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Deoxynivalenol and its conjugated glucoside forms in imported dry wheat *(Triticum aestivum* L.*)* grains for Brazilian consumption

V.M. Scussel[1,2*], M. Rokka[2], G.D. Savi[1] and M. Jestoi[2]

[1]*Food Science and Technology Department, Centre of Agricultural Sciences, Federal University of Santa Catarina, Florianopolis, SC, Brazil;*

[2]*Finnish Food Safety Authority, Chemistry and Toxicology Unit, Mustialankatu 3, Helsinki , Finland*

Abstract

DON and its Phase II conjugate DON-3-β-D-glucopyranoside (DON-3-β-D-Glc) determination in imported wheat grains was carried out by ultra-performance liquid chromatography with quadrupole orthogonal acceleration time-of-flight mass spectrometry. The quantification limits were 8.5 and 8.0 µg/kg with mean recoveries of 89 and 79% for DON and DON-3-β-D-Glc. They were detected in 51 and 64% of the wheat grains surveyed and levels ranged from 8.5/8.0 µg/kg to 6926.0/4001.3 µg/kg, respectively, with 14% of the positive samples only DON-3-β-D-Glc contaminated. Other DON metabolite forms were also checked through Metabolynx Browser. The wheat grains moisture content and water activity were low (from 10.1 to 15.9% and 0.42 to 0.82) as samples were collected in grain storage unities after passing through drying process. When the DON levels data of contaminated wheat samples were compared to the maximum levels established by the national and international regulations, a total of 33 and 26% did not accomplish to the Brazilian and EU regulations, specifically for flour production (either, whole or white flour). There is a need of consistent DON monitoring of import wheat grain coming to Brazil in order to keep consumers wheat diet based, safe.

Keywords Masked. Deoxynivalenol. Conjugate. Mass spectrometry. Time-of-flight.

I. INTRODUCTION

Deoxynivalenol (DON), also called vomitoxin (Fig. 1), is a field *Fusarium* (*F. graminearum*, *F. culmorum* and *F. avenaceum*) toxin and has been reported contaminating wheat worldwide with varied levels including the Americas [1-12]. That contamination means consumers exposure, especially those on wheat based diet [13-14].

