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Animal feeding stuffs: Methods of sampling and analysis — Determination of T-2 and HT-2 toxins, Deoxynivalenol and Zearalenone, in feed materials and compound feed by LC-MS

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National foreword

This British Standard is the UK implementation of EN 16877:2016.

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A list of organizations represented on this committee can be obtained on request to its secretary.

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Animal feeding stuffs: Methods of sampling and analysis - Determination of T-2 and HT-2 toxins, Deoxynivalenol and Zearalenone, in feed materials and compound feed by LC-MS

Aliments des animaux - Méthodes d'échantillonnage et d'analyse - Dosage par CL-SM des toxines T-2 et HT-2, du déoxynivalénol et de la zéaralénone dans les matières premières pour aliments et les aliments composés

Futtermittel - Probenahme- und Untersuchungsverfahren - Bestimmung von T-2- und HT-2-Toxinen, Deoxynivalenol und Zearalenon in Einzelfuttermitteln und Mischfuttermitteln mittels LC-MS

This European Standard was approved by CEN on 26 September 2016.

EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
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European foreword

This document (EN 16877:2016) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs - Methods of sampling and analysis”, the secretariat of which is held by NEN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 2017, and conflicting national standards shall be withdrawn at the latest by May 2017.

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Introduction

WARNING — The method described in this standard implies the use of reagents that pose a hazard to health. The standard does not claim to address all associated safety problems. It is the responsibility of the user of this standard to take appropriate measures for the health and safety protection of the personnel prior to use of the standard and to ensure that regulatory and legal requirements are complied with.

1 Scope

This method of analysis is applicable to the determination of HT-2 toxin (HT2) in the tested range of 22 µg/kg to 178 µg/kg, T-2 toxin (T2) in the tested range of 7 µg/kg to 50 µg/kg, Deoxynivalenol (DON) in the tested range of 88 µg/kg to 559 µg/kg, and Zearalenone (ZON) in the tested range of 14 µg/kg to 430 µg/kg in cereals and cereal-based compound animal feed. The actual working ranges may extend beyond the tested ranges. It is the responsibility of the laboratory to prove that the limit of quantitation (LOQ) for HT-2 and T-2 toxin is ≤ 10 µg/kg, for DON ≤ 100 µg/kg, and for ZON ≤ 20µg/kg.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696:1995, *Water for analytical laboratory use - Specification and test methods (ISO 3696:1987)*

3 Principle

Finely ground and homogeneous test material is suspended in water. After addition of ethyl acetate the sample is agitated. Then sodium sulphate is added to facilitate phase separation and after a delay the sample is centrifuged to pellet particulate matter at the bottom of the extraction tube. The organic phase is transferred to a clean vial for possible storage. An aliquote of the organic phase is mixed with stable-isotope labelled analogues of the analytes and evaporated to dryness in deactivated glass vials. After reconstitution of the dry extract with organic mobile phase modifier and water, and thorough mixing, the analytes are quantified with a Liquid Chromatography-Mass Spectrometry (LC-MS) system.

4 Reagents

WARNING The method described in this standard implies the use of reagents that pose a hazard to health. The standard does not claim to address all associated safety problems. It is the responsibility of the user of this standard to take appropriate measures for the health and safety protection of the personnel prior to use of the standard and to ensure that regulatory and legal requirements are complied with.

4.1 Water (deionized).

4.2 Water (LC-MS grade, double-distilled or water of grade 1 as defined in EN ISO 3696:1995).

4.3 Methanol (LC-MS grade).

4.4 Methanol (p.a.).

4.5 Ethyl acetate (p.a.).

4.6 Formic acid (98-100 %, LC-MS grade).

4.7 Acetonitrile (LC-MS grade).

4.8 Sodium sulfate, anhydrous, granulated.

4.9 Deoxynivalenol (DON).

4.10 HT-2 toxin (HT2).

4.11 T-2 toxin (T2).

4.12 Zearalenone (ZON).

4.13 $^{13}C_{15}$-Deoxynivalenol ($^{13}C_{15}$-DON).

4.14 $^{13}C_{22}$-HT-2 toxin ($^{13}C_{22}$-HT2).

4.15 $^{13}C_{24}$-T-2 toxin ($^{13}C_{24}$-T2).

4.16 $^{13}C_{18}$-Zearalenone ($^{13}C_{18}$-ZON).

4.17 Multitoxin stock solution:

A mixture containing Deoxynivalenol (4.9), HT-2 toxin (4.10), T-2 toxin (4.11), and Zearalenone (4.12) in neat acetonitrile (4.7) at relevant concentrations.

When preparing this solution the certified purities of the mycotoxin reference materials need to be properly accounted for. In any case the purities shall be ≥ 95 %.

NOTE 1 3,2 µg/ml DON, 0,5 µg/ml HT-2 toxin, 0,3 µg/ml T-2 toxin, and 0,3 µg/ml ZON in neat acetonitrile have been used during the collaborative study. This solution is stable for three months in the dark at 2–8 °C.

To compare a new stock solution against an old one add 25 µl of each into separate deactivated vials (5.6) and proceed as described in "Test solution" (6.3).

NOTE 2 If 6.4"Spiking procedure" is executed at least 6 ml of the stock solution are needed.

4.18 Multitoxin working solution:

Dilute Multitoxin stock solution (4.17) with Methanol (4.3) such that the resulting concentration in the working solution is applicable to the calibration range of the different compounds. Only prepare enough volume for one full calibration.

NOTE Adding 188 µl of the Multitoxin stock solution described in 4.17, Note 1 to a 3 ml volumetric flask and making up to the mark with methanol will result in a solution containing 0,2 µg/ml DON, 0,031 µg/ml HT-2 toxin, 0,019 µg/ml T-2 toxin, and 0,019 µg/ml ZON in methanol/acetonitrile (94/6, v/v).

