A Validated LC-HRMS Method for the Detection of T-2 and HT-2 Toxins in Cereals

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Summary

The tricothocene mycotoxins T-2 Toxin and HT-2 Toxin have been shown to be toxic to a range of species and, as a consequence, limits have been set on their consumption. The toxins have been found in a range of grain varieties and grain-containing products which each have specific limits above which further investigation must be carried out. Here we describe the development and partial validation of a method for the detection of T-2 and HT-2 Toxins in cereals. Samples of oat flour and corn flour were spiked with the mycotoxins at various levels between 5 and 500ng/g before being extracted using a modified QuEChERS approach. Analysis of the subsequent extracts was carried out via LC-MS using a high resolution quadrupole time-of-flight mass spectrometer. Using this method the limits of detection for the two analytes were at least 5ng/g in each matrix with limits of quantitation of each set as 10ng/g. The method was validated across four batches (two per matrix) at three levels (10, 25 and 100ng/g) with excellent recovery and low variability across the levels.

The use of high resolution mass spectrometry and accurate mass for this type of analysis is a departure from a more typical approach which generally involves low resolution, triple quadrupole mass spectrometry operating in selected reaction monitoring mode. We therefore also describe the development of specific analyte identification criteria for such technology.

Keywords

High resolution MS, mycotoxins, HT-2 Toxin, T-2 Toxin, LC/MS, QuEChERS, cereals

Introduction

T-2 Toxin and HT-2 Toxin are tricothecene mycotoxins produced by various fusarium species which are known to be toxic to humans and animals[1-3]. The Scientific Panel on Contaminants in the Food Chain (CONTAM) of the European Food Safety Authority (EFSA) has established a tolerable daily intake (TDI) of 100ng/kg body weight for the sum of T-2 and HT-2 Toxins[4]. While CONTAM's investigation of these toxins concluded that the risk of exposure for humans and most animals is low and therefore unlikely to be a health hazard, there is a desire to better understand when and where they occur. The European Commission have therefore recommended that member states should monitor cereals and cereal products for the presence of T-2 and HT-2 Toxins as outlined in Commission Recommendation 2013/165/EU. The annex to this recommendation sets indicative maxima for the sum of T-2 and HT-2 above which further investigation must be carried out (eg 200µg/kg for unprocessed barley and maize)[5]. In order to support potential Government Chemist referee cases relating to these analytes a validated LC-MS method was therefore required.

Routine approaches for LC-MS quantitation utilise triple-quadrupole mass spectrometry (QqQ-MS) analysis. QqQ instruments are low resolution, typically only capable of nominal mass determination and therefore must be run in MS/MS mode monitoring for specific fragmentation in order to achieve analyte-specificity (typically in selected reaction monitoring mode – SRM). High resolution mass spectrometry (HRMS) has various definitions depending on the context in which it is being used; for screening, however, it is generally defined as a mass spectrometer capable of >10,000 FWHM (Full Width at Half Maximum) mass resolution and mass accuracy of <5ppm[6-9]. With such resolution and mass accuracy it is possible to achieve specificity (for small molecules) based on mass measurement alone[10].

A key advantage of HRMS over standard MS approaches is that HRMS instruments tend to be operated in full scan mode meaning data can be collected for the entire mass range simultaneously thereby allowing later interrogation of results if required eg for identification of subsequently identified mycotoxin species. In the past time-of-flight mass spectrometers (ToF-MS) were somewhat unreliable for quantitation due to issues such as mass resolution, mass accuracy, sensitivity and, particularly, linear range[11]. However, recent developments in ToF instrumentation have begun to eliminate these drawbacks[12]. ToF resolution has been improved thanks to developments in ion optics[13]; mass accuracy has been improved in part due to the increase in resolution but also the use of lockmass for on-the-fly mass correction[14]; sensitivity has been improved by the developments in source design and the use of differentially pumped ion optics[15,16] whilst linear range has been improved by the switch from the use of time-to-digital converters (TDCs) to analogue-to-digital converters (ADCs)[17,18].

The recent purchase by LGC of a Waters Xevo G2-XS quadrupole time-of-flight (QToF) mass spectrometer (Waters, Wilmslow, UK) allowed the authors to benefit from these advances. Using this instrument mass resolution of up to 50,000 is achievable, mass accuracy of <5ppm is typical and a linear dynamic range of up to 4 orders of magnitude is possible. With these specifications the instrument should be capable of providing high analyte specificity and excellent quantitative data, hence it was deployed for development and validation of the method described herein.

Materials and Methods

Chemicals

The following solvent standards were obtained from LGC Standards (Teddington, UK):

B-MYC0540-1	T-2 Toxin, 100µg/mL in acetonitrile (certified concentration 100.2 ±0.6µg/mL, k=2)
B-MYC0560-1	HT-2 Toxin, 100µg/mL in acetonitrile (certified concentration 100.4 ±0.8µg/mL, k=2)
B-MYC0550-1.2	T-2 Toxin (13C24), 25µg/mL in acetonitrile (internal standard)
B-MYC0565-1.2	HT-2 Toxin (13C22), 25µg/mL in acetonitrile (internal standard)
Mycotoxin Mix 2	Deoxynivalenol, Nivalenol, 3-Acetyldeoxynivalenol and 15-Acetyldeoxynivalenol, 100µg/mL each in acetonitrile
Mycotoxin Mix 3	Fumonisin B1 and Fumonisin B2, 50µg/mL each in acetonitrile
B-MYC0335-1	Deoxynivalenol-3-glucoside, 50µg/mL in acetonitrile

The chosen internal standards were the [13]C labelled analogues of T-2 Toxin and HT-2 Toxin. In both cases the molecules were fully labelled with carbon-13. The labelled T-2 Toxin was enriched with carbon-13 to a level of 99.3% and the HT-2 Toxin was enriched to a level of 99.1%.

Methanol and acetonitrile were obtained from LGC Standards, formic acid was obtained from Thermo Fisher Scientific (Loughborough, UK) and 18 $M\Omega cm^{-1}$ ultrapure water was obtained in-house using an Elga water purification unit (Veolia Water Technologies UK, High Wycombe, UK).

Quechers extraction salts and dispersive SPE (solid phase extraction) kits were obtained from Crawford Scientific (Strathaven, UK).

Oat flour and maize flour were obtained from Healthy Supplies Ltd. (Lancing, UK) and Buy Wholefoods Online Ltd (Canterbury, UK) respectively.

The FAPAS QC material T2296QC (Oat Flour containing T-2 Toxin (149µg/mL) and HT-2 Toxin (187µg/mL)) was purchased from FERA Science (York, UK).

Standard Preparation

The five stock natural mycotoxin standards were diluted in acetonitrile to 50µg/mL where required then mixed and further diluted to give a working standard at 5µg/mL containing nine mycotoxins: T-2 Toxin (T-2), HT-2 Toxin (HT-2), Deoxynivalenol (DON), Nivalenol (NIV), 3-Acetyldeoxynivalenol (3Ac-DON), 15-Acetyldeoxynivalenol (15Ac-DON), Fumonisin B1 (FB1), Fumonisin B2 (FB2) and Deoxynivalenol-3-glucoside (DON-3G). This standard was diluted 1 in 10 in acetonitrile to give a second spiking standard at 0.5µg/mL. The two labelled mycotoxin standards (T-2*and HT-2*) were diluted 1 in 10 in acetonitrile to give spiking solutions at 2.5µg/mL.

