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# Occurrence of deoxynivalenol and deoxynivalenol-3-glucoside in durum wheat from Argentina

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ABSTRACT

The occurrence of deoxynivalenol, 3- and 15-deoxynivalenol and deoxynivalenol-3-glucoside in 84 durum wheat samples, from the Argentinean main growing area, was investigated during 2012/13 and 2013/14 using LC-MS/MS. Deoxynivalenol was found in all samples at concentrations varying between <LOQ (50 μg/kg) and 9480 μg/kg. Deoxynivalenol-3-glucoside was detected in 94% of the samples at concentrations ranging from <LOQ (50 μg/kg) to 850 μg/kg. Moreover, the acetylated derivatives were also detected but at lower frequency (49%). To the best of our knowledge, this is the first report of deoxynivalenol-3-glucoside in wheat in Argentina. All the commercial cultivars transformed deoxynivalenol to its glucosylated form at conversion rates between 6 and 22%. The results obtained alert of the potential risk present in durum wheat for Argentinean consumers but also show that some of the commercial cultivars currently on used could be promising candidates for breeding programs intended to obtained *Fusarium* head blight resistance.



## 1. Introduction

*Fusarium* head blight (FBH) is one of the most destructive and economically important fungal diseases of wheat and other small grain cereals worldwide. FHB is caused primarily by species within the *Fusarium graminearum* species complex (FGSC), mainly *F. graminearum*. Other species which may appear as major pathogens depending on the geographic region are *Fusarium culmorum, Fusarium avenaceum* and *Fusarium poae* (Becher, Miedaner, & Wirsel, 2013; Parry, Jenkinson, & MCleod, 1995). The disease can severely reduce grain yield and quality, and under favorable conditions (high humidity and warm temperatures) yield losses can reach up to 50% (Parry et al., 1995). Moreover, the infected grains could be contaminated with mycotoxins such as trichothecenes which results in difficulties for wheat trading, besides the threat to human and animal health. Trichothecenes have been classified into four groups: types A–D, according to their chemical structure (WHO, 1990), the most important in cereals are types A and B (Desjardins, 2006). The B-trichothecenes include the mycotoxins deoxynivalenol (DON), its acetylated derivatives, 3-acetyldeoxynivalenol (3ADON) and 15-acetyldeoxynivalenol (15ADON), and nivalenol (NIV). DON can be found worldwide and it is the most frequent type-B trichothecene. SCOOP (2003) reported trichothecene incidence in samples from 11 European countries. DON incidence was 57% while NIV, fusarenon X (FUS-X), 15ADON and 3ADON were found in the 16, 10, 20 and 8% of the samples analyzed. DON inhibits protein synthesis by interference with peptidyl transferase function on the ribosome, and it has been associated with intoxication of humans and animals through consumption of contaminated food and feed (Pestka, 2010). In Argentina, there are not maximum permitted levels set for DON, however, being its toxicity well characterized, the maximum permitted levels set by the European Commission (EC) for DON in wheat and derived products are often taken as reference. In particular, in durum wheat for human consumption the EC maximum permitted level is 1750 μg/kg (Commission Regulation, EC N° 1881/2006). Durum wheat (*Triticum turgidum* L. var. *durum*) is an important small grain cereal, used for human consumption. In Argentina durum wheat is mainly used for pasta production. In the last few years the national production of pasta has increased to 40% and in 2013 it reached 352,062 tons. The pasta consumption per capita was estimated in 8.27 kg/year (UIFRA, 2014). Although previous studies showed that the retention level of DON from unprocessed wheat grains to cooked pasta in the plate can be of 25% or less (Visconti, Haidukowski, Pascale, & Silvestri, 2004), according to the Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluations, the incidence of DON in pasta (30% of

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positive samples) can be considered of concern (JECFA, 2001). Several studies have reported DON contamination of pasta but at levels below the maximum permitted level established by the EC of 750 μg/kg for adults (Commission Regulation, EC N° 1881/2006; Brera et al., 2013; EFSA, 2013; González-Osnaya, Cortés, Soriano, Moltó, & Mañes, 2011). However, Raiola, Meca, Mañes, and Ritieni (2012) analyzed twenty-seven pasta samples intended for young children consumption and reported that seven of them exceeded the maximum tolerable limit of 200 μg/kg for DON fixed for processed cereal-based baby foods by the EC (Commission Regulation, EC N° 1881/2006).Therefore, DON contamination in wheat, mainly in those varieties intended for pasta production, is of concern for the Argentinean consumers.

