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Simple Validated Method for Determination of Deoxynivalenol and Zearalenone in Some Cereals Using High Performance Liquid Chromatography

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ABSTRACT

Deoxynivalenol (DON) and zearalenone (ZON) are two of the five most important naturally occurring mycotoxins in human foods and animal feeds produced by Fusarium species. The aim of the present study was to develop a fast extraction and a cheap method that can be applied for the determination of DON and ZON in cereals using High Performance Liquid Chromatography (HPLC) using diode array and fluorescence detectors. The method validation performance was tested on three varieties of cereal samples (maize, rice and wheat). The within-laboratory reproducibility expressed as relative standard deviation (RSDWR) was less than 10% for both mycotoxins (9% for DON and 7% for ZON). The method is linear from the Limit of Quantification (LOQ) from 200 to 2000 μg kg^{-1} levels for DON and 20 to 400 μg kg^{-1} levels for ZON. The Limits of Detections (LOD) for maize, rice and wheat samples were 24, 32 and 40 μg kg^{-1} for DON, respectively and 3 μg kg^{-1} for ZON. The measurement of uncertainty in terms of expanded uncertainty expressed as relative standard deviation (at 95% confidence level and coverage factor of k = 2) is in the range of ±31% for DON and ±25% for ZON, the method is economically useful in ZON and DON determination in safety and quality assurance programs.

Key words: Deoxynivalenol, zearalenone, method validation, cereals, mycotoxins

INTRODUCTION

Mycotoxins are secondary metabolites of molds, their exposure cause toxicity for humans, animals and crops. The most worldwide mycotoxins are aflatoxins, ochratoxins, trichothecenes, zearalenone and fumonisins (Zain, 2011). Mycotoxins can produce both acute and chronic toxicities ranging from death to other dangerous effects and may also be carcinogenic, mutagenic, teratogenic and immunosuppressive (WHO, 2001a). Their occurrence being more detected under tropical conditions due to environmental conditions, improper harvesting and bad storage (Rivka and Nachman, 2008). Fusarium mycotoxins occur worldwide in cereal grains and animal feeds and cause outbreaks of Fusarium fungal symptoms in humans and animals (Cetin and Bullerman, 2005). DON (Vomitoxin) is a type B trichothecene mycotoxin. DON is stable at 120°C and is not decomposed under mildly acidic conditions. It is thermally stable and soluble even in water and in polar solvents (WHO, 2001b). It is stable in organic solvents (Shepherd and Gilbert, 1988) with structural formula shown in Fig. 1. Ethyl acetate and acetonitrile are the most suitable

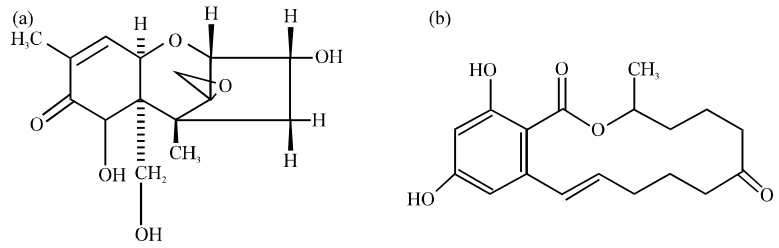

Fig. 1(a-b): Structural formula for (a) Deoxynivalenol (DON) and (b) Zearalenone (ZON)

solvents, particularly for long-term storage (Widestrand and Pettersson, 2001). The survey presented by Joint FAO (Food and Agriculture Organization)/WHO (World Health Organization) Expert Committee on Food Additives (WHO, 2001b) showed that DON was the most abundant trichothecene in cereals. Its occurrence is in cereals particularly in wheat and maize but also present in barley, flour, oats, popcorn, rice, rye, sorghum, other cereals and feed.

ZON is an estrogenic compound produced by several different *Fusarium* species. ZON is usually produced pre-harvest but can also be produced under extremely bad storage conditions (e.g., high moisture content) (EC, 2003). It is stable both during storage, milling, processing and cooking of food. It does not degrade at high temperatures due to its large lactone ring (Urry *et al.*, 1966).

