HR-MS profiling and distribution of native and modified Fusarium mycotoxins in tritordeum, wheat and barley whole grains and corresponding pearled fractions

HR-MS profiling and distribution of native and modified *Fusarium* mycotoxins in cereal grains and corresponding pearled fractions

How does sequential pearling affect the content of Fusarium mycotoxins in grains?

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Abstract

Mycotoxins are one of the most important contaminants in cereal grains. Besides parent forms, the presence and identification of structurally modified mycotoxins is nowadays recognized as a challenging food safety-related issue and contribute to increase the human and animal exposure to these compounds. The aim of this study was to follow the distribution of *Fusarium* toxins and their main modified forms in the pearled fractions of several grain species (i.e. tritordeum, durum and bread wheat, and barley), using high-resolution mass spectrometry technique (HR-MS). A significant decreasing trend in mycotoxins concentration was observed from the outer layer to the inner kernel, along the sequential pearling process. Among modified forms, deoxynivalenol (DON) -oligoglucosides were described for the first time in naturally infected grains, while zearalenone (ZEN) -sulfate was the only ZEN-related form detected in pearling fractions. HR-MS could be confirmed as useful technique to study and characterize modified forms of mycotoxins.

Key words: modified mycotoxins, pearled fractions, tritordeum, wheat, barley, high resolution mass spectrometry

1. Introduction

Whole grain cereals are an important source of bioactive compounds, micronutrients, dietary fibre and they are considered as staple food worldwide. Cereal grains are rarely consumed as whole kernel, in fact they undergo to several processes, such as the separation of the outer layers of the seed to the endosperm. These fraction are usually discarded because of their reduced sensory and technological value of the end-use products compared to those obtained by refined one (Zhang and Moore, 1999). However, it has been shown that bioactive compounds are mainly concentrated in the outer layers of the grain (Sovrani et al., 2012). An increasing evidence from clinical and epidemiological studies suggests that the regular consumption of wheat, as whole grain, might reduce the risk of developing chronic diseases (Bach Knudsen et al., 2017; Dykes and Rooney, 2007; Fardet et al., 2008). Consequently, the conventional roller-milling process, which promote the removal of the outer layers of the kernel in the bran fraction, causes a great decrease in the nutritional value of the refined flour (Felizardo and Freire, 2018). To overcome the drawbacks, several grain fractionation technologies have been developed over years, to obtain flour mixes and ingredients with technologically optimized functional and nutritional attributes (Giambanelli et al., 2018; Giordano et al., 2017). Among them, sequential pearling effectively allows the separation of external bran fractions, which contain coarse fiber and are potentially subjected to contamination, from underlying fractions with potential health benefits due to their high content of bioactive compounds (Sovrani et al., 2012). These fractions can be efficiently employed as functional ingredients in bakery and particularly, as previously suggested, for bread-making (Blandino et al., 2015a, 2015b, 2013). On the other hand, the outer layers of the wheat kernel are mostly subjected to contamination by pesticides or natural contaminants, such as heavy metals and mycotoxins, mainly those produced by *Fusarium* spp. (Cheli et al., 2013; Giordano and Blandino, 2018). In particular mycotoxins are generally found in cereal grains and overall, more than one type of mycotoxin can be present in the same foodstuff (Freire and Sant'Ana, 2018). Deoxynivalenol (DON), belonging to

69 the trichothecenes class and zearalenone (ZEN) are commonly accepted as the main mycotoxins
70 occurring in wheat worldwide. These compounds gather several toxic effects, thus represent a threat
71 for humans and animal health. Moreover, they can act as a virulence factor for Fusarium Head
72 Blight (FHB) in cereals (Audenaert et al., 2013; Kazan et al., 2012) making them responsible for
73 relevant economical losses due to low yield and crop withdrawal. Besides native mycotoxins,
74 several structurally modified forms produced in plants have been reported (Berthiller et al., 2013).
75 Recently, EFSA has reconsidered the toxicological relevance of DON and its main modified forms,
76 stating that a thoroughly assessment of the sum of DON, 3- and 15-Acetyl-DON (Ac-DON) and
77 DON-3-Glucoside (DON-3-Glc) is highly recommended (Knutsen et al., 2017).

78 These compounds may present also different chemical properties (i.e. solubility, polarity), and
79 arguably different toxicity. Furthermore, the combination of unknown structure, lack of analytical
80 standard and trace concentration, their analysis using conventional techniques is challenging, but
81 necessary as it constitutes an important information during the risk assessment process.

82 The ability of sequential pearling to decrease the content of *Fusarium* mycotoxins from the outer to
83 the inner layers of wheat, has been reported by several authors (Cheli et al., 2013; Ríos et al., 2009;
84 Sovrani et al., 2012). However, the distribution of the modified forms of DON, DON-3Glc, 3- and
85 15Ac-DON, has never been tested so far. Therefore, the aim of this study was to follow the
86 distribution of trichothecenes, zearalenone (ZEN) and its main modified forms into sequential
87 pearled fractions of two tritordeum (X *Tritordeum* Ascherson et Graebner), one durum wheat
88 (*Triticum turgidum L. subsp. durum (Desf.) Husnot*), one bread wheat (*Triticum aestivum* L.) and
89 one barley (*Hordeum vulgare* L.) varieties, using two different approaches. As first, a target
90 quantification was run using liquid chromatography coupled with tandem mass spectrometry (LC-
91 MS/MS). Then high-resolution mass spectrometry (HRMS) was used for a full profiling of
92 modified mycotoxins in the samples.

93

2. Material and Methods

2.1.Experimental design and raw materials

Two varieties of tritordeum (cvs. Aucan and Bulel), a durum wheat variety (cv. Saragolla), a bread wheat variety (cv. Illico) and six-row barley varieties (cv. Ketos) were cultivated side by side on the same field in northwestern of Italy (Cigliano, 45° 31' 97''N, 8°04'77''E) during the 2015-2016 growing season. The experiment was carried out in natural infection conditions, but the choice of the growing area (frequent rainfall during wheat anthesis) and of the agronomic techniques (previous crop, no fungicide application) was carried out to guarantee a medium-high level of *Fusarium* infection, although the adopted crop practices are commonly used by farmer in the areas. Briefly, the previous crop was maize, and the mechanical sowing was carried out on 6 November 2015, following an autumn plowing (30 cm) and disk harrowing to prepare a proper seedbed. Planting was conducted in 12 cm wide rows at a seeding rate of 450 seeds m^{-2}. A total of 140 kg N ha^{-1} was applied as a granular ammonium nitrate fertilizer, split into 60 kg N ha^{-1} at wheat tillering (GS 23), 80 kg N ha^{-1} at stem elongation (GS 32). No fungicide has been applied to control foliar and head disease. Harvesting was conducted with a combine-harvester on 21 June for the barley variety and on 4 July 2016 for the tritordeum and wheat varieties.

