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Occurrence of deoxynivalenol and nivalenol in Spanish corn-based food products

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Runnig tittle: Mycotoxins in Spanish corn-based foods

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Abstract

The aim of the present work was to evaluate the occurrence of trichothecenes toxins, deoxynivalenol (DON) and nivalenol (NIV), in samples of corn-based foods (breakfast cereals and snacks) consumed by the Spanish population. A total of 175 commercially available samples were randomly collected during 2005. Trichothecenes were determined by gas chromatography-electron capture detector. The estimated limit of quantification was 25.4 µg/kg for DON and 15.9 µg/kg for NIV. DON was detected in 22 of the 55 samples of breakfast cereals, in 13 of the 57 samples of baked corn snacks and in 12 of the 63 samples of fried corn snacks. NIV was detected in 6 samples of breakfast cereals and 1 sample of snacks. Based on total of samples, the median concentrations of DON and NIV found were 53.9 and 60.2 µg/kg, respectively. The influence of different factors, such as the presence of additional ingredients and the type of commercial brand on the toxin incidence and content levels were also studied. The values of both mycotoxin intake found in this study are lower than the proposed Tolerable Daily Intake for the respective toxin (1 and 0.7 µg/kg bw/day for DON and NIV, respectively).

Keywords: deoxynivalenol; nivalenol; trichothecenes; breakfast cereals; snacks

1. Introduction

Deoxynivalenol (DON) and nivalenol (NIV) are type-B trichothecene mycotoxins, that is, secondary metabolites produced by several fungal genera, most notably *Fusarium*, which is known to attack various cereals. Surveys have shown that DON occurs frequently in grains like wheat, barley, and maize, and that it is also the most common toxin, occurring concomitantly with 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol and NIV (Tanaka et al., 1990; Trucksess et al., 1995; Placinta et al., 1999). Trichothecenes cause a wide range of toxic effects in animal and humans such as feed refusal, vomiting, diarrhea, hemorrhage, anemia and immunosuppression (Hussein and Brasel, 2001).

As the incidence of *Fusarium* toxins has been reported for cereals on a global scale (Placinta et al., 1999; JEFCA 2000, 2001; Schollenberger et al., 2007), regular contamination can be expected for grain-based foods as the food processing of cereal crops does not completely eliminate mycotoxins (Scott, 1991; Hazel and Patel, 2004; Cetin and Bullerman, 2006). This has been confirmed for a variety of foodstuffs, including breakfast foods, snack foods, bread, pasta, etc. (FSA, 2003, 2005; Schollenberger et al., 2005a,b; Samar et al., 2007). As a result, humans are exposed to mycotoxins in their diet. It is clear that the presence of mycotoxins in the human diet, and especially in the diet of vulnerable populations like children, is a matter of concern.

In recent years the EU's Scientific Committee on Food (SCF) has evaluated the *Fusarium* toxins DON (SCF, 1999), NIV (SCF, 2000), and a group of trichothecenes (SCF, 2002). Likewise, the SCF established a tolerable daily intake (TDI) per kg of bodyweight and day of 1 and 0.7 µg/kg for DON and NIV, respectively. In 2005, the

European Commission (EC) set maximum levels of DON and zearalenone, which were applied from July 2006 (EC, 2005a). No limits are established for NIV.

Despite corn being one of the more susceptible substrates to this type of contamination, there is a little information available on the occurrence of trichothecenes in corn-derived foods in Spain (Cerveró et al., 2007). The purpose of this study was to examine the occurrence and concentration levels of DON and NIV in Spanish corn-based foods, specifically breakfast cereals and snacks. Although there are currently no regulatory limits for NIV, their co-occurrence with DON and its toxicity justifies their inclusion in our studies. Other aims of this study were to estimate the daily intake of DON and NIV, and to assess the real contribution of cereal-derived foods to the TDI proposed by the SCF in the Spanish population.

