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**Characterisation and application of isotope substituted ( $^{13}\text{C}_{15}$ )-DON as internal standard for the determination of DON**

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**Characterisation and application of isotope substituted  
(<sup>13</sup>C<sub>15</sub>)-deoxynivalenol (DON) as internal standard for the  
determination of DON**

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## Abstract

The powerful combination of liquid chromatography with mass spectrometry is often limited by matrix effects during ionisation in the MS ion source. The use of fully isotope substituted ( $^{13}\text{C}_{15}$ )-deoxynivalenol ( $(^{13}\text{C}_{15})$ -DON) as internal standard (IS) corrects matrix effects and improves the accuracy of analytical methods using mass spectrometry for the quantitative determination of the *Fusarium* mycotoxin deoxynivalenol (DON). The IS was characterized with respect to its chromatographic purity by LC-UV and its isotope distribution by time of flight mass spectrometry. Its low-energy collision induced dissociation behaviour was compared to DON. Moreover, this work describes the successful application of  $(^{13}\text{C}_{15})$ -DON as IS for the determination of DON in maize using HPLC electrospray (ESI) MS/MS. The results demonstrate that the IS can successfully correct for fluctuations during extraction and clean-up of the sample as well as the ionization of DON in the MS ion source. Random variations in ionization affect the IS in the same way as the analyte. Recoveries for DON in maize of  $76\% \pm 1.9\%$  (external calibration) or  $101\% \pm 2.4\%$  (internal calibration) were reached respectively after sample clean-up.

## Keywords

deoxynivalenol, mass spectrometry, internal standard, maize

## Introduction

The plant pathogenic moulds of the genus *Fusarium* are growing on agricultural commodities in the field and produce several toxic secondary metabolites (mycotoxins) (Bennet 2003, Jelinek 1989, Megalla 1986, Pathre 1978, Sydenham 1991). They grow parasitically on living plants and lead to lower crop yields and poor seedling quality. *Fusarium* species are ubiquitous fungi occurring on a broad variety of hosts including barley, maize and wheat (Krska 2001, Luo 1990, Tanaka 1988). Contamination of cereals with mycotoxins causes food- and feed borne intoxications in man and animals. One of the most prevalent *Fusarium* toxins is deoxynivalenol,

1  
2 3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one (DON), also known as vomitoxin  
3 (figure 1).  
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7 [insert figure 1]  
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11 Maximum levels for DON in food were regulated by the European Commission and  
12 will be put in force from 1st July, 2006 (Commission Regulation EC 856/2005). These  
13 levels range from 200 µg/kg for processed cereal-based food for infants, young  
14 children and babies up to 1750 µg/kg for unprocessed durum wheat, oats and maize.  
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19 No other technique in the area of chemical analysis developed so rapidly during the  
20 past ten years than liquid chromatography-mass spectrometry (LC-MS). Analytical  
21 methods for the determination of trichothecenes have been extensively reviewed  
22 (Langseth and Rundbergert 1998), (Lawrence and Scott, 1999), (Yoshizawa 2000),  
23 (Krska et al. 2001), (Lombaert 2002), (Koch, 2004).  
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29 Mass spectrometric methods for DON determination utilizes both GC-MS (Onji et al.,  
30 1998), (Tanaka et al. 2000), (Lombaert et al. 2003) and HPLC-MS (Kostiainen 1991),  
31 (Huopalahti et al. 1997), (Berger et al. 1999), (Razzazi-Fazeli et al. 1999), (Razzazi-  
32 Fazeli et al. 2003), (Laganà et al. 2003), (Dall'Asta et al. 2004), (Royer et al. 2004),  
33 (Berthiller et al., 2005), (Biselli and Hummert 2005), (Cavaliere et al. 2005),  
34 (Biancardi et al. 2005). None of these methods employs isotope labelled internal  
35 standards. However, in order to achieve a reliable quantification, the use of a suitable  
36 internal standard substance (IS) is highly recommended since differences in  
37 ionization efficiencies and hence in signal intensities between calibrants and analytes  
38 in matrix limit LC/MS methods. Only recently, the successful use of a (<sup>13</sup>C<sub>15</sub>)-DON  
39 as IS for the determination of DON in maize and wheat without clean-up was  
40 demonstrated for the first time (Häubl 2005)  
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47 In the present study (<sup>13</sup>C<sub>15</sub>)-DON was characterised with respect to its purity, identity  
48 and isotope distribution. Furthermore, (<sup>13</sup>C<sub>15</sub>)-DON was successfully used as IS for  
49 the quantification subsequent to the application of a well established routine in-house  
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extraction and sample preparation procedure for DON in maize. External and internal calibration was employed to show the suitability of ( $^{13}\text{C}_{15}$ )-DON as IS.