Moreover, DON may acts as a virulence factor of the fungus, affecting the protein synthesis in plant cells. As a consequence, plants have developed detoxification processes which include DON glucosylation to a less toxic compound, DON-3-β-d-glucoside (D3G). This product is known as a modified mycotoxin and it is less active as a protein synthesis inhibitor than DON in vitro (Poppenberger et al., 2003). So far, D3G has been reported in naturally contaminated samples of bread and durum wheat, maize, oats, barley, malt, triticale and also derived products such as flour, breakfast cereals, snacks and beer (Berthiller et al., 2013; Dall'Asta, Dall'Erta, Mantovani, Massi, & Galaverna, 2013; Lancova et al., 2008; Malachova et al., 2011; Rasmussen et al., 2012).

Currently, there are not regulatory levels for the most frequently occurring modified forms of DON, i.e. 3 and 15ADON and D3G, in cereals and cereal-based foods. However, the JECFA considered that D3G and the acetylated derivatives, might contribute to exposure to DON. Based on the available toxicity data, the Committee considered the toxicity of the acetylated derivatives equal to that of DON (JECFA, 2011). Some studies indicate that D3G can be released during food processing as a consequence of enzymatic degradation of polysaccharides and also it could be cleaved during mammalian digestion, thus contributing to the overall toxicity of DON (Franz Berthiller et al., 2013; Nagl et al., 2014). Furthermore, the JECFA experts pointed out that additional data on the occurrence of and the effects of processing on 3ADON, 15ADON and D3G are needed, as well as their co-occurrence with DON (JECFA, 2011).

Updated information on the occurrence of DON and its modified forms are reported in the recent report issued by the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (EFSA, 2013). Results from the EFSA survey showed that the DON derivatives (3ADON, 15ADON) were far less frequently found and at lower levels than DON. The Panel highlighted that further data should be collected on D3G, 3ADON and 15ADON in order to better characterize their potential contribution to the total exposure to DON (EFSA, 2013).

Up to now, in Argentina only DON has been reported as grain contaminant in durum wheat, there are not data available on D3G and the acetylated DON derivatives contamination in this cereal. The aim of this study was, therefore, to measure the contamination by deoxynivalenol, 3- and 15-deoxynivalenol and deoxynivalenol-3-glucoside in different durum wheat commercial cultivars collected in the Argentinean main growing area. Furthermore, in order to get insights into the possible correlation between the glucosylation ability and the resistance to FHB, the level of glucosylation in the field was assessed.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Methanol, and acetonitrile (both HPLC grade) were purchased from Mallinckrodt Baker (Milan, Italy). Ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Ammonium acetate (for mass spectrometry) was from Sigma-Aldrich (Milan, Italy). Oasis® HLB (3 ml, 60 mg) columns were purchased from Waters (Milan, Italy). Filter papers (Whatman no. 4) were obtained from Whatman International Ltd (Maidstone, UK). Standard mycotoxins (DON, 3ADON, 15ADON, and D3G) were purchased from BiopureReferenzensubstanzen GmbH (Tulln, Austria). Stock solutions of each trichothecene were prepared by dissolving the solid commercial toxin in acetonitrile at concentration of 1 mg/ml. D3G was purchased as reference solution, 50 μg/ml acetonitrile, from Biopure™ (Romer Labs, Tulln, Austria). A mixed standard solution of DON, 3ADON, 15ADON, and D3G was prepared in acetonitrile at concentrations of 6.25 μg/ml for DON, and 0.75 μg/ml for 3ADON, 15ADON, and D3G, to be used for preparation of matrix assisted calibrant solutions for LC-MS/MS analysis. All mycotoxin solutions were stored at 4 °C until use.