2.2. Pearling process

Nine fractions of kernels from each variety were obtained through incremental pearling. The pearling consisted of consecutive passages of kernels or pearled kernels in an abrasive-type grain testing mill (Model TM-05C, Satake, Tokyo, Japan). Starting from unprocessed grain samples, the kernels were initially pearled to remove 5% of the original weight, and this resulted in a first fraction (0-5% w/w). The same process was also performed to remove another seven fractions (designated fractions 5-10%, 10-15%, 15-20%, 20-25%, 25-30%, 30-35%, 35-40% w/w). The residual 60% of the kernel (40-100% w/w) was also collected and milled by means of a laboratory centrifugal mill (Model ZM-100, Retsch, Haan, Germany) equipped with a 1-mm sieve. The same

process was performed also for the unprocessed grain samples in order to obtain a wholegrain flour. Before chemical analyses, samples were ground to a fine powder (particle size < 300 μm) with a Cyclotec 1093 sample mill (Foss, Padova, Italy), and stored at -25°C until analyses were performed.

2.3.Chemicals and reagents

Analytical standards of DON (100 μg mL^{-1} in acetonitrile), DON-3Glc (solution in acetonitrile 50.6 μg ml^{-1}), 3Ac-ADON (50 μg mL^{-1} in acetonitrile), T-2 and HT-2 toxins (50 μg mL^{-1} in acetonitrile), nivalenol (NIV) (50 μg mL^{-1} in acetonitrile) and ZEN (100 μg mL^{-1} in acetonitrile) were purchased from Romer Labs®. HPLC-grade methanol, acetonitrile and acetic acid were purchased from Sigma-Aldrich (Taufkirchen, Germany); bidistilled water was obtained using a Milli-Q System (Millipore, Bedford, MA, USA). MS-grade formic acid from Fisher Chemical (Thermo Fisher Scientific Inc., San Jose, CA, USA) and ammonium acetate (Fluka, Chemika-Biochemika, Basil, Switzerland) were also used.

2.4. Sample preparation for LC-MS analysis.

Samples were prepared according to Malachová et al., 2014 procedure, with slight modifications. Briefly, 1 g of ground cereal was stirred for 90 min at 200 strokes/min on a shaker with 4 mL of acetonitrile/water (80/20, v/v) mixture acidified with 0.1% of formic acid. An aliquot of the extract was collected and centrifuged for 10 min at 14,000 rpm at room temperature then 1 mL of supernatant was evaporated to dryness under a gentle stream of nitrogen. Finally, the residues were re-dissolved in 1 mL of water/methanol (80/20 v/v) prior to LC- MS/MS and LC-HRMS injection.

2.5. LC-MS/MS quantification of Fusarium mycotoxins and their modified forms

The LC-MS/MS analysis was performed on a UHPLC Dionex Ultimate 3000 instrument coupled with a triple quadrupole mass spectrometer (TSQ Vantage; Thermo Fisher Scientific Inc., San Jose,

CA, USA) equipped with an electrospray source (ESI). For the chromatographic separation, a RP-C18 Kinetex EVO column 2.10×100 mm and a particle size of 2.6 µm (Phenomenex, Torrance, CA, USA) heated to 40 °C was used. 2 µL of sample extract was injected into the system; the flow rate was 0.350 mL min^{-1}. Gradient elution was performed by using 5 mM ammonium acetate in water (eluent A) and methanol (eluent B) both acidified with 0.2% acetic acid. Initial conditions were set at 2% B for 1 min, then eluent B was increased to 20% in 1 min; after an isocratic step (6 min), eluent B was increased to 90% in 9 min; after a 3 min isocratic step, the system was re-equilibrated to initial conditions for 8 min. The total run time was 28 min. MS parameters: the ESI source was operated in negative ionization mode for DON, DON-3Glc, 3Ac-DON, NIV and ZEN, and in positive ionization mode for T-2 and HT-2 toxins; spray voltage was 3,000 V, capillary temperature at 270 °C, vaporizer temperature was kept at 200 °C, sheath gas flow was set at 50 units and the auxiliary gas flow at 5 units. The S-Lens RF amplitude value and collision energies (CE) were optimized during infusion of analyte standard solutions (1 mg kg^{-1}, in methanol). Detection was performed in SRM mode, monitoring the $[M + CH_3COO]^-$ adducts for DON, NIV and modified forms, $[M-H]^-$ for ZEN and $[M+NH_4]^+$ adducts for T-2 and HT-2 toxins. The following transitions were measured: DON *m/z* 355→295 (CE = 13 eV) and *m/z* 355→265 (CE = 17 eV); DON-3Glc *m/z* 517→457 (CE = 16 eV) and *m/z* 517→427 (CE = 23 eV), 3Ac-DON *m/z* 397→337 (CE = 16 eV), *m/z* 397→307 (CE = 18 eV) and *m/z* 397→59 (CE = 20 eV), ZEN *m/z* 317→175 (CE = 26 eV) and *m/z* 317→131 (CE = 32 eV), HT-2 toxin *m/z* 442→263 (CE = 11 eV), *m/z* 442→215 (CE = 4 eV) and T-2 toxin *m/z* 484→215 (CE = 19 eV), *m/z* 484→185 (CE = 22 eV), NIV *m/z* 371→59 (CE = 48 eV), *m/z* 371→281 (CE = 32 eV) and *m/z* 371→311 (CE = 11 eV). Matrix-matched calibration curves (calibration range for DON, DON-3-Glc, 3Ac-DON, NIV 100–2500 µg kg^{-1}, for ZEN, T-2 and HT-2 toxins 1-2500 µg kg^{-1},) were used for target analyte quantification. A good linearity was obtained for all the considered mycotoxins ($R^2 > 0.99$).

DON-3Glc/DON molar ratio was calculated from the values of DON and DON-3Glc by the following equation (Nakagawa et al., 2017):

171 $$\frac{DON3Glc}{DON}\ molar\ ratio = (DON3Glc\ (\mu g/kg\ )/MWa)/(DON\ (\mu g/kg)/MWb)\ \times 100$$

172 where the *MWa* represent the molar weight of the DON-3Glc (458 Da) and *MWb* that of DON (296
173 Da).

174 **2.6.HR-MS profiling of Fusarium mycotoxins and their modified forms**

175 LC-HRMS analysis was performed on a UHPLC Dionex UltiMate 3000 instrument coupled to a Q-
176 Exactive™ high resolution mass spectrometer (Thermo Scientific, Bremen, Germany) equipped
177 with electrospray ionization.