## Experimental

**Chemicals and Reagents:** DON and the internal standard ( $^{13}\text{C}_{15}$ )-DON were purchased from biopure Referenzsubstanzen GmbH (Tulln, Austria) as liquid calibrants (25mg/L) in acetonitrile.

LC-grade methanol and acetonitrile was obtained from J.T. Baker (Deventer, The Netherlands). Water, which was used for the preparation of extraction solvents, sample dilution and as LC mobile phase was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France).

**Purity check of  $^{13}\text{C}_{15}$ -DON with HPLC-UV:** To determine the purity of the IS, HPLC-UV chromatograms were recorded at 200nm, 218nm and 230nm using a diode array detector. The separation was performed on a Phenomenex Luna 5 $\mu$  C18(2) column (250mm x 3mm) after injecting 100 $\mu$ L of a 80mg/L solution of ( $^{13}\text{C}_{15}$ )DON. Flow rate: 0.5mL/min, temperature 25°C. The following linear solvent gradient was applied: After 5min hold time with acetonitrile/water 10+90 (v+v) the gradient reached 100% acetonitrile after 25min. After another 5min hold time the gradient was switched back to acetonitrile/water 10+90 in 1min, before the column finally was re-equilibrated for 10min.

**TOF-MS:** An ESI  $\mu$ TOF (Bruker Daltonik, Bremen, Germany) has been used to determine the relative intensities of the different isotopes present in the ( $^{13}\text{C}_{15}$ )-IS and to check for possible contamination with natural DON. The instrument was operated in the negative ion mode and the following MS parameters were applied: ion spray voltage 4500V, nebulizer gas 5L/min, drying gas 0.5bar, 200°C, end plate 500V. The hexapole transfer parameters were: capillary exit voltage 80.0V, hexapole 1: 25.0V, hexapole 2: 22.3V, hexapole RF 60Vpp, skimmer 1: 50.0V, skimmer 2: 24.0V transfer time 48.0 $\mu$ s, pre plus storage 5 $\mu$ s, lens 1 storage: 40.0V, extraction 22.6V, lens 2: 0.2V, lens 3: 22.0V, lens 4: 1.0V, lens 5: 40.0V, detector 1200V. The flight tube

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2 voltage was 9000V and reflector voltage was 1300V. 20000 scans were summarised  
3 over a time range of 0.5min.  
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5 5µg DON and 5µg (<sup>13</sup>C<sub>15</sub>)-DON respectively were dissolved in 1mL of water/methanol  
6 (80+20, v+v). Additionally, the pure solvent mixture was taken for blank  
7 measurements. Each of the solutions was introduced into the mass spectrometer  
8 using a syringe pump at a flow rate of 350µL/h (n=7 measurements per solution).  
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10 The precision of the determination of the <sup>12</sup>C / <sup>13</sup>C isotope ratios were evaluated for  
11 both natural DON and (<sup>13</sup>C<sub>15</sub>)-DON. Additionally, for natural DON the trueness of the  
12 determined isotope distribution was estimated by comparing the theoretical isotope  
13 ratios (calculated from natural abundances of different C/H/O isotopes) with those  
14 obtained for ESI TOF measurements. For the determination of the isotope ratios,  
15 standard deviations of the individual isotope intensities were combined according to  
16 the rules of error propagation (Eurachem 2000) and expressed as confidence  
17 intervals (α=0.05).  
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26 **MS/MS:** Stable isotope dilution mass spectrometry and MS/MS characterization of  
27 DON and the IS were performed on a QTrap triple quadrupole linear ion trap MS/MS  
28 instrument from Applied Biosystems (Foster City, US). The Turbo Ionspray (ESI)  
29 interface was used for generation of ions.  
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32 Enhanced Product Ion (EPI) spectra were recorded both for DON and (<sup>13</sup>C<sub>15</sub>)-DON.  
33 Solutions containing 5mg/L in MeOH/H<sub>2</sub>O (20+80, v+v) were injected in the Turbo Ion  
34 Spray (ESI) interface at a flow rate of 10µl/min using a syringe pump. The following  
35 MS settings were used: ion spray voltage -4200V, curtain gas 20psi (137.9kPa),  
36 source gas 20psi (137.9kPa), collision activated dissociation gas high, declustering  
37 potential -40V, collision energy -25eV, Q1 resolution unit, linear ion trap (LIT) fill time  
38 50ms, LIT scan speed 1000amu/s. 100 consecutive (MCA) scans were added for the  
39 final spectrum.  
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46 **HPLC-MS/MS:** A 1100 Series LC system from Agilent Technologies (Waldbronn,  
47 Germany) was used for liquid chromatography. The QTrap equipment described  
48 above was used for MS measurements. Chromatographic separation was achieved  
49 on an Aquasil RP-18 column (3µm, 50mm x 2.1mm) from Thermo Electron (Woburn,  
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MA, USA). Injection volume: 10 $\mu$ L, flow rate: 0.3mL/min, temperature: 25°C. The duration of a HPLC run was 15min, starting with methanol/water (15+85, v+v) as mobile phase. After an initial hold time of 2.5min, a linear gradient was used to reach 100% methanol in 1.5min (hold time: 3.5min), and back to methanol/water 15+85 (v+v) in 1.5min (hold time: 6.0min). In order to minimize contamination of the MS ion source with matrix components the HPLC eluate was allowed to enter the MS interface only between 2min and 4min after LC injection.