2. Material and methods

2.1. Reagents

The trichothecene standards DON and NIV, lindane (Riedel-de-Haën®) (internal standard) and derivatization reagent, Tri-Sil TBT (Supelco), a mixture of N-trimethylsilylimidazole–N,O-bis(trimethysilyl) acetamide–trimethylchlorosilane (3:3:2) were purchased from Sigma-Aldrich (Madrid, Spain). Deionized water was purified with a Millipore Milli-Q Plus system (Millipore, Billerica, MA, USA). Potassium dihydrogen phosphate (KH_2PO_4) and sodium hydroxide (NaOH), used to prepare phosphate buffer, were purchased from Panreac (Barcelona, Spain). All solvents (acetonitrile and hexane) were analytical grade and purchased from J.T. Baker (Deventer, Holland). Stock and working standards of DON and NIV were prepared by

100 appropriate dilution in acetonitrile to assess the linearity of method and spiked samples.
101 All stock solutions were stored at – 20 ºC when not in use. The internal standard was
102 dissolved in hexane at 1 mg/l for GC-ECD measurements. The Mycosep^{TM} 227 columns
103 were purchased from Romer Labs, Inc., USA.

104 The standards of DON and NIV were purchased from Sigma-Aldrich as pure
105 mycotoxins. Krska et al. (2004) calculated the purity of commercially available DON
106 from Sigma, resulting in 496%, and they concluded that these products can be
107 considered sufficiently pure for routine analysis of these mycotoxins in food and feed.
108 So we have used the standards as they were provided.

109

110 *2.2. Samples*

111

112 A total of 175 packaged samples of commercial corn foods were randomly collected
113 during the first 8 months of 2005 from supermarkets and retail outlets located in the city
114 of Valencia (Spain). A wide range of brands were covered to ensure that the survey was
115 representative of the range of products available to consumers in Spain. At least 0.5 kg
116 was collected for each commercial sample, which was finely ground for 3 min using an
117 Osterizer mill (Oster Co., USA) and was stored at -20 ºC until the moment of the
118 analysis, according to Cirillo et al. (2003a,b).

119 The following food items were collected: corn-based breakfast cereals (n = 55),
120 baked corn snacks (n = 57) and fried corn snacks (n = 63). The sample collection was
121 divided to convenience of discussion into different groups on the basis of possible
122 ingredients and of the commercial brand type. The possible additional ingredients were
123 chocolate, butter, honey, wheat, oat, and aromatic flavours such as cheese, jam and
124 barbecue flavour.

125

126 *2.3. Apparatus*

127

128 Mycotoxin analysis was performed using an Agilent 6890N gas chromatograph,
129 equipped with a ^{63}Ni ECD (Agilent Technologies, Waldbrom, Germany). A fused-silica
130 capillary column HP-5 [5% methyl phenyl siloxane column (30 m x 0.32 mm i.d., 0.25
131 μm film thickness, Agilent Technologies)] was used. The carrier gas was nitrogen at a
132 flow of 1.8 ml/min. The temperatures of splitless injection port and ECD were 250 ºC
133 and 300 ºC, respectively. The injection volume was 2 μl. The column temperature
134 program, following an initial period of 80 ºC for 1 min, was 30 ºC/min to 140 ºC, 5
135 ºC/min to 280 ºC and hold 31 min. Signals were processed by HP GC ChemStation
136 software Version A.09.01 [1206] (Agilent Technologies, 1990-2001).

137

138 *2.4. Extraction and clean-up*

139

140 The mycotoxins were extracted and purified as described previously (Eskola et al.,
141 2001) with few modifications. A 25 g subsample was extracted with 100 ml of
142 acetonitrile:water (84:16 v/v) into a 500 ml Erlenmeyer flask. The flask was shaken
143 with a rotary shaker at 170 rpm for 80 min at room temperature, and filtered through a
144 filter paper (Whatman grade 2V) from Whatman (Maidstone, UK). The filtrate was
145 defatted with n-hexane (2 x 20 ml). A total of 8 ml of defatted extract was purified
146 through MycoSep 227 column, according to the instructions of the manufacturer. The
147 purified extract that passed through the column (~ 4-5 ml) was collected, and the
148 procedure was repeated by eluting the column with 8 ml of acetonitrile:water (84:16

v/v). The combined fractions were evaporated to dryness under a gentle stream of nitrogen.