Sample Preparation

As no certified reference materials are currently available for T-2 and HT-2 Toxins in the chosen matrices the method was developed and validated using a pre-extraction spiking strategy. For each batch (one matrix material per batch) n=6 replicates of the material were spiked with the mixed mycotoxin standards at three different levels and with a fixed amount of each internal standard compound. These pre-extraction matrix spikes (PrEMS) samples were extracted alongside a group of matrix blanks (n=6) and, where appropriate, a QC material (n=6). Aliquots of the blank extracts were then spiked with the mixed standards and internal standards at appropriate levels to give a set of post-extraction matrix extracts (PoEMS) which were used for calibration.

The combination of PrEMS and PoEMS samples were then used to assess recovery, sensitivity, linearity and repeatability.

For each validation batch three sets of PrEMS samples were prepared at three different levels, 10, 25 and 100µg/kg (PrEMS 10, PrEMS 25 and PrEMS 100 respectively) along with a calibration PoEMS sample set at concentrations ranging from 0 to 500µg/kg (PoEMS 0 to PoEMS 500). These samples were prepared using a set of four standard solutions: T-2 Toxin Label Dilution 1 (2.5µg/mL in acetonitrile (ACN)), HT-2 Toxin Label Dilution 1 (2.5µg/mL in ACN), Mixed Mycotoxin Spiking Standard Dilution 2 (5µg/mL in ACN) and Mixed Mycotoxin Spiking Standard Dilution 3 (0.5µg/mL in ACN).

The PrEMS samples were spiked as follows:

PrEMS 10:	40 µL T-2 Toxin Label Dilution 1; 40 µL HT-2 Toxin Label Dilution 1; 40 µL Mixed Myco Spiking Standard Dilution 3
PrEMS 25:	40 µL T-2 Toxin Label Dilution 1; 40 µL HT-2 Toxin Label Dilution 1; 10 µL Mixed Myco Spiking Standard Dilution 2
PrEMS 100:	40 µL T-2 Toxin Label Dilution 1; 40 µL HT-2 Toxin Label Dilution 1; 40 µL Mixed Myco Spiking Standard Dilution 2

The PoEMS samples were prepared by spiking 750µL of cleaned-up blank extract with the same standard solutions as shown in table 1.

Table 1: Spiking Volumes for PoEMS Calibration Samples

Standard	Concentration (µg/kg)	Volume T-2 Toxin Label Dilution 1 (µL)	Volume HT-2 Toxin Label Dilution 1 (µL)	Volume Mixed Myco Spiking Solution Dilution 2 (µL)	Volume Mixed Myco Spiking Solution Dilution 3 (µL)
PoEMS 0	0	3	3	0	0
PoEMS 10	10	3	3	0	3
PoEMS 25	25	3	3	0	7.5
PoEMS 50	50	3	3	0	15
PoEMS 75	75	3	3	0	22.5
PoEMS 100	100	3	3	0	30
PoEMS 200	200	3	3	6	0
PoEMS 300	300	3	3	9	0
PoEMS 400	400	3	3	12	0
PoEMS 500	500	3	3	15	0

nb this calibration set is intended as a two-stage calibration covering two ranges, low (10 to 100ng/g) and high (100 to 500ng/g)

Extraction Procedure

Several approaches exist in the literature for the extraction of mycotoxins from cereals using a number of techniques including immunoaffinity purification, mycotoxin-specific SPE, liquid-liquid extraction and gel permeation chromatography[19]. However, due to the authors' recent positive experience in the analysis of pesticides the chosen extraction strategy for this project utilises QuEChERS ("Quick, Easy, Cheap, Effective, Rugged and Safe") methodology. QuEChERS is a simplified sample extraction and clean-up methodology originally designed for the analysis of veterinary residues but now widely used in a number of areas for multi-residue analysis methods particularly in pesticide analysis[20-24].

In QuEChERS a homogenised sample is extracted into mixed solvent (eg 50% ACN) to which salts ($MgSO_4$ and NaCl) are then added in order to induce solvent partitioning (usually aided by centrifugation). A portion of the appropriate solvent layer is then subject to dispersive solid phase extraction clean-up (dSPE) followed by solvent exchange prior to analysis[20]. Using this approach far greater sample throughput can be achieved compared with traditional approaches. The QuEChERS method used for mycotoxin analysis described here is a modified version of the method described by Oplatowska-Stachowiak *et al.* for the analysis of multiple mycotoxins in distiller's dried grain with solubles (DDGS)[25].

Samples were prepared for analysis as follows:

1	2.00±0.02 g of material weighed into 50mL polypropylene tube (weight recorded)
2	Sample rehydrated for 30 minutes with 10mL of 1% formic acid in water
3	Sample spiked with internal standard (PrEMS and QC samples only)
4	Sample spiked with mixed mycotoxin standard (PrEMS samples only)
5	Sample allowed to equilibrate for 30 minutes
6	10mL of acetonitrile added to sample
7	Sample mixed on rotary shaker for 30 minutes to extract analytes
8	1 sachet of QuEChERS extraction salts (1g NaCl, 5g $MgSO_4$) added to tube
9	Sample vortexed immediately for 30 seconds
10	Tube centrifuged at 2147 x *g* rpm for 10 minutes to separate phases
11	1.6mL of upper (organic) layer transferred to dSPE tube
12	dSPE tube vortexed for 30 seconds then centrifuged at 4000 x *g* rpm for 5 minutes
13	15mL polypropylene tubes spiked with internal standards and mixed mycotoxin standard (PoEMS only, see table 1)
14	750µL of supernatant transferred to corresponding 15mL polypropylene tube
15	Sample evaporated on Turbovap (Biotage, Uppsala, Sweden) at 40°C for 30 minutes
16	Sample reconstituted in 250µL of 25% acetonitrile + 0.1% formic acid in water then transferred to maximum recovery vial (Waters, Wilmslow, UK) for analysis

LC-Method

The finalised LC method was as follows:

Instrument:	Waters Acquity H-Class UPLC
Column:	Waters Acquity UPLC BEH C18 1.7µm, 2.1 x 50mm with in-line 0.2 µm filter
Column Temp:	40°C
Sample Temp:	10°C
Injection Vol:	20µL
Solvent A:	Water + 0.1% formic acid
Solvent B:	50:50 Methanol/Acetonitrile + 0.1% formic acid
Flow Rate:	0.5 mL/min

Gradient Table:

0.0 min	5% B
1.0 min	5% B
8.0 min	95% B
10.0 min	95% B
10.5 min	5% B
15.0 min	5% B

MS Method

Instrument:	Waters Xevo G2-XS QToF
Ion Source:	ESI
Ionisation Mode:	Positive
Acquisition Mode:	Sensitivity, continuum
Capillary Voltage:	3kV
Cone Voltage:	40V
Source Temp:	100°C
Drying Gas Temp:	500°C
Cone Gas:	100L/h
Drying Gas:	600L/h
Mass Range:	m/z 100 - 1200
Scan Rate:	0.5s/scan
Lock Mass:	LeuEnk (m/z 556.2771; 1.5kV; 10µL/min; 1s/scan; 1scan every 30s)

Divert Valve Timetable:

0 min	Waste
4 min	LC
7 min	Waste

Data Analysis Method

Data analysis was carried out using two different software packages: Targetlynx (Waters, Wilmslow, UK) – the quantitation software provided with the instrument; and Skyline

(University of Washington, Seattle, USA) a freeware quantitation software produced by the MacCoss group at the University of Washington[26].

For Targetlynx analysis the following parameters were used:

Mass Window:	20ppm (±10ppm)
Retention Time Window:	0.3 minutes
Smoothing:	Mean, 3 iterations, width 2

Using these settings the software automatically generated extracted ion chromatograms for the species of interest and peak areas for these species. However, mass accuracies and peak resolution was required to be extracted manually using this method.

