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Determination of Zearalenone in Cereals by High-Performance Liquid Chromatography and Liquid Chromatography–Electrospray Tandem Mass Spectrometry

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ABSTRACT

Zearalenone is a mycotoxin produced mainly by fungi belonging to the genus *Fusarium* in foods and feeds. A rapid and accurate method to quantify zearalenone in cereals was described. The determination of zearalenone was performed using immunoaffinity column for clean-up, high-performance liquid chromatography (HPLC)/fluorescence for quantification and liquid chromatography/electrospray tandem mass spectrometry (LC/ESI-MS-MS) for confirmation. Cereal samples were extracted with acetonitrile-water (90:10, v/v) prior to a Vicam ZearalaTest^{TM} immunoaffinity column clean-up. Zearalenone was eluted from the column with methanol and quantified by HPLC with fluorescence detection (λ_{ex} = 274 nm, λ_{em} = 440 nm). A mobile phase of acetonitrile-water (50:50, v/v) and a flow rate of 1.0 mL/min^{-1} were used. Average recoveries of zearalenone from cereals spiked at levels of 5, 20, 100 and 200 ppb ranged from 66.4% to 96.1%. The limit of quantitation was 5 ppb. The selective determination of zearalenone was achieved by LC/MS-MS with an electrospray (ESI) ionization interface. Using the negative ion mode, the parent ion of *m/z* 317 and the product ion of *m/z* 175 were selected. The quantification limit of 3 ppb was achieved. Seven mixed cereals samples, seven corn samples, five wheat samples, five rice samples and two oat samples were analyzed. The results showed that zearalenone was detected in four corn samples ranging from 7.9 to 9.0 ppb. Using the consumption data from "Nutrition and Health Survey in Taiwan 1993~1996", the zearalenone consumption for Taiwanese adults (male and female are 0.00297 and 0.00478 μg/kg b.w. respectively) was much lower than the tolerable daily intake of 0.5 μg/kg b.w. established by JECFA.

Key words: zearalenone, cereals, HPLC, LC/ESI-MS-MS, immunoaffinity column

INTRODUCTION

Zearalenone (ZON; F-2 toxin) is a secondary fungal metabolite produced by several species of *Fusarium*, mainly by *Fusarium graminearum* and *Fusarium culmorum*[1,2]. ZON is a resorcyclic acid lactone, chemically described as 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-B-resorcyclic acid lactone[2]. Storage of *Fusarium*-contaminated cereals under humid conditions can result in high levels of ZON. Besides, ZON production has been reported on grains in the field, during harvest, commercial grain processing and storage[1-3]. It is prevalent in many cereals such as wheat, barley, oat, sorghum, corn and rice. Among different grains, corn is the most frequently contaminated one. In addition, ZON has been detected in cereals products, such as flour, malt, soybeans and beer[3]. In the past few years, the contamination of foods with ZON worldwide has been reported in many countries and the levels varied between ppb and ppm[2,3]. Most important of all, the presence ZON in cereals is often accompanied with other *Fusarium* toxins including trichothecenes and fumonisins. According to the report, rice collected from Korea was found ZON contamination with the co-occurance of aflatoxin B_1, ochratoxin (OTA), deoxynivalenol (DON) and nivalenol (NIV)[4].

ZON is a strong estrogenic compound which causes reproductive problems in animals, such as cattle, swine and poultry. Among them, pig is the most sensitive animal species[1]. ZON may affect the uterus by decreasing progesterone secretion and altering the morphology of uterine tissues[5]. In male pigs, ZON can depress serum testosterone, weight of testes and spermatogenesis. Both DON and ZON from toxic *Fusaria* have been linked to scabby grain toxicoses in USA, Japan, China and Australia, symptoms including nausea, vomiting and diarrhea[6]. Besides, ZON has been reported to be genotoxic, and to induce DNA-adduct formation[7]. In addition, ZON was also shown to be hepatotoxic[8], and may contribute to the increasing occurrence of cancer[9]. In 1993 the International Agency for Research on Cancer

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Journal of Food and Drug Analysis, Vol. 17, No. 1, 2009

(IARC) classified ZON as group 3[10].