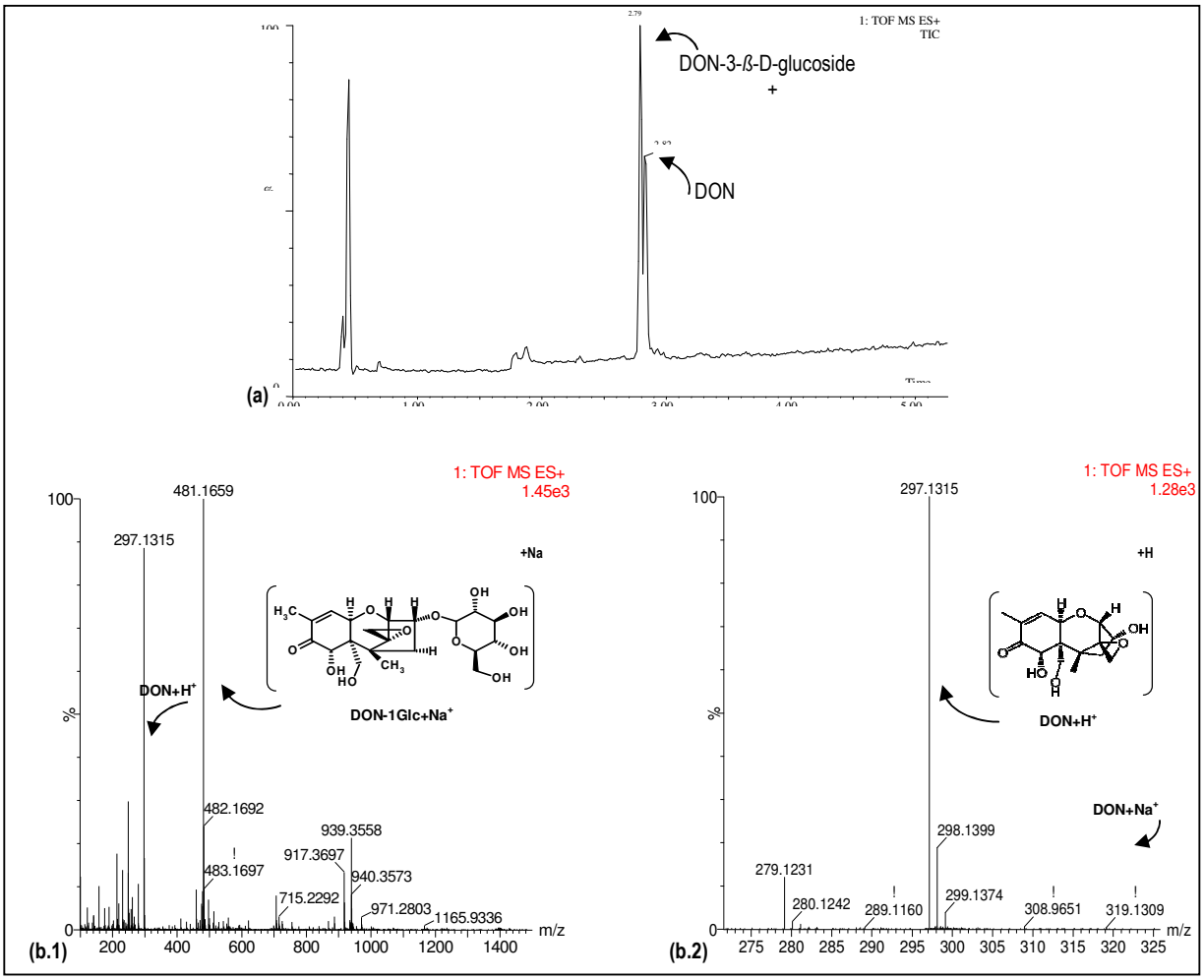
Regarding Brazil and its changing food habits to wheat, there is quite a few data about the consumers DON exposure reported [14]. Despite of its high agricultural production, the country does not produce enough wheat, thus importing about a half of it from other American countries such as Argentina, Canada, Paraguay, Uruguay and United States of America [15].

DON also has been reported in conjugated form, i.e., a DON mono-glucoside, the DON-3-*β*-D-glucopyranoside (DON-3-*β*-D-Glc; Fig. 1) detected in different cereals including wheat [16-24]. It is a very usual DON conjugate present in that grain, can increase during food processing and be hydrolyzed to DON by the bacteria glucosidases in the colon. The FAO/WHO Experts in 2010/2011 have considered DON monoglucoside to be an additional contributing factor to total DON dietary exposure. Indeed, toxin conjugate forms are produced by plants (at grain development stage) when infected by toxigenic *Fusarium* strains in order to protect themselves against the harmful effects of their toxins. That detoxification process involves several reactions such as hydrolysis, reduction and oxidation of toxins - Phase I and/or glucosylation, sulfation, acetylation and methylation - Phase II [19]. The main DON conjugates that have been detected in plants are the hydrosoluble glucoside

forms, which are also called masked toxin, as they are difficult to be detected by the conventional methods [25].

Among the techniques for masked toxins, the mass tandem (MS/MS) and quadrupole time-of-flight (Q-TOF) mass spectrometry are the ones currently utilized. Berthiller et al. [18-19,26] as well as Sulyok et al. [27-28] have registered their presence in different grains and processed food, by applying mass tandem spectrometry (MS/MS) detection. Regarding Q-TOF, it gives inclusive useful data for chemical structures identification of unknown compounds [25,29-31]. Scussel et al. [25] were also able to identify and characterize DON conjugate form (including the most abundant adducts and their isotopes) by applying that technique, both at negative ($[M-H]^-$) and positive ($[M+H]^+$) modes.