For the determination of DON, the MRM transitions from the deprotonated molecular ion [M-H]<sup>-</sup>  $m/z$  = 295.1 to  $m/z$  = 265.1 (collision energy (CE) -12eV) and  $m/z$  = 138.0 (CE -26 eV) were monitored. For (<sup>13</sup>C<sub>15</sub>)-DON, two MRM transitions referring to fragments of the molecular ion [M-H]<sup>-</sup> ( $m/z$  = 310.1 to  $m/z$  = 279.1 (CE -12 eV)) and  $m/z$  = 310.1 to  $m/z$  = 145.0 (CE -26eV) were used. The transitions  $m/z$  = 295.1 to  $m/z$  = 265.1 and  $m/z$  = 310.1 to  $m/z$  = 279.1 correspond to the loss of formaldehyde from the [M-H]<sup>-</sup> ions and were used for quantification, while the other two transitions were used as qualifiers to confirm the identity of the analytes.

**Matrix calibration:** 50g of blank maize were extracted with 200mL of acetonitrile/water, 84+16, v+v) on a rotary shaker for 90 minutes. After filtration 4mL aliquots of the raw extract were spiked with (<sup>13</sup>C<sub>15</sub>)-DON as IS (0.25mL of a 1.0mg/L solution in acetonitrile to each aliquot) and DON (2.0mg/L in acetonitrile). The following spiking levels were achieved: 0, 30, 50, 100, 300, 500, 1000 $\mu$ g DON/kg maize (n=3 per concentration level).

Subsequently, the fortified extracts were purified with Mycosep<sup>®</sup>225 clean up columns (Romer Labs, Tulln, Austria) and volumes corresponding to 0.5g of maize were evaporated to dryness under a gentle stream of nitrogen. The dried extracts were re-constituted with 0.5mL of starting mobile phase. Aliquots of 10 $\mu$ L were injected into the HPLC instrument.

**Calibrants:** A blank and 7 standard solutions with DON concentrations of 10, 30, 50, 100, 300, 500 and 1000 $\mu$ g/L in pure water were prepared. Each of these solutions also contained 250 $\mu$ g/L (<sup>13</sup>C<sub>15</sub>)-DON as IS.



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**Data evaluation:** Linear regression lines without considering the IS were constructed by plotting the signal intensity (peak area) as a function of the DON concentrations. For the stable isotope dilution, the method of IS calibration was applied. According to this method the calibration line was established by plotting the ratio of the DON detector signal / IS signal against the DON concentrations of the calibration solutions. Recoveries were estimated from the slope of the regression curve according to both, the method of external standards and the method of IS calibration. Linear regression data were calculated according to the DIN standard 32645 using the validation software program ValiData® (Wegscheider 1999).