### 2.2. Sampling

A total of 84 samples (7 cultivars × 2 reps × 3 geographic locations × 2 years) of durum wheat were collected during 2012/13 and 2013/14 harvest season, from three different geographic locations in the South of Buenos Aires province (Pampas region), Argentina, including Miramar, Balcarce and La Dulce. The samples belonged to seven commercial cultivars: Buck Platino (BPLT), Buck Granate (BGNT), Buck Topacio (BTOP), Bonaerense INTA Carilo (BICRL), Bonaerense INTA Facon (BIFAC), ACA1801F and ACA1901F. The plant material was collected along transects, with sampling stations within fields being 5 m from each other. At each sampling station, five or six wheat heads were selected. From these samples, a subsample of 500 g was taken and immediately stored at 4 °C. All samples were ground to a fine powder and stored in paper bags at 4 °C prior to analyses.

### 2.3. Sample preparation (extraction and clean-up)

Samples were prepared according to Lattanzio, Gatta, Suman, and Visconti (2011) with a minor modification in the clean up step (i.e. in the column washing solvent) to avoid D3G losses at this stage. Briefly, ground samples (5.00 +/− 0.01 g) were extracted by 60 min shaking with 25 ml acetonitrile/water (84:16, v/v). After filtration (filter paper, Whatman no. 4), 5 ml of filtrate were evaporated to dryness at 40 °C under a stream of air. The residue was completely dissolved by adding 1 ml of a mixture of methanol/water 10:90 (v/v). The reconstituted extract was purified through Oasis® HLB, after column activation and conditioning according to manufacturer instructions. After passing the reconstituted sample extract, the column was washed with 1 ml water and dried. Afterwards the toxins were eluted with 1 ml methanol. The eluate was dried at 40 °C under a stream of air, and the residue redissolved in 400 μl of methanol/water (20:80). Volumes of 20 μl were analyzed by LC-MS/MS.

### 2.4. LC-MS/MS equipment and parameters

LC–MS/MS analyses were performed on a QTrap MS/MS system, from Applied Biosystems (Foster City, CA, USA), equipped with an ESI interface and a 1100 series micro-LC system comprising a binary pump and a microautosampler from Agilent Technologies (Waldbronn, Germany). The analytical column was a Gemini® C18 column (150 mm × 2 mm, 5 μm particles) (Phenomenex, Torrance, CA, USA), preceded by a Gemini C18 guard column (4 mm × 2 mm, 5 μm particles). The column oven was set at 40 °C. The flow rate of the mobile phase was 0.2 ml/min and the injection volume was 20 μl. Eluent A was water and eluent B was

methanol, both containing 5 mM ammonium acetate. A gradient elution was performed by changing the mobile phase composition as follows. After 3 min at 10% eluent B, the proportion was linearly increased to 83% in 15 min, then increased to 100% in 1 min, and kept constant for 4 min. The column was re-equilibrated with 10% eluent B for 6 min. For LC-MS/MS analyses, the ESI interface was used in negative ion mode, with the following settings: temperature (TEM) 350 °C; curtain gas (CUR), nitrogen, 30 psi; nebulizer gas (GS1), air, 10 psi; heater gas (GS2), air, 30 psi; ion spray voltage (IS), −4500V. The mass spectrometer operated in SRM (selected reaction monitoring) mode, by monitoring 2 transitions (1 quantifier, 1 qualifier) for each compound, with a dwell time of 100 ms (Table 1). Quantification of mycotoxins was performed by matrix matched calibration. Limits of quantification (LOQ) determined as the lowest amount of each analyte which could be determined with a precision <20%, were: 50 μg/kg for DON, 20 μg/kg for the sum of 3 and 15ADON and 50 μg/kg for D3G. Linearity of the method was proven in the range LOQ – 2500 μg/kg for DON and LOQ – 300 μg/kg for 3 and 15ADON, and D3G. Samples resulting contaminated above the highest calibration level were diluted and re-analyzed.

### 2.5. Statistical analysis of data

Results are reported as the mean ± SD. To compare among durum wheat cultivars a one-way ANOVA was used since the data passed both the normality and the equal variance tests. All pairwise multiple comparisons were carried out using the Holm-Sidak method. The correlations between DON and D3G were performed by the Pearson Correlation test. A P-value $\leq 0.05$ indicated statistical significance. All statistical procedures were performed using the software package SigmaStat (SigmaStat for Windows, v. 2.03).