Currently many countries such as Armenia, Austria, Belarus, Bulgaria, Chile, Columbia, France, Estonia, Hungary, Iran, Italy, Latvia, Morocco, Russia, Ukraine and Uruguay have made specific regulations for ZON in foods with maximum tolerated levels ranging from 20 to 1000 ppb[3]. European Community (EC) has made the regulatory limits as follows: 200 ppb for unprocessed maize, 100 ppb for unprocessed cereals other than maize, 50 ppb for cereal snacks and breakfast cereals, 20 ppb for processed cereal-based foods and baby foods for infants and young children[11]. Besides, the tolerable intakes have also been estimated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) as 0.5 μg/kg b.w./day[12]. Up to now, Taiwan has not made any regulatory limits for ZON.

In this paper, the determination of ZON was established using immunoaffinity column for clean-up, high-performance liquid chromatography (HPLC) for quantification and liquid chromatography/electrospray tandem mass spectrometry (LC/ESI-MS-MS) for confirmation. In addition, the established methods were applied to determine the ZON contamination in cereal samples in Taiwan.

MATERIALS AND METHODS

I. *Materials*

(I) *Sample Collection*

The samples were collected from supermarkets in Taiwan, including seven mixed cereal samples, seven corn samples, five wheat samples, five rice samples and two oat samples. The minimum sample size was 200 g.

(II) *Reagents*

The immunoaffinity columns ZearalaTest™ were purchased from Vicam (Watertown, MA, USA). The ZON standard was from Supelco (Bellefonte, PA, USA). Potassium chloride (reagent grade) was from J. T. Baker (Phillipsburg, NJ, USA). Acetonitrile and methanol (LC grade) used for the liquid chromatographic mobile phases were purchased from Merck (Darmstadt, Germany). Milli-Q plus water (Millipore, Bedford, MA, USA) was used throughout this study.

II. *Methods*

(I) *Sample Preparation*

The procedures were adopted from Visconti and Pascale with modification[2]. Cereal samples were milled before extraction. Twenty grams of test portion were weighted into a 250 mL blender jar with 2 g KCl and extracted with 50 mL of extraction solution (acetonitrile/water = 90/10, v/v). The mixture was blended for 2 min and centrifuged for 3 min at 3,000 rpm. The extract was filtered through filter paper (Whatman No.1). 10 mL filtrate was mixed with 90 mL water, followed by filtration through a glass microfibre filter.

(II) *Immunoaffinity Clean-up*

Ten milliliter of filtrate was passed through Zearalatest™ immunoaffinity columns at about 1-2 drops/s. 10 mL water was used to wash the loaded immunoaffinity column at a steady flow rate. ZON was eluted with 1 mL of methanol. The methanol eluate was filtered through a 0.45 μm microfilter and collected in a clean vial for the following HPLC/fluorescence and LC/ESI-MS-MS analysis.

(III) *HPLC/fluorescence Analysis*

The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-10AT pump, a Hitachi L-2480 fluorescence detector (Tokyo, Japan), and a Shimadzu SIL-10AF autosampler (Kyoto, Japan). Data acquisition was performed with a SISC program (Scientific Information Service Corporation Inc., Taipei, Taiwan). The column (4.6 x 250 mm, 5 μm, Cosmosil 5C18-AR-II, Nacalai, Japan) was maintained at 30°C. Injection volume was 50 μL. The detector wavelength was 274 nm (excitation) and 440 nm (emission). The mobile phase, acetonitrile/water (50/50, v/v), was pumped at a flow rate of 1.0 mL/min.

Linearity of the method was verified by analyzing six standard solutions in the range of 3-100 ppb for ZON (3, 6, 12.5, 25, 50 and 100 ppb). Each concentration was analyzed in triplicate.