Figure 1. (a) Total ion mass MRM chromatogram of DON (2.82 min) and DON-3-*β*-D-Glc (2.79 min) using UPLC-Q-TOF/MS ESI $[M+H]^+$ with mobile phase of acetonitrile:water at 0.5 mL/min (capillary & cone voltages of 3.0 kV and 20 V) and (b) TOF/MS $[M+H]^+$ spectra (b.1) DON-1Glc* (*DON-1Glc+H*$^+$, *DON-1Glc+Na*$^+$) and (b.2) DON (*DON+H*$^+$, *DON+Na*$^+$) at same capillary and cone voltages as in (a), respectively [*DON-3-*β*-D-Glc].



Regarding DON contamination from the wheat exporting countries to Brazil, several studies have reported its detection [2,6,9-11,32-34]. By analysing different crops including wheat (total: 1056) in Argentina, Quiroga et al. [32], reported 100% of wheat samples DON contaminated. Gonzáles et al. [33] also reported freshly harvested wheat grains DON contaminated with levels ranging from 2 to 30,000 µg/kg in the same country. Marasas [34] reported up to 1000 µg/kg in Canada and in addition, Wegulo and Dowell [4] registered the concern on *Fusarium* head blight wheat epidemic and DON in Nebraska in USA. On the other hand, Pan et al. [11] reported that the toxigenic *F. graminearum* have been causing destructive epidemics on wheat in Uruguay. Regarding data from

Paraguay, Santos et al. [12] reported also wheat DON contamination. Despite that, to our knowledge no work has been carried out on DON conjugates presence in the South American grains.

As far as DON-3-*β*-D-Glc is concerned, it has been detected abroad in raw and processed food [24], including different cereals, cereals based food and beverage such as *maize and barley* [21,23,26,35], *cereal based products* [22,36] and *beer & malt* [35,37-38]. The methodology applied has been mainly the LC-MS/MS [18,27-28,39-40].

Considering the (a) country's low wheat production and wheat import dependence (although its large and productive agricultural area - mainly for soy and maize); (b) the very usual DON conjugate wheat presence; (c) its increase during food processing [41] and (d) high incidence of DON contamination reported from the exporting countries, this study reports a survey on DON and its possible conjugate form contamination from imported wheat grains utilized in Southern Brazil (for flour milling), by UPLC-Q-oa-TOF/MS.

II. MATERIALS AND METHODS

SAMPLES

Dry whole wheat grains (1kg each) imported by Brazil from other American countries and commercialized in the Southern region (States of Parana / Santa Catarina / Rio Grande do Sul) for flour production (total: 23). They were collected from silos during their grain stream transfer to four mill processing facilities [composite sample (100 g): n=10 per silo]. To find out the moisture content (mc), the wheat grains were dried in an oven (105±5°C) up to a constant weight using gravimetric method. Water activity (aw) was determined using a Aqua-Lab 4TE Decagon equipment. All these analyses were carried out in triplicate and according to the Association of Official Analytical Chemists - AOAC [42] guidelines.

CHEMICALS AND REAGENTS

DON and DON-3-*β*-D-Glc standards were purchase from Sigma (St. Louis, USA) and Biopure (Tulln, Austria), respectively. Acetonitrile (ACN) and methanol (MeOH), HPLC grade; formic acid and sodium hydroxide, analytical grade, from J.T. Baker (Deventer, Holland). Ultrapure water was obtained from a Milli-Q system (Billerica, USA). The DON and DON conjugate solutions (individually and mix), as well as the accurate mass (leucine-enkephaline) and mass scale (sodium formate) solutions were carried out as reported by Scussel et al. [25].

WHEAT SAMPLES PREPARATION

The method applied was of Scussel et al. [20,25]. Briefly, wheat grains were milled (particle size 400 mm) and 10 g portions added of ACN:water, followed by clean-up through Mycosep column (Romer Labs, Austria), centrifuged and extract evaporated to dryness in a concentration work station (Turbo VapLc - Life Sciences), redissolved in 10% ACN and filtered into conical bottom vials (1 mL) with special pre-lit PTFE/silicone septa caps (Waters Corp. Mildford, USA). The clean extract was injected into an UPLC-Q-TOF/MS system through an Acquity BEH C_{18} UPLC reversed phase (100 x 2.1 mm; 1.7 µm) column with column heater and autosampler (10 µl loop) (Waters, Milford, USA). The Q-TOF/MS detector (Premier Analyser) was equipped with ZSpray electrospray ionization (ESI) interface (MicroMass, Manchester, UK). Analysis was carried out in triplicate. Detection limit (LOD) was defined by three times the signal/noise (S/N) ratio (3 S/N) and the quantification limit (LOQ) by ten times S/N (10 S/N) ratio.

