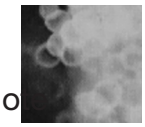

Determination of trichothecenes, zearalenone and zearalenols in commercially available corn-based foods in Spain

María Carmen Cerveró, María Ángeles Castillo, Rosa Montes and Enrique Hernández

Departamento de Biotecnología, Universidad Politécnica de Valencia, Valencia, Spain

Summary

Among the main Spanish commercially available trademarks, we have selected a total of 25 samples of corn-based foods, which have the highest consume rate, to carry out the analysis of deoxynivalenol (DON), T-2 toxin, zearalenone (ZEA) and zearalenols (ZOL). The contents of mycotoxins were determined by gas chromatography with flame ionization detection, and those of ZEA were confirmed by HPLC with fluorescence detection. Of the 25 analyzed samples, the incidence of DON, ZEA and α-ZOL was 68, 44 and 24%, respectively; levels detected ranged from 29-195, 34-216, and 36-71 µg/kg, respectively. T-2 toxin was only detected in one sample (< 50 µg/kg). ß-ZOL was not present in excess of the detection limit in the investigated samples.
The results suggest a risk for consumers of corn products and the need to monitor the final products before consumption. This is the first report in Spain on natural contamination with these mycotoxins in corn-based foods.

Key words Trichothecenes, Deoxynivalenol, T-2 Toxin, Zearalenone, Zearalenols, Corn-based foods

Determinación de tricotecenos, zearalenona y zearalenoles en alimentos derivados de maíz del mercado español

Resumen

Se han seleccionado 25 muestras de alimentos derivados de maíz entre las principales marcas y las de mayor consumo del mercado español.
Éstas se han analizado para detectar la presencia natural de deoxinivalenol (DON), toxina T-2, zearalenona (ZEA) y α- y ß-zearalenoles (ZOL). Los contenidos de micotoxinas se han determinado por cromatografía de gases con detector de ionización de llama, confirmando la presencia de zearalenona por cromatografía líquida de alta resolución con detector de fluorescencia.
La incidencia de DON, ZEA y α-ZOL en las 25 muestras fue del 68, 44 y 24%, respectivamente; los niveles detectados variaron entre 29-195, 34-216, y 36-71 µg/kg, respectivamente. La toxina T-2 sólo se detectó en una de las muestras (< 50 µg/kg). Por último la micotoxina ß-ZOL no fue detectada por encima del límite de detección en ninguna de las muestras analizadas.
La presencia de micotoxinas en este tipo de productos supone un riesgo para la salud de los consumidores e indica la necesidad de controlar estos productos finales antes de ser consumidos. Este trabajo constituye el primer estudio sobre la presencia natural de estas micotoxinas en alimentos derivados de maíz presentes en el mercado español.

Palabras clave Tricotecenos, Deoxinivalenol, Toxina T-2, Zearalenona, Zearalenoles, Alimentos derivados de maíz

Corresponding Author:
Dra. María Ángeles Castillo
Departamento de Biotecnología
Área de Microbiología
Universidad Politécnica de Valencia
Camino de Vera, s/n
46022 Valencia, Spain
Tel.: +34 96 387 7423
Fax: +34 96 387 7429
E-mail: mcastill@btc.upv.es

Aceptado para publicación el 9 de marzo de 2006

1130-1406/01/10.00 €

Fusarium is an ubiquitous fungi, with several species involved as important pathogens of cereal crops [19]. *Fusarium* species are also known to produce several types of secondary metabolites, such as trichothecenes, zearalenone, and other mycotoxins, which are contaminants of cereal grains such as wheat, barley, oats and corn [24].

Trichothecenes are a diverse range of structurally related compounds derived from 12,13-epoxytrichothec-9-ene, distinguishing two groups according to chemical structure at carbon 8: group A trichothecenes, having a functional group other than a ketone at C-8, and group B trichothecenes, characterized by a ketone at C-8 [31]. The trichothecenes are responsible for several types of mycotoxicoses in farm animals, causing a variety of adverse health side effects such as feed refusal, diarrhoea, vomiting, skin irritation, hemorrhaging, immunosuppression and death [10, 33]. They are also implicated in some diseases of man [9,26,31]. Among the trichothecenes, deoxynivalenol (DON) and T-2 toxin are the most potent toxins [11].