178 The chromatographic separation was obtained on a Synergi 4U Hydro-RP 150 x 2.0 mm
179 (Phenomenex, Torrance, CA, USA) heated to 30 °C. 10 µL of sample extract was injected into the
180 system; the flow rate was 0.3 mL min^{-1}. Gradient elution was performed by using 1 mM ammonium
181 acetate in water (eluent A) and methanol (eluent B) both acidified with 0.5% acetic acid. Initial
182 conditions were set at 5% B followed by a linear change to 10% B in 2 min. After 2 min of isocratic
183 step (10% B) B% increased up to 65% in 16 min. Column was then washed for 4 min with 100% B
184 followed by a reconditioning step for 5 min using initial composition of mobile phases. The total
185 run time was 29 min.

186 The Q-Exactive mass analyser was operated under negative ionization mode. The full MS/data
187 dependent MS/MS mode (full MS–dd-MS/MS) was set at following parameters: sheath and
188 auxiliary gas flow rates 32 and 7 arbitrary units, respectively; spray voltage 3.3 kV; heater
189 temperature 220 °C; capillary temperature 250 °C, and S-lens RF level 60. Following parameters
190 were used in full MS mode: resolution 70,000 FWHM (defined for *m/z* 200; 3 Hz), scan range 100–
191 1000 *m/z*, automatic gain control (AGC) target 3e6, maximum inject time (IT) 200 ms. Parameters
192 for dd-MS/MS mode: intensity threshold 1e4, resolution 17,500 FWHM (defined for *m/z* 200; 12
193 Hz), scan range 50 – fragmented mass *m/z* (*m/z* +25), AGC target 2e5, maximum IT 50 ms,
194 normalized collision energy (NCE) 35% with ±25% step.

Only in few cases, fragmentation spectra could not be collected, due to parent ion abundance below the threshold. In this case, a tentative annotation based on accurate mass and elemental formula was performed, as already proposed (Righetti et al., 2017)

2.7. Statistical analysis

All the analyses of wheat samples (whole-grain flour, pearled fractions and residual pearled kernel) were performed in triplicate. Analysis of variance (ANOVA) was applied in order to compare the mycotoxins content in the whole-grain flours and in the different pearled fractions. The Tukey-b's post-hoc test was performed for multiple comparisons. A $p<0.05$ threshold was used to reject the null hypothesis. Statistical analyses were carried out by means of SPSS for Windows, statistical package Version 25 (SPSS Inc., Chicago, Illinois).

3. Results and Discussion

3.1. Quantification of parent and modified Fusarium mycotoxins in wholegrains and pearled fractions

Due to the higher association of mycotoxins with outer layers of grains, the effect of pearling in decreasing DON content and the distribution of their modified forms into pearled fractions can be of relevance for food safety. The occurrence and distribution of main trichothecenes (NIV, DON, DON-3Glc, 3Ac-DON, T-2, HT-2 and ZEN) were analysed in durum wheat cv. Saragolla (**Fig. S1**) as well as in two varieties of tritordeum, in bread wheat and in barley.

In terms of whole grain contamination, the overall content of DON and its modified forms was higher in durum wheat (cv. Saragolla) compared to tritordeum (cv. Aucan and cv. Bulel), bread wheat (cv. Illico) and barley (cv. Ketos) (**Table 1**). The latter is easily explainable since the cv. Saragolla is genetically susceptible to fungi contamination and for that reason was selected for the subsequent metabolites analysis. Other *Fusarium* toxins, such as T-2, HT-2 and NIV as well as

ZEN were also detected only in durum wheat. In bread wheat and barley whole grains the DON content was significantly lower than those of previous reported cereals, while its modified forms or other *Fusarium* toxins resulted lower than LOD. In fact, Illico is recognized to be a strong resistant variety of wheat grains. It is worth to mention that the extent of contamination depends on climatic conditions prevailing in any given growing season at any given geographic location of the cultivation site. The content of DON-3Glc, natural product of metabolism of DON under the influence of plant enzymes, directly depends on the wheat genotype (Dall'Asta et al 2012).

The main modified form of deoxynivalenol, DON-3-Glc, was found at a concentration of 1130 µg/Kg, 1060 µg/Kg, and 1210 µg/Kg in cv. Aucan, Bulel and Saragolla, respectively, while it was < LOD in cv. Illico and cv. Ketos samples, likely in consideration of the lower accumulation of the parent form compared to the other cultivars. Considering the DON-3-Glc/DON ratio, it was found in the range 10-30%, in agreement with data reported in the literature (Berthiller et al., 2013).

However, it should be noticed that DON-3-Glc/DON ratio in cereal grains can vary in relation to many factors like genotype, environmental conditions or climatic conditions, as already discussed by several authors (Berthiller et al., 2013; Cirlini et al., 2013; Nagl et al., 2014). In addition, also high variances among the same wheat species have been found as reported in the study of (Bryła et al., 2018), in which they monitored the occurrence of these toxins along 92 polish winter wheat cultivars (growing season 2016) and the molar ratio ranged between 5 to 37 %.

Being DON the main contaminant of all the grain samples, its distribution together with the distribution of DON-3Glc was evaluated over nine sequential pearled fractions, obtained from each cultivar considered within this study. Results are reported in **Figure 1**.

As expected, a decreasing trend of DON and DON-3Glc was observed throughout the pearling fractions, moving from outer to inner layers. In particular, the outer fraction, mainly composed by the outer and inner pericarp (fr 0-5%) showed the higher contamination in all the considered samples.

244 The effectiveness of sequential pearling in decreasing mycotoxin content is clearly demonstrated by
245 the strong decrease observed in the inner pearled kernel, representing about the 60% in weight of
246 the initial wholegrain (**Figure 1A**). In particular, DON concentration dropped below LOQ in cv.
247 Bulel, cv. Illico, and cv. Ketos, while it was in the range 30-40% of the DON concentration level
248 found in the whole grain in cv. Saragolla and cv. Aucan. DON-3-Glc showed the same trend of
249 DON (**Figure 1B**). These results are in agreement with other studies, in which the accumulation of
250 DON is higher in the outermost fractions compared to the starchy endosperm (Šliková et al., 2010).
251 However, variances regarding the DON-3-Glc content in respect to its native form and the
252 distribution trend could be also explained by the different metabolic properties of each cereal
253 species in relationship with that of fungi. In fact, when the plant goes to the senescence period, its
254 metabolism is almost deactivated, thus unable to produce the glucoside form of DON. Furthermore,
255 the fungi developed in more extent on the peripheral tissues of the caryopsis, can still produce
256 mycotoxins, as long as the moisture content during the dry-down process persists above the 20%.