The intra-day precision and accuracy were determined at four various levels (3, 50, 100 and 200 ppb) of ZON standard solution in five replicates each. The inter-day precision and accuracy were determined at four levels of ZON standard solution on five independent occasions. The precision was calculated as the relative standard deviation of the mean (R.S.D.) with R.S.D. (%) = (standard deviation of the mean/mean) × 100. The accuracy was calculated as the relative mean error (R.M.E.) with R.M.E.(%) = [(mean concentration−theoretical concentration)/theoretical concentration] × 100.

For the recovery test, corn, wheat, rice and oat samples were spiked with ZON standard at a concentration of 5, 20, 100 and 200 ppb in triplicate. The detection and quantification limit was determined as the concentration with peak area ratio of signal to noise (S/N ratio) no less than 3 and 10, respectively.

(Ⅳ) *LC/ESI-MS-MS Analysis*

LC/ESI-MS-MS analysis was performed on a TSQ Ultra MS system (Thermo Electron Co., MA, USA)

equipped with a Surveyor Plus LC pump (Model 68649), a Surveyor Plus autosampler (Model 76598) and an electrospray ionization (ESI) interface. Data acquisition was performed with a Xcalibur software system. Chromatographic separation was achieved using a column (4.6 × 250 mm, 2.5 µm, XTerra MS C18, Waters, USA). Injection volume was 20 µL. The mobile phase, acetonitrile/water containing 0.1% formic acid (75/25, v/v), was pumped at a flow rate of 0.5 mL/min. The ESI interface was operated in the negative ion mode. The parent ion of 317 *m/z* and the product ion of 175 *m/z* were selected. The parameters for ESI operation were as follows: capillary voltage, 4 kV; capillary temperature, 270°C; source CID, 16 V; sheath gas pressure, 43 psi; ion gas pressure, 2 psi; collision pressure, 2 psi; tube lens offset, 102 V; collision energy, 32 V.

RESULTS AND DISCUSSION

I. *Method Validation*

(I) *Linearity*

Standard curves were made in triplicate for each concentration in HPLC/fluorescence analysis. A good linearity was achieved in the concentration of 3, 6, 12.5, 25, 50 and 100 ppb (Figure 1). The regression equation and correlation coefficient were y = 3653.4x + 11083 (R^2 = 0.9996). The retention time was 12.2 min.

(Ⅱ) *Limit of Detection (LOD) and Limit of Quantification (LOQ)*

The LOD and LOQ were 3 and 5 ppb, respectively. They were determined as the concentration with peak area ratio of signal to noise (S/N ratio) no less than 3 and 10 respectively. The LOQ of this method was lower than those reported in the literatures[13,14]. According to the maximum tolerance levels and guideline levels, reliable determination between 10 and 100 ppb is needed in food and feedstuff. This method provided excellent sensitivity in the low ppb range which was well below the present guideline and maximum residue levels of ZON.

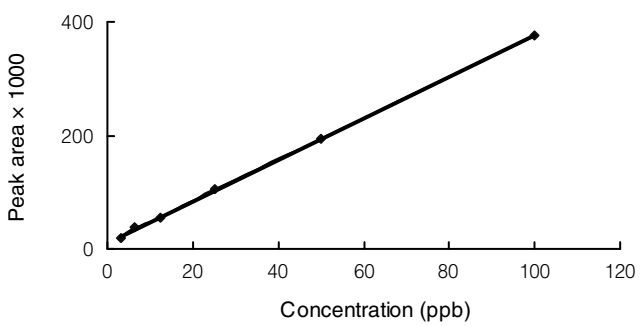

Figure 1. Standard curve of ZON analyzed by HPLC

(III) *Precision and Accuracy*

The precision and accuracy were calculated from four different levels (3, 50, 100 and 200 ppb) of ZON standard solution in five replicates. The inter-day precision and accuracy were determined at four different levels of concentrations on five independent occasions. The results were summarized in Table 1. The precision and accuracy were satisfactory with an overall value < 10% for all samples. It indicated high precision with R.S.D. of intra-day and inter-day test ranging from 0.1 to 5.9% and from 0.5 to 7.7%, respectively. Besides, it also showed high accuracy with R.M.E. of intra-day and inter-day test ranging from -3.0 to -1.1% and from -5.7 to -1.6%, respectively. Good precision and accuracy of the instrument was obtained.