**Results and Discussion**

To control the purity of the (<sup>13</sup>C<sub>15</sub>)-DON standard, a concentrated solution (80mg/L) was analysed by HPLC/DAD and chromatograms at different wavelengths were recorded. Figure 2 shows a typical chromatogram at the maximum absorbance for DON in acetonitrile (218nm). The relative peak area of the peak corresponding to (<sup>13</sup>C<sub>15</sub>)-DON compared to the sum of all peak areas in the respective chromatogram was 99.5% or higher, depending on the UV wavelength chosen for data evaluation. Therefore, it was concluded that, based on the UV measurements, the impurities were ≤ 0.5% in total.

[insert figure 2]

The TOF-MS spectra show no detectable impurities of naturally occurring DON. For DON the seven replicate measurements yielded the following relative intensities for the different isotopes, measured as the deprotonated molecular ions: monoisotopic [<sup>12</sup>C<sub>15</sub>]-DON-H]<sup>-</sup> *m/z* = 295.12: 83.5%±0.15%; [<sup>12</sup>C<sub>14</sub><sup>13</sup>C<sub>1</sub>]-DON-H]<sup>-</sup> *m/z* = 296.12: 13.4%±0.27%; [<sup>12</sup>C<sub>13</sub><sup>13</sup>C<sub>2</sub>]-DON-H]<sup>-</sup> *m/z* = 297.13: 2.5%±0.24% and [<sup>12</sup>C<sub>12</sub><sup>13</sup>C<sub>3</sub>]-DON-H]<sup>-</sup> *m/z* = 298.11: 0.6%±0.15%. The measurement results corresponded to a relative amount of <sup>12</sup>C atoms in natural DON of 98.7%±0.72% (α=0.05) when the sum of these four isotopes were considered. This value is in good agreement with the actual relative abundance of <sup>12</sup>C isotope which amounts to of 98.9% of the total

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carbon. Therefore, no significant difference between calculated and measured isotope ratios was detected.

For the IS four different isotopes were detected (figure 3). The following relative signal intensities were obtained for the deprotonated molecular ions with ESI TOF MS:  $[(^{13}\text{C}_{15})\text{-DON-H}]^-$   $m/z = 310.17$ :  $81.7\% \pm 0.17\%$ ;  $[(^{12}\text{C}_1^{13}\text{C}_{14})\text{-DON-H}]^-$   $m/z = 309.17$ :  $15.6\% \pm 0.20\%$ ;  $[(^{12}\text{C}_2^{13}\text{C}_{13})\text{-DON-H}]^-$   $m/z = 308.17$ :  $2.4\% \pm 0.09\%$  and  $[(^{12}\text{C}_3^{13}\text{C}_{12})\text{-DON-H}]^-$   $m/z = 307.16$ :  $0.4\% \pm 0.16\%$ . These values resulted in a relative amount of  $^{13}\text{C}$  atoms of  $98.6\% \pm 0.58\%$  ( $\alpha=0.05$ ) in the isotope substituted ( $^{13}\text{C}_{15}$ )-DON.

[insert figure 3]

MS/MS spectra of DON and ( $^{13}\text{C}_{15}$ )-DON were recorded (Figure 4) at a collision energy of 25eV and compared to each other. Both the observed  $m/z$  values of the MS/MS fragments as well as their relative intensities were identical, except for the mass shift resulting from the  $^{13}\text{C}$  atoms in ( $^{13}\text{C}_{15}$ )-DON. Figure 4 also contains a description of the most abundant fragments with respect to the functional groups cleaved off during CID. Typical for DON is the loss of -30amu ( $\text{H}_2\text{CO}$ ) during fragmentation (Berthiller 2003 and Razzazi-Fazeli 2003). Exact the same cleavage of formaldehyde, but one neutron heavier can be observed in the MS/MS spectra of the fully isotope substituted DON. The comparison of the two MS/MS spectra also demonstrates the great value of isotope labelled compounds for structural characterisation of DON by indicating the number of C-atoms in the cleaved fragments

[insert figure 4]

To demonstrate the potential of ( $^{13}\text{C}_{15}$ )-DON to be used as IS for the determination of DON, a non-contaminated maize sample was extracted and aliquots of the raw extract were spiked with DON and IS. It has been demonstrated before that spiked DON can be extracted quantitatively from blank maize with acetonitrile / water