DON AND ITS CONJUGATES DETERMINATION

The separation of compounds from wheat extracts was carried out through the UPLC column (with in-line filter - 0.2 mm, 2.1 mm and eCord) at 40 °C. The mobile phase gradient elution used was 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B) at a flow rate of 0.5 mL/min (after 4 min of a liner gradient from 100% A to 44.4% A and 55.6% B, for 0.1 min and then to 100% B).

The data acquisition was performed with the instrument operating by ESI in the positive $[M+H]^+$ mode. The ionization and response of both compounds applied were: capillary voltage 3.0 kV, cone voltage 20 V; also source temperature of 120°C, desolvation temperature 400 °C, desolvation gas flow 1000 L/h and collision energy 5 eV (total MS scan range for DON and DON-3-*β*-D-Glc was of 100 to 1000 Da) (Table 1).

The identification, confirmation and quantification of analytes were carried out by utilizing MassLynx (Waters, Milford, USA). From the total ion chromatogram obtained, expected analytes exact masses were extracted and the retention time (r_t) established (Table 1). Accurate mass measurements were obtained applying elemental composition (EC) search [features: calculated mass; mDa error; ppm error (part per million error); DBE (double bond equivalent); and i-Fit (isotope fit value)]. Difference between mass and calculated mass was also used for the ion mass found identification and confirmation. The quantification was carried out by QuanLynx (Waters, Milford, USA).

Table 1. DON and DON-3-*β*-D-Glc characteristic ions and parameters for UPLC-oa-Q-TOF/MS[a] at $[M+H]^+$ unequivocal identification

Analyte	Expected molecular and adduct ions	Most abundant molecular and adduct ions formed*					TIC[b]	TOF/MS[c]*	Spectrum[d]*	Instrumental	
	Reaction	Calculated mass**(*m/z*)	mDa[e]	PPM[f]	DBE[g]	i-FIT[h]	r_t[k,l]*** (min)	Peak intensity (x10⁴)	Ion abundance (x10³)	Cone[j] (V)	Capillary[j] (kV)
DON[m]											
	$+H]^+$	297.1338⁺	2.0	4.1	5.5	6.5	2.82	2.10	4.176	20	3.0
	$+Na]^+$	319.1158⁺	-0.8	2.5	5.5	3.3			LA[n]		
	$+K]^+$	LA	>5	>5	<-1.5	HV[o]			LA		
	$+H+CH_3CN]^+$	LA	>5	>5	>50.0	HV			LA		
	$+H+(CH_3CN_2)\ ]^+$	LA	>5	>5	<-1.5	HV			LA		
DON-3-*β*-D-glucoside[p]											
	$+H]^+$	459.1866⁺	-0.5	2.2	6.5	15.0	2.79	3.98	0.80	20	3.0
	$+Na]^+$	481.1686⁺	-1.6	-3.3	6.5	3.6			0.49		
	$+K]^+$	LA	>5	>5	>50.0	HV			LA		
	$+CH\ CN]^+$	LA	>5	>5	>50.0	HV			LA		
	$+(CH\ CN)\ ]^+$	LA	>5	>5	<-1.5	HV			LA		
	$DON+H]^+$	297.1338⁺	0.1	1.9	5.5	8.3			3.06		
	$Glucoside+H]^+$	ND[r]	ND	ND	ND	ND			ND		