## 3. Results and discussion

Occurrence of DON, its acetylated derivatives and D3G were assessed in samples of durum wheat (n = 84) produced in three different geographic locations of the main durum wheat growing area during 2012/13 and 2013/14 harvest seasons. All samples were found positive for DON at concentrations varying between <LOQ and 9480 μg/kg, with an average of 1750 μg/kg for quantified samples, there was only one sample below the LOQ. D3G was detected in 94% of the analyzed samples at concentrations ranging from <LOQ to 850 μg/kg, there were five samples that were below the LOQ. Acetylated derivatives of DON were also found in 49% of the samples in levels ranging from <LOQ to 190 μg/kg, there were eleven samples that were below the LOQ. Therefore, with respect to the contribution to the total mycotoxin content, the D3G resulted to be more relevant that acetylated DON derivatives, both in terms of incidence and contamination levels.

All the investigated samples showed DON contamination, 30% of them showed levels above the maximum one established by Commission Regulation, EC N° 1881/2006 of 1750 μg/kg for unprocessed durum wheat grains. There are few reports of DON incidence in Argentinean durum wheat. González, Martínez, Pacin, and Resnik (1999) analyzed sixty durum wheat samples originated from the same region and found levels between 26 and 6400 μg/kg. In 2003, Lori et al. studied DON incidence during two harvest years and found levels up to 8000 μg/kg, they also found differences in DON contamination among locations within the durum wheat growing area. Recently, Cendoya et al. (2014) reported the co-occurrence of DON and fumonisins in durum wheat grains and found DON in levels ranging between 194 and 15,141 μg/kg. Several epidemics have occurred in the main durum wheat growing area in 1963, 1976, 1978 and 1985, with crop losses as high as 70% (Moschini & Fortugno, 1996). The results here presented are in agreement with the previous reports.

Considering D3G, it was detected in most samples. Similar results were obtained by Berthiller et al. (2009) in wheat samples from Germany, Austria and Slovakia and Dall'Asta et al. (2013) in durum wheat samples from Italy, in these samples acetylated derivatives were also found.

Statistical analysis showed that there were significant differences between harvest years ($p = 0.005$) and among geographic locations ($p < 0.001$), indicating that durum wheat samples collected during different harvest years and area might have different DON levels (Fig. 1). During 2012/13, DON contamination ranged between 300 and 7760 μg/kg with a mean level of 1560 μg/kg while in 2013/14 the DON levels in the samples ranged between <LOQ and 9480 μg/kg with a mean level of 1960 μg/kg (quantified samples). Regarding the geographic locations, samples collected from Miramar (mean value = 2400 μg/kg) and Balcarce (mean value = 2280 μg/kg) presented higher values of DON than La Dulce (mean value for quantified samples = 580 μg/kg). Samples from Miramar in 2012/13 showed a DON mean contamination value of 2030 μg/kg, in Balcarce the mean contamination value obtained for DON was 2000 μg/kg while in La Dulce, the mean level was 630 μg/kg. For the 2013/14 samples, those collected from Miramar showed a mean value of DON contamination of 2780 μg/kg, the ones obtained in Balcarce showed a mean value of 2560 μg/kg while in La Dulce, the samples collected presented a mean contamination value of 570 μg/kg (quantified samples) (Table 2). As it is shown in Table 2, D3G was detected in the three geographic locations in both years, in levels ranging from <LOQ to 850 μg/kg (mean = 190 μg/kg for quantified samples) in 2012/13 and from <LOQ to 650 μg/kg (mean quantified samples = 160 μg/kg) in 2013/14. There were not significant differences between D3G contamination between years or geographic locations.

FHB development is dependent of several factors being the weather conditions such as rainfall and temperature prevailing during anthesis period, the most important. In the Argentinean main growing area which belongs to the Pampas region, both

**Table 1**
LC–MS/MS parameters for mycotoxin detection.

| Analyte | Q1 (*m/z*) | Q3 (*m/z*) | DP (V) | EP (V) | CE (V) | CXP (V) |
|---|---|---|---|---|---|---|
| DON | 355.1 | 295 | −50 | −4 | −16 | −6 |
| | **355.1** | **59** | **−50** | **−4** | **−28** | **−6** |
| D3G | 517.1 | 247.2 | −18 | −5 | −33 | −2 |
| | **517.1** | **427.4** | **−18** | **−5** | **−29** | **−6** |
| | 517.1 | 59 | −18 | −5 | −80 | −12 |
| ADON | 397 | 307 | −70 | −6 | −23 | −5 |
| | 397 | 134.8 | −70 | −4 | −26 | −2 |
| | **397** | **59** | **−70** | **−6** | **−35** | **−8** |

Transitions used for quantitation in bold characters. Q1: Fist quadrupole; Q3: third quadrupole; DP: declustering potential; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential.