This mycotoxin is practically insoluble in water, while it is soluble in aqueous alkaline solutions, ethyl acetate, acetonitrile, alcohols, diethyl ether, benzene, chloroform and methylene chloride which makes it difficult to remove and/or decompose from food (Betina, 1989). Its structural formula is given in Fig. 1. ZON has been found worldwide in a range of cereals and other crops, including wheat, barley, maize, rice, oats, sorghum and some legumes. High levels have also been reported in bananas grown in India. The level of contamination in cereal crops varies widely depending on climatic conditions. Foods reported to be contaminated wheat and corn flour, bread, breakfast cereals, noodles, biscuits, snacks and corn beer (Richard *et al.*, 2008).

The most of screening analyses employed for DON and ZON determination are included Enzyme-linked Immunosorbent Assay (ELISA) (Trucksess *et al.*, 1995; MacDougald *et al.*, 1990), Fluorescence Polarization Immunoassay (FPIA) (Maragos *et al.*, 2002; Chun *et al.*, 2009), Thin-layer Chromatography (TLC) (Fernandez *et al.*, 1994; Ostry and Skarkova, 2003) and bioassays have also been used (Koshinsky and Khachatourians, 1992; Mitterbauer *et al.*, 2003). Generally, chromatographic separation using Gas Chromatography (GC) or High-performance Liquid Chromatography (HPLC) coupled with a specific detector are employed to obtain accurate and precise results. The types of detectors normally used with such chromatographic techniques are Flame-ionization Detection (FID), Electron Capture (ECD), Ultraviolet (UV), UV diode array, fluorescence, Mass Spectrometry (MS) and tandem mass spectrometry (MS/MS) (Jimenez and Mateo, 1997; Valle-Algarra *et al.*, 2005; Czerwiecki and Wilczynska, 2003; Raters and Matissek, 2008; Visconti and Pascale, 1998; Tanaka *et al.*, 2000; Romagnoli *et al.*, 2010). The aim of this study is to develop simple, validated, fast, cheap, available and ready HPLC method for determination of DON with UV detection and ZON with fluorescence detection and one unique extraction step for the two mycotoxins. This method is considered to be a modification to the quick, easy, cheap, effective, rugged and safe method as dispersive solid phase extraction method (dSPE)

(QuEChERS method) (Anastassiades *et al*., 2003) to be applicable for determination of DON and ZON mycotoxins in cereals. There are some publications that use QuEChERS approach for the extraction of mycotoxins from different matrices using mass techniques (Rasmussen *et al*., 2010; Zachariasova *et al*., 2010; Sospedra *et al*., 2010). The method accurately and economically can determine ZON and DON with rapid and unique extraction from cereals and sufficient clean up which permit analysis of them slightly below 20 and 200 μg kg^{-1}, respectively.

MATERIALS AND METHODS

Reagents and materials: All reagents were analytical and HPLC grade. De-ionized water is generated by Milli-Q A10 FOCN53824k. Acetonitrile, dichloromethane, ethyl acetate (Lab-scan) (HPLC), assay >99%, Sodium chloride, sodium sulphate anhydrous (minimum assay after drying 99%) supplied from EL-Nasr Pharmaceutical Chemicals Company, Cairo, Egypt. Activated charcoal (CAS number: 7440-44-0) from Alpha Chemica®, India. Activated Basic Alumina (150 mesh) from Sigma Aldrich company. Celite (545) course supplied from Fluka®, Switzerland. Magnesium sulphate anhydrous supplied from J.T. Baker®, Japan. Primary Secondary Amine (PSA) bonded silica was supplied from Supelco®, USA. deoxynivalenol and zearalenone standards (99%); 100 μg mL^{-1} purchased in acetonitrile form Sigma-Aldrich company.

Standard preparation: 2 mL of 100 μg mL^{-1} in acetonitrile of DON and ZON purchased as stock standard solution is diluted in 20 mL volumetric flask with acetonitrile to obtain a diluted solution (10 μg mL^{-1}) as a working solution. Stock and working solutions were kept in refrigerator at -20°C.