For Skyline the following parameters were used:

Mass Window:	10ppm (±10ppm)
Retention Time Window:	0.2 minutes

The software automatically generated extracted-ion chromatograms (EICs) for each species along with peak areas and mass accuracies.

Both software packages allow concentration calculation within their quantitation modules. However, for consistency and ease of comparison all calculations were carried out in Microsoft Excel.

Results and Discussion

Species Confirmation

In a typical QqQ-MS method species are confirmed when they meet a set of criteria relating to retention time, presence of a detectable signal in more than one SRM channel and the peak area ratio between different SRM transitions[27]. Clearly all these criteria cannot be used for full scan Q-ToF-MS data as generated using the method described above; a new set of criteria specific to HRMS was therefore developed.

Specific regulations for the use of HRMS for the confirmation of the presence of mycotoxin species are not currently available. However, advice relating to chemical residue analysis in food using HRMS does exist in EU Commission Decision 2002/657/EC[28] and SANCO Guidance SANCO/10684/2009[29]. These two documents were used as a framework for the development of confirmation criteria for this work.

The criteria for LC-Q-ToF-MS confirmation of the presence of an analyte were as follows:

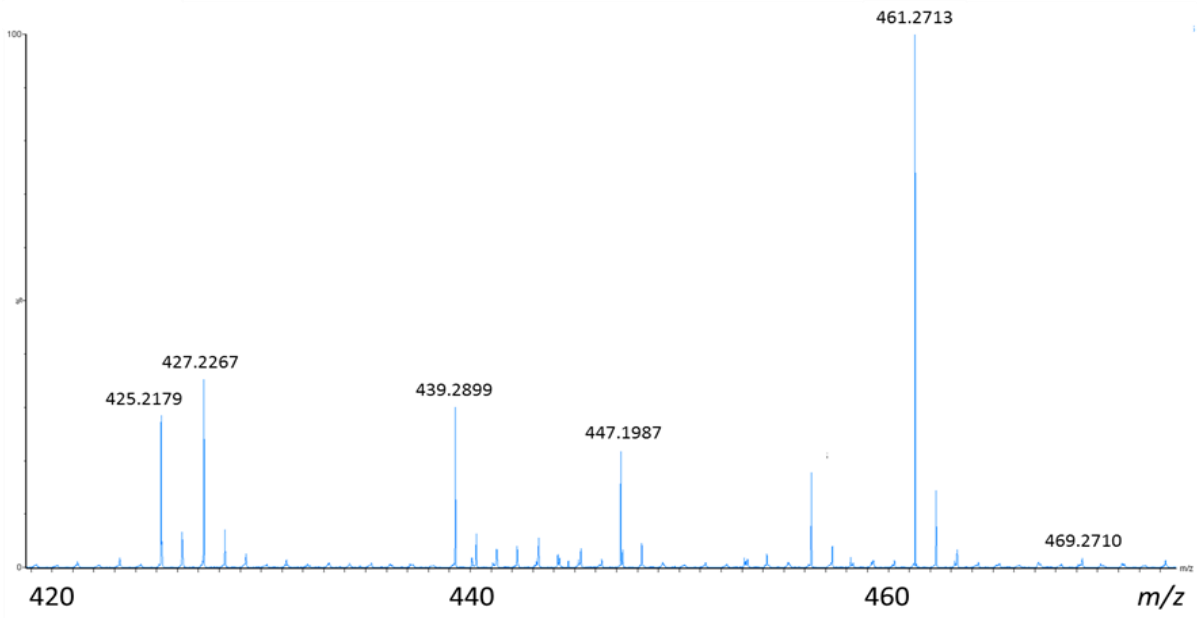
1. Presence of ≥2 diagnostic ions (including the ionised molecule) at mass resolving power >10,000 and mass accuracy <5 ppm with S/N >3 (peak-to-peak).
2. Peak area/intensity ratio between the diagnostic ions matching with that of reference spectra acquired in the same run (see Table 2 for matching criteria)

3 Retention time within ±0.1min of reference standards

Table 2: Matching Criteria for Peak Area/Intensity Ratios

Relative Intensity (% of base peak)	Ratio Tolerance
>50%	±20%
20% to 50%	±25%
10% to 20%	±30%

Figure 1 – an example Mass Spectrum obtained for HT-2 for an Oat Flour Extract PoEMS 500



This shows a Centroid mass spectrum of HT-2 Toxin in oat flour extract at 500µg/kg. Potential HT-2 and HT-2 label-related species are at nominal masses *m/z* 425, *m/z* 442, *m/z* 447, *m/z* 464 and *m/z* 569.

Clearly there are many species present in the spectrum including a number with nominal mass which can be assigned to HT-2 or its labelled analogue. Table 3 shows the mass-to-charge ratios of the peaks observed in this spectrum along with potential assignments and the associated mass difference between the two species.

Table 3 – Detected Ions for PoEMS 500 in Oat Flour and Corresponding Potential HT-2 Toxin (M) and HT-2 Label (M*) species

Measured *m/z*	Assignment	Theoretical *m/z*	delta M (m/z)	delta M (ppm)	Resolution	Intensity	S/N
425.2208	$[M+H]^+$	425.2175	0.0033	7.8	17717	344533	68.3
442.2443	$[M+NH_4]^+$	442.2441	0.0002	0.5	13162	46579	9.6
447.1987	$[M+Na]^+$	447.1995	-0.0008	-1.8	25265	262816	103.8
447.2913	$[M^*+H]^+$	447.2914	-0.0001	-0.2	13312	41540	20.6
464.3023	$[M^*+NH_4]^+$	464.3179	-0.0156	-33.6	8646	12443	3.2
469.2710	$[M^*+Na]^+$	469.2733	-0.0023	-4.9	11120	23813	6.8

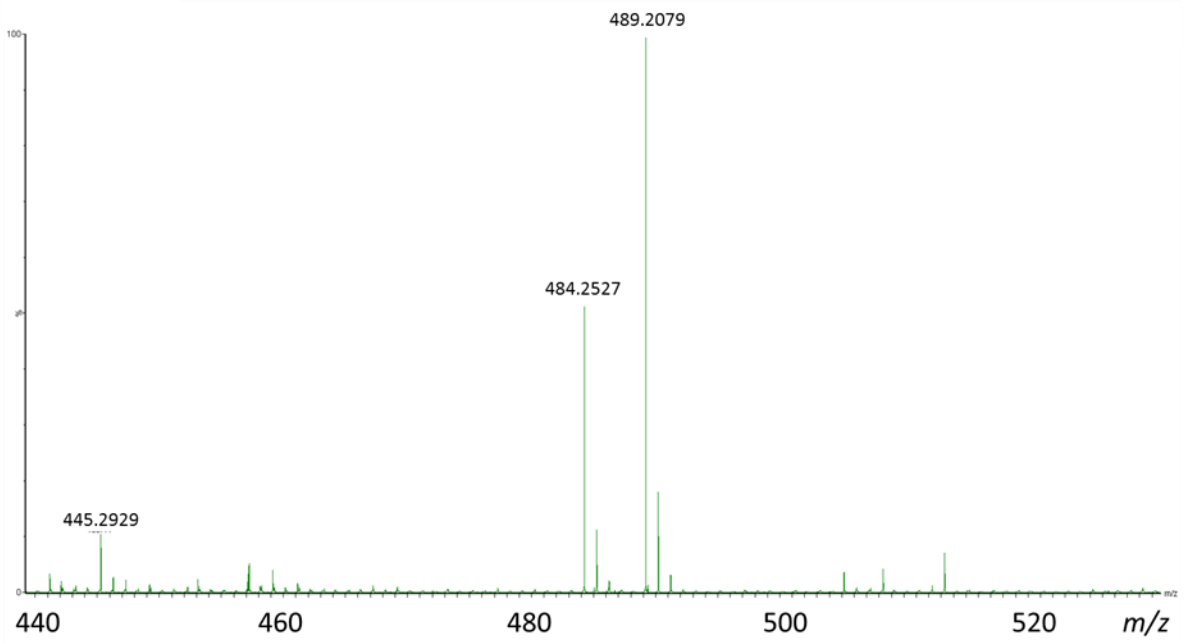
Cells highlighted green fall within the defined limits of mass accuracy, signal-to-noise or resolution while cells highlighted red fall outside of these limits