[a]ultra performance liquid chromatography-quadrupole orthogonal acceleration time-of-flight mass spectrometry [b]total ion chromatogram [c] extracted time of flight ion mass chromatogram at specific r_t [d]TOF/MS extracted spectrum [e]difference between the calculated mass and the mass in mili Dalton (max tolerance: 5.0) [f]difference between the calculated mass and the mass in part per million error (acceptable: less than 5) [g]double bond equivalent for the suggested formula of selected mass (min -1.5 and max 50.0) [h]isotope-fit value (acceptable: the lowest the best) [i]cone voltage [j]cappilary voltage [k]retention time [l] mobile phase: acetonitrile:water (in 0.1% formic acid)–gradient [m]DON ($C_{15}H_{20}O_6$; MW 296.1260) [n]very low abundance ion [o]high value [p]DON-3-*β*-D-glucoside ($C_{21}H_{30}O_{11}$; MW 458.1788) *from mix solution of DON and DON-3-*β*-D-glucoside (conc. 10 and 8.5 µg/mL^{-1}, respectively) **most abundant ions detected in spectra per precursor at each ionization condition *** analytes r_t evaluated separately / mix solution MetaboLynx Browser applied further to check DON conjugate presence by matching mass similarities to the analytes studied: in solvent/wheat matrix (Scussel *et al.,* 2011b modified).

The DON and its conjugate acquisition data were also checked by browsing through Metabolynx for further confirmation and to search for other unknown conjugates. That was carried out after setting the right method program i.e., by filling the exact mass of the possible conjugates to be detected (Table 2). *Note:* for that purpose the UPLC run took 18 min in order to allow any possible compound to be chromatographically separated and identified.

Table 2. DON and its possible conjugates molecular ions with calculated masses at $[M+H]^+$search

Analyte	Possible DON conjugates	Molecular ions[b]	Exact mass *(m/z)*[b]
		+H	$[M+H]^+$
DON[a]			
	NA[c]	$C_{15}H_{21}O_6^+$	297.1338
DON conjugate			
	+S	$C_{15}H_{21}O_9S^+$	377.0906
	+OH-S	$C_{15}H_{21}O_{10}S^+$	393.0855
	+3-*β*-D-Glc[d]	$C_{21}H_{31}O_{11}^+$	459.1866
	+2Glc	$C_{27}H_{41}O_{16}^+$	621.2394
	+3Glc	$C_{33}H_{51}O_{21}^+$	783.2923
	+4Glc	$C_{39}H_{61}O_{26}^+$	945.3451
	+5Glc	$C_{45}H_{71}O_{31}$	1107.3879
	+6Glc	$C_{51}H_{81}O_{36}^+$	1269.4507

[a] deoxynivalenol [b] for Q-TOF identification by MassLynx & MetaboLynx Browser [c] not applicable [d] DON-1-glucoside (DON-3-*β*-D-Glc)

For matrix effect and R^2, blank sample extracts were previous spiked with the standards mix at different concentrations (1, 2, 4, 6, 8, 10 and 12 µg/kg) and injected into the UPLC-Q-oa-TOF/MS

(n=3). At the same time, for recovery, the wheat sample was grinded and spiked prior extraction with both toxins at six concentrations (1 to 10 µg/kg, n=3) and extracted as previously mentioned.

III. RESULTS AND DISCUSSION

By applying the extraction and clean-up steps with UPLC-Q-oa-TOF/MS (ESI) at $[M+H]^+$ mode for DON and DON-3-*β*-D-Glc separation and detection, it was possible to simultaneously identify and quantify both target compounds in the imported wheat grains for flour processing. Table 3 presents the wheat grain samples levels of both compounds and other conjugates.

Table 3. DON[a] and DON-3-*β*-D-Glc levels[b] detected in imported wheat (*Triticum aestivum* L.) grains for flour milling commercialized in Brazil Southern *versus* regulation and their humidity conditions

Dry wheat grain contamination number	DON[a] µg/kg (samples%)			DON conjugates[b]		Humidity	
	Positive	>ML[c,d]		DON-3-*β*-D-Glc[e] µg/kg (samples %)	Others[f]	mc[g] (%)	aw[h]
		1000 (BR / USA / FAO)	1250 (EU)				
POSITIVE*: 15	**(51%)**	**(33%)**	**(26%)**	**(64%)***	**(15%)**	NA	NA
Mean	1.072	1711.7	1711.7	236	DON-4Glc[j]	12.5	0.57
Min[i]	8.5	1000	1500	8.0	DON-6 Glc	10.1	0.42
Max[j]	6926.0	6926.0	6926.0	4001.3	DON-S	15.9	0.82
SD[k]	103.5	83.0	74.9	10	DON-SO_4	0.9	0.32
RSD%[l]	68.1	43.3	42.7	39.7	NA[m]	1.6	2.3
NEGATIVE: 8 (34.7%)**							
	<LOQ[n]	NA	NA	NA	NA	11.0***	0.46
TOTAL GENERAL: 23	711.7(51)	9(8)	11(NA)	14.7 (64)	19.9(87)	12.5	5.7