Sample preparation: All stages for sample preparation are illustrated in Fig. 2 in which five grams of homogenized cereal sample weighed in 50 mL tube and then 10 mL water was added. The tube was shacked and 10 mL acetonitrile was added and the tube was reshaked. Then the mixture (6 g $MgSO_4$+1.5 g NaCl) was added. The tube was shaked and centrifugated at 4000 rpm for 5 min. The supernatant was collected into 15 mL tube containing (1 g $MgSO_4$+0.2 g PSA) and shaked for 1 min then centrifugated. For ZON determination, a volume of 4 mL from the supernatant evaporated using rotary evaporator then diluted with 2 mL with mobil phase. For DON determination, 4 mL from the supernatant was taken into SPE column and the eluent evaporated then diluted with 2 mL mobil phase.

HPLC analysis: High performance liquid chromatography instrument model HP 1100 series from Germany equipped with quaternary pump (G1311A), vacuum degasser (G1322), autosampler (G1313), photodiode array detection Agilent 1200 series (G1315D), fluorescence detector Agilent 1200 series (G1321A), analytical column: Phenomenex C18 (2) 5 μm 250×3 mm; or similar quality was used. Software: Chemistation for LC, Rev. A. 09.03 [1417]. HPLC-pump flow rate: 0.5 mL min^{-1}. DON mobile phase: Acetonitrile 10: water 90 (v/v), ZON mobile phase: Acetonitrile 50: water 50 (v/v). HPLC column: Phenomenex C18 (2) 5 μm 250×3 mm or similar specifications. Injection volume: 20 μL. Software: Chemistation for LC, Rev. A. 09.03 (1417). Detector parameters: photodiode array detection accepted at a wavelength of 220 nm and fluorescence detector at (274 nm excitation, 410 nm emission) and (236 nm excitation, 410 nm emission) used for confirmation.