2.5. Derivatization

A 50 µl volume of the derivatization mixture (TMSI-BSA-TCMS, 3:3:2) was placed into a vial containing the dry residue. The mixture was allowed to react for 30 min at 80 ºC. After cooling, the derivatized sample was diluted to 480 µl with hexane and mixed thoroughly on vortex for 30 s. The hexane was then washed with 1 ml of phosphate buffer (0.1 M, pH 7.2) by mixing for about 30 s and, finally 20 µl of internal standard (1 mg/l) was added and shaken on vortex. The two layers were allowed to separate. The upper hexane layer with the trimethylsilyl derivatives was transferred to a vial for the gas chromatographic analysis.

2.6. Recovery assays, detection and quantification limits

For recovery studies, mycotoxin-free samples were artificially fortified at three levels of each mycotoxin (50, 500, and 1000 µg/kg of DON, and 300, 500, and 2000 µg/kg of NIV), as follows. Twenty-five grams of ground sample, previously defrosted, were soaked in 0.5 ml of acetonitrile solution containing a suitable amount of each mycotoxin. Then the sample was submitted to dissolvent evaporation for 2 h at room temperature. Finally, the spiked sample was extracted and analyzed by GC as described above. All tests were done in triplicate using different matrices.

The detection and quantification limits for both mycotoxins were assessed at a signal to noise ratio of 3:1 and of 6:1, respectively.

2.7. Statistical analyses

The results from mycotoxin analyses were subjected to statistical analysis using STAGRAPHICS PLUS software (Statistical Graphics Corp. version 5.1) and SPSS (version 14.0.1 for Windows, SPSS Inc., Chicago). The toxin content data were checked for normal distribution (Shapiro-Wilks test) and variance homogeneity (Cochran test). As the data did not fulfill these conditions, it was subsequently subjected to the Kruskal-Wallis test. When comparing the toxin levels in the different sample groups, the Mann-Whitney U-test applied to ranks was used to determine the statistical significance of the differences. The toxin incidence data were also analyzed in order to establish a significant association between different sample groups and the presence of mycotoxin (Fisher's test). A probability value of 0.05 was used to determine the statistical significance.

3. Results and discussion

3.1. Analytical quality control

Mycotoxins were quantified by an internal calibration procedure. Calibration curves with internal standard were linear from 0.05 to 5 mg/l for DON and NIV (regression coefficients r = 0.9993 and r = 0.9990 for DON and NIV, respectively). The estimated limits of detection and quantification were 14.4 and 25.4 µg/kg, respectively, for DON, 9.6 and 15.9 µg/kg for NIV.

198 The recoveries of DON and NIV in different samples are summarized in Table 1.
199 The recoveries of mycotoxins at the lower spiking level varied between 102.7% and
200 107.3% for DON and 93.0% to 113.2% for NIV. At the higher spiking level, the
201 recoveries varied between 70.5% and 97.0% for DON and 77.9% and 94.1% for NIV.
202 The lowest recoveries corresponded to fried corn snack samples. These differences
203 could be attributable to the differences of matrix as described by Jestoi et al. (2004) and
204 it is possible that specific extraction analysis protocols should be used for each
205 combination of mycotoxin and food. The RSD_r (%) of the mean recoveries for DON
206 ranged from 2.2% at the higher spiking level to 12.8% at the lower spiking level,
207 whereas these values for NIV varied between 0.3% and 19.5%, both at the lower
208 spiking level. The recovery and RSD_r obtained for DON are in line with the legislation
209 levels for the DON determination methods (EC, 2005b) thus the method is acceptable
210 according to EU criteria. Specific measures for NIV have not been considered by
211 legislation because a certain degree of co-occurrence with DON is generally observed.
212
213 *3.2. Occurrence of DON and NIV in corn-based foods*
214
215 The results obtained from the analysis of DON and NIV in the samples are
216 presented in Table 2 where the predominant mycotoxin for all analyzed samples was
217 DON.
218 The incidence of DON and NIV in all the samples was 26.8% and 4%, respectively,
219 while the median content of the positive samples was 53.9 and 60.2 µg/kg. The
220 occurrence of DON varied between 25.5%, 27.6% and 46.8% for fried snacks, baked
221 snacks and breakfast cereals, respectively. The incidence of DON was significantly
222 different in breakfast cereals (P = 0.026). With regard to NIV occurrence, 3.4% of the

