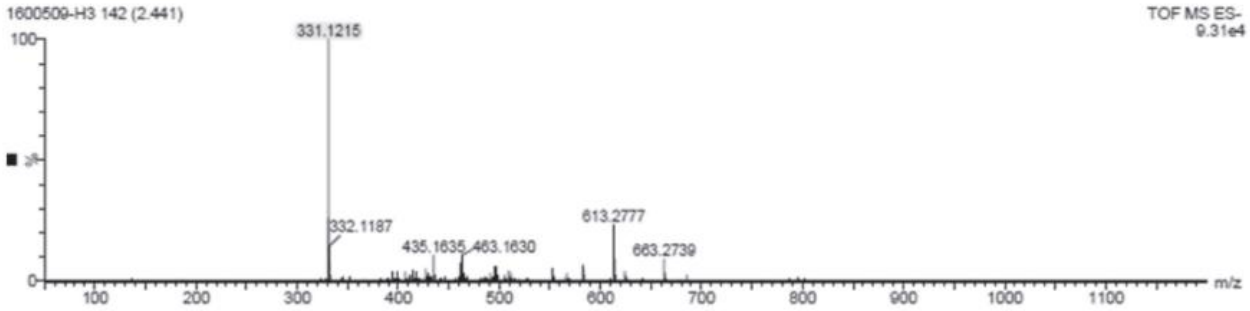

Fig. 2. LC-MS Human urine samples analysed for the presence of DON

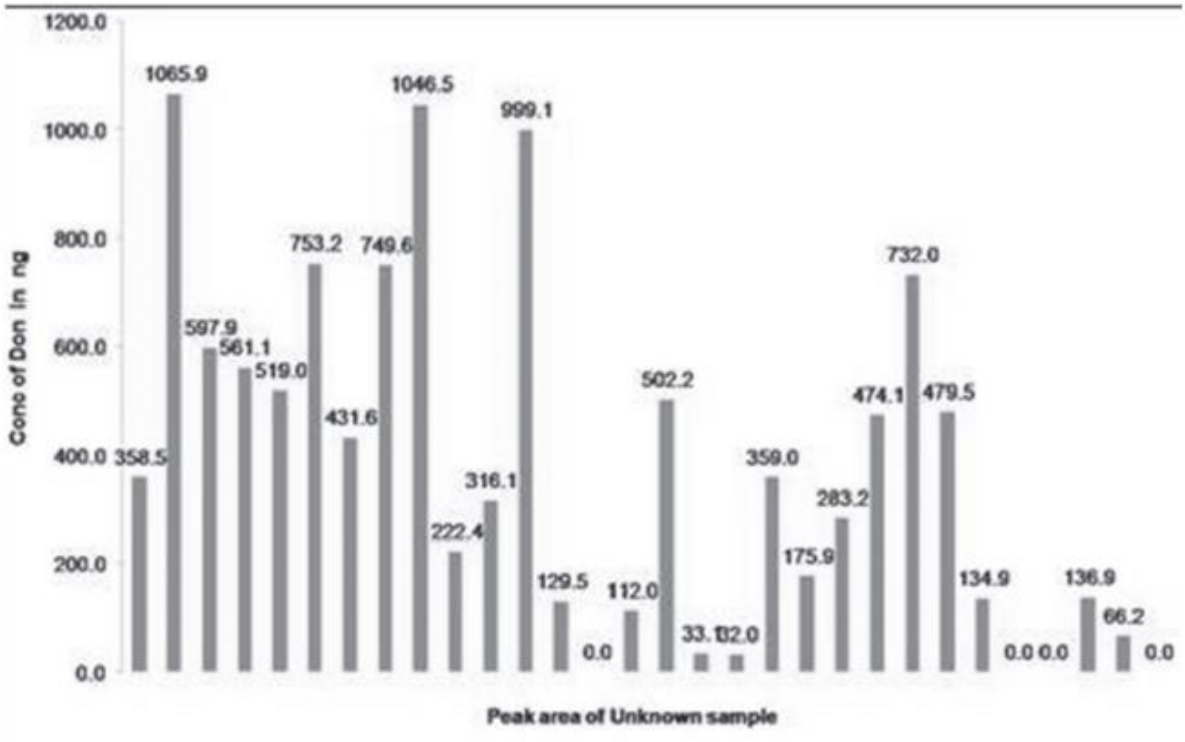

Fig. 3. The mean urinary levels of DON in urine samples analysed

Table 3. Concentration of DON and Creatinine among urine samples analyzed

Sample Number	Geographical area (All places within State of Karnataka, India)	DON Conc. in ng	Creatinine In mg/dl	Sample Number
H1	Mysore	358.5	2.2	1
H 2	Mysore	1065.9	2.89	2
H 3	Mysore	597.9	2.10	3
H 4	Mysore	561.1	1.23	4
H 5	Mysore	519.0	1.50	5
H 6	Mysore	753.2	2.79	6
H 7	Mysore	431.6	1.15	7
H 8	Mysore	749.6	0.93	8
H 9	Chamarajanagara	1046.5	2.50	9
H 10	Chamarajanagara	222.4	0.52	10
H 11	Chamarajanagara	316.1	0.64	11
H 12	Chamarajanagara	999.1	1.11	12
H 13	Chamarajanagara	129.5	0.20	13
H 14	Chamarajanagara	0.0	0.06	14
H 15	Chamarajanagara	112.0	0.19	15
H 16	Mandya	502.2	1.32	16
H 17	Mandya	33.1	0.41	17
H 18	Mandya	32.0	0.15	18
H 19	Mandya	359.0	0.44	19
H 20	Mandya	175.9	0.55	20
H 21	Mandya	283.2	1.50	21
H 22	Hassan	474.1	0.84	22
H 23	Hassan	732.0	0.28	23
H 24	Hassan	479.5	0.56	24
H 25	Hassan	134.9	0.23	25
H 26	Hassan	0.0	0.05	26
H 27	Shivmoga	0.0	0.14	27
H 28	Shivmoga	136.9	0.41	28
H 29	Shivmoga	66.2	0.40	29
H 30	Shivmoga	0.0	0.05	30

We found out from the Urine sample subjected to the LCMS, that Deoxynivalenol was present as formic acid molecule adduction. In the chromatogram of Deoxynivalenol m/z 296 showed corresponding adduct (DON + Formic Acid). This showed the fragmentation pattern with the peak at m/z 341 respectively.

Conclusion:

In the present work 30 urine samples from the volunteers from different geographical regions of Southern Karnataka were obtained and subjected to the analysis through Liquid Chromatography-Mass spectrometry. Astonishgly Deoxynivalenol was detected at a concentration of 32 to 1065.9ng/mL of human urine sample and 90% of the samples analysed were positive for Deoxynivalenol. The method used in the sample process was restricted to centrifugation and dilution process. This has given good result in the identification of deoxynivalenol in both Human and Cattle urine samples.

Further the urinary creatinine was also analysed which increased with the presence of Deoxynivalenol toxin in the urine samples. A high concentration of creatinine was reported that is 2.89 and 2.79 mg/dL of urine. **This is the first survey that has diagnosed Deoxynivalenol in human urine samples from the state of Karnataka population and also the first demonstration of creatinine imbalance with an increase in Deoxynivalenol among Indian population.** Therefore the present study is of immense help in understanding etiology of gastrointestinal disturbances and immunosupression among humans.

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