From the table it can be seen that HT-2 toxin and the HT-2 label can be observed as the protonated ion, the ammonium adduct and the sodium adduct. For both molecules the respective protonated species can be seen to be the most abundant ions, however, for HT-2 Toxin the mass accuracy can be seen to be insufficient for confident assignment. This is most likely due to an isobaric interference at this *m/z* which cannot be isolated at the achieved resolution; this is also reflected in the lower signal-to-noise measured for this ion despite being present at greater intensity. For the label it can be seen that the peak detected at *m/z* 464 is unlikely to be the ammonium adduct due to the large mass error.

The chosen quantitation ions were therefore $[M+Na]^+$ at *m/z* 447.1995 and $[M^*+H]^+$ at *m/z* 447.2913. Whilst the ammonium adduct ion for HT-2 toxin does pass criteria for this example the low relative intensity and signal-to-noise means it is unlikely to be useful as a qualifier ion. The first isotope peak at *m/z* 448.2023 was therefore chosen as the qualifier. Whilst this is not ideal the inclusion of an internal standard at the same retention time means this peak can be used as a qualifier with high confidence. The qualifier ion for the internal standard was chosen to be the sodium adduct at *m/z* 469.2710.

Figure 2 shows the mass spectrum obtained for the T-2 Toxin peak from the same experiment.

Figure 2 – Centroid Mass Spectrum for T-2Toxin and T-2 Toxin label for an Oat Flour PoEMS 500 sample



As with the HT-2 data, several potential T-2 Toxin (M) and T-2 Toxin Label (M*) species can be potentially assigned from the spectrum. Table 4 shows the assignments and associated metrics.

Table 4 – Detected Ions for PoEMS 500 in Oat Flour and Corresponding Potential T-2 Toxin (M) and T-2 Label (M*) Species

Measured *m/z*	Assignment	Theoretical *m/z*	delta M (m/z)	delta M (ppm)	Resolution	Intensity	S/N
467.2281	[M+H]+	467.2281	0	0.0	12136	21983	2.5
484.2527	$[M+NH_4]+$	484.2547	-0.002	-4.1	30456	975945	222.9
489.2079	[M+Na]+	489.2101	-0.0022	-4.5	33739	1843279	192
491.2136	[M*+H]+	491.3087	-0.0951	-193.6	18893	41540	<1
508.3327	$[M^*+NH_4]+$	508.3352	-0.0025	-4.9	18552	12443	55.7
513.2885	[M*+Na]+	513.2906	-0.0021	-4.1	19666	23813	53.6

Cells highlighted green fall within the defined limits of mass accuracy, signal-to-noise or resolution while cells highlighted red fall outside of these limits

From the table it can be seen that T-2 toxin and the T-2 label can be observed as the protonated ion, the ammonium adduct and the sodium adduct. For the natural molecule all three species are present with the required resolution and mass accuracy. However, the signal-to-noise achieved for the protonated species is below the required level and therefore this ion cannot be confidently assigned. For the labelled molecule the mass accuracy of the protonated species was very low indicating that the detected ion is unlikely to be protonated T2 label (the detected peak is most likely the third isotope peak of the ammonium adduct of the natural T2).

The chosen quantitation ions were therefore $[M+Na]^+$ at *m/z* 489.2079 and $[M^*+Na]^+$ at *m/z* 513.3327. The qualifier ions in each case were the ammonium adduct ions: $[M+NH_4]^+$ at *m/z* 484.2527 and $[M+NH_4]^+$ at *m/z* 508.3327. It should be noted that in both cases the ammonium ion has a greater signal-to-noise than the sodium adduct ion despite having a lower intensity.

The quan/qual ratios for each detected species will vary with the experiment and matrix (eg when using solvent standards little or no sodium adduct species were detected for either analyte) and the tolerances are therefore determined on a batch-to-batch basis with reference to Table 2.

Table 5 shows the identification data obtained for the first T-2 Toxin calibration curve from batch 3 (T-2 and HT-2 Toxins in corn flour). The table shows the retention times obtained for the quantifier ion (*m/z* 489.2079, $[M+Na]^+$), the IS quantifier ion (*m/z* 513.2885; $[M^*+Na]^+$) and the qualifier ion (*m/z* 484.2527, $[M+NH_4]^+$) along with the mass errors obtained for each and the quantifier/qualifier ratios obtained for each ion. In this case the average retention time for the standards was 5.97 minutes giving an allowed range of 5.87 to 6.07 minutes; the mass error range was from -5 to +5 ppm; and the average quan/qual ratio was 0.171 giving an allowed range of 0.120 to 0.223. All PoEMS samples in this set meet these criteria.

Table 5 – Confirmation Data for the Presence of T-2 Data in the PoEMS Calibration Curve for Batch 3 (T-2 and HT-2 Toxins in Corn Flour)

Sample	Analyte Retention Time (min)	Analyte Mass Error (ppm)	IS Retention Time (min)	IS Mass Error (ppm)	Qualifier Retention Time (min)	Qualifier Mass Error (ppm)	Quan/Qual Ratio
PoEMS 10	5.97	2.3	5.97	-0.2	5.97	3.1	0.146
PoEMS 25	5.97	-0.1	5.97	-3.4	5.97	-0.8	0.173
PoEMS 50	5.97	-1.2	5.97	-2.9	5.97	-1.3	0.186
PoEMS 75	5.98	0.4	5.98	-1.2	5.98	-0.1	0.192
PoEMS 100	5.98	-2	5.98	-2.5	5.98	-2.2	0.194
PoEMS 200	5.97	-1.8	5.97	-2.5	5.97	-1.9	0.184
PoEMS 300	5.97	0.4	5.97	-1.1	5.97	0.7	0.177
PoEMS 400	5.98	-2.2	5.98	-2.7	5.98	-2.2	0.174
PoEMS 500	5.97	-3.1	5.97	-4.2	5.97	-3.3	0.165

The table shows the retention times and mass errors for T-2 toxin, its labelled analogue and the chosen qualifier ion along with the quantifier/qualifier peak area ratio.

The advantage of this analysis approach is that the data acquisition process is non-specific and therefore the data can be re-interrogated at a later stage if necessary eg if the matrix causes a shift in the observed ions a different set of ions can be used for identification and quantitation without the need to re-run the samples.

Sensitivity

Sensitivity in each matrix was assessed using calibration curves comprised of post-extraction matrix spikes at 0, 5, 10, 25, 50, 75 and 100ng/mL. The calibration curves were run in duplicate and the peak area and signal-to-noise were assessed using Targetlynx.

Table 6 shows the results obtained for the calibration curves in corn showing the signal-to-noise of the 5ng/g sample to be >5 for both T-2 Toxin and HT-2 Toxin in both calibration curves obtained (nb the data shown is for the quantifier in each case).

Table 7 shows the equivalent data obtained for T-2 and HT-2 toxins in corn across the same calibration range. In this case it can be seen that the signal-to-noise ratio interpolated at 5ng/g is > 5 for both HT-2 Toxin samples and ≥3 for both T-2 Toxin samples. It should be noted that the oat calibration curves were run at the end of a long analytical run and the sensitivity was therefore lower than might be achieved for a typical run with a clean instrument.