4.19 Multi internal standard (ISTD) stock solution:

A mixture containing $^{13}C_{15}$-DON (4.13), $^{13}C_{22}$-HT-2 toxin (4.14), $^{13}C_{24}$-T-2 toxin (4.15), and $^{13}C_{18}$-ZON (4.16) in neat acetonitrile (4.7) at the same concentrations as the respective native compounds in the Multitoxin stock solution (4.17).

NOTE This solution is stable for three months in the dark at (2–8) °C.

4.20 Calibration:

To six deactivated glass vials (5.6) add different volumes of the Multitoxin working solution (4.18) such that six equidistant calibration levels across the calibration range result. Proceed as described in 6.3, "Test solution".

Table 1 below shows example calibration levels using the solution described in the Note to 4.18 above.

Once it has been shown that there is linearity the number of levels may be adjusted to local needs and requirements.

Table 1 — Example calibration solutions

Volume of Multitoxin working solution (4.18.) [µl]	**Total mass of analyte per vial** [ng]			
	DON	**HT-2**	**T-2**	**ZON**
25	5	0,78	0,48	0,48
180	36	5,6	3,4	3,4
335	67	10	6,4	6,4
490	98	15	9,3	9,3
645	129	20	12	12
800	160	25	15	15

4.21 Quality control material:

An appropriate material with natural contamination or fortification of the tested mycotoxins which is sufficiently stable.

5 Apparatus

5.1 Mill:

Single mill or multiple mills capable of comminuting test materials to particle sizes of < 500 µm.

5.2 Mixer capable of sufficiently homogenizing the comminuted test materials.

NOTE A tumble mixer that uses a folding action either through moving paddles or fins, or an end-over-end movement has shown to work well.

5.3 Conical polypropylen screw-cap centrifuge tubes, 50 ml with caps.

5.4 Volumetric flasks: 3, 5, and 10 ml.

5.5 Pipettors: adjustable (10-100) µl and adjustable (100-1 000) µl.

5.6 Deactivated glass vials:

Silanized glass vials, minimum volume 1,5 ml.

5.7 Auto Liquid Sampler (ALS) vials of appropriate size for the Auto Liquid Sampler in use.

5.8 Shaker or Sonicator.

5.9 Evaporator capable of maintaining a stable temperature in the range of 30 - 60 °C with a constant flow of dry nitrogen.

5.10 Centrifuge capable of generating a relative centrifugal force (RCF) of 3 000 g.

5.11 Syringe filter:

Small internal volume, Nylon, Pore size: 0,2 µm Nylon.

5.12 LC-MS:

5.12.1 Solvent delivery system capable of delivering a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy.

5.12.2 Auto liquid sampler (ALS) capable of injecting an appropriate volume of injection solution with sufficient accuracy, cross-contamination below 0,1 %.

5.12.3 Analytical column capable of separating the four analytes with the following performance:

Peak asymmetry factor at 10 % height: 0,9 < As < 1,4; minimum apparent retention factor for any of the four analytes: k ≥ 2; minimum plate number for any of the four analytes: $N \geq 1200$; minimum resolution between two adjacent analyte peaks: Rs ≥ 4.

5.12.4 Mass spectrometer:

An instrument capable of performing selected reaction monitoring (SRM) with a sufficiently wide dynamic range. Any ionization source giving sufficient yield may be employed.

5.13 Balance with readability d = 0,001 g or better.

6 Procedures

6.1 Sample preparation

Laboratory samples should be taken and prepared in accordance with European legislation ([1], [2]) where applicable or, in any other case, with EN ISO 6498. The laboratory sample should be finely ground and thoroughly mixed using a mill (5.1) and a mixer (5.2) or another process for which complete homogenization has been demonstrated before a test portion is removed for analysis.

The recommended way is to comminute the laboratory sample in several steps. Beginning with the totality of the laboratory sample each step consists of taking a representative aliquot of the previous step after sufficient homogenization. This aliquot is then comminuted to the next smaller particle size until a subsample of ca. 50 g of the final particle size is obtained. It is of utmost importance that the test portion is taken from a subsample which is sufficiently homogenous with a particle size of ≤ 500 µm. Care should be taken to not overheat the sample during this process.

In all instances everything should be at room temperature before any kind of manipulation takes place.

6.2 Extraction

Some of the steps described below are more critical for the accuracy of the results than others. These steps are marked as such and should be carried out with the necessary attention. A scale-up of the test portion size is deemed to be acceptable if such a need is assumed. In that case the amounts of added water, ethyl acetate, and sodium sulphate need to be increased at the same rate, f.i. scale-up by factor of 2: 4 g test portion, 16 ml water, 32 ml ethyl acetate, 16 g sodium sulphate. In no way shall a scale-up be seen as replacement for proper sample preparation (6.1).

— For the test portion weigh 1,9 to 2,1 g of the homogeneous sample into a conical polypropylene screw-cap tube (5.3), round and record the weight to the second decimal (the accuracy of this weight is critical for the accuracy of the final result!).

— Add 7,2 to 8,8 ml of deionized water (4.1).

— Vortex thoroughly until test portion is completely suspended. Do not let this suspension stand for more than 15 min to prevent effects due to enzymatic activities.

— Add 16,0 ml of ethyl acetate (4.5).

The accuracy of this volume is critical for the accuracy of the final result!

— Extract for 27 to 33 min in a sonicator or by vigorously shaking (5.8).

— Add between 7,2 and 8,8 g of sodium sulphate (4.8).

— Instantly shake hard for 5 s.

— Let stand for 10 to 20 min.

— Centrifuge (5.10) at RCF 3 000 g for at least 1 min to aid settlement of particulate matter and phase separation.

— If wanted for possible repeats: Transfer the extract (organic layer) into clean glass vial for storage of up to 7 d at 2 °C to 10 °C in the dark.

— Transfer 500 µl of the extract (organic layer) into a deactivated glass vial (5.6) for further processing (the accuracy of this volume is critical for the accuracy of the final result!).

6.3 Test solution

— Add 25 µl of the Multi ISTD stock solution (4.19) to the aliquot of the extract and/or the calibration solutions (4.20) (the accuracy of this volume is critical for the accuracy of the final result!).