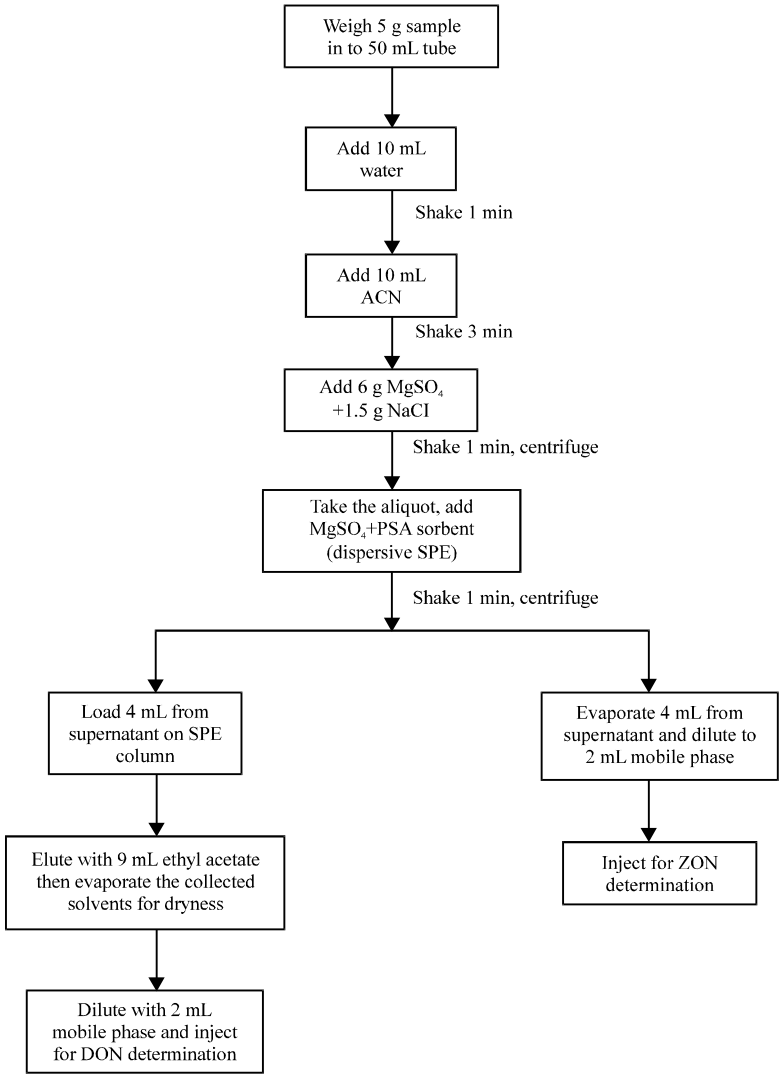

Fig. 2: Sample preparation scheme of cereal samples for DON and ZON analysis

RESULTS AND DISCUSSION

Method development and optimization: The method modifies the quick, easy, cheap, effective, rugged and safe method as Dispersive Solid Phase Extraction (DSPE) method (QuEChERS method). Water added is to homogenize the sample and also swells the cereal matrix and/or influences the analyte-matrix interactions, making the analytes more available to the extracting solvent (Turner, 2006) which facilities the extraction of DON and ZON from cereals using acetonitrile. The extraction in this method Fig. 2 (step 3 to 5) was carried out using one efficient extraction step better than those reported by Sancho *et al.* (2012) and Pleadin *et al.* (2012) and similar to those reported by Berthiller *et al.* (2005) and Soleimany *et al.* (2012a). The mean recovery reproducibility was carried out over three cereal commodities (maize, rice and wheat) for DON and ZON at spiking levels 500 and 100 μg kg^{-1}, respectively. It is relatively high (80.11% for DON and 92.93% for ZON) compared with the acceptability of analytical methods for ZON and DON, i.e., recoveries between 70 and 120% reasonable with (EC, 2005) guideline and some current

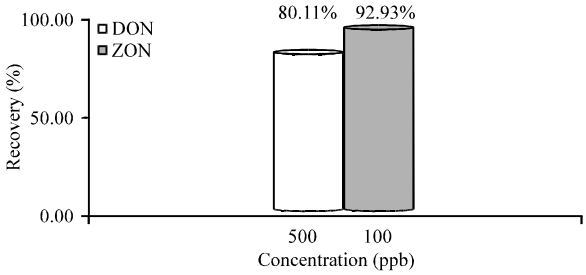

Fig. 3: Mean reproducibility recovery experiments for DON and ZON at spiking levels 500 and 100 ppb, respectively

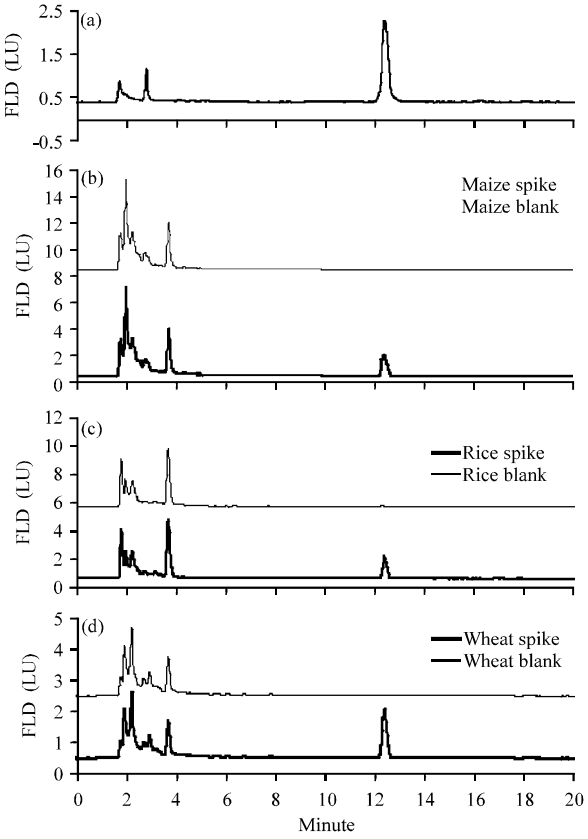

Fig. 4(a-d): Chromatograms of ZON (a) ZON standard (100 μg L^{-1}), (b) Blank and spiked maize samples, (c) Blank and spiked rice samples and (d) Blank and spiked wheat samples