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(84+16, v+v) (Häubl 2005). Therefore, the spiking of the raw extracts can be regarded sufficient to test the suitability of the IS. The measurement results obtained for the spiking experiments were evaluated by plotting the found DON concentrations versus true DON concentrations after fortification of blank maize material. Figure 5 illustrates the linear regression lines which were obtained. When DON was quantified by use of (<sup>13</sup>C<sub>15</sub>)-DON as IS, a correlation coefficient ( $R^2$ ) of 0.9977 and a slope of  $1.01 \pm 0.024$  ( $\alpha=0.05$ , degrees of freedom = 18) were obtained. This corresponds to an estimated recovery of  $101 \pm 2.4\%$ . In case of no consideration of the IS, a slope of  $0.76 \pm 0.019$  ( $\alpha=0.05$ , degrees of freedom = 18) was indicating a recovery of  $76 \pm 1.9\%$ . In this case  $R^2=0.9974$  was obtained. For none of the two evaluation methods there was any evidence of disturbing background signals. This finding is in good agreement with the low values for the Y-intercepts of the recovery functions ( $2.94 \mu\text{g/kg}$  (with IS) and  $2.74 \mu\text{g/kg}$  (no IS)), none of them being significantly different from zero ( $\alpha=0.05$ ).

The results of the spiking experiments demonstrate that (<sup>13</sup>C<sub>15</sub>)-DON can be used to efficiently correct for losses of DON during sample preparation as well as ion suppression effects in the ESI source of the mass spectrometer (resulting in an average recovery of 76% when no IS was used for quantification of DON).

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[insert figure 5]

Conclusion

In this paper we analytically characterized (<sup>13</sup>C<sub>15</sub>)-DON for the use as IS for quantification of DON in cereals using LC-MS/MS. The purity checks of (<sup>13</sup>C<sub>15</sub>)-DON resulted in UV absorbing impurities of less than 0.5% relative to DON. No naturally occurring DON was found in the IS. The <sup>13</sup>C isotope enrichment of the IS was calculated to be  $98.6\% \pm 0.6\%$ . Both, the MS/MS product ion scans of DON and the (<sup>13</sup>C<sub>15</sub>)-DON were recorded and are presented. Whole method recovery for DON was  $76\% \pm 1.9\%$  after clean-up by using external standard calibration. The IS successfully compensated losses in sample preparation and ion suppression effects overall yielding  $101\% \pm 2.4\%$  recovery.

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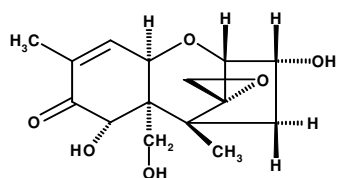


Figure 1 : Chemical structure of DON



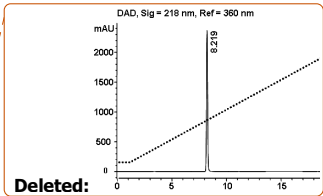
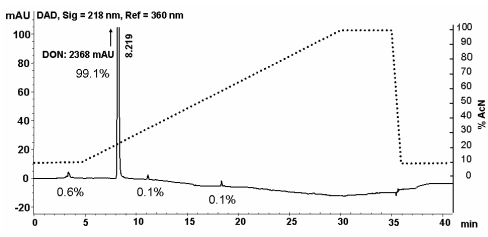


Figure 2: HPLC-DAD chromatogram of a 80 mg/L solution of  $(^{13}\text{C}_{15})$ -DON at 218nm (blank run is subtracted). %-values correspond to the peak area of the respective HPLC peak relative to the sum of all peaks detected. Dotted line visualizes eluent gradient.

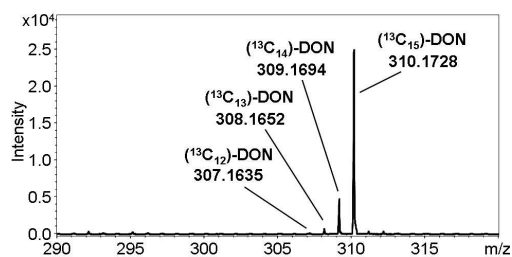


Figure 3: TOF-MS Spectra of (<sup>13</sup>C<sub>15</sub>)-DON.