— Dry down the aliquot of the extract and/or the calibration solutions in an evaporator (5.9) with a gentle stream of dry nitrogen at 60 °C.

— Add 250 µl of the organic mobile phase modifier used for LC-MS to the dry residue for reconstitution.

— Vortex thoroughly for at least 10 s.

— Add 250 µl deionized water (4.1) to the reconstituted extract.

— Vortex thoroughly for at least 5 s.

— Transfer the test solution into an ALS vial (5.7); if solution is turbid it may be filtered through a syringe filter (5.11).

NOTE It has been shown that even very turbid samples can be injected without any negative effects to the life time of column and LC provided that appropriate in-line filters or guard columns are used.

6.4 Spiking procedure

If recovery needs to be determined execute the following in duplicate:

To three times 2 g of a material free of DON, HT2, T2, and ZON add three different volumes of the Multitoxin stock solution (4.17) such that 3 contamination levels across the calibration range result. Distribute the solutions evenly over the materials, mix to further distribute the spike, and leave for a minimum of 5 h to a maximum of 18 h. Proceed to 6.2 “Extraction” second step.

NOTE Addition of 360, 980, and 1 600 µl of the Multitoxin stock solution described in 4.17, Note 1 has been shown to work well.

7 Measurements

7.1 General

The LC-MS system shall meet the requirements laid out in 5.12 and its sub-entries.

7.2 LC conditions

Choose an analytical column, mobile phase, gradient settings, and injection volume that let you meet the requirements in 5.12.3 (for examples see Annex B).

7.3 MS conditions

Choose an ion source with sufficient ionization yield for the four analytes and ion source settings such that a stable spray is achieved.

Choose for each analyte an appropriate precursor ion (adducts of the molecule with a Proton, Sodium, Ammonia, etc. in positive mode, or deprotonation, etc. in negative mode). If more than one precursor ion per analyte is detectable choosing the strongest is a good starting point. But one shall be aware that the choice of precursor ion will affect repeatability and, by that, LOD and LOQ.

For SRM select two product ions in the MS/MS spectrum for each selected precursor ion of each analyte. Set up SRM transitions with these precursor/ product ion combinations (for SRM examples see Annex B).

The selected MS settings shall be such that for a relevant feed material with a contamination of ca. 90 µg/kg DON, 10 µg/kg HT-2 toxin, 10 µg/kg T-2 toxin, and 10 µg/kg ZON, prepared according to Clause 6, signal-to-noise ratios (peak-to-peak) of larger than 10 are obtained (see Annex C).

7.4 Batch composition

Always start a batch of measurements with a reagent blank run to prove non-contamination of the system. Then inject the calibration solutions once again followed by a reagent blank to check for possible carry-over. Subsequently inject the test solutions. At the end of the batch reinject the calibration solutions for a second run.

7.5 Peak identification

Identify an analyte peak in the test solution by plotting the extracted ion currents of the analyte and its respective labelled analogue and then A) comparing the retention time of the analyte with the retention time of the respective labelled analogue (difference shall be smaller than 0,25 times peak width (FWHM)), and B) comparing the ratio of the two measured transitions of the analyte with that of a calibration solution of comparable signal intensity.

For example chromatograms see Annex C.

7.6 Determination of DON, HT2, T2, and ZON in calibration or and test solutions

Inject aliquots of the calibration and test solutions (6.3) onto the column using identical conditions. For each injection calculate the ratio of the peak area of the analyte divided by the peak area of the respective labelled analogue. These peak area ratios will be used in all subsequent calculations. For peak area determination integrate the extracted ion current of the transition with the least interferences (best signal-to-noise ratio). If both transitions of a given analyte are equally well suited integrate the sum of both.

7.7 Calibration

Plot the peak area ratios of all the measured calibration solutions against the corresponding total masses in the calibration solution of DON, HT2, T2, and ZON separately. Do not use means of the

multiple injections! With weighted least-square regression over all data estimate slope and possible intercept of each of the four calibration functions (DON, HT2, T2, ZON). Check for significance of the intercept (95 % confidence level) and test for linearity (use e.g. a residuals vs fitted-values plot, a lack-of-fit test (95 % confidence), Mandel's fitting test (95 % confidence)). If a nonlinearity is indicated identify the cause and, if necessary, rerun the analyses.

8 Determination of mass fraction

To calculate the mass fractions ($w_{An,S}$) of a specific analyte in the test portion use the following model formula:

$$w_{An,S} = \left(\frac{\overline{R}}{\beta_1} - \frac{\beta_0}{\beta_1} \right) \times \frac{m_{ISTD,S}}{m_{ISTD,C}} \times \frac{V_{EtOAc}}{V_{Aliq} \times m_S} \quad (1)$$

where

$w_{An,S}$ is the mass fraction of analyte in the test portion [ng/g];

$\overline{R}$ is the mean of the peak area ratios of replicate injections;

β_1 is the slope, estimated with weighted least-square regression from calibration data (7.7) [area ratio/ng];

β_0 is the intercept, estimated with weighted least-square regression from calibration data (becomes zero if not significant (see 7.7)) [area ratio];

$m_{ISTD,S}$ is the mass of the labelled analogue in the test solution [g];

$m_{ISTD,C}$ is the mass of the labelled analogue in the calibration solution [g];

V_{Aliq} is the volume of the aliquot taken from the raw extract [ml];

V_{EtOAc} is the volume of the ethyl acetate used for extraction [ml];

m_S is the mass of the test portion [g].