257 Regarding T-2 and HT-2 toxins content, they were detected only in whole grain and pearled
258 fractions of durum wheat cv. Saragolla. The distribution pattern of T-2 and HT-2 toxins was similar
259 to the one of DON, decreasing toward the inner part of the kernel (**Figure 2**). The dominating
260 analogue was the deacetylated form (HT-2) as reported by other studies (Lindblad et al., 2013;
261 Pascale et al., 2012).

262 Concerning the content of DON and DON-3-Glc, a significant correlation along the pearled
263 fractions was observed in cv. Aucan ($p = 0.0002$), cv. Bulel ($p = 0.0000$), and cv. Saragolla ($p =$
264 0.0158), while the low contamination found in cv. Illico and cv. Ketos did not allow any
265 calculation.

266 However, it should be noticed that a different toxin redistribution within the kernel fractions could
267 be influenced by many factors. For example, the latter can be related to the fact that cereals differ
268 for their size and shape, and consequently the progressive removal of the outer part of the grains by

means of the pearling process is not homogeneous. In fact, regarding barley grains the two first pearling passages (0-5 and 5-10%) were responsible for an almost total dehulling of the kernel, while for other cereals these steps could abrade the peripheral tissues of seed.

Overall, the present study clearly showed that the removal of the first two fractions (0-5% and 5-10%) could significantly reduce the content of DON and DON-3-Glc in all the considered samples. Since these two fractions correspond to the outermost layers (inner and outer pericarp ~ 12 % of kernel weight), the pearling process could be as far as comparable to the traditional milling process, even considering a very high contaminated sample.

XX However, despite the initial high concentration of mycotoxins, these sample have to be considered as not suitable for human consumption (DON law limit 1750 ppb in unprocessed durum wheat, Regulation (EC) No. 1881/2006 and Regulation (EC) No. 1126/2007).

3.2.Qualitative profiling of *Fusarium* mycotoxins in pearled fractions

To get a full picture of mycotoxin modified forms occurring in pearled fractions and to evaluate possible changes in distribution moving from the outer layer to the inner kernel, a LC-HRMS profiling was performed on durum wheat. The experiment was performed on cv. Saragolla pearled fractions in consideration of the higher amount of DON found in whole grain.

The same profile in terms of modified forms was observed throughout the sequential fractions, returning 4 putative metabolites and two modified forms confirmed by analytical standard comparison (**Table 2**). Besides DON, 3-Ac-DON and DON-3-Glc, the occurrence of di- and tri-glucoside forms of DON together with 3-Ac-DON-15-Glc were observed in all the considered fractions. In addition, ZEN and ZEN-Sulf were identified as well.

According to (Righetti et al., 2017), the identification process used for metabolite putative assignment starts from the extracted ion chromatogram; then the parent ion molecular formula is assigned, and theoretical and experimental isotopic pattern are compared to reduce the number of

possible candidates. In the last step, the HR-MS fragmentation pattern, obtained by using data dependent acquisition (DDA), facilitate compound identification.

Oligoglycosides were annotated according to the in-source fragmentation pattern, as reported by (Zachariasova et al., 2012). The sugar moieties were bound indeed to C3 of DON, resulting in the formation of a peak at *m/z* 427.1610 $[M\text{-}CH_2O\text{-}H]^-$. It is worth of notice that this is the first study showing the occurrence of DON-oligoglycosides in grains. These forms have been reported before as resulting from the malting process in brewing, as the effect of enzymatic release from cell wall polysaccharides (Maul et al., 2012).

Besides DON-oligoglycosides, one of the acetylated forms of DON (3-Ac-DON) was detected in the full scan mass spectrum (*m/z* 397.1504) (**Figure 4**). The acetylation of DON can take place on two sites of the backbone; therefore, two isomeric forms might be expected. Nevertheless, as reported by (Schmeitzl et al., 2015), the fragment ion at *m/z* 173.0462 is characteristic for the 3-Ac-DON. In our study only the 3-Ac-DON isomer was found and confirmed by comparison with analytical standard.

Together with 3-Ac-DON, its glycosylated form was found in kernel fractions, where the 3Ac-DON contamination was detected. Due to its low intensity no HR-MS/MS spectrum was obtained. However, it was possible to putatively confirm the 3-Ac-DON-Glc identity by the low mass error (-0.9 Δ ppm) (**Table 2**), the isotopic pattern and the retention time, anticipated in respect to its aglycone (-3Ac-DON). In addition, also the in-source fragmentation was reported, in which the breakage of the ether bond released the 3-Ac-DON moiety. Moreover, no molecular ion (*m/z* 397.1504) was found in both standard reference and sample (**Figure S2**). This means that even with low collision energy (CE, 10v) the $[M+H_3C_2O_2]^-$ it was completely fragmented.

Among sulfated forms, DON-Sulf was not observed in wheat pearling fractions, in agreement with studies reporting that sulfation is a minor biotransformation route in plants for DON (Knutsen et al.,

2017). On the other hand, ZEN-Sulf was annotated together with ZEN, on the basis of its molecular ion $[M-H]^-$ at *m/z* 397.0952, and consistent fragmentation (**Figure S3**).

In terms of concentration of the modified forms of mycotoxins, their abundance was calculated as the total peak areas of the mycotoxins detected and the peak area of each modified form, thus expressed as percentage (%). For example, in first pearled fraction (0.5%) the di-glycoside of DON accounted for 0.685 %, while a 0.047 % for the DON-3-triGlc and 0.120 % for 3-Ac-DON-Glc. In the rest of pearled fractions, the relative abundance ranged between 0.317-0.012, 0.038-0.06 and 0.090-0.052 %, for DON-3-diGlc, DON-3-triGlc and 3-Ac-DON-Glc, respectively. Moreover, for the ZEN-S the relative abundance in fraction 0-5 %, in respect to its native form, was 15.527 % and ranged between 20.43-9.068 % in the other seed fractions.

Structural modification of the native form of mycotoxins, could be interpreted as an opposition mechanism of the plant, aimed to struggle the infection (FHB) (Buerstmayr and Lemmens, 2015). In which the conjugation of the DON toxin to a sugar or/and sulphates increases the polarity of the molecule that can then be stored in cell vacuole (Berthiller et al., 2005)

More studies have to be conducted in order to increase the information regarding the toxicity of modified mycotoxins. Nevertheless, they are extremely important and must be taken into account in food safety areas since the native, and toxic, form might be released during digestion (Dall'Erta et al 2014). Although the occurrence of these compounds is usually lower than the parent forms, they should be monitored in food chain otherwise this could lead to an under estimation of the real mycotoxin human and animal intake, as suggested by the recent EFSA opinion (EFSA 2014).