studies methods (Huang *et al.*, 2012; Zaied *et al.*, 2012; Ferreira *et al.*, 2012; Soleimany *et al.*, 2012b) which represented in Fig. 3. ZON peaks appearing in Fig. 4 are separated and integrated well in the standard chromatogram (4a) and also in the three spiked cereal samples (Fig. 4b-d) even in the maize samples as animal feed contains colour pigments (yellow) and heavy matrix. This indicates that the DSPE clean up technique is enough with the more effective mobile phase elution solvents (acetonitrile: water 1/1 v/v) for minimizing the matrix interfering peaks which do not exist in rice and wheat matrix. SPE column subjected for DON clean up (steps 9 and 10) Fig. 4 was modified to provide selective absorption of matrix existing in the

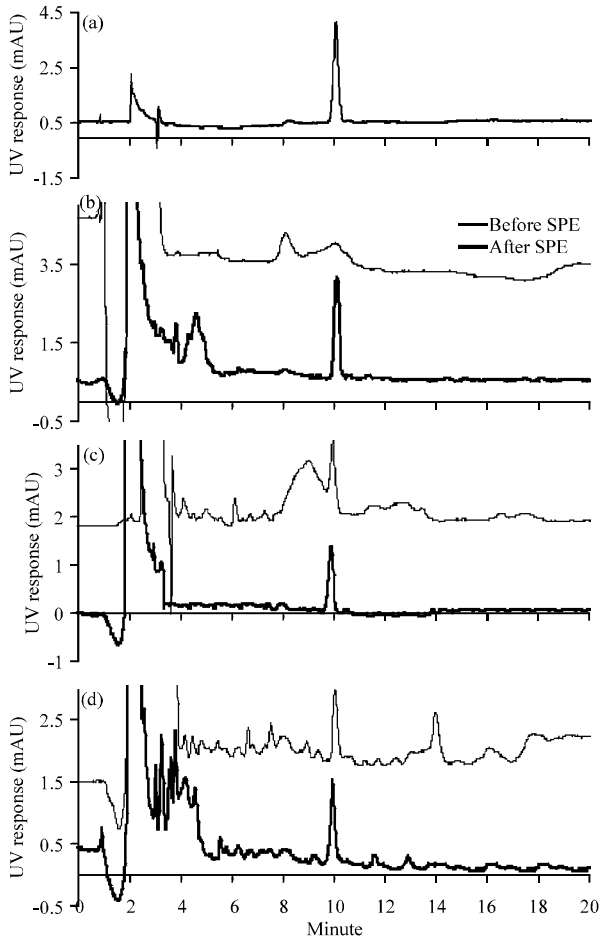

Fig. 5(a-d): Chromatograms of DON (a) DON standard (500 μg L^{-1}) in (b) Blank maize sample after and before clean up, (c) Spiked rice sample and (d) Spiked wheat sample

cereals in which the SPE column was designed to trap impurities and permit the analyte to pass through the column with more selective DON eluting solvent (ethyl acetate).

DON has been determined by HPLC with UV detection so that time consuming clean up procedure was required for the determination of DON in complex matrices such as food and feed (FAO/WHO, 2001). The efficiency and performance of this SPE column in the clean up of DON in cereals from matrices substances appears and were proved from the comparisons between the chromatograms gained from validation data after and before clean up step (Fig. 5).

Method validation: Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results. It is an integral part of any good analytical practice.

Codex (CODEX, 2001, 2006) and Eurachem guidelines (EURACHEM, 1998) were followed in performing the different validation parameters and uncertainty estimation as far as possible.

LOQ and LOD: The Limit of Quantitation (LOQ) is the minimum concentration of analyte in the test sample that can be determined with acceptable precision (repeatability) and recovery under the stated conditions of the test. The Limit of Detection (LOD) is estimated as three divided by standard deviation (3Sd) of sample blanks fortified at lowest acceptable concentration measured once each. This approach assumes that a signal more than (3Sd) above the sample blank value could only arise from the blank much less than 1% of the time and therefore is likely to have arises from something else, such as the measured. Data obtained for LOQ and LOD for ZON and DON are summarized in Table 1.