[a]deoxynivalenol [b] masked toxins [c]maximum level [d] Brazil, 2013 (for whole grains, flour and bran), European Union, 2007 (for unprocessed cereals), and United States of America, 2010 (for human consumption) [e] DON-1Glc [f] other DON glucosides (*Metabolynx Browser*) only detected [g] moisture content [h] water activity [i] minimum [j]maximum [k]standard deviation [l] relative SD% [m] not applicable [n] limit of quantification * LOQ: >8.5/8.0 µg/kg for DON / DON-1Glc, respectively ** <LOQ *** mean mc/aw for the negative samples

METHOD VALIDATION

The LOQ values obtained from the application of UPLC-QTOF method for both compounds quantification in wheat were adequate (8.5 and 8.0 µg/kg) as well as the recovery (between 89 and 79%, respectively), reaching relative standard deviation values lower than 5%. Linearity was from 2.0 to 12.0 µg/kg and 2.5 to 12.0 µg/kg with R^2 values of 0.998 and 0.9976 for both compounds, respectively.

WHEAT DON CONTAMINATION AND CONJUGATES DETECTED

Deoxynivalenol: the DON levels detected by QTOF/MS ranged from 8.5 (LOQ) to 6926.0 µg/kg in 51% of the wheat grains surveyed. Regarding the country of origin, from where those DON positive grains samples came to the country, it was not possible to identify. That was because most of the grain storage unities (GSUs) in Brazil have their silos loaded with the wheat batches straight after passing through the first three processes (classification, drying and cleaning), despite the wheat origin. Thus, same silo has loaded different batches of similar quality. DON analysis is not a routine at the GSUs Reception (classification step), but at harbours (during ship downloading by choice i.e., not all ships wheat load are sample checked at arrival, but exporters present mycotoxins report). Some researchers have reported the effect of time and low temperatures during storage on both compounds ratio changes, and that should be our further study [43]. Important to emphasize that the wheat batches do not stay longer stored in the GSUs due to the country food industries high demand of utilization. Data obtained in the current work was low when compared to some levels reported in the literature. Studies on wheat from exporting American countries reported DON contamination levels reaching up to 30,000 µg/kg [8,32-33,44].

Deoxynivalenol conjugates: in the current study, the DON *Fusarium* toxin conjugate (DON-3-*β*-D-Glc), produced by the plant for its own protection, was detected in 64% of the dry whole wheat

samples surveyed, including blank DON. Levels ranged from 8.0 to 4001.3 µg/kg. It was observed that 14% of the samples reported negative (<LOQ) for DON contamination, had that conjugate detected. Therefore, 37% of the wheat were positive for both compounds.

Apart from that, some samples also had other DON masked forms that were possible only to be detected through the screening method. They were DON-4Glc and DON-6Glc apart from DON-S [45]. Despite that, they were only possible to identify, and more studies need to be carried out in order to confirm them. Their detection in different grains (oats wheat and maize) have been reported in the literature mainly by Tandem MS [20,25-28,39].

As far as the methodology applied for unknowns conjugates identification is concerned, the exact mass was a quite important tool to confirm the detected components in the wheat samples with the help of EC calculator. The i-FitTM worked on the basis of exact mass and matched with the EC suggested with the isotopic pattern. The lowest i-FitTM value, the most likely correct answer [25].