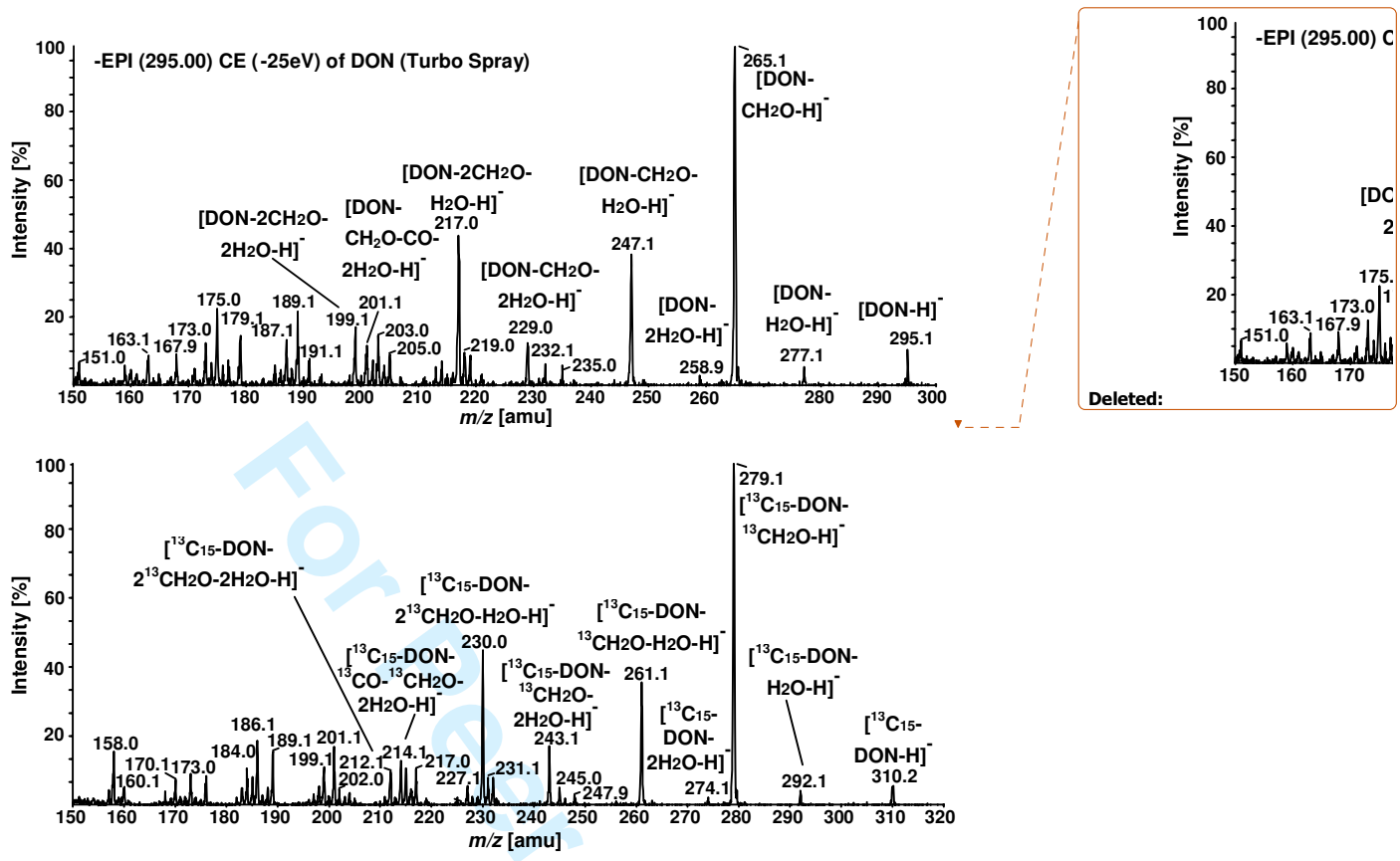


Figure 4a and 4b: Comparison of the MS/MS Spectra of DON and (<sup>13</sup>C<sub>15</sub>)-DON

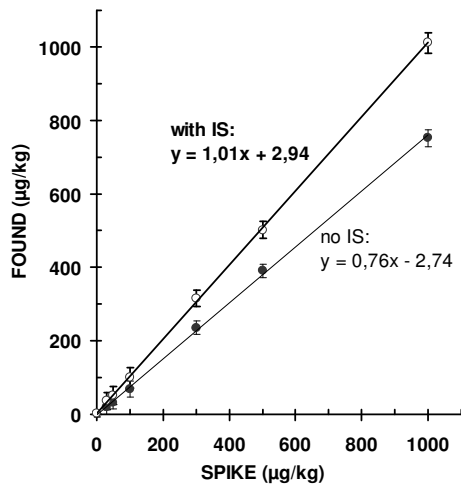


Figure 5: Comparison of recovery functions for a blank maize extract, fortified with different concentration levels of DON. For each level the toxin was spiked in triplicate.