Under the assumption that test and calibration solutions are treated identically (same volume of Multi ISTD stock solution added, $m_{ISTD,S} = m_{ISTD,C}$) the model formula reduces to:

$$w_{An,S} = \left(\frac{\overline{R}}{\beta_1} - \frac{\beta_0}{\beta_1} \right) \times 1 \times \frac{V_{EtOAc}}{V_{Aliq} \times m_S} \text{ [ng/g]} \quad (2)$$

The term in parentheses is the total mass of the analyte ($m_{An,S}$) in the test solution obtained from applying the calibration function. So a reduced model formula may be written as:

$$w_{An,S} = m_{An,S} \times \frac{V_{EtOAc}}{V_{Aliq} \times m_S} \text{ [ng/g]} \quad (3)$$

For a test portion of 2,0 g, 16,0 ml of ethyl acetate, and a 0,5 ml aliquot of the extract the second term becomes 16 and Formula (3) may be written as:

$$w_{An,S} = m_{An,S} \times 16 \text{ [ng/g]} \quad (4)$$

Because of the use of peak area ratios the total volumes of the test or calibration solutions and the injected volumes have no direct influence on the result and do not appear in the model formula.

In cases where it is required, the determined mass fraction $w_{An,S}$ may be corrected for recovery according to Formula (5).

$$w_{An,S,corr} = \frac{w_{An,S}}{rec} \text{ [ng/g]} \quad (5)$$

where

rec is the recovery.

For T-2 toxin and Zearalenone the recovery has been shown to not be different from 1 while for HT-2 toxin the average recovery over the tested range is 0,93 and for deoxynivalenol 0,91 (see Annex A).

9 Precision

9.1 Interlaboratory study

Details of an interlaboratory study on the precision of the method are shown in [3]. The values derived from this interlaboratory study may not be applicable to concentration ranges and/or matrices other than those stated.

9.2 Repeatability

9.2.1 General

The absolute difference between two single test results found on identical test materials by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit r in not more than 5 % of the cases. Below the functional relationships between the measured mass fraction $w_{An,S}$ for the four analytes and the repeatability standard deviation s_r are listed. From this the repeatability limit *r* can be calculated as:

$$r = 2{,}8 \times s_r \quad (6)$$

For more information see tables in Annex A.

9.2.2 HT-2 toxin

s_r = 0,11 $w_{An,S}$ [ng/g]

9.2.3 T-2 toxin

s_r = 0,04 $w_{An,S}$ + 1,2 [ng/g]

9.2.4 DON

s_r = 0,05 $w_{An,S}$ + 3,8 [ng/g]

9.2.5 ZON

s_r = 0,05 $w_{An,S}$ + 1 [ng/g]

9.3 Reproducibility

9.3.1 General

The absolute difference between two single test results on identical test materials reported by two laboratories will exceed the reproducibility limit R in not more than 5 % of the cases. Below the

functional relationships between the measured mass fraction $w_{An,S}$ for the four analytes and the repeatability standard deviation s_R are listed. From this the reproducibility limit R can be calculated as:

$$R = 2{,}8 \times s_R \quad (7)$$

For more information see tables in Annex A.

9.3.2 HT-2 toxin

s_R = 0,22 $w_{An,S}$ [ng/g]

9.3.3 T-2 toxin

s_R = 0,08 $w_{An,S}$ + 2,8 [ng/g]

9.3.4 DON

s_R = 0,10 $w_{An,S}$ + 8,7 [ng/g]

9.3.5 ZON

s_R = 0,1 $w_{An,S}$ + *4,3* [ng/g]

10 Test report

The test report shall contain at least the following data:

a) information necessary for the identification of the sample;

b) a reference to this European Standard;

c) the date of sample receipt;

d) the test results and the units in which they have been expressed; where necessary the recoveries shall be stated along with the test results and whether the test results were corrected with those recoveries.

Annex A
(informative)

Precision data

The following data were obtained in an interlaboratory study [3] according to the AOAC Guidelines for collaborative study procedures [4] to validate characteristics of a method of analysis. Robust statistical methods, as described in ISO 5725-5 [5], were used to avoid the need to exclude individual "outlying" results. The functional relationships between the repeatability / reproducibility standard deviation and the measured value were calculated as described in ISO 5725-2 [6]. The labels in the table headers below refer to the materials used in the interlaboratory study [3] as follows:

— Compound feed 1 is EFL1;

— Compound feed 2 is IRMMFEED;

— Compound feed 3 is EFL3;

— Cereal Mix 1 is EFL2;

— Cereal Mix 2 is IRMMCER.

Table A.1 — Precision data HT-2 toxin

Sample	Compound feed 1	Compound feed 2	Compound feed 3	Cereal Mix 1	Cereal Mix 2
Year of inter-laboratory study	2011	2011	2011	2011	2011
Number of laboratories	21	21	21	21	21
Number of laboratories retained after eliminating non-compliant ones	16	16	16	16	15
Number of non-compliant laboratories	5	5	5	5	6
Number of accepted results	16	16	16	16	15
Mean value $\bar{x}$ [a], µg/kg	38,0	22,0	177,6	49,1	53,1
Repeatability standard deviation s_r, µg/kg	3,4	3,3	13,5	3,4	8,1
Repeatability relative standard deviation RSD_r, %	9	15	8	7	15
Functional relationship between s_r and $\bar{x}$	$s_r = 0{,}11\ \bar{x}$				
Repeatability limit r [b], µg/kg	10	9	38	10	23
Reproducibility standard deviation s_R, µg/kg	6,2	6,3	23,2	12	12,4
Reproducibility relative standard deviation RSD_R, %	16	29	13	25	24
Functional relationship between s_R and $\bar{x}$	$s_R = 0{,}22\ \bar{x}$				
Horwitz ratio	0,7	1,3	0,6	1,1	1,1
Reproducibility limit R [c], µg/kg	17	18	65	34	35
Trueness, %	n.a [d]	n.a [d]	89	96 [e]	n.a. [d]

[a] Means where not corrected for recovery.

[b] $r = 2{,}8 \times s_{r,}$

[c] $R = 2{,}8 \times s_{R,}$

[d] Not applicable.

[e] Not significantly different from 100 %.