223 breakfast cereals and 0.6% of the baked snack samples were contaminated by this
224 mycotoxin, while no fried snack samples was contaminated with NIV. The statistical
225 analyses showed a significant dependence between the type of food and the presence of
226 NIV at the 99% confidence level ($P = 0.006$).

227 The DON contamination levels varied from 30.1 to 121.1 µg/kg in breakfast
228 cereals, from 36.4 to 131.7 µg/kg in baked snacks, and from 26.1 to 80.4 µg/kg in fried
229 snacks. No significant differences were found among the median DON contents for all
230 food items ($P > 0.05$). The highest level of DON was found among the baked snack
231 samples (131.7 µg/kg) but no samples exceeded the legally established DON limit (500
232 µg/kg) (EC, 2005a).

233 The NIV contamination levels fluctuated between 51.1 and 106.5 µg/kg in breakfast
234 cereals. Only one sample of baked snacks was contaminated with NIV (55.7 µg/kg). No
235 significant differences were found among the median NIV contents for all positive food
236 items ($P > 0.05$). The results show the natural co-occurrence of both toxins in two
237 samples at concentrations of 64.9 µg/kg of DON and 55.7 µg/kg of NIV for a baked
238 snack sample, and of 35.6 and 106.5 µg/kg, respectively, for a breakfast cereal sample.

239 Comparison between Spanish surveillance and literature data indicates that the
240 incidence of DON contamination in Spanish corn-based foods is lower than in similar
241 foods analyzed in Italy (64% of breakfast cereals and 93% of maize-based foodstuffs)
242 (Cirillo et al., 2003a,b), Germany (67% of breakfast cereals) (Schollenberger et al.,
243 2005b), and the United Kingdom (35% of breakfast cereals and 83.3% of snacks) (FSA,
244 2005). Differences between these data and the results of the present study may be
245 attributed, among others, to a different origin of basing corn; it is well known that cereal
246 infection with *Fusarium* and toxin production depend strongly on environmental
247 conditions (damp climate, cool temperatures). *Fusarium* and trichothecene

248 contamination is more likely under the wet and cold weather conditions of Northern and
249 Central European regions. This would explain the relative low percentage of DON/NIV
250 positive samples. Moisture levels during harvest, transporting and storing the grain, and
251 differences in food production methods (Larsen et al., 2004; Cavaliere et al., 2005) are
252 also believed to be contributing factors. Conversely however, Spanish corn-based
253 contamination is greater than that reported by Milanez et al. (2006) in Brazil, where
254 only one out of 78 samples of corn-based products (cornflakes, corn grits) was found to
255 present traces of DON and NIV. This may attribute either to a different origin of corn,
256 as mentioned above, or to the high detection and quantification limits estimated by the
257 authors (40 and 170 µg/kg for DON and 40 and 200 µg/kg for NIV).

258 With regard to the median mycotoxin levels detected in this study, they were similar
259 to both those found in Italy by Cirillo et al. (2003a,b) and in Germany by
260 Schollenberger et al. (2005b), but lower than those registered in samples surveyed in the
261 United Kingdom (FSA, 2005). The results from a previous study on the occurrence of
262 *Fusarium* toxins in 25 samples of corn-based foods marketed in Spain (Cerveró et al.,
263 2007) showed a higher incidence of deoxynivalenol (68%) and mean content (91 µg/kg)
264 of DON. This discrepancy between these data and present results may partly be
265 attributed to the limited number of samples analyzed in the former study.