Table 1. Precision and accuracy data of the HPLC method for the determination of ZON

ZON (ppb)	Ceoncentration measured (ppb)		R.S.D. (%)		R.M.E. (%)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
3	3.1	2.3	5.9	7.7	-3.0	-5.7
	2.6	3.0				
	2.9	3.1				
	3.6	3.6				
	2.9	2.8				
50	46.6	46.5	4.8	4.0	-1.3	-1.9
	52.9	50.2				
	50.7	47.8				
	47.6	49.0				
	48.9	51.8				
100	96.8	92.0	0.4	3.1	-2.9	-3.3
	96.7	100.1				
	97.4	95.3				
	97.3	98.6				
	97.4	97.5				
200	197.7	195.5	0.1	0.5	-1.1	-1.6
	198.3	196.9				
	197.9	197.9				
	197.8	196.7				
	197.7	197.5				

The precision was calculated as the relative standard deviation of the mean (R.S.D.) with R.S.D. (%) = (standard deviation of the mean / mean) × 100. The accuracy was calculated as the relative mean error (R.M.E.) with R.M.E. (%) = [(mean - theoretical concentration)/ theoretical concentration] × 100.

Journal of Food and Drug Analysis, Vol. 17, No. 1, 2009

Table 2. Recoveries of ZON in different samples

Spiking level (ppb)	Recovery ± S.D. (%)			
	Corn	Wheat	Rice	Oat
5	73.9 ± 3.9	68.5 ± 7.1	66.4 ± 4.1	71.0 ± 4.7
20	96.1 ± 2.9	89.8 ± 3.1	83.3 ± 1.7	83.1 ± 4.6
100	87.8 ± 4.3	82.7 ± 2.4	80.7 ± 4.1	81.7 ± 4.3
200	81.6 ± 2.0	85.8 ± 2.3	85.6 ± 2.4	87.1 ± 1.4

(Ⅳ) *Recovery*

The recovery was determined by measuring the concentration of spiked samples. As shown in Table 2, the recovery was good except for the low concentration of 5 ppb. The average recovery ranged from 66.4% to 96.1% indicating reliable experimental procedures. At the concentration of 20, 100 and 200 ppb, the recovery was over 80%, which suggested it was a suitable method. Based on the commission regulation (EC) No 401/2006, recovery of ZON should be 70 to 120% for concentration above 50 ppb, and 60 to 120% for concentration below 50 ppb. Our results met the criteria[15].

From the validation results, the developed HPLC/fluorescence method with the aforementioned extraction and clean-up procedure could be used for reliable analysis of ZON in cereal samples. Recently, antibody-based immunoaffinity columns have become popular in mycotoxin analysis. They are selective and time-saving. It is better to use an immunoaffinity column rather than solid phase extraction (SPE) column to purify mycotoxin concerning the specificity. We found that Zearalatest^{TM} immunoaffinity column is a suitable tool for purifying ZON-contaminated cereal samples.

Ⅱ. *Survey Results*

ZON was present in 4 of 7 (57.1%) corn samples with ZON levels ranging from 7.9 to 9.0 ppb (Table 3 and Figure 2). In addition, no ZON was detected in 7 mixed cereal samples, 5 wheat samples, 5 rice samples and 2 oat samples. Overall, ZON was present in 4 of 26 (15.4%) cereal samples, and the ZON levels were very low compared to those reported in the literatures[2,14,16-18]. In this survey, ZON was only detected in corn samples. The result was similar to many studies from other countries, indicating that corn is the most prominent cereal at risk with high incidence and high levels of contamination with ZON.