Taken altogether, profiling data reported within this study confirm the occurrence of a complex mixture of parent and modified forms of *Fusarium* mycotoxins in grains and although the modified mycotoxin forms represent a very little in terms of concentration they could lead to a huge problem for human and animal safety.

4. Conclusion

In conclusion, results obtained in this work increase the knowledge on the distribution of trichothecenes and zearalenone among the cereal grain tissues. DON was the mycotoxins found in higher concentration in all the cereal species and in the corresponding pearled fractions. Among them, the peripheral layers were the most contaminated (from 0-15 % of total kernel weight), underlying the importance of considering mycotoxin contamination when milling by-products are used in food formulation, with the final aim to increase the nutritional value of the products. The sequential pearling process showed a good potential for the mitigation of mycotoxin concentration in the endosperm, through the controlled removal of the outer layers. Considering the genetic susceptibility to fungi contamination of the wheat varieties taken under study, the pearling process achieve a 60 % of DON reduction and total absence of modified forms in inner part of cereal grains. Finally, the HR-MS analysis could elucidate clearly few of nowadays non-regulated modified forms of mycotoxins, laying the groundwork for future studies focused on the in plant metabolism or/and studies regarding the biological activity of these compounds in human or animal organisms.-

Abbreviation used

15-Acetyl-Deoxynivalenol (15-Ac-DON), 3-Acetyl-Deoxynivalenol (3-Ac-DON), analysis of variance (ANOVA), below the limit of detection (<LOD).below the limit of quantification (<LOQ), deoxynivalenol (DON), deoxynivalenol-3-Glucoside (DON-3-Glc), dependent acquisition (DDA), electrospray source (ESI), high-resolution mass spectrometry (HR-MS), liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), nivalenol (NIV), zearalenone (ZEN), zearalenone sulphate (ZEN-Sulf).

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Supporting information description

Appendix 1

385

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Tables

Table 1. Trichothecenes and ZEN content in the whole grain of tritordeum, durum wheat, bread wheat and barley, expressed in µg kg^{-1} d.w (dry weight).

Crop	Cultivar	Mycotoxins							
		DON	DON-3-Glc[1]	3-Ac-DON[1]	NIV[1]	T-2[2]	HT-2[2]	ZEN[2]	DON-3-Glc/DON *
Tritordeum	Aucan	6354±152ab	1130±20a	<LOD	<LOD	<LOD	<LOD	2±0b	11.5
Tritordeum	Bulel	3209±1460bc	1060±70a	<LOD	<LOD	<LOD	<LOD	2±0b	21.3
Durum wheat	Saragolla	6920±160a	1210±20a	387±5	859±188	3±0	11±1	58±5a	11.3
Bread wheat	Illico	379±67c	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	-
Barley	Ketos	241±60c	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	-
p-value		0.002	-					0.001	-

*Results were expressed as mean±standard deviation µg kg^{-1}. Different letters indicate a statistical difference ($p<0.05$). * expressed as molar ratio (%); [1] <LOD: <7 µg kg^{-1}; [2] <LOD: < 1 µg kg^{-1}, <LOQ: 1 µg kg^{-1}; - not determined.*

Table 2. Metabolites of DON and ZEN found in the outermost fraction (0-5%) of durum wheat cv. Saragolla. Mass deviation ppm is calculated by the values detected by full scan spectrum (resolving power 70,000 FWHM, extraction window 5 mg kg^{-1}).

Rt (min)	Formula	Detected mass (*m/z*)	Theoretic mass (*m/z*)	Ion species	Mass error (Δ ppm)	Putative metabolite
8.75	C15 H20 O6	355.1405	355.1382	$[M+H_3C_2O_2]^-$	1.9	DON
9.11	C21 H30 O11	517.1939	517.1910	$[M+H_3C_2O_2]^-$	2.4	DON-3-Glc
10.44	C27 H40 O16	589.2159	589.2138	$[M-CH_2O-H]^-$	3.6	DON-3-diGlc
10.21	C31 H44 O19	751.2677	751.2666	$[M-CH_2O-H]^-$	1.5	DON-3-triGlc
12.23	C23 H32 O12	499.1816	499.1821	$[M-H]^-$	-0.9	3-Ac-DON-Glc
14.44	C17 H22 O7	397.1516	397.1504	$[M+H_3C_2O_2]^-$	3.1	3-Ac-DON
10.58	C18 H22 O5	317.1398	317.1394	$[M-H]^-$	1.2	ZEN
9.13	C18 H22 O8 S	397.0968	397.0963	$[M-H]^-$	1.2	ZEN-Sulf

Figures captions

Figure 1. DON (A) and DON-3-Glc (B) concentration (µg kg^{-1} d.w.) among pearled fractions of tritordeum (cv. Aucan and Bulel) durum wheat (cv. Saragolla), bread wheat (cv. Illico) and barley (cv. Ketos). Different letters on top of each bar indicate a significant difference ($p<0.05$) using Tukey-b's post-hoc test.

Figure 2. HT-2 and T-2 toxins concentration (µg kg^{-1} d.w.) among pearled fractions of durum wheat (cv. Saragolla.). Different letters on top of each bar indicate a significant difference ($p<0.05$) using Tukey-b's post-hoc test.

Figure 3. Extracted ion chromatogram (EIC) of DON and DON oligoglycosides. Due to low abundance, EIC intensities of DON-3-Glc, DON-3di-Glc and DON-3tri-Glc were multiplied by a factor of 10, 100 and 1000 respectively.

Figure 4. EIC of 3-Ac-DON (*m/z* 397.1504) and 3-Ac-DON-Glc (*m/z* 499.1821), in-source fragmentation and loss of glucose from 3-Ac-DON-Glc. Due to low abundance, EIC intensities of 3-Ac-DON-Glc were multiplied by a factor of 10.