Zearalenone (ZEA), widely distributed throughout the world in cereals and feeds, has also been detected in corn intended for food use [13,23,34]. This toxin has estrogenic effects on animals including infertility, enlargement of uterus and mammary glands, vaginal prolapse and atrophy of testicles and ovaries [8,12,15]. It had also been implicated in some incidents of precocious puberty changes in children [17]. The same activity is shown by the two isomers (alpha and beta) of zearalenol (α- and ß-ZOL), although α-ZOL is 2-4 times more active than ZEA or ß-ZOL [1].

The presence of this kind of mycotoxins in foods is potentially harmful to human health. However, there is limited information on the general occurrence of these toxins in cereal-based foods, which are of prime importance in human nutrition. Monitoring of this kind of products has been mainly directed towards DON, and ZEA [18,22,29,30], whereas other *Fusarium* mycotoxins such as T-2 have received only little attention [21,25,32].

The main objective of this study was to determine trichothecenes of both the A-type (T-2) and B-type (DON) as well as ZEA and its alcohol derivatives (α- and ß-ZOL) in samples of corn-based products for human consumption in order to establish the most frequent levels found in this kind of foods in the Spanish market. Although, there are several previous reports of the presence of *Fusarium* species and their toxins in harvested grains of cereals, feeds, etc., published in Spain [4-7,20], none of these studies investigated the presence of mycotoxins in cereal-based foods. Therefore, this is the first report in Spain on natural contamination with these mycotoxins in corn-based foods. Besides, our work provides evidence of the presence of *Fusarium* mycotoxins in this kind of products for human consumption.

Materials and methods

Sampling and sample preparation. Twenty-five commercially available corn-based food samples were purchased in different supermarkets in Valencia, Spain. The products were cornflakes, tinned sweet corn, frozen corn, baked corn snack, and fried corn snack. They are among the most consumed by the Spanish population and represent the main trademarks in Spain. Representative (500 g) samples were finely grounded in a laboratory mill and thoroughly mixed before taking aliquots (50 g) which were stored at 0-4 °C until required for mycotoxin analysis.

Chemicals and reagents. Organic solvents were HPLC grade. Standards of DON, T-2 toxin, ZEA, α-ZOL, ß-ZOL, 19-nortestosterone, and Tri-Sil TBT, a mixture of N-trimethylsilylimidazole-N,O-bis(trimethysilyl)acetamide-trimethylchlorosilane (3:3:2), were purchased from Sigma-Aldrich (Madrid, Spain). Florisil (60-100 mesh) was obtained from Merck (Darmstadt, Germany) and Sep-Pak C_{18} cartridge was a product from Waters (Milford, MA, USA).

Extraction and clean-up procedure. The mycotoxins were extracted and purified as described previously [14] with slight modifications. A 20 g subsample was extracted with 100 ml of methanol-1% aqueous NaCl (95:5, vol/vol), 1 ml (10 µg/ml) of 19-nortestosterone as an internal standard was added; the flask was shaken with a rotary shaker for 1 h at 230 rpm and filtered through a filter paper with suction. The filtrate was defatted with n-hexane (2 x 50 ml), and evaporated to dryness. The residue was transferred to a Florisil column with 2 x 2 ml methanol.

The Florisil column was filled as follows: 5 g Na_2SO_4, 10 g Florisil (previously activated at 130 °C, 2 h) and towards the top 5 g Na_2SO_4. *n*-Hexane was used to fill the column. The mycotoxins were eluted from the column with 200 ml chloroform-methanol (90:10, vol/vol). The eluate was evaporated to dryness on the rotary evaporator and the residue was dissolved in 1 ml methanol-water (60:40, vol/vol) and filtered through Sep-Pak C_{18} cartridges. The mycotoxins were eluted from the cartridge with 15 ml methanol-water (60:40, vol/vol). The eluate was evaporated to dryness under N_2 and dissolved in 1 ml of methanol and reserved for chromatographic analysis.