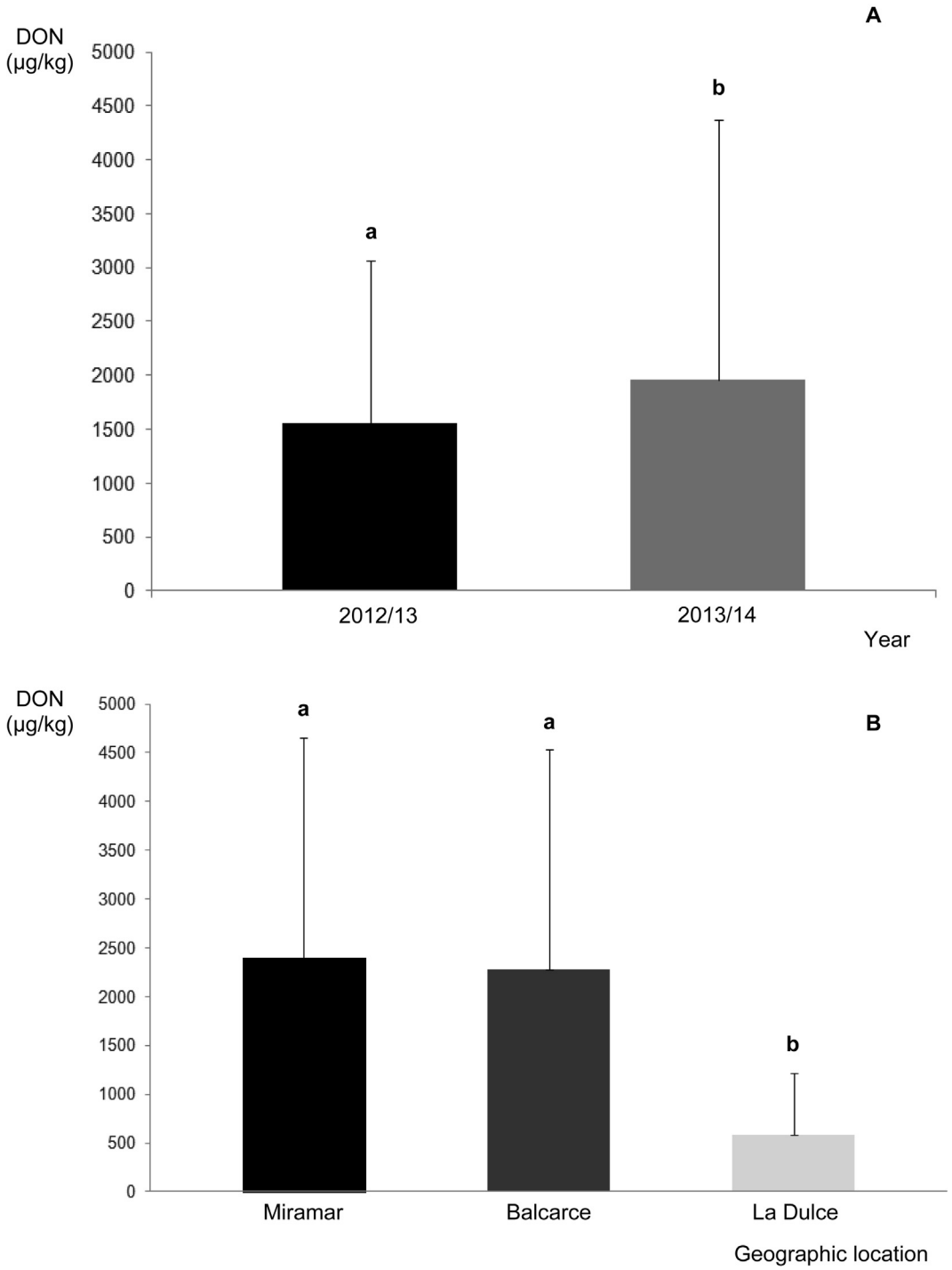


**Fig. 1.** DON contamination between harvest years (A) and among geographic locations from the main durum wheat growing area in Argentina (B). Different letters on the top of the columns indicate statistically significant differences ($p = 0.005$ for A and $p < 0.001$ for B).