Table 6: Peak Areas and Signal-to-noise Measurements

		HT-2 Toxin				T-2 Toxin			
Sample	**Conc (ng/g)**	**Peak 1 Area**	**Peak 1 S/N**	**Peak 2 Area**	**Peak 2 S/N**	**Peak 1 Area**	**Peak 1 S/N**	**Peak 2 Area**	**Peak 2 S/N**
PoEMS 5	5	96.2	6	73.6	5	230.8	6	187.1	5
PoEMS 10	10	148.5	8	130.9	9	374.5	10	299.8	11
PoEMS 25	25	385.8	34	266	13	821.5	33	666.4	28
PoEMS 50	50	895.7	70	525.8	103	1943.1	69	1399.4	58
PoEMS 75	75	1073.6	62	949.8	60	2428.1	89	1943.6	85
PoEMS 100	100	1698.4	104	1225.3	117	4220.4	196	3196.3	127

The above results are for two repeat analyses of calibration curves of HT-2 Toxin and T-2 Toxin in oat flour in the 5-100ng/g range. Signal-to-noise ratios were calculated using the peak-to-peak method.

Table 7: Peak Areas and Signal-to-noise Measurements

		HT-2 Toxin				T-2 Toxin			
Sample	**Conc (ng/g)**	**Peak 1 Area**	**Peak 1 S/N**	**Peak 2 Area**	**Peak 2 S/N**	**Peak 1 Area**	**Peak 1 S/N**	**Peak 2 Area**	**Peak 2 S/N**
PoEMS 5	5	144.1	6	158.0	5	122.0	7	68.9	3
PoEMS 10	10	166.9	5	176.4	7	253.6	10	246.0	7
PoEMS 25	25	340.8	14	356.0	17	573.5	25	509.5	20
PoEMS 50	50	607.5	25	577.6	28	1014.3	39	896.2	45
PoEMS 75	75	856.5	37	844.8	32	1481.5	56	1342.9	47
PoEMS 100	100	1414.4	70	1163.8	51	2587.5	148	2422.0	117

The above results are for two repeat analyses of calibration curves of HT-2 Toxin and T-2 Toxin in oat flour in the 5-100 ng/g range. Signal-to-noise ratios were calculated using the peak-to-peak method.

For both matrices it can therefore be seen that the limit of detection (LOD) of the method is at least 5ng/g for each analyte (as defined by S/N>3). The LOQ of the method is taken to be the lowest calibration point, which in the case of this validation was 10ng/g.

Validation Results

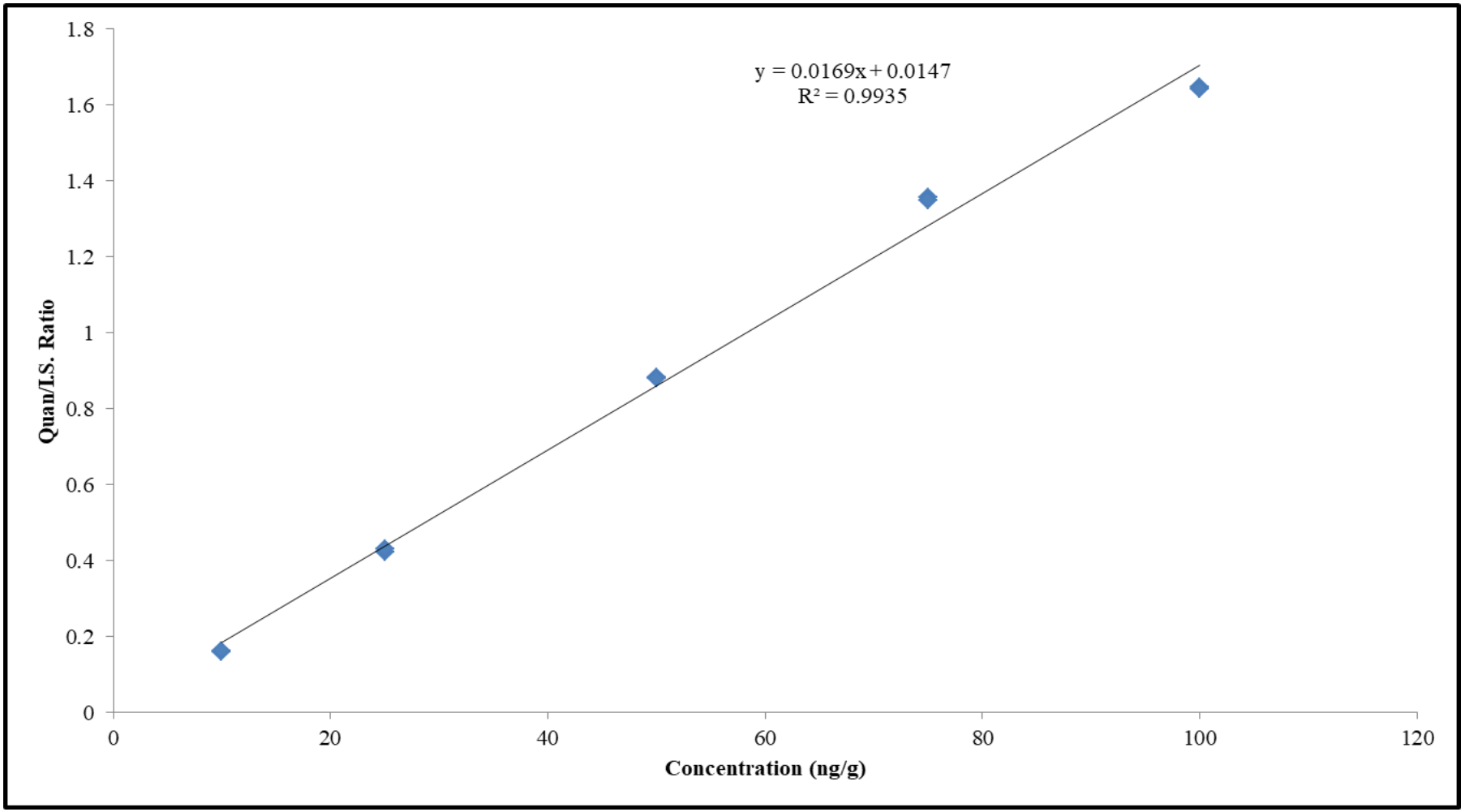
Four separate batches of samples were prepared across four days for the validation. For batches 1 and 2 samples were prepared in oat flour while for batches 3 and 4 samples were prepared in corn (maize) flour. Each batch consisted of a calibration set of PoEMS samples (0 to 500ng/g), three sets (N=6) of pre-extraction matrix spikes at 10, 25 and 100ng/g and a set of matrix and solvent blanks. Samples were run in the following order: PoEMS Set – PrEMS 10 Set – PrEMS 25 Set – PrEMS 100 Set – PoEMS Set with each sample set bracketed with appropriate blanks. Additionally each PrEMS sample set was proceeded with its corresponding PoEMS sample as a QC.

For batch 2 an additional set of samples was run comprising of extracts of the FAPAS QC material T2296QC (T-2 and HT-2 Toxins in Oat Flour). These samples were run between the PrEMS 50 samples and the second set of PrEMS samples.