Linearity: Linearity was estimated by injection of ZON standard from 10 to 200 μg L^{-1} and DON from 150 to 2000 μg L^{-1}. The correlation coefficient must be greater than 0.99. The accepted standard linearity for ZON and DON plotted using calibration solutions and the correlation coefficient was found to be 0.99994 for ZON and 0.99995 for DON. Method linearity for ZON was checked by making recovery tests for six replicates at different four levels of 20, 100, 200 and 300 μg kg^{-1} on maize samples. Maize samples for ZON were found to be linear from the limit of quantitation 20 up to 400 μg kg^{-1}. For DON, the method linearity was checked by making recovery tests for six replicates at different four levels of 200, 500, 1000 and 1500 μg kg^{-1} on maize samples. Maize samples for DON are linear from the limit of quantitation 200 up to 1500 μg kg^{-1}. Table 1 shows an acceptable recovery values for the different four levels for ZON and DON on maize samples. Method linearity was done to give relation curves with strong correlation coefficients ($r = 0.9992$ for ZON and $r = 0.9990$ for DON) which denotes a fairly good regression.

Accuracy and precision: Accuracy is expressed in terms of (Trueness) where the trueness of a method is an expression of how close the mean of a set of results (produced by the method) is to the true value. The method trueness also was tested using Food Analysis Performance Assessment Scheme (FAPAS®) test reference material or Proficiency Test (PT). To check trueness of ZON and

Table 1: Trueness calculations, recoveries (Mean±SD, n = 6), LOD and LOQ for levels of fortification for ZON and DON from spiked cereals

Mycotoxin	Commodity	Spiking level (μg kg^{-1})	Recovery	X-T	Bias (RD %)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
ZON	Maize	20	99.33±5.530	0.13	1	3	20
		100	84.68±4.640	15.32	15		
		200	91.30±5.270	17.40	9		
		300	88.74±6.850	36.77	12		
	Rice	20	20.46±4.210	0.46	2	3	20
		100	93.43±3.370	6.57	7		
	Wheat	20	20.38±4.300	0.38	2	3	20
		100	90.58±6.360	9.42	9		
DON	Maize	200	91.49±4.030	17.02	9	24	200
		500	79.52±2.950	118.90	20		
		1000	85.18±10.34	148.20	15		
		1500	81.60±11.72	276.00	18		
	Rice	200	170.9±5.2900	29.08	15	32	200
		500	366.6±3.4500	133.40	19		
	Wheat	200	163.7±6.7000	36.36	18	40	200
		500	391.1±3.6300	109.00	20		

X: Expected value, T: Mean value, RD: Relative difference

DON method, spiked samples are used at different levels on maize, rice and wheat samples. Bias expressed as absolute relative difference percent (RD%) must not exceed 20% (≤20%). Table 1 shows the results of the trueness calculations for ZON and DON.

The two most common precision measures are (repeatability) and (reproducibility). Less precision is reflected by a larger standard deviation. Repeatability Relative Standard Deviation (RSDr) determined for both spiking levels for ZON and DON must be ≤20% and was found to be 5% for ZON and for DON is found to be 3%. Within-laboratory reproducibility (RSD_{WR}) must not exceed 20% (≤20%) and was found to be 7% for ZON and for DON was found to be 9%.

Uncertainty measurement: It is a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measure. The parameter may be, for example, a standard deviation (or a given multiple of it) or the width of a confidence interval. Uncertainty was found to be in the range of ±25% for ZON and ±31% for DON.

CONCLUSION

Because ZON and DON contamination of wheat and other cereals is widespread and considered as two of the five most important naturally occurring mycotoxins in human foods and animal feeds, reliable and accurate analytical techniques are required for quality and safety assurance of these grains and their food and feed products. For this reason, a simple and reliable high performance liquid chromatography method was validated over three cereals (maize, rice and wheat). The method resolves the two mycotoxins well and substantially reduced solvent volume which would be economically useful in ZON and DON determination in safety and quality assurance programs. The method LOQ for ZON and DON was represented at the lowest European Union MRL's (20 μg kg^{-1} for ZON and 200 μg kg^{-1} for DON) for the baby food.

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