HUMIDITY *VERSUS Fusarium* GROWTH CONDITIONS

As far as wheat grains humidity (mc and aw) and conditions for *Fusarium* growth are concerned, the mc ranged from 10.1 to 15.9% (mean: 12.5%) and aw from 0.42 to 0.82 (mean: 0.57) (Table 3), levels that can be considered safe *Fusarium* growth wise. The conditions for field fungi genera development and toxins production are mc 20 to 25% with threshold of ca. 18% which depend also on the crop characteristics and the optimal surrounding environment with high relative humidity (RH) and temperature [13]. It is necessary a much higher mc than those of the current study obtained in the GSUs wheat samples surveyed for *Fusarium* spores growth.

Therefore, the DON toxin and respective conjugate detected in the imported wheat samples were already there prior reaching the Brazilian storage facilities. That means, they were shipped to the country already field toxin contaminated. In fact, data show that the drying and storage processes applied at the home GSU facilities kept those grains safe, both for field and storage fungi spores development (low humidity/temperature).

Another factor that may lead to field fungi development and toxin contamination could be the ship grain downloading conditions, at the importing country's harbour i.e., the temperature difference between the (a) grains stored *inside the ship* (rather low) and the (b) warm environment in sub-tropical/tropical countries *harbours* at downloading (warm and high RH). That can lead to high moist condensation on grains surface, with subsequent high mc increase. Also, there is the possibility of the ship traveling from the country of origin, exposed to long journey and high humidity (water drippings down into the ship compartments stored grains).

WHEAT DON AND DON-3-B-D-GLC DATA *VERSUS* NATIONAL & INTERNATIONAL REGULATIONS

When the DON levels data of contaminated wheat samples were compared to the maximum levels (ML) established by the *national* [46,47] and *international* [48-50] regulations, it was observed that some levels detected were above the recommended. A total of 33 and 26% did not accomplish to the Brazilian and EU regulations, specifically for flour production (either, *whole* or *white* flour).

Brazil, set DON MLs for wheat and its products as follows: for *whole* grains & flour and its *bran* of 1000 µg/kg; for *white* flour & pasta / biscuits products of 750 µg/kg; for children food cereal based of 200 µg/kg and for wheat for further processing of 3000 µg/kg [47]. Similarly, the USA, set DON ML in food for human consumption (general) of 1000 µg/kg [49,51].

On the other hand, regarding EU, it set MLs for wheat (durum), cereal (unprocessed), cereal (for direct consumption) and baby food of 1750, 1250, 750 and 200 µg/kg, respectively. The provisional maximum tolerable daily intake calculation is based on the tolerable intake (1 µg/kg body weight/day) for DON and its acetylated derivatives [52].

Therefore, although for some of the regulations, the ML is set for unprocessed wheat; one would imply that the wheat ML is for *whole* grain, as they do not specify the wheat characteristics (whether *whole* or *white* flour/product made with).

Considering that the levels of the dry whole wheat grains for flour milling application, detected in the current study varied and some were high, it is important that, both the (a) government at harbour (ship downloading) and (b) the flour mills (prior food industries flour utilization) carry out mycotoxin analysis (rapid /fast results delivery / sensitive and accurate tests), including DON and other *Fusarium* toxins. Also to submit wheat grains (as soon as possible after ship harbour grain downloading) to humidity reduction (drying) or other procedure to avoid and control that downloading temperature contrast, which could be difficult to implement. Keep monitoring programs for continuous evaluation of grains getting into the country, in order to protect consumers for a worldwide DON problem is necessary, specifically due to the increasing Brazilian dependency on import wheat.

IV. CONCLUSIONS

DON and DON-3-*β*-D-Glc were identified and quantified in about a half (51 and 64%) of the imported wheat grains samples commercialized in Southern Brazil for flour milling. A total of 14% of wheat samples had only DON-3-*β*-D-Glc detected and some of them also other glucosides (DON-4Glc, DON-6Glc).

Considering the country's dependence on imported wheat, it is necessary a consistent DON levels monitoring in those grains, prior being accepted by the Brazilian costumers, despite of documents brought with the batches from the country of origin. The same also for the national production to keep consumers wheat based diet, safe.

More studies need to be carried out utilizing other methodologies, including NMR for the other DON conjugates detected confirmation and quantification. It will be also important to make other conjugates reference standards available.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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