low and high temperatures may occur during October and November, when anthesis generally takes place. These temperatures may influence the infection process (Moschini & Fortugno, 1996). Another factor influencing *F. graminearum* infection is crop residue and weeds since they serve as reservoir of inoculum of the fungus (Osborne & Stein, 2007). During 2012/13 harvest season, a severe epidemic occurred in the north central part of the Pampas region (Palazzini et al., 2015) which was previously predicted by a disease forecasting system (Moschini, Martínez, & Cazenave G., 2013), however in the south of the Pampas region (where durum wheat is cultivated) the environmental conditions were not favorable for the development of the FHB epidemic and the incidence of the disease was less than 20% (Moschini et al., 2013).

Furthermore, an analysis of correlation between DON and D3G in the samples was performed (Fig. 2). The Pearson coefficient was significant with $R^2 = 0.84$. This means that the D3G production is related positively to the DON content and increasing DON levels also increase the D3G level in wheat. This is in accordance with other studies (Ovando-Martínez et al., 2013; Rasmussen et al., 2012; Simsek, Ovando-Martínez, Ozsisli, Whitney, & Ohm, 2013).

**Table 2**
Mean values and concentration range of mycotoxin contents in positive samples of all studied cultivars from 3 geographic locations from the main durum wheat growing area in Argentina during 2012/13 and 2013/14 harvest years.

| Locality | 2012/2013 | | | 2013/2014 | | |
|---|---|---|---|---|---|---|
| | DON | ADON | D3G | DON | ADON | D3G |
| | Number of samples<br>Mean (μg/kg)<br>(Range) | | | Number of samples<br>Mean (μg/kg)<br>(Range) | | |
| Miramar | 14 | 6 | 14 | 14 | 13 | 14 |
| | 2030 | - | 190 | 2780 | 52 | 210 |
| | (880–7760) | (<LOQ – 52) | (110–500) | (720–8820) | (<LOQ-150) | (80–540) |
| Balcarce | 14 | 3 | 14 | 14 | 14 | 14 |
| | 2000 | - | 280 | 2560 | - | - |
| | (360–6950) | (<LOQ – 30) | (90–850) | (580–9480) | (<LOQ – 190) | (<LOQ – 650) |
| La Dulce | 14 | 1 | 14 | 14 | 4 | 9 |
| | 630 | - | 110 | 570 | - | - |
| | (300–1700) | <LOQ | (<LOQ – 230) | (<LOQ – 2840) | (<LOQ – 40) | (<LOQ – 190) |

LOQ: Limit of quantification 50 μg/kg for DON and D3G and 20 μg/kg for ADON.

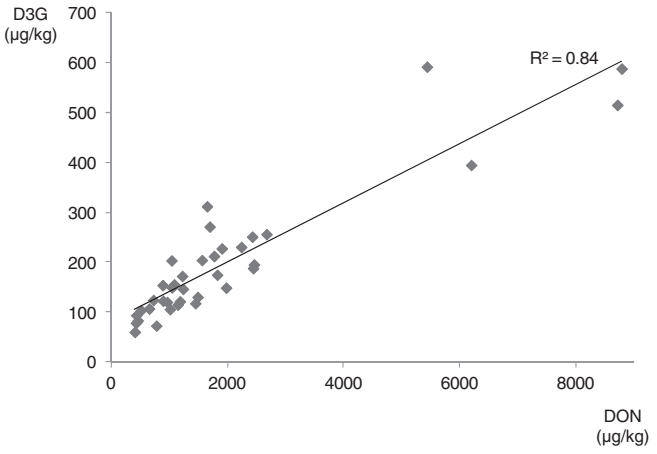


**Fig. 2.** Correlation between DON and D3G levels in different durum wheat cultivars. Pearson coefficient: 0.84, $p \leq 0.05$.