Figure 1

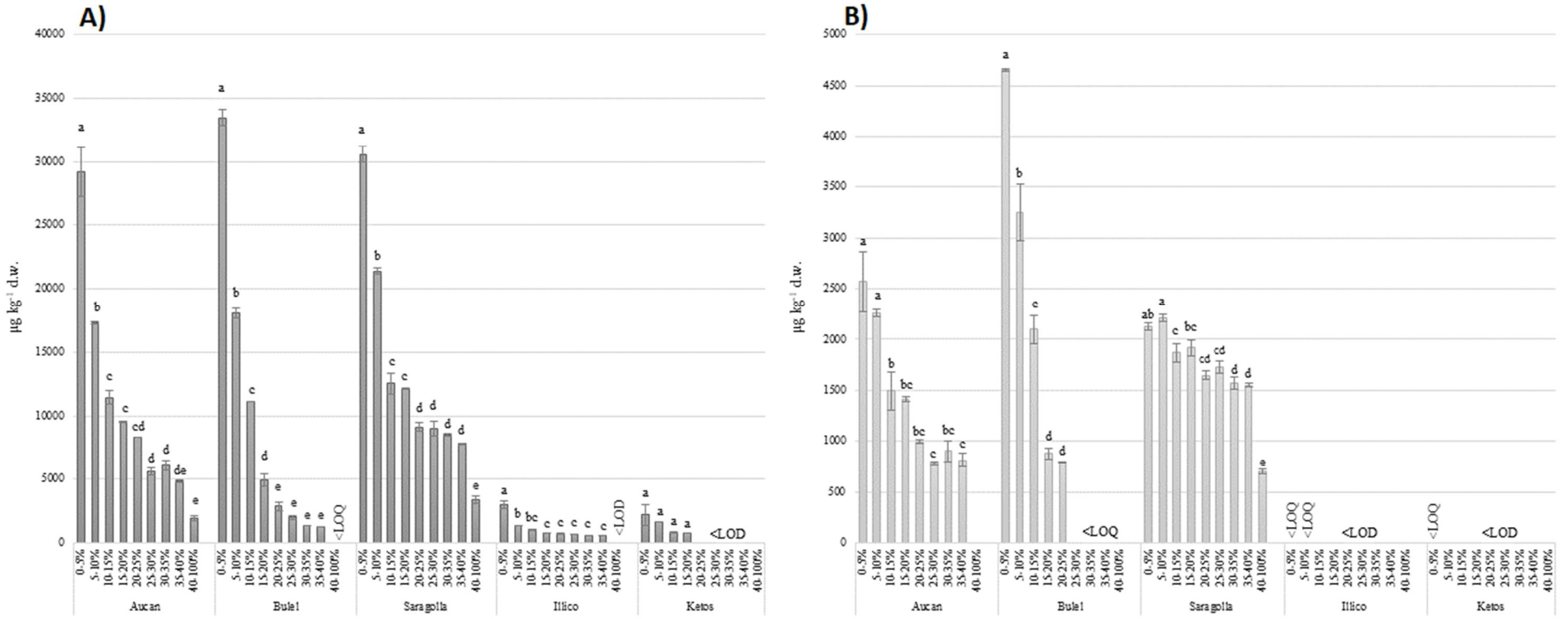

A)
B)
µg kg-1 d.w.
0-5%
5-10%
10-15%
15-20%
20-25%
25-30%
30-35%
35-40%
40-100%
<LOQ
<LOD
Aucan
Bula1
Saragolla
Illico
Ketos