Table A.2 — Precision data T-2 toxin

Sample	Compound feed 1	Compound feed 2	Compound feed 3	Cereal Mix 1	Cereal Mix 2
Year of inter-laboratory study	2011	2011	2011	2011	2011
Number of laboratories	21	21	21	21	21
Number of laboratories retained after eliminating non-compliant ones	16	16	16	16	15
Number of non-compliant laboratories	5	5	5	5	6
Number of accepted results	16	16	16	16	15
Mean value $\bar{x}$ [a], µg/kg	12,1	3,5	50,3	17,7	7,0
Repeatability standard deviation s_r, µg/kg	1,7	1,2	3,1	1,6	1,8
Repeatability relative standard deviation RSD_r, %	14	35	6	9	27
Functional relationship between s_r and $\bar{x}$	$s_r = 0{,}04\,\bar{x} + 1{,}2$				
Repeatability limit r [b], µg/kg	5	3	9	5	5
Reproducibility standard deviation s_R, µg/kg	3,9	3,1	6,5	4,4	3,1
Reproducibility relative standard deviation RSD_R, %	32	88	13	25	44
Functional relationship between s_R and $\bar{x}$	$s_R = 0{,}08\,\bar{x} + 2{,}8$				
Horwitz ratio	1,5	4	0,6	1,1	2
Reproducibility limit R [c], µg/kg	11	9	18	12	9
Trueness, %	n.a [d]	n.a [d]	96[e]	100[e]	n.a. [d]

[a] Means where not corrected for recovery.

[b] $r = 2{,}8 \times s_{r,,}$

[c] $R = 2{,}8 \times s_{R,}$

[d] Not applicable.

[e] Not significantly different from 100 %.

Table A.3 — Precision data Deoxynivalenol

Sample	Compound feed 1	Compound feed 2	Compound feed 3	Cereal Mix 1	Cereal Mix 2
Year of inter-laboratory study	2011	2011	2011	2011	2011
Number of laboratories	21	21	21	21	21
Number of laboratories retained after eliminating non-compliant ones	16	16	16	16	15
Number of non-compliant laboratories	5	5	5	5	6
Number of accepted results	16	16	16	16	15
Mean value $\bar{x}$ [a], µg/kg	88,5	281,8	558,6	250,0	135,8
Repeatability standard deviation s_r, µg/kg	9,5	19,9	30,1	13,6	8,2
Repeatability relative standard deviation RSD_r, %	11	7	5	6	6
Functional relationship between s_r and $\bar{x}$	$s_r = 0{,}05\ \bar{x} + 3{,}8$				
Repeatability limit r [b], µg/kg	27	56	84	38	23
Reproducibility standard deviation s_R, µg/kg	17,0	33,1	66,9	33,3	23,0
Reproducibility relative standard deviation RSD_R, %	19	12	17	13	17
Functional relationship between s_R and $\bar{x}$	$s_R = 0{,}10\ \bar{x} + 8{,}7$				
Horwitz ratio	0,9	0,6	0,7	0,7	0,8
Reproducibility limit R [c], µg/kg	48	93	187	93	64
Trueness, %	n.a [d]	n.a [d]	93	89	n.a. [d]

[a] Means where not corrected for recovery.

[b] $r = 2{,}8 \times s_{r,}$

[c] $R = 2{,}8 \times s_{R,}$

[d] Not applicable.

Table A.4 — Precision data Zearalenone

Sample	Compound feed 1	Compound feed 2	Compound feed 3	Cereal Mix 1	Cereal Mix 2
Year of inter-laboratory study	2011	2011	2011	2011	2011
Number of laboratories	21	21	21	21	21
Number of laboratories retained after eliminating non-compliant ones	16	16	16	16	15
Number of non-compliant laboratories	5	5	5	5	6
Number of accepted results	16	16	16	16	15
Mean value $\bar{x}$ [a], µg/kg	13,9	15,9	430,0	30,5	3,4
Repeatability standard deviation s_r, µg/kg	2,0	1,7	25,0	2,9	1,1
Repeatability relative standard deviation RSD_r, %	15	11	6	10	32
Functional relationship between s_r and $\bar{x}$	$s_r = 0{,}05\ \bar{x} + 1$				
Repeatability limit r [b], µg/kg	6	5	70	8	3
Reproducibility standard deviation s_R, µg/kg	4,3	10,4	49,3	6,0	3,3
Reproducibility relative standard deviation RSD_R, %	31	65	12	20	98
Functional relationship between s_R and $\bar{x}$	$s_R = 0{,}1\ \bar{x} + 4{,}3$				
Horwitz ratio	1,4	3,0	0,6	0,9	4,4
Reproducibility limit R [c], µg/kg	12	29	138	17	9
Trueness, %	n.a [d]	n.a [d]	97[e]	107[e]	n.a. [d]

[a] Means where not corrected for recovery.

[b] $r = 2{,}8 \times s_{r,}$

[c] $R = 2{,}8 \times s_{R,}$

[d] Not applicable.

[e] Not significantly different from 100 %.

Annex B
(informative)

Examples

B.1 Example 1

B.1.1 General

With a LC-MS system consisting of two Shimadzu LC-20AD pumps, Thermo Scientific Accela Auto Liquid Sampler, and a Thermo Scientific TSQ Quantum Ultra MS with IonMax HESI2 interface the following settings have shown to satisfy the performance requirements and provide overall acceptable results (see Figure C.1 and Figure C.2 for chromatograms).

B.1.2 LC conditions

— Dwell volume: 60 µl

— Injection volume: 5 µl full loop

— Column: Supelco Ascentis Express C18, 75 × 2,1 mm, particle size 2,7 µm fused-core

— Column temperature: 40 °C

— Flow rate: 0,3 ml/min

— Mobile phase A: 0,1 % formic acid (4.6.) in water (4.2.)

— Mobile phase B: 0,1 % formic acid (4.6.) in methanol (4.3.)

The mobile phase was chosen to be very generic. It is permissible to add Ammonium ions to the mobile phase if this leads to suppression of sodiation and you want to measure the ammonium adducts.