For each batch a combination of Targetlynx and Skyline was use to obtain the required information for analyte confirmation and quantitation (for each of the quantifier, qualifier and internal standard): mass resolution, mass accuracy, retention time, peak area and peak signal-to-noise ratio. Each sample was then assessed against the identification criteria outlined above in order to confirm the presence of the analytes and internal standard. For batches 1, 2 and 4 every sample in the batch passed the identification criteria and the presence of HT-2 and T-2 toxins was confirmed. For batch 3, however, the HT-2 Toxin quantifier/qualifier ratio for several of the PoEMS 10 samples did not match the expected ratio and these samples were therefore removed from the validation (see Appendix for details). All other samples met the identification criteria.

The peak areas of the quantifier and internal standard ions for each sample (as generated by Skyline) were then taken into Excel. For each batch, calibration curves were generated for the PoEMS samples by plotting the qualifier-to-internal standard area against concentration in each of the two concentration ranges (0-100ng/g and 100-500ng/g). Calibration curves were taken to be valid where the coefficient of variation (R^2) was greater than 0.99 which it was in all cases. Figure 3 shows an example low range calibration curve for T-2 toxin generated from batch 1 data.

Figure 3: Calibration Curve for T-2 Toxin in Oat Flour



The results are in the 0-100 ng/g range generated from two sets of PoEMS data for batch 1. The data shows a plot of the peak area ratio of the quantifier ion and the internal standard ion plotted against concentration.

Using these calibration curves measured concentrations were calculated for each of the PrEMS samples. These calculated concentrations were then used to assess whether or not the batch in question had passed the validation for each analyte. The criteria for passing the validation were:

1	Analyte confirmed as present in all samples
2	Individual and average recoveries all within the 60-130% range
3	RSD of recoveries less than 40%

Table 8 shows some example data as obtained for batch 2 showing the recoveries obtained for HT-2 and T-2 toxins for the three PrEMS samples sets and for the FAPAS QC material.

Table 8: Data Obtained for the Analysis of HT-2 and T-2 Toxins in Oat Flour

	HT-2 Toxin				T-2 Toxin			
Replicate	**PrEMS 10**	**PrEMS 25**	**PrEMS 100**	**QC**	**PrEMS 10**	**PrEMS 25**	**PrEMS 100**	**QC**
1	101.8	82.1	80.9	85.4	123.1	102.1	90.6	85.8
2	98.6	78.4	83.8	97.5	123.3	73.6	92.2	92.2
3	83.8	63.9	87.7	92.5	110.3	60.1	84.4	89.7
4	119.1	97.7	83.8	85.7	120.3	116.4	89.4	89.8
5	124.6	95.6	85.6	104.2	87.1	102.0	84.7	98.0
6	100.1	98.3	88.6	105.8	119.1	108.1	90.2	92.7
Average	**104.7**	**86.0**	**85.1**	**95.2**	**113.9**	**93.7**	**88.6**	**91.4**
S.D.	**14.9**	**13.7**	**2.9**	**8.9**	**13.9**	**21.9**	**3.2**	**4.1**
%R.S.D.	**14.2**	**16.0**	**3.4**	**9.3**	**12.2**	**23.4**	**3.7**	**4.4**

The data shows the obtained recoveries for each set of pre-extraction spiked material and for the FAPAS QC material. Note: the values stated are recoveries not measured concentrations

From the data in Table 8 it can be seen that each of the PrEMS sample sets and the QC material sample set pass the validation.

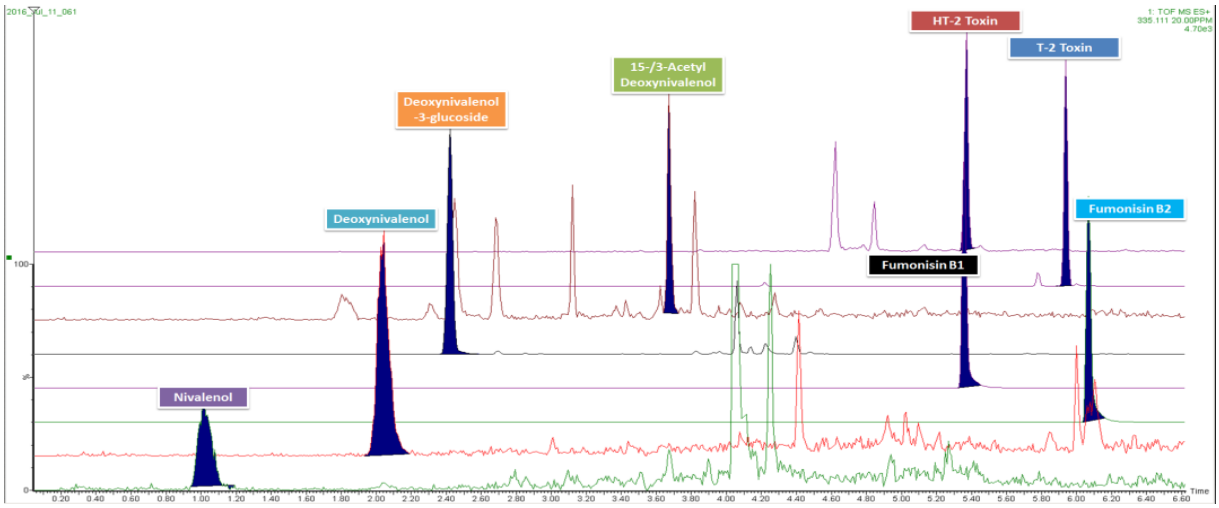
Across the four batches all the matrix recovery and QC samples passed the validation criteria apart from the PrEMS 10 set for batch 2 (see above) with recoveries for HT-2 toxin falling in the range 85.1-117.1% with RSDs between 3.4% and 23.4%. For T-2 Toxin recoveries were in the range 88.6-113.9% with RSDs between 1.5% and 23.4%. Details of the recoveries for each of the batches can be found in the Appendix. As the method passed validation at two or more levels for all batches it can therefore be considered to have passed validation for HT-2 Toxin and T-2 Toxin in the two matrices studied.

Screening for Other Mycotoxins

A key reason for choosing a full scan, accurate mass approach for this analysis was its non-targeted nature – data is collected across the mass range with no specific selection. The result of this is that a method designed for quantitation of one analyte can also be used to screen for the presence of another[30].

To demonstrate this each of the PrEMS and PoEMS samples were spiked with an additional seven mycotoxin species at the same level as HT-2 and T-2 Toxins. The additional mycotoxin species were Deoxynivalenol, Nivalenol, 3-Acetyldeoxynivalenol, 15-Acetyldeoxynivalenol, Fumonisin B1, Fumonisin B2 and Deoxynivalenol-3-glucoside. Figure 4 shows the extracted ion chromatograms for the different species from corn extract spiked post-extraction[1].

Figure 4: Extracted Ion Chromatograms of Nine Mycotoxin Species spiked at 400 ng/g in Corn Extract



[1] nb – In order to detect all the species present in the sample the time during which the LC output was sent to the mass spectrometer (as opposed to waste) was changed to the range 1 to 10 minutes for the generation of this data.

An extraction window of 20 ppm (±10 ppm) was used to generate the data. The EICs are (front to back): nivalenol, deoxynivalenol, fuminosin B2, fuminosin B1, deoxynivalenol-3-glucoside, 15-/3-acetyl deoxynivalenol (isobaric species), T-2 Toxin, HT-2 Toxin.

In this case the peak retention times were identified by analysis of individual solvent standards. However, from the data it is clear that there are multiple peaks in several of the EICs making confirmation of these species unclear for unknowns. The confirmation can be improved somewhat by use of mass accuracy as a filter. To this end mass spectra were generated for the major peaks in each EIC and the mass of the appropriate diagnostic ion or ions was compared to the theoretical mass of the species in question. Table 9 shows mass errors for the detected ions at each retention time, in each case the peak identified as the mycotoxin is highlighted in yellow.