266 According to the information on the package, samples have been classified into two
267 groups to associate mycotoxin incidence and content with the presence of additional
268 ingredients: (1) samples with additional ingredients (92 samples), (2) samples without
269 additional ingredients (83 samples). The percentage of positive samples and the content
270 of toxins are listed in Table 3. The occurrence of DON and NIV was similar for both
271 groups of samples, and the Fisher exact test showed no association between the
272 variables considered (P = 0.225 for DON; P = 0.182 for NIV). The median content of

DON was significantly higher in samples without additional ingredients (63.7 µg/kg) (P = 0.004), while no significant differences were found for the median NIV content, although the highest levels of mycotoxins were detected in samples with additional ingredients. Moreover, fifty per cent of samples containing another type of cereal (wheat or oats) presented detectable levels of DON. The highest level of NIV was detected in one sample containing oats. It was clear from this work that contamination by DON and NIV is not only exclusive of corn, but also of oat and wheat contents as some authors have reported for these ingredients (Müller et al., 1997, 1998, 2001; Langseth and Rundberget 1999; Schollenberger et al., 2002, 2005a,b).

In the same way, samples were classified into two groups to compare mycotoxin occurrence and content according to the commercial brand: (1) private label (52 samples), and (2) company brand (123 samples). The results of the occurrence and levels of toxins are shown in Table 3. Approximately 37% of private label brand samples and 22.7% of company brand samples were positive for DON. With regard to NIV occurrence, 7.7% of the private brands and 2.4% of the company brands were contaminated with this mycotoxin. The statistical study showed a weak association between DON occurrence and type of commercial brand (P = 0.047) while no significant association was found for NIV. The statistical comparison between the mycotoxin level for both sample groups indicated no significant differences between the levels of both mycotoxins (P = 0.259). Although private labels have been seen as low-priced and low-quality products in a social context, the results obtained herein indicate that the quality of both types of commercial brands, in terms of mycotoxin content, is approximately the same. In recent years, a significant increase in private label brands has been observed, and companies have started using them to market higher quality

297 products. Nowadys in Europe, private label goods account for around 45% of the
298 products sold in supermarkets.

299 In order to assess the public health risk of consuming DON and NIV in the Spanish
300 population, the exposure of consumers to these toxins can be compared to safety
301 guidelines, such as the Tolerable Daily Intake (TDI). Table 4 shows the calculated
302 human DON and NIV intake from the mean mycotoxin levels found in the samples
303 analyzed in this study. The relationship between each mycotoxin intake and the TDI
304 levels proposed by the SCF of the European Union (1µg/kg bw/day for DON and 0.7
305 µg/kg bw/day – temporary TDI for NIV), has been expressed as a percentage. The
306 mycotoxin intake values found in this study are less than those proposed in the TDI for
307 the respective toxin, and they represented a fraction which does not exceed 7.1% for
308 adults. However, these percentages increase to 12% for DON and 15.7% for NIV for
309 risk groups, such as children. The results of a large-scale European study on the
310 occurrence of *Fusarium* toxins and dietary intake in the European population (EC,
311 2003) demonstrated that while the dietary intakes of DON and NIV were often less than
312 the TDI's for the respective toxin for the entire population and adults, higher intakes
313 values were observed for infants and children.

314 Overall, although the mycotoxin levels found in this survey were low, it is
315 important, however, to bear in mind that cereals are only one of the many possible
316 sources of these mycotoxins for humans, specially children or young people. Moreover,
317 *Fusarium* contamination in grains may differ among the years of harvest (Langseth and
318 Elen, 1997; Müller et al., 1997, 2001; Scott, 1997). Thus, different levels of toxin
319 contamination can also be expected for cereal-based foods.

320 Although longer extensive studies including more *Fusarium* toxins are advisable,
321 the results of the present study provide evidence of the presence of these mycotoxins in

the corn-based foods marketed in Spain. This study confirms the importance of continued surveillance of mycotoxin occurrence in cereal-processed foods in Spain, especially if they are mainly consumed by children and young people. A considerable contribution of these products to *Fusarium* toxin intake of the Spanish consumers may be assumed.

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Table 1

Relation of analyzed samples according to different categories

	Commercial brand		Additional Ingredients[a]		Total
	Company brand	Private label	With	Without	
Breakfast cereals	25	30	30	25	55
Baked corn snacks	49	8	29	28	57
Fried corn snacks	49	14	33	30	63

[a]: Samples with or without another ingredients such as chocolate, butter, honey, wheat, oat, and aromatic flavours.