Table B.1 — Gradient settings

Run time [min]	Mobile phase A [%]	Mobile phase B [%]
0	92	8
2	43	57
6	39	61
6,1	5	95
7,6	5	95
7,7	92	8
8,7	92	8

B.1.3 MS conditions

The run is divided into four segments around the four analyte peaks. The ion transitions in "selected reaction monitoring" mode as in Table B.2 are measured.

Table B.2 — Ion transitions for Example 1

Item	Segment 1	Segment 2	Segment 3	Segment 4
Run time [min]	0 – 2,6	2,6 – 4,1	4,1 – 4,9	4,9 – 8,7
Analyte	DON + $^{13}C_{15}$-DON	HT2 + $^{13}C_{22}$-HT2	T2 + $^{13}C_{24}$-T2	ZON + $^{13}C_{18}$-ZON
Adduct	Protonated	Sodium	Sodium	Deprotonated
Transitions (Collision Energy [eV])	297- > 231 (16), 297- > 249 (13), 312- > 263 (9), 312- > 276 (9)	447- > 285 (22), 447- > 345 (20), 469- > 300 (19), 469- > 362 (18)	489- > 245 (30), 489- > 327 (25), 513- > 260 (26), 513- > 344 (23)	317- > 131 (25), 317- > 175 (22), 335- > 185 (26), 335- > 290 (21)
Tube Lens [V]	80	110	140	80
Polarity	Pos	Pos	Pos	Neg
Spray Voltage [V]	2800	2800	2400	2000
Vaporizer temperature [°C]	350	350	350	350
Sheath Gas Pressure [arbitrary units]	30	30	30	30
Aux Gas Pressure [arbitrary units]	10	10	10	10
Transfer Capillary temperature [°C]	320	320	320	320

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B.2 Example 2

B.2.1 General

With a LC-MS system consisting of a HP1100 HPLC and a Micromass Quattro Ultima PT with ESI interface the following settings have shown to satisfy the performance requirements and provide overall acceptable results (see Figure C.3 and Figure C.4 for chromatograms).

B.2.2 LC conditions

— Dwell volume: the original static mixer was replaced by a low-volume peek mixing Tee;

— Injection volume: 5 µl;

— Column Supelco Ascentis Express C18, 75 × 2,1 mm, particle size 2,7 µm fused-core;

— Column temperature: 40 °C;

— Flow rate: 0,3 ml/min;

— Mobile phase A: 0,1 % formic acid (4.6.) in water (4.2);

— Mobile phase B: 0,1 % formic acid (4.6.) in methanol (4.3).

Table B.3 — Gradient settings

Run time [min]	Mobile phase A [%]	Mobile phase B [%]
0	92	8
0,67	50	50
8	33	67
8,01	5	95
9,5	5	95
9,51	92	8
11,5	92	8

B.2.3 MS conditions

The run is divided in to four segments around the four analyte peaks. The ion transitions in “selected reaction monitoring” mode as in Table B.4 are measured.

Table B.4 — Ion transitions for Example 2

Item	Segment 1	Segment 2	Segment 3	Segment 4
Run time [min]	0 – 4,0	4,0 – 6,2	6,2 – 7,2	7,2 – 11,5
Analyte	DON + $^{13}C_{15}$-DON	HT2 + $^{13}C_{22}$-HT2	T2 + $^{13}C_{24}$-T2	ZON + $^{13}C_{18}$-ZON
Adduct	Protonated	Sodium	Sodium	Deprotonated
Transitions (Collision Energy [eV])	297- > 231 (18), 297- > 249 (18), 312- > 263 (18), 312- > 276 (18)	447- > 285 (21), 447- > 345 (18), 469- > 300 (17), 469- > 362 (17)	489- > 245 (24), 489- > 327 (21), 513- > 260 (20), 513- > 344 (19)	317- > 131 (18), 317- > 175 (18), 335- > 185 (18), 335- > 290 (18)
Cone voltage [V]	50	85	80	60
Polarity	Pos	Pos	Pos	Neg
Spray voltage [V]	2,500	2,500	2,500	2,500
Desolvation temperature [°C]	350	350	350	350
Desolvation Gas Flow [L/h]	700	700	700	700
Cone Gas Flow [L/h]	100	100	100	100
Source temperature [°C]	120	120	120	120

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B.3 Example 3

B.3.1 General

With a LC-MS system consisting of an Agilent 1200 SL HPLC and an Applied Biosystems/ MDSciex API 4000 with Turbospray interface the following settings have shown to satisfy the performance requirements and provide overall acceptable results (see Figure C.5 (Annex C) for chromatogram).

B.3.2 LC conditions

— Injection volume: 30 µl;

— Column Phenomenex Luna C18, 150 × 4,6 mm, particle size 5 µm;

— Column temperature: 40 °C;

— Flow rate: 0,3 ml/min;

— Mobile phase A: Water/ Methanol/ Formic Acid (950/ 50/ 0,025, v/v/v), 1 mmol/L Ammonium carbonate;

— Mobile phase B: Methanol (4.3).

Table B.5 — Gradient settings

Run time [min]	Mobile phase A [%]	Mobile phase B [%]
0	100	0
5	20	80
6,9	20	80
7	0	100
10	0	100
10,1	100	0
13	100	0

B.3.3 MS conditions

The transitions as in Table B.6 were monitored.