Also, the glucosylation capacity of the commercial durum wheat cultivars was calculated. The percentages obtained during both years and the three geographic locations ranged between 6 and 22%. Mean glucosylation percentages, and DON and D3G contamination for each cultivar are shown in Fig. 3.There were significant differences ($p < 0.05$) in the glucosylation capacity among cultivars, being BGNT the cultivar with the maximum glucosylation capacity and ACA1901F the one with the minimum glucosylation percentage.

Lemmens et al. (2005) showed that the conversion of DON to D3G as part of a detoxification process is implicated in the FHB resistance of wheat. All the commercial cultivars analyzed in this study were able to transform DON to its glucosylated form; however the glucosylation capacity varied from 6 to 22%. These percentages are in agreement with those reported for artificially and naturally contaminated samples (Audenaert et al., 2013; Cirlini et al., 2014; Rasmussen et al., 2012). Therefore, the commercial cultivars here analyzed could present different levels of FHB resis-

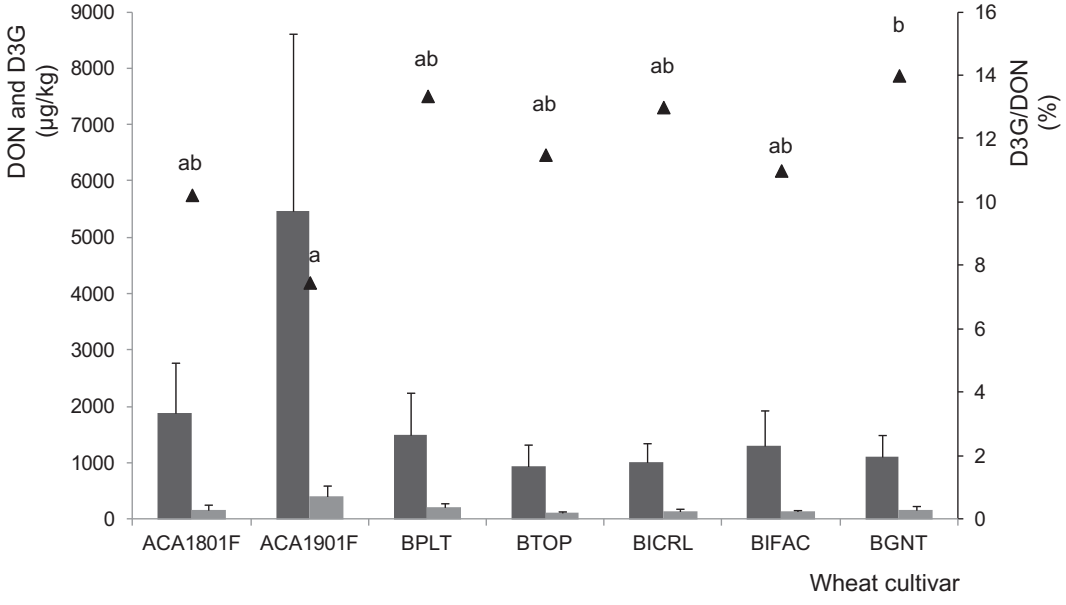


**Fig. 3.** DON and D3G concentrations (bars) in seven commercial durum wheat cultivars under natural conditions. Discrete data points represent glucosylation capacity, calculated as the relative amount of DON that was glucosylated to D3G. Different letters indicate statistically significant differences ($p < 0.05$).

tance, in particular, the cultivar BGNT which showed the highest glucosylation rate among the investigated ones.

## 4. Conclusion

In the present study, the contamination by deoxynivalenol, 3- and 15-deoxynivalenol and deoxynivalenol-3-glucoside in different durum wheat commercial cultivars from the Argentinean main growing area was investigated. All analyzed samples were found to be contaminated with DON, and 30% of them exceeded the maximum permitted level established by the EC, presenting a potential risk for Argentinean consumers. It was also observed that DON contamination was dependent on the harvest year and the geographic location; these differences could be attributed to the weather conditions prevailing during anthesis period. Moreover, the samples were also contaminated with the acetylated DON derivatives and D3G, the latter contributing up to 22% of the total DON content. To the best of our knowledge, this is the first report of this modified mycotoxin in durum wheat grains in Argentina. Among the commercial wheat cultivars under study there were some of them that presented a good conversion rate of DON to D3G and they could be promising candidates for breeding programs intended to obtain local durum wheat lines resistant to FHB.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.03.085.

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