According to the "Nutrition and Health Survey in Taiwan 1993~1996", the daily intake of starch from roots and tubers were 21.19 and 28.96 g for adult male and female[19]. The average body weight of adult male and female was 64.3 and 54.5 kg, respectively. Based on the highest ZON level of 9.0 ppb found in this survey, the daily possible ZON intake was estimated as follows:

(The intake sum of starch from roots and tubers) × ZON level / mean body weight

The results were 0.00297 and 0.00478 (μg/kg bw/day) for adult male and female, respectively. The intakes were much lower than the tolerable intakes estimated by JECFA (0.5 μg/kg bw/day), indicating that it should not cause any high risk to Taiwanese.

Table 3. Results of ZON in different samples

Samples	No. of samples	No. of positive samples (%) by HPLC	No. of positive samples (%) by LC/MS/MS
Mixed cereals	7	0	0
Corn	7	4(57.1%)[1]	4(57.1%)[2]
Wheat	5	0	0
Rice	5	0	0
Oat	2	0	0
Total	26	4(15.4%)	4(15.4%)

[1]ZON content was 7.9~9.0 ppb.
[2]ZON content was 8.1~8.9 ppb.

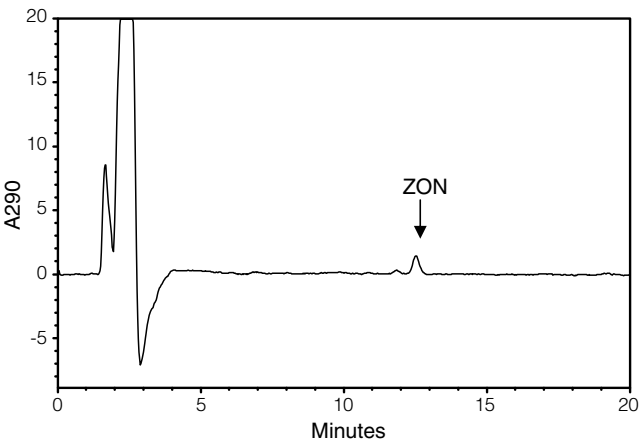

Figure 2. HPLC chromatogram of ZON-contaminated corn (7.9 ppb)

III. *Confirmation by LC/ESI-MS-MS*

Trace analysis of ZON can be performed with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in the positive and negative ion modes[20]. According to the literatures[17,21,22], negative ion mode was more selective and sensitive than the positive ion mode due to exclusive formation of deprotonated molecules $[M-H]^-$. This may be due to the acidic phenolic groups and the instability of the protonated molecule $[M+H]^+$ in the positive ion mode. In this study, we also select negative ion mode because of its higher sensitivity than the positive ion mode. We also found that acid

addition in mobile phase can enhance the ionization of analytes and thus improve sensitivity.

For SRM (selected reaction monitoring) mode, the parent ion of *m/z* 317 and the product ion of m/z 175 were selected as reported in the literatures[17,22-24]. The total ion chromatogram (TIC) and ESI-MS spectrum of ZON were shown in Figure 3. The optimum parameters were as follows: capillary voltage, 4 kV; capillary temperature, 270°C; source CID, 16 V; sheath gas pressure, 43 psi; ion gas pressure, 2 psi; collision pressure, 2 psi; tube lens offset, 102 V; collision energy, 32 V.

Standard curves were performed in triplicate for each concentration. A good linearity was achieved in the concentrations of 0.5, 1, 3, 12.5, 25 and 50 ppb. The regression equation and correlation coefficient were [y = 29375x – 8568.5] (R^2 = 0.9996). The TIC was shown in Figure 3. Among the mobile phases, acetonitrile/water (75/25, v/v) exhibited the best resolution. Among the columns we tested, best chromatographic separation was achieved using the column (4.6 × 50 mm, 2.5 µm, XTerra MS C18, Waters, USA). The retention time of 1.54 min was much shorter than that by HPLC (12.2 min). The LOD and LOQ were 0.5 and 3 ppb, respectively, which were lower than those by HPLC. The LC/ESI-MS-MS method provided excellent sensitivity in the low ppb range, which was well below the present guideline and maximum residue levels of ZON. The LOQ was lower compared to those reported in the literatures[20,21,25-27]. Linear regression, LOD and LOQ obtained by HPLC/fluorescence and LC/ESI-MS-MS are summarized in Table 4.