Table B.6 — Ion transitions for Example 3

Analyte	MS1	MS 3	Polarity
DON	295,000	265,000	negative
DON	295,000	138,000	negative
DON C13	310,200	279,000	negative
DON C13	310,200	145,000	negative
ZON	317,000	131,000	negative
ZON	317,000	175,000	negative
ZON C13	335,000	185,000	negative
ZON C13	335,000	290,000	negative
HT2	442,000	263,000	positive
HT2	442,000	215,000	positive
HT2 C13	464,000	340,000	positive
HT2 C13	464,000	322,000	positive

Analyte	MS1	MS 3	Polarity
T2	484,000	215,000	positive
T2	484,000	185,000	positive
T2 C13	508,000	322,000	positive
T2 C13	508,000	260,000	positive

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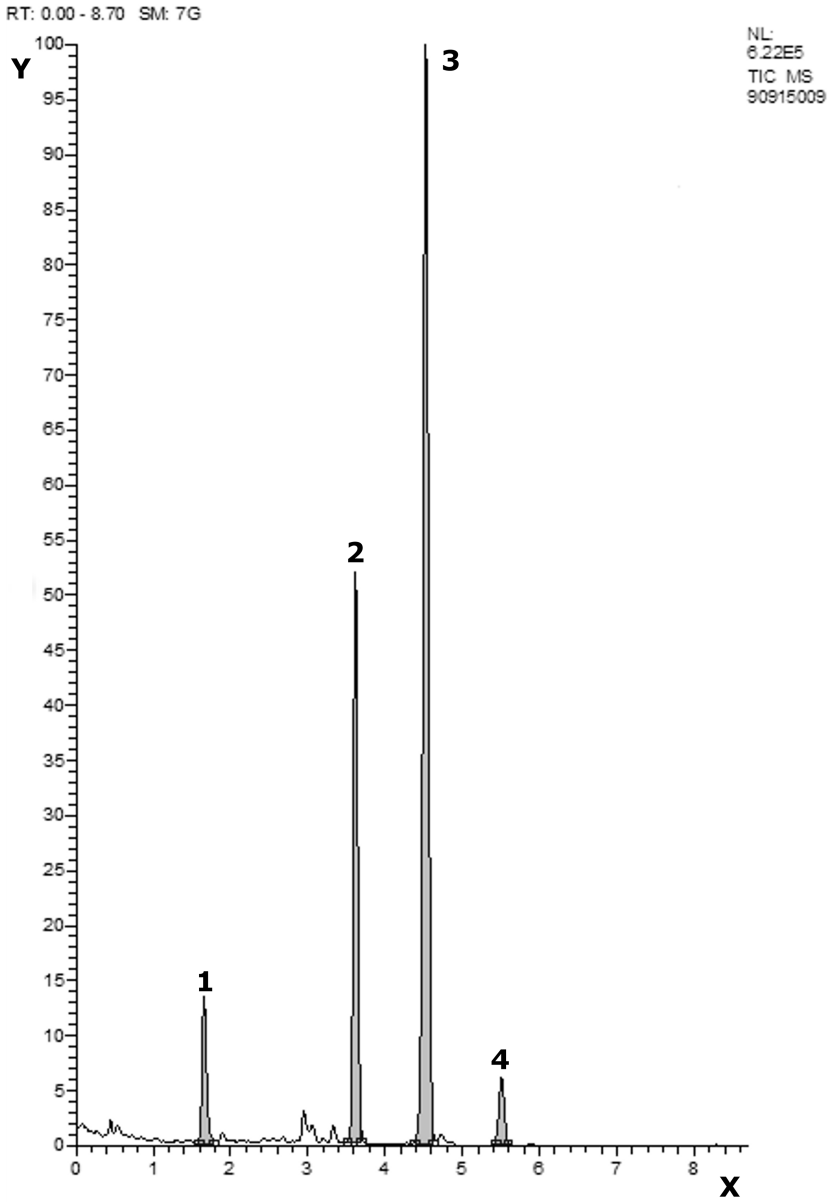
Annex C
(informative)