Table 2

Recoveries and relative standard deviations (%) for trichothecenes in spiked samples[a]

	Spiking levels (μg/kg)	Breakfast cereals	Baked corn snacks	Fried corn snack
DON	1000	94.7 ± 10.7	97.0 ± 12.8	70.5 ± 7.3
	500	95.2 ± 4.2	100.2 ± 6.6	94.9 ± 4.9
	50	107.3 ± 2.2	106.7 ± 8.8	102.7 ± 2.3
NIV	2000	94.1 ± 8.5	93.2 ± 18.7	77.9 ± 4.7
	1000	96.8 ± 4.1	105.4 ± 9.7	106.2 ± 9.7
	300	93.0 ± 7.8	113.2 ± 19.5	101.6 ± 0.3

DON, deoxynivalenol; NIV, nivalenol

[a]:Number of samples = 3

458

459 **Table 3**

460 **Natural occurrence of mycotoxins in corn-based foods**

			Toxin in positive samples (μg/kg)	
	Mycotoxin	Samples positive/total (%)	Range	Median
Breakfast cereals	DON	22/55 (40.0)	30.1 –121.1	44.5
	NIV	6/55 (11.0)	51.1 – 106.5	67.8
Baked snacks	DON	13/57 (22.8)	36.4 – 131.7	62.5
	NIV	1/57 (1.7)	55.7	55.7
Fried snacks	DON	12/63 (18.2)	26.1 – 80.4	55.5
	NIV	0/63	-	-
Total samples	DON	47/175 (26.8)	26.1 – 131.7	53.9
	NIV	7/175 (4.0)	51.1 – 106.5	60.2

461 DON, deoxynivalenol; NIV, nivalenol

462 **Table 4**

463 **Natural occurrence of mycotoxins in corn-based foods according to their composition**

			Toxin in positive samples (μg/kg)	
	Mycotoxin	Samples positive/total (%)	Range	Median
With additional ingredients	DON	22/92 (23.9)	26.4 – 131.7	38.7
	NIV	2/92 (2.2)	51.0 – 106.5	78.7
Without additional ingredients	DON	25/83 (30.1)	34.8 – 105.7	63.7
	NIV	5/83 (6.0)	53.9 – 90.2	60.2

464 DON, deoxynivalenol; NIV, nivalenol

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Table 5

Natural occurrence of mycotoxins in corn-based foods according to the type of commercial brand

			Toxin in positive samples (μg/kg)	
	Mycotoxin	Samples positive/total (%)	Range	Median
Company brand	DON	28/123 (22.7)	26.1 – 131.7	59.7
	NIV	3/123 (2.4)	53.9 – 106.5	55.7
Private label brand	DON	19/52 (36.5)	30.1 – 121.1	46.7
	NIV	4/52 (7.7)	51.0 – 90.2	67.8

DON, deoxynivalenol; NIV, nivalenol

Table 6

Estimated DON and NIV daily intake (µg/kg bw/day) and the percentage that it represents of the proposed tolerable daily intake (TDI) by the Scientific Committee on Food of European Union (SCF)

		DON			NIV		
		Mean level (µg/kg)	Intake (µg/kg bw/day)	SCF (%)	Mean level (µg/kg)	Intake (µg/kg bw/day)	SCF (%)
Breakfast cereals [a]	Adults [c]	53.3	0.03	3.0	72.8	0.04	5.7
	Children [d]		0.06	6.0		0.09	12.8
Corn snacks [b]	Adults	61.9	0.05	5.0	55.7	0.05	7.1
	Children		0.12	12.0		0.11	15.7

DON, deoxynivalenol; NIV, nivalenol

[a] Recommended intake indicated on the label of the package (30 g) is assumed.

[b] A mean weight by package of 50 g is assumed.

[c] A body weight (bw) of 60 kg is assumed for adults.

[d] A body weight (bw) of 25 kg is assumed for children.