Table 9: Detected Ions for Most Abundant Peaks in EICs for Nine Mycotoxin Species and Mass Errors for Each

Putative Species	RT	Ion 1 Expected *m/z*	Ion 1 Measured *m/z*	Ion 1 Error (ppm)	Ion 2 Expected *m/z*	Ion 2 Measured *m/z*	Ion 2 Error (ppm)
HT-2	4.62	447.1995	447.1896	-22.1	425.2175	425.2073	-24.0
HT-2	4.85	447.1995	447.1978	-3.8	425.2175	425.2472	69.8
HT-2	**5.37**	**447.1995**	**447.1985**	**-2.2**	**425.2175**	**425.2501**	**76.7**
HT-2	6.74	447.1995	447.1989	-1.3	425.2175	425.1961	-50.3
HT-2	6.79	447.1995	447.1985	-2.2	425.2175	425.3077	212.1
HT-2	7.08	447.1995	447.2057	13.9	425.2175	425.2933	178.3
T-2	4.22	489.2101	489.218	16.1			
T-2	5.78	489.2101	489.2188	17.8			
T-2	**5.94**	**489.2101**	**489.2092**	**-1.8**			
DON	**2.04**	**319.1158**	**319.1141**	**-5.3**	**297.1338**	**297.1328**	**-3.4**
DON	4.41	319.1158	319.1143	-4.7	297.1338	297.2168	279.3
DON	6	319.1158	319.1142	-5.0	297.1338	297.2608	427.4
DON	6.1	319.1158	319.1154	-1.3	297.1338	297.1296	-14.1
DON	7.26	319.1158	319.1121	-11.6	297.1338	interfered	
NIV	**1.02**	**335.1107**	**335.1091**	**-4.8**			
NIV	4.05	335.1107	335.1193	25.7			
NIV	4.25	335.1107	335.1066	-12.2			
FB2	**6.07**	**706.4014**	**706.4003**	**-1.6**			
FB1	**5.35**	**722.3962**	**722.3951**	**-1.5**			
D3G	**2.42**	**481.1686**	**481.1683**	**-0.6**			
D3G	4.06	481.1686	481.1684	-0.4			

Putative Species	RT	Ion 1 Expected *m/z*	Ion 1 Measured *m/z*	Ion 1 Error (ppm)	Ion 2 Expected *m/z*	Ion 2 Measured *m/z*	Ion 2 Error (ppm)
D3G	4.4	481.1686	481.1551	-28.1			
AcDON	1.86	361.1263	361.1077	-51.5			
AcDON	2.3	361.1263	361.1078	-51.2			
AcDON	2.45	361.1263	361.1078	-51.2			
AcDON	2.68	361.1263	361.1078	-51.2			
AcDON	3.12	361.1263	361.1257	-1.7			
AcDON	**3.67**	**361.1263**	**361.1245**	**-5.0**			
AcDON	3.82	361.1263	361.1232	-8.6			

Studied species are HT-2 Toxin (HT-2; $[M+Na]^+$ and $[M+H]^+$), T-2 Toxin (T-2; $[M+Na]^+$), Deoxynivalenol (DON; $[M+Na]^+$ and $[M+H]^+$), Nivalenol (NIV; $[M+Na]^+$), Fumonisin B2 (FB2; $[M+H]^+$), Fumonisin B1 (FB1; $[M+H]^+$), Deoxynivalenol-3-glucoside (D3G; $[M+Na]^+$), and 3-/15-Acetyl Deoxynivalenol (AcDON; $[M+Na]^+$). All peaks of sufficient signal-to-noise to be considered detected are reported here with the peak identified as the species of interest (by use of retention time etc.) highlighted in yellow.

In some cases (e.g. T-2 Toxin) it can be seen that only one detected peak falls within the defined ±5ppm mass accuracy tolerance range thereby providing good evidence for further investigation were this data to be used for screening. For other species, however, the situation is more complex. For HT-2 Toxin, for example, four different peaks fall within the ±5ppm limit and the mass accuracies of all secondary peaks studied fall outside the limit (as with other data shown this is primarily due to an interference around *m/z* 425.2). For deoxynivalenol (DON) the mass accuracy of the peak known to be DON is outside the 5ppm limit for the $[M+Na]^+$ species but inside the limit for the $[M+H]^+$ species (again due to an incompletely resolved interference at *m/z* 319.1). The ability to screen for particular species with confidence using this method therefore requires a degree of knowledge about expected retention times with this particular method which is typically how screening methods are operated meaning that they require standards in order to provide a confident identification.

An area of interest in the field of mycotoxin analysis is the identification of masked mycotoxins (mycotoxin species which have been chemically modified eg by glycosylation). These species are of interest because they may return to the active mycotoxin species *in vivo* but would be missed by targeted analyses[31-33]. As many of these masked mycotoxins are not yet identified and there are few standards available for those which have been identified a screening approach is required. One potential approach is to use an MS/MS analysis combined with a mycotoxin library. The traditional approach of targeted MS/MS would not be appropriate here due in part to the (potentially) large number of ions that would be included in a screen but also because QToF-MS was chosen for its ability to analyse the full mass range in each scan; including an isolation step removes this advantage as only chosen ions are transmitted to the collision cell by the quad. An alternative approach which does not have this drawback is MS^E (also known as all ions MS/MS)[34]. In MS^E, collision induced dissociation (CID) is performed without any pre-selection using the quadrupole such that all species present are fragmented simultaneously yielding mass spectra which are an amalgam of the CID spectra of all species present at any one time. By sequentially recording MS and MS/MS spectra across a chromatographic run fragment ions can be assigned to each precursor using software tools and individual MS/MS spectra for each component can be constructed then used for identification of the original mycotoxin and its modification. This approach to screening will be investigated in the future.

Conclusions

We have presented here the development of a method for the quantification of HT-2 Toxin and T-2 Toxin in cereals. In the developed method analytes are extracted using a modified QuEChERS methodology followed by LC-MS analysis on a quadrupole time of flight instrument. The development of the method included selection of a set of criteria for analyte identification and confirmation using a full scan, accurate mass approach with high resolution mass spectrometry.

The method was validated for both analytes using two matrices (oat flour and corn flour) with two batches analysed for each matrix. Limits of detection for both analytes were at least 5ng/g in each matrix with limits of quantitation set as 10ng/g (the lowest calibration point). All four batches passed the defined criteria for analyte detection, recovery and intra-batch variability; the method can therefore be considered to be useful for both analytes in both matrices and by extension for cereals in general.

A key reason for using Q-ToF HRMS over a more typical QqQ-MS approach was the ability of HRMS to provide qualitative screening data alongside quantitative data. Accordingly an initial assessment of this method for mycotoxin screening has been completed using a set of nine mycotoxins with encouraging results. Further work is required to develop this into a robust method.

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Appendix: Recoveries of HT-2 and T-2 Toxins for Batches 1, 2, 3 and 4

Batch 1 – HT-2 and T-2 in Oat Flour

All PoEMS and PrEMS samples met the identification criteria outlined above. Calibration curves were generated in Excel using the low calibration range (0-100ng/g) by plotting the analyte/internal standard ratio against concentration. The obtained R-squared values were 0.993 for the HT-2 calibration curve and 0.993 for the T-2 calibration curve. Concentrations and subsequent recoveries were calculated in Excel using the calibration curves. Table A1 shows the obtained recovery percentages and subsequent averages for each PoEMS sample in batch 1.