Examples of chromatograms according to the settings of the examples in Annex B

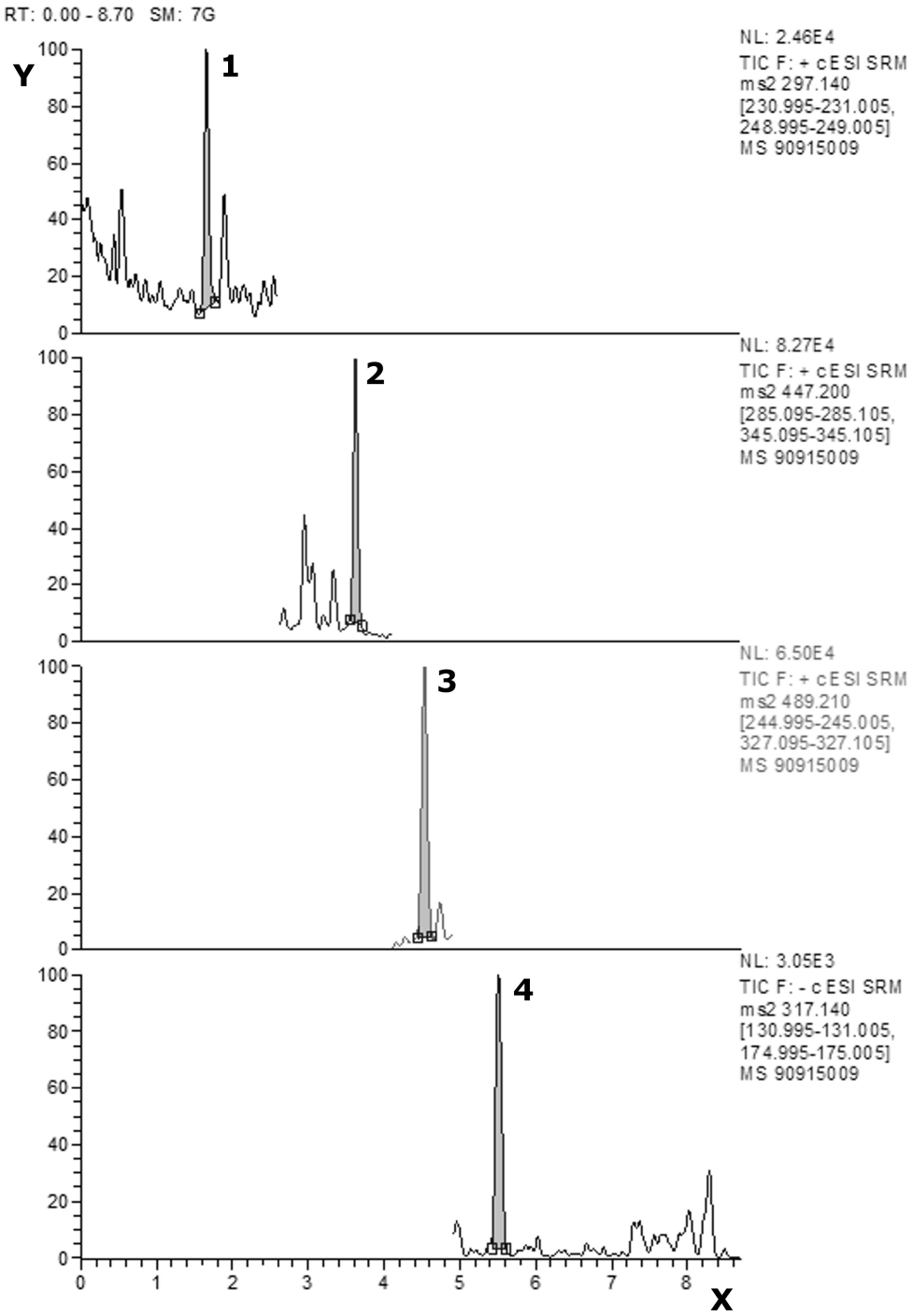


Key

X	time (min)	2	30 µg/kg HT-2 toxin	RT = 3,62
Y	relative abundance (%)	3	10 µg/kg T-2 toxin	RT = 4,53
1	90 µg/kg DON; RT = 1,65	4	10 µg/kg ZON	RT = 5,51

The peak area is mostly representing the ^{13}C-labelled ISTDs.

Figure C.1 — Total Ion Current (TIC) of a QC sample, acquired with the settings of Example 1 in Annex B

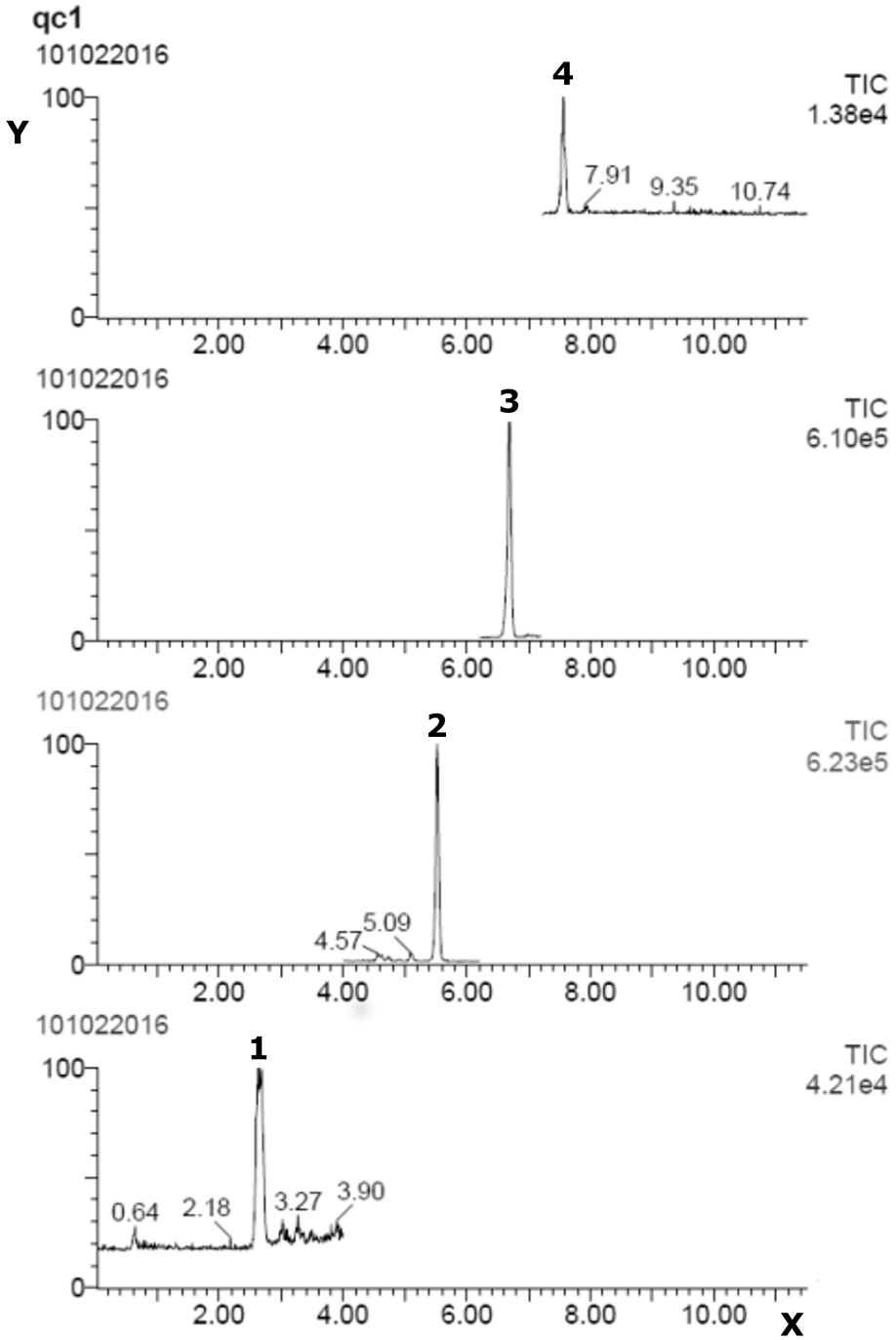


Key

X	time (min)	2	30 µg/kg HT-2 toxin	RT = 3,62
Y	relative abundance (%)	3	10 µg/kg T-2 toxin	RT = 4,53
1	90 µg/kg DON RT = 1,65	4	10 µg/kg ZON	RT = 5,51

The ion traces represent the transitions of the native analytes.

Figure C.2 — Extracted Ion Currents (XIC) of the same QC sample as above, acquired with the settings of Example 1 in Annex B



Key

X time (min)

Y relative abundance (%)