In the survey results (Table 3), ZON was present in 4 of 7 (57.1%) corn samples ranging from 8.1 to 8.9 ppb. There was no ZON present in 7 mixed cereal samples, 5 wheat samples, 5 rice samples and 2 oat samples. Overall, ZON was detected in 4 of 26 (15.4%) cereal samples. The ZON contents found in corn samples by LC/ESI-MS-MS were closed to those by HPLC/fluorescence, indicating that both HPLC/fluorescence and LC/ESI-MS-MS methods are consistent.

Table 4. Linear regression, LOD and LOQ obtained by HPLC and LC/ESI-MS-MS

Method	Linearity range (ppb)	R^2	LOD (ppb)	LOQ (ppb)
HPLC	3-100	0.9996	3	5
LC-MS-MS	0.5-50	0.9996	0.5	3

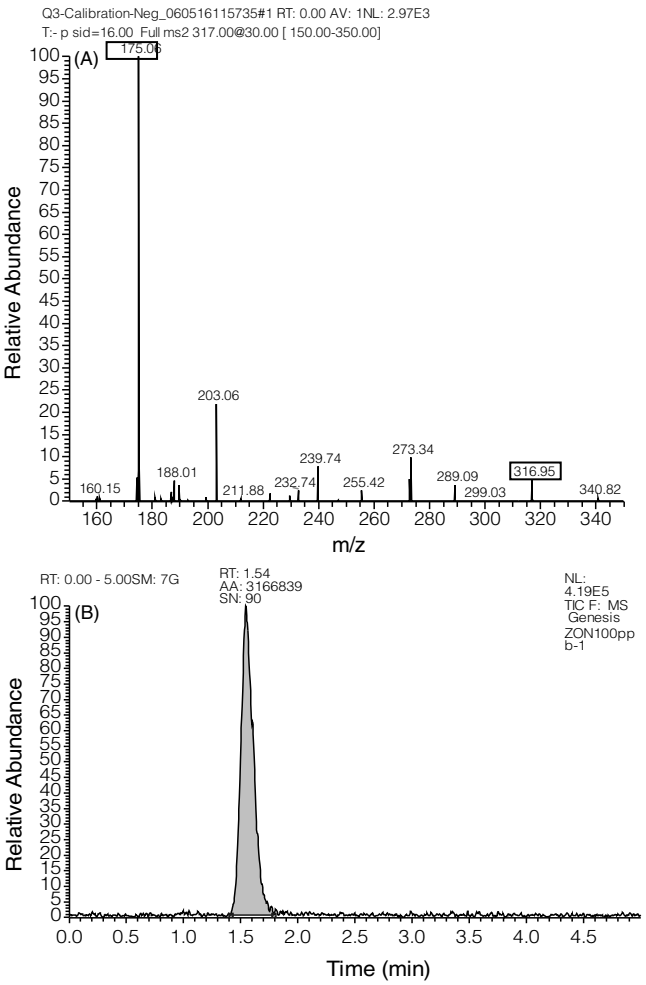

Figure 3. LC/ESI-MS-MS product ion spectra in the negative mode (A) and total ion chromatogram (B) of ZON (100 ppb)

CONCLUSIONS

In this study, a method for detecting ZON in cereals was developed. The determination of ZON was performed using immunoaffinity column for clean-up, HPLC/fluorescence for quantification and LC/ESI-MS-MS for confirmation. It showed good linearity, recoveries, precision and accuracy. The method has been used to analyze twenty-six cereal samples. The results showed that ZON was detected in four corn samples at 7.9 to 9.0 ppb levels. Using the consumption data from "Nutrition and Health Survey in Taiwan 1993~1996", the probable ZON consumption by Taiwanese adults (male and female are 0.00297 and 0.00478 µg/kg b.w. respectively) was much lower than the tolerable daily intake of 0.5 µg/kg b.w. established by JECFA.

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