Figure 2

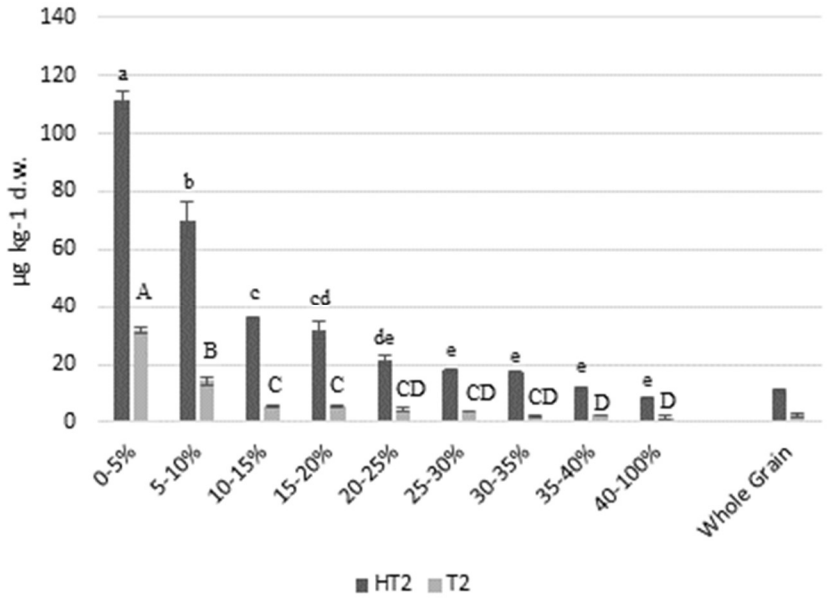


Figure 3

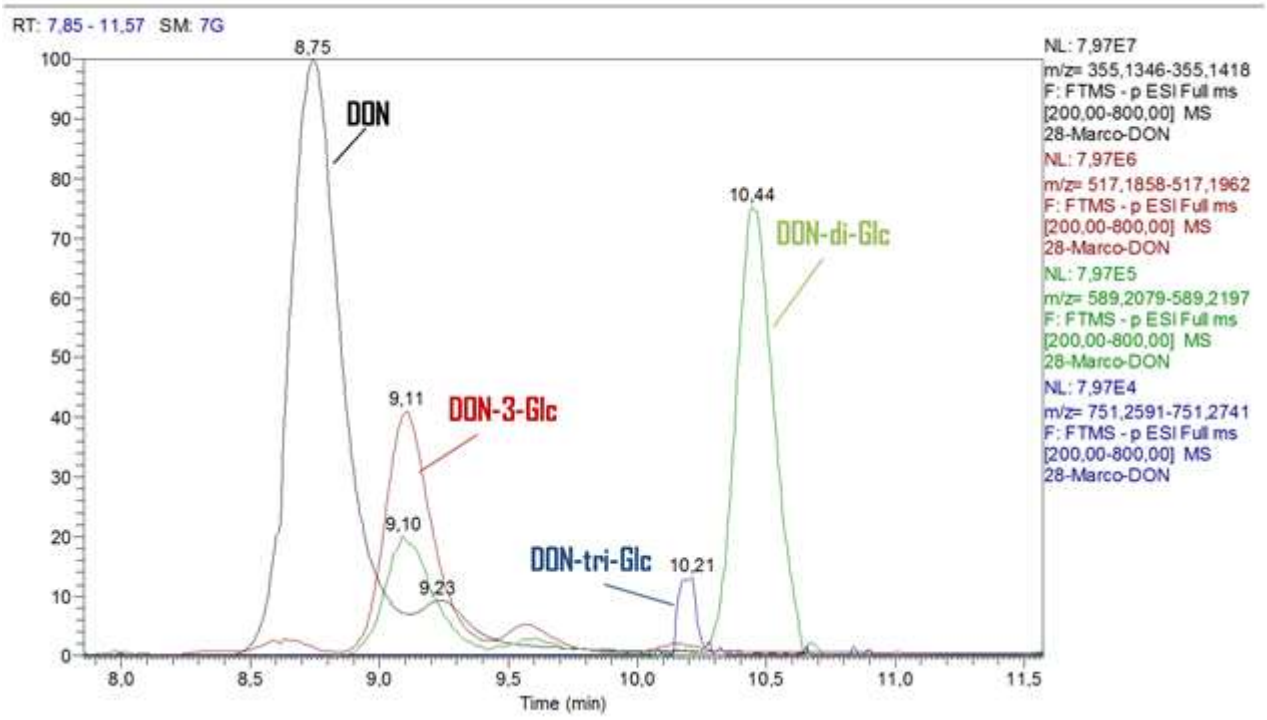
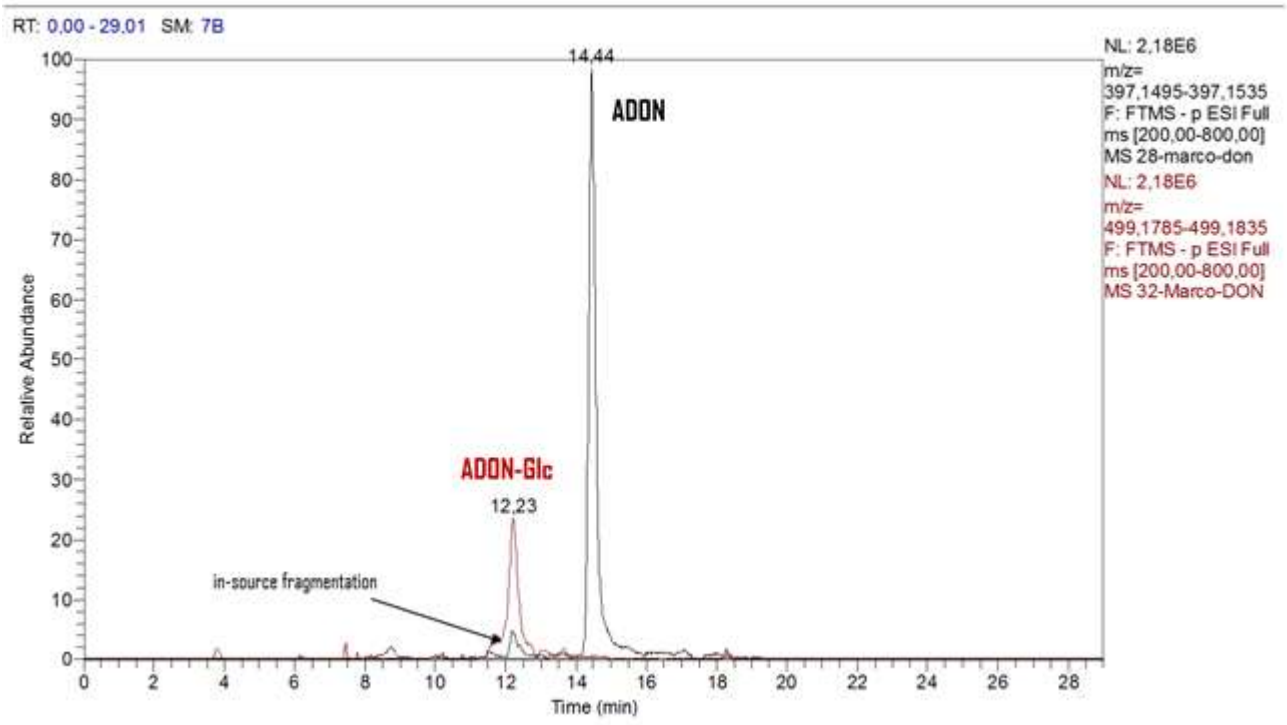


Figure 4


RT: 0,00 - 29,01 SM: 7B
14,44
ADON
ADON-Glc
12,23
in-source fragmentation
Relative Abundance
Time (min)
NL: 2,18E6
m/z=
397,1495-397,1535
F: FTMS - p ESI Full
ms [200,00-800,00]
MS 28-marco-don
NL: 2,18E6
m/z=
499,1785-499,1835
F: FTMS - p ESI Full
ms [200,00-800,00]
MS 32-Marco-DON

Appendix A. Supplementary material

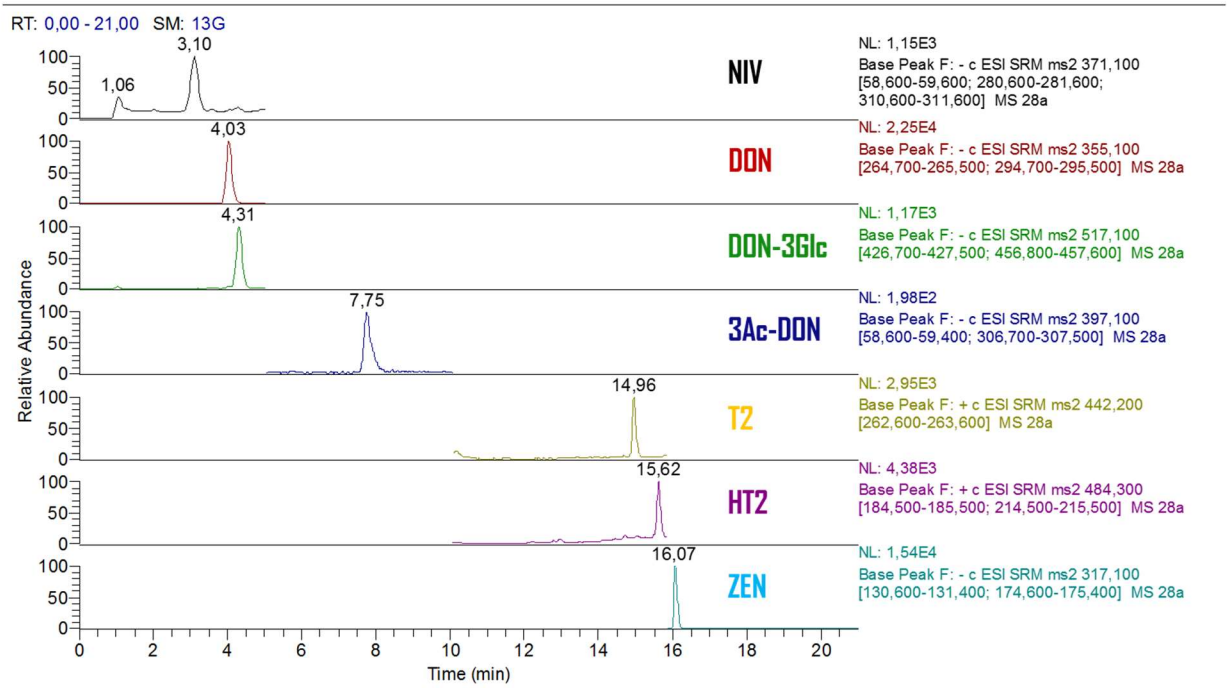


Figure S1. SRM (Selected Reaction Monitoring) of the monitored toxins in 0-5% pearled fraction of durum wheat cv. Saragolla.