Table A1 – Recoveries for HT-2 Toxin and T-2 Toxin in Oat Flour obtained from Validation Batch 1

	HT-2 Toxin			T-2 Toxin		
Replicate	**PrEMS 10**	**PrEMS 25**	**PrEMS 100**	**PrEMS 10**	**PrEMS 25**	**PrEMS 100**
1	103.2	97.4	93.9	112.2	104.7	90.8
2	92.0	104.2	99.3	110.3	94.1	93.4
3	93.1	111.4	101.3	95.5	115.0	89.9
4	108.8	102.8	94.0	124.6	100.0	93.6
5	95.6	102.4	101.9	126.4	100.4	94.7
6	97.4	102.1	96.6	114.4	99.8	94.0
Average	**98.4**	**103.4**	**97.8**	**113.9**	**102.3**	**92.7**
S.D.	**6.5**	**4.6**	**3.5**	**11.2**	**7.1**	**1.9**
%R.S.D.	**6.6**	**4.4**	**3.6**	**9.8**	**6.9**	**2.1**

From the table it can be seen that in all cases the recoveries and average recoveries fall in the required 60-130% range with relative standard deviations within the required <40% range. This batch can therefore be said to have passed validation.

Batch 2 – HT-2 and T-2 Toxins in Oat Flour

Analytes and internal standards were spiked into oat flour as per batch 1 and additionally 6 replicates of the QC material T2296QC were weighed out and spiked with internal standard as outlined in the sample preparation section. The prepared samples were then extracted and analysed as per the previous batch.

The PoEMS, PrEMS and QC samples all met the defined identification criteria for both analytes. Calibration curves were generated in Excel. Correlation coefficients for HT-2 toxin were 0.992 in the low calibration range (0-100ng/g) and 0.990 in the high calibration range (100-500ng/g). The equivalent correlation coefficients for T-2 toxin were 0.992 (low range) and 0.992 (high range). PrEMS samples were quantified using the low range calibration while the QC samples were quantified using the high range calibration. Table A2

shows the recoveries obtained for the three PrEMS samples and the QC sample (using the consensus values from the QC material certificate).

Table A2 – Pecentage Recoveries for HT-2 Toxin and T-2 Toxin Obtained for Batch 2

	HT-2 Toxin				T-2 Toxin			
Replicate	PrEMS 10	PrEMS 25	PrEMS 100	QC	PrEMS 10	PrEMS 25	PrEMS 100	QC
1	101.8	82.1	80.9	85.4	123.1	102.1	90.6	85.8
2	98.6	78.4	83.8	97.5	123.3	73.6	92.2	92.2
3	83.8	63.9	87.7	92.5	110.3	60.1	84.4	89.7
4	119.1	97.7	83.8	85.7	120.3	116.4	89.4	89.8
5	124.6	95.6	85.6	104.2	87.1	102.0	84.7	98.0
6	100.1	98.3	88.6	105.8	119.1	108.1	90.2	92.7
Average	**104.7**	**86.0**	**85.1**	**95.2**	**113.9**	**93.7**	**88.6**	**91.4**
S.D.	**14.9**	**13.7**	**2.9**	**8.9**	**13.9**	**21.9**	**3.2**	**4.1**
%R.S.D.	**14.2**	**16.0**	**3.4**	**9.3**	**12.2**	**23.4**	**3.7**	**4.4**

The above shows results from a set of three pre-extraction matrix spikes (PrEMS) in oat flour and a FAPAS QC material.

Once again all individual and average recoveries fall within the 60-130% limits required for this method with RSDs of <40%. Batch 2 can therefore be said to have met the validation criteria.

Batch 3 – HT-2 and T-2 Toxins in Corn Flour

Analytes and internal standards were spiked into corn flour as per the method outlined above. The prepared samples were then extracted and analysed as per batches 1 and 2.

The obtained data was analysed via Masslynx and Skyline as before. Recoveries for both analytes were within the expected range and variability at 10, 25 and 100ng/g. From these results all samples met the required mass accuracy apart from PrEMS 25 (HT-2 only, both channels) and PoEMS 400 (T-2, qualifier channel only). For the PrEMS 10 sample set four of the six samples did not meet the quan/qual ratio requirements for HT-2 Toxin and therefore the batch did not pass validation at this level, however, the batch did meet the ID criteria at 25ng/g (4 of 6 ratios within range) and 100ng/g (all ratios within range). The second PoEMS 25 sample was removed from the calibration curve due to not meeting mass accuracy requirements.

The correlation coefficient for HT-2 toxin was 0.993 and for T-2 toxin was 0.994 (both in the 0 to 100ng/g range). The recoveries obtained for each set of PrEMS samples using these calibration curves are shown in table A3.

Table A3 – Percentage Recoveries Obtained for HT-2 Toxin and T-2 Toxin in Corn Flour for Batch 3

	HT-2 Toxin			T-2 Toxin		
Replicate	**PrEMS 10**	**PrEMS 25**	**PrEMS 100**	**PrEMS 10**	**PrEMS 25**	**PrEMS 100**
1	100.3	124.1	94.3	98.2	113.4	86.9
2	118.1	119.3	102.8	100.2	111.2	97.4
3	141.0	116.1	101.5	114.3	102.8	97.3
4	124.0	111.7	96.3	106.0	102.7	96.2
5	106.2	104.2	105.1	108.4	98.5	97.4
6	113.0	116.3	120.9	113.0	108.9	105.8
Average	**117.1**	**115.3**	**103.5**	**106.7**	**106.3**	**96.8**
S.D.	**14.4**	**6.8**	**9.5**	**6.6**	**5.8**	**6.0**
%R.S.D.	**12.3**	**5.9**	**9.1**	**6.2**	**5.4**	**6.2**

Recoveries for HT-2 Toxin in the PrEMS 10 samples are for information only as these samples did not meet the required peak identification criteria.

For each of the sample sets the recoveries can be seen to fall within the defined criteria apart from HT-2 PrEMS 10 3 although those samples had been failed anyway due to quan/qual ratio (see above). As each batch is required to pass validation at two levels, the batch can be considered to have passed validation for both analytes.

Batch 4 – HT-2 and T-2 Toxins in Corn Flour

Analytes and internal standards were spiked into corn flour as per batch 3. The prepared samples were then extracted and analysed as per above.

Table A4 – Recoveries Obtained from Batch 4 for HT-2 Toxin and T-2 Toxin in Corn Flour

	HT-2 Toxin			T-2 Toxin		
Replicate	**PrEMS 10**	**PrEMS 25**	**PrEMS 100**	**PrEMS 10**	**PrEMS 25**	**PrEMS 100**
1	94.4	98.5	94.6	110.6	88.2	106.0
2	89.3	113.5	103.6	104.6	97.1	106.3
3	86.2	104.6	96.0	84.8	104.5	109.0
4	116.1	116.1	100.7	84.9	99.8	105.5
5	123.4	83.3	100.3	85.9	70.4	108.7
6	128.1	109.6	94.1	97.3	90.9	108.8
Average	**106.3**	**104.3**	**98.2**	**94.7**	**91.8**	**107.4**
S.D.	**18.4**	**12.0**	**3.9**	**11.2**	**12.0**	**1.6**
%R.S.D.	**17.3**	**11.5**	**3.9**	**11.9**	**13.1**	**1.5**

The PoEMS and PrEMS samples all met the defined identification criteria for both analytes. Calibration curves were generated in Excel. The correlation coefficients for HT-2 toxin in the low range (0-100ng/g) was 0.998 and the equivalent correlation coefficient for T-2 toxin was 0.994. Table A4 shows the recoveries obtained for the three PrEMS samples.

For each of the three sample sets the recoveries can be seen to fall within the required 60-140% recovery range with relative standard deviations of the recoveries all within the 40% limit. This batch can therefore be said to have passed the validation criteria.