Analysis of mycotoxins by GC-FID. A 900 µl aliquot of the evaporated residue was treated with 100 µl silylating reagent Tri-Sil TBT for 1 h at 45 °C. After reaction, 0.5 ml n-hexane and 1 ml phosphate buffer (0.1 M, pH 7) were added to the cooled mixture. The sample was mixed by shaking and the organic layer was transferred to another vial. Mycotoxins were detected using a Hewlett-Packard gas chromatograph Model 6890 Series II provided with flame ionization detection (FID) and split/splitless injector. Data were analyzed with a HP Chem-Station software. Separation was carried out on a HP5 (30 m x 0.32 mm I.D., 0.25 mm d_f) fused-silica capillary column. Operating conditions were as follows: helium carrier gas 1.76 ml/min; injector and detector temperature 275 °C and 300 °C, respectively; temperature programme: 150 °C (1 min), 150-280 °C (8 °C/min), 280 °C (10 min); injection volume 2 ml. Split injections at split ratio 1: 37 were made. Mycotoxins were determined by the internal standard procedure.

Detection limits (signal-to-noise 3:1) were 25 µg/kg of sample for DON, ZEA, and α- and ß-ZOL, and 30 µg/kg of sample for T-2 toxin. These values are considered good and perfectly comparable with previous reports [16,28].

Analysis of ZEA by HPLC. A 100 µl aliquot of the extract dissolved in methanol was submitted to HPLC to confirm ZEA. A Waters 600E system provided with a programmable fluorescence detector Model 470 (Waters, Milford, MA, USA) was used. The sample loop was 20 µl. A reverse-phase column Lichrochart C_{18} (250 x 4 mm I.D., 5 mm d_p) (Merck) protected with a guard precolumn packed with the same phase was used throughout. Chromatograms were recorded and integrated by a Waters 745 integrator. The mobile phase was methanol-water (70:30, vol/vol) with a flow rate of 1 ml/min. Fluorescence was recorded at excitation and emission wavelengths of 280 and 460 nm, respectively. The detection limit for ZEA was 3 µg/kg.

Statistical analysis. Mycotoxin content data were all analyzed with one-way analysis of variance (ANOVA) and the least significant difference (LSD) test was used to compare means (STATGRAPHICS PLUS 5.1).

Results and discussion

Table 1 lists the recovery percentages of mycotoxins found in the spiked samples (spiking level was 100 µg/kg). By GC-FID, the recoveries of trichothecenes varied between 67 and 90%. The higher value for DON was obtained in tinned sweet corn sample; T-2 toxin yielded recoveries nearly 89% in all products excepting cornflakes. Moreover, recoveries of ZEA, α-ZOL, and ß-ZOL were very high, ranging from 93 to 96%, excepting for ZEA in frozen corn (86.5%), which could be explained by the high humidity find on this kind of product, being more difficult for the extraction of this toxin. Good recoveries of ZEA measured by HPLC (87-94%) were also obtained, which are in agreement with the GC results.

The naturally contaminated samples were analyzed in triplicate. The occurrence of mycotoxins in corn-based foods is presented in table 2. The most frequent mycotoxins detected were DON and ZEA, which were detected in all types of products. Both, DON and ZEA were present in high percentages in cornflakes, baked and fried corn snack. Seventeen out of 25 samples analyzed (68%) showed to contain DON. The highest mean content of this mycotoxin was found in cornflakes (91 µg/kg). A total of 44% samples were positive for ZEA with a mean content ranging from 37-149 µg/kg. Cornflakes contained the highest mean value of this toxin (149 µg/kg), but the maximum content was detected in fried corn snack samples (216 µg/kg). α-ZOL was detected in 24% of the samples at percentages and mean contents lower in all of groups. T-2 toxin was present in only one sample (frozen corn), and finally, ß-ZOL was not found in any of the samples analyzed.

Analysis by HPLC indicated a higher incidence of ZEA (64%), what may have been due to the lower detection limit obtained by this technique.

In the present study DON was the predominant toxin based on incidence in the products examined. This is consistent with results described previously in most cereal-based foods [3,18,22,27]. In most cases the percentage of

Table 1. Recovery of mycotoxins added to corn-based products measured by GC-FID and HPLC.

Sample	Recovery of mycotoxins (%) ± SD[a]					
	GC-FID					HPLC
	Deoxynivalenol	T-2 toxin	Zearalenone	α-Zearalenol	ß-Zearalenol	Zearalenone
Cornflakes	74.8 ± 4.0	85.2 ± 11.2	93.2 ± 9.4	94.6 ± 4.6	95.9 ± 7.6	91.3 ± 3.1
Tinned sweet corn	80.1 ± 3.9	89.5 ± 11.7	93.3 ± 1.3	95.3 ± 4.7	96.1 ± 3.9	92.3 ± 2.2
Frozen corn	73.5 ± 1.4	88.8 ± 8.7	86.5 ± 3.4	95.2 ± 6.2	95.7 ± 5.2	87.7 ± 1.7
Baked corn snack	66.8 ± 2.9	89.3 ± 4.0	95.6 ± 7.7	94.1 ± 7.7	95.7 ± 2.0	94.2 ± 0.9
Fried corn snack	71.6 ± 7.4	89.0 ± 8.6	92.6 ± 8.1	95.1 ± 6.3	96.4 ± 8.8	93.0 ± 7.5

[a] Data are mean ± standard deviation value from the analysis of three replicate spiked samples
Note: spiking level of mycotoxins was 100 µg/kg

Table 2. Natural occurrence of mycotoxins in corn-based products measured by GC-FID and HPLC.

Toxin	Product	Samples positive (%)[1]	Toxin in positive samples (µg/kg)	
			Range	Mean ± SD[2]
Deoxynivalenol	Cornflakes	4 (80)	37.6 – 195.1	91.3 ± 52.6[a]
	Tinned sweet corn	2 (40)	35.1 – 60.6	47.5 ± 10.7[b]
	Frozen corn	3 (60)	32.8 – 85.7	51.2 ± 22.3[b]
	Baked corn snack	4 (80)	46.2 – 175.6	80.0 ± 53.9[ab]
	Fried corn snack	4 (80)	28.3 – 109.1	53.1 ± 32.8[b]
T- 2 Toxin	Cornflakes	0		
	Tinned sweet corn	0		
	Frozen corn	1 (20)	41.3 – 47.6	45.2 ± 3.4
	Baked corn snack	0		
	Fried corn snack	0		
Zearalenone	Cornflakes	3 (60)	125.6 – 186.8	149.2 ± 21.6[a]
	Tinned sweet corn	1 (20)	34.2 – 40.3	37.3 ± 3.1[b]
	Frozen corn	1 (20)	58.7 – 68.2	63.1 ± 4.8[b]
	Baked corn snack	3 (60)	35.1 – 111.6	61.2 ± 32.4[b]
	Fried corn snack	3 (60)	79.6 – 216.6	143.1 ± 54.1[a]
α-Zearalenol	Cornflakes	0		
	Tinned sweet corn	1 (20)	40.6 – 44.2	42.1 ± 1.9[a]
	Frozen corn	2 (40)	36.8 – 48.2	43.1 ± 4.4[a]
	Baked corn snack	1 (20)	42.3 – 53.1	47.7 ± 5.4[a]
	Fried corn snack	2 (40)	36.4 – 71.3	53.5 ± 17.1[a]
Zearalenone (HPLC)	Cornflakes	3 (60)	99.1 – 127.8	114.1 ± 10.6[a]
	Tinned sweet corn	2 (40)	4.8 – 17.5	11.1 ± 6.5[b]
	Frozen corn	2 (40)	8.9 – 60.5	34.0 ± 26.2[b]
	Baked corn snack	5 (100)	12.5 – 90.4	33.7 ± 27.5[b]
	Fried corn snack	4 (80)	19.9 – 144.9	91.2 ± 45.2[a]

[1] Number of analyzed samples was five for each product in triplicate.
[2] Values within a column with different superscripts differ at $p < 0.05$ between products.

positive samples exceeded 50%. Other mycotoxins such as T-2 toxin were not or only seldom detected [25,27,32].

Two or more of the toxins analyzed in this study were found in 12 out of a total of 25 samples. Of these, 7, 4, and 1 samples contained 2, 3, and 4 different toxins, respectively. This co-occurrence has been frequently found in cereals [19,35] and cereal-based products [27].

Due to the high incidence of DON, all samples with co-occurring toxins contained this toxin. In samples containing two toxins, the most frequent combination was DON/ZEA (6 out of 7 samples). The most frequent toxin combination of three toxins was DON/ZEA/α-ZOL (4 out of 4 samples). Finally, four toxins were found in one sample containing DON, T-2 toxin, ZEA and α-ZOL.

The frequent occurrence of DON and ZEA in corn-based foods raises the question of health risks for humans. Thus, for ZEA, a tolerance limits between 30 and 200 µg/kg for cereals have been proposed in different countries [2]. In the present study, over 30% samples exceeded the Austrian guideline (60 µg/kg). Besides, the results of DON, although were below recommended tolerance limits (500 µg/kg), indicate an elevated incidence of this mycotoxin.

Although the number of analyzed sample were not high, the findings of this study indicate some contamination of corn-based products for the studied mycotoxins, especially ZEA and DON, and emphasizes the need for further and regular studies of cereal-based foods for the *Fusarium* toxins for the prevention and assessment of risk human.

This work as been financed by the Spanish Comisión Interministerial de Ciencia y Tecnología (Project Ref. AGL2001-2974-C05-04) whose support is gratefully acknowledged.

References

1. Arukwe A, Grotmol T, Haugen TB, Knudsen FR, Goksoyr A. Fish model for assessing the in vivo estrogenic potency of the mycotoxins zearalenone and its metabolites. Sci Total Environ 1999; 236: 153-161.
2. Boutrif E, Canet C. Mycotoxin prevention and control: FAO programmes. Rev Med Vet 1998; 149: 681-694.
3. Brumley W C, Trucksess MW, Adler SH, Cohen CK, White KD, Sphon JA. Negative ion chemical ionization mass spectrometry of deoxynivalenol (DON): Application to identification of DON in grains and snacks foods after quantitation/isolation by thin layer chromatography. J Agric Food Chem 1985; 33: 326-330.
4. Cantalejo MJ, Carrasco JM, Hernández E. Fusarin C production by *Fusarium* spp. from Spain. J Food Prot 1997; 60: 837-842.
5. Cantalejo MJ, Torondel P, Amate L, Carrasco JM, Hernández E. Detection of fusarin C and trichothecenes in *Fusarium* strains from Spain. J Basic Microbiol 1999; 39: 143-153.
6. Castella G, Bragulat MR, Cabanes FJ. Fumonisin production by *Fusarium* species isolated from cereals and feeds in Spain. J Food Prot 1999; 62: 811-813.
7. Castella G, Bragulat MR, Cabanes FJ. Surveillance of fumonisins in maize-based feeds and cereals from Spain. J Agric Food Chem 1999; 47: 4707-4710.
8. D'Mello JPF, Placinta CM, Macdonald AMC. *Fusarium* mycotoxins: a review of global implications for animal health: welfare and productivity. Anim Feed Sci Technol 1999; 80: 183-205.
9. Ehling G, Cockburn A, Snowdon P, Buschhaus H. The significance of the *Fusarium* toxin deoxynivalenol (DON) for human and animal health. Cereal Res Comm 1997; 25: 443-447.
10. Eriksen GS, Alexander J. *Fusarium* toxins in cereals – a risk assessment. Copenhagen, Nordic Council of Ministers, Tema-Nord, 1998.
11. Froquet R, Sibiril Y, Parent-Massin D. Trichothecene toxicity on human megakaryocyte progenitors (CFU-MK). Hum Exp Toxicol 2001; 20: 84-89.
12. Gaumy JL, Bailly JD, Bernard G, Guerre P. Zearalenone: origin and effects on farm animals. Rev Med Vet 2001; 1152: 123-136.
13. Gonzalez HH, Martinez EJ, Pacin AM, Resnik SL, Sydenham EW. Natural co-occurrence of fumonisins, deoxynivalenol, zearalenone and aflatoxins in field trial corn in Argentina. Food Addit Contam 1999; 16: 565-569.
14. Hietaniemi V, Kumpulainen J. Contents of *Fusarium* toxins in Finnish and imported grains and feeds. Food Addit Contam 1991; 8: 171-182.
15. Hollinger K, Ekperigin HE. Mycotoxins in food producing animals. Vet Clin N Am: Food Anim Pract 1999; 15: 133-165.
16. Jiménez M, Mateo R. Determination of mycotoxins produced by *Fusarium* isolates from banana fruits by capillary gas chromatography and high-performance liquid chromatography. J Chromatogr A 1997; 778: 363-372.
17. Kuiper-Goodman T, Scott PM, Watanabe H. Risk assessment of the mycotoxin zearalenone. Regul Toxicol Pharmacol 1987; 7: 253-306.
18. Martins ML, Martins HM. Determination of deoxynivalenol in wheat-based breakfast cereals marketed in Portugal. J Food Prot 2001; 64: 1848-1850.
19. Miller JD. In: Miller JD, Trenholm HL (Eds.) Epidemiology of *Fusarium* ear diseases of cereals. St. Paul MN, Eagen Press, 1994: 19-36.
20. Muñoz L, Cardelle M, Pereiro M, Riguera R. Occurrence of corn mycotoxins in Galicia (Northwest Spain). J Agric Food Chem 1990; 38: 1004-1006.
21. Omurtag GZ, Yazicioglu D. Occurrence of T-2 toxin in processed cereals and pulses in Turkey determined by HPLC and TLC. Food Addit Contam 2001; 18: 844-849.
22. Pacin AM, Resnik SL, Neira MS, Molto G, Martinez E. Natural occurrence of deoxynivalenol in wheat, wheat flour and bakery products in Argentina. Food Addit Contam 1997; 14: 327-331.
23. Park JJ, Smalley EB, Chu FS. Natural occurrence of *Fusarium* mycotoxins in field samples from the 1992 Wisconsin corn crop. Appl Environ Microbiol 1996; 62: 1642-1648.
24. Placinta CM, D'Mello JPF, Macdonald AMC. A review of world-wide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. Anim Feed Sci Technol 1999; 78: 21-37.
25. Ramakrishna Y, Bhat RV, Vasanthi S. Natural occurrence of mycotoxins in staple foods in India. J Agric Food Chem 1990; 38: 1857-1859.
26. Richard JL, Thurston R. Diagnosis of mycotoxicoses. Dordrecht, M Nijhoff Publ, 1986.
27. Schollenberger M, Suchy S, Jara HT, Drochner W, Müller HM. A survey of *Fusarium* toxins in cereal-based foods marketed in an area of southwest Germany. Mycopathologia 1999; 147: 49-57.
28. Schothorst RR, Jekel AA. Determination of trichothecenes in wheat by capillary gas chromatography with flame ionisation detection. Food Chem 2001; 73: 111-117.
29. Tanaka T, Hasegawa A, Matsuki Y, Ueno Y. A survey of the occurrence of nivalenol, deoxynivalenol and zearalenone in foodstuffs and health foods in Japan. Food Addit Contam 1985; 2: 259-265.
30. Tanaka T, Yamamoto S, Hasegawa A, Aoki N, Besling JR, Sugiura Y, Ueno Y. A survey of the natural occurrence of *Fusarium* mycotoxins, deoxynivalenol, nivalenol, and zearalenone in cereals harvested in The Netherlands. Mycopathologia 1990; 110: 19-22.
31. Ueno Y. Trichothecens – chemical, biological and toxicological aspects. Amsterdam, Elsevier, 1983.
32. Valente-Soares LM, Furlani RPZ. Survey of mycotoxins in wheat and wheat products sold in health food stores of the city of Campinas, state of Sao Paulo. Rev Microbiol Sao Paulo 1996; 27: 41-45.
33. Van Egmond HP, Speijers GJA. In: Van der Heijden K, Younes M, Fisbein L, Miller S (Eds.) Natural Toxins I. Mycotoxins. New York, Marcel Dekker Inc, 1999: 341-355.
34. Vrabcheva T, Gessler R, Usleber E, Martlbauer E. First survey on the natural occurrence of *Fusarium* mycotoxins in Bulgarian wheat. Mycopathologia 1996; 136: 47-52.
35. Yoshizawa T. In: Smith JE, Henderson RS (Eds.). Mycotoxins and animal foods. Boca Raton, Florida, CRC Press, 1991: 301-324.