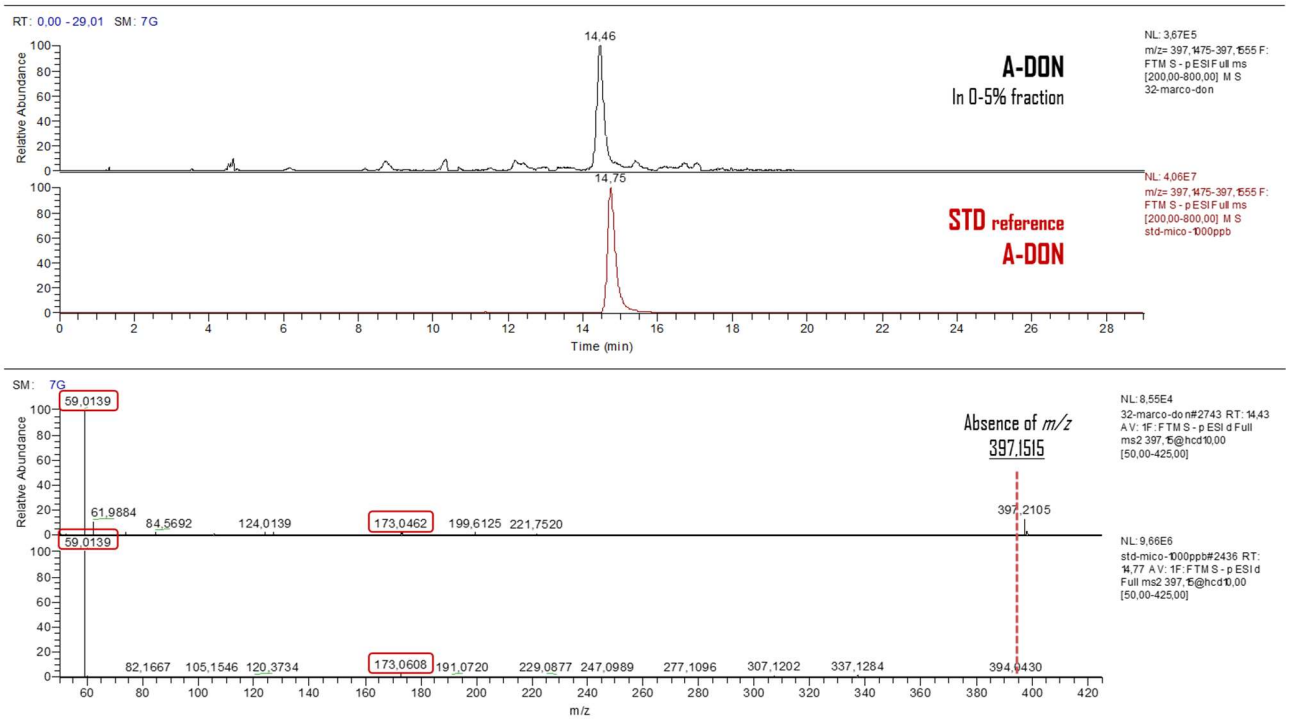


Figure S2. EIC of ADON in sample and in standard reference at 1000 µg kg^{-1}. Below chromatograms are reported the mass spectra, emphasizing the absence of the molecular ion and the characteristic fragmentation pattern of 3Ac-DON (red circles).

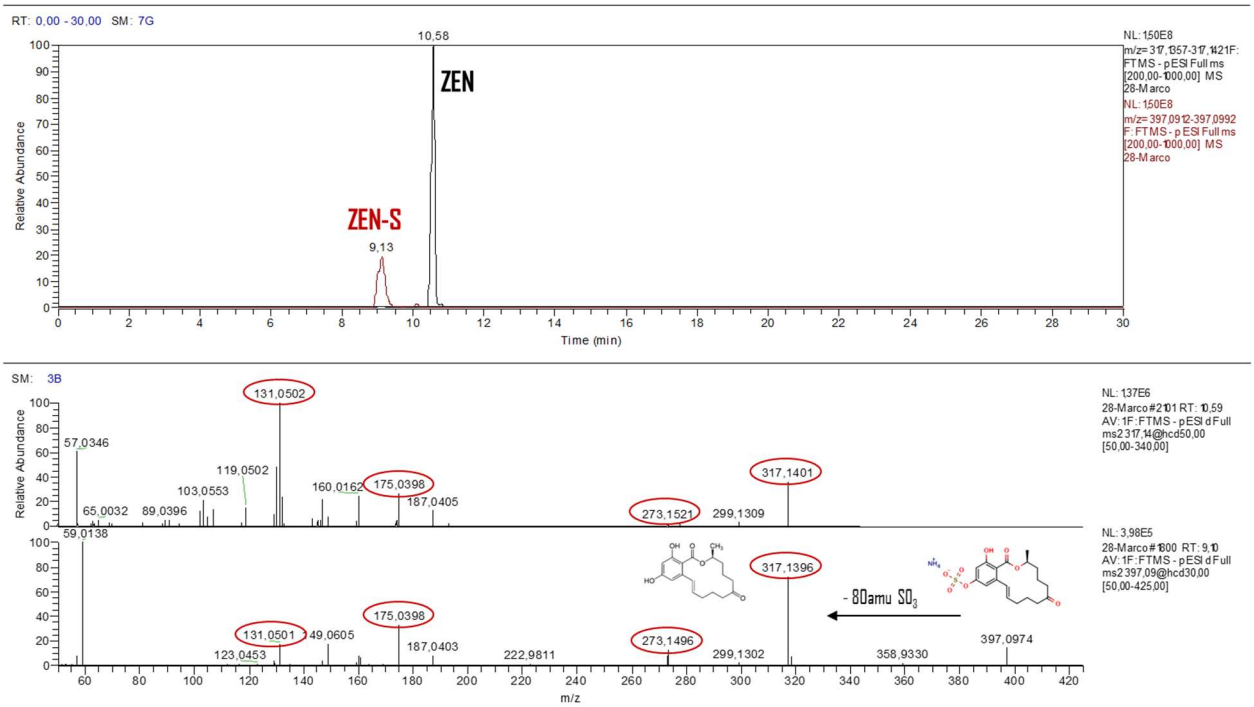


Figure S3. EIC of ZEN (*m/z* 317.1389) and ZEN-Sulphate (*m/z* 397.0952) and correspondent LC-HRMS/MS spectra. The loss of SO_3 from the ZEN molecule is clearly appreciable in the lower mass spectra, in which the ZEN molecular ion is generated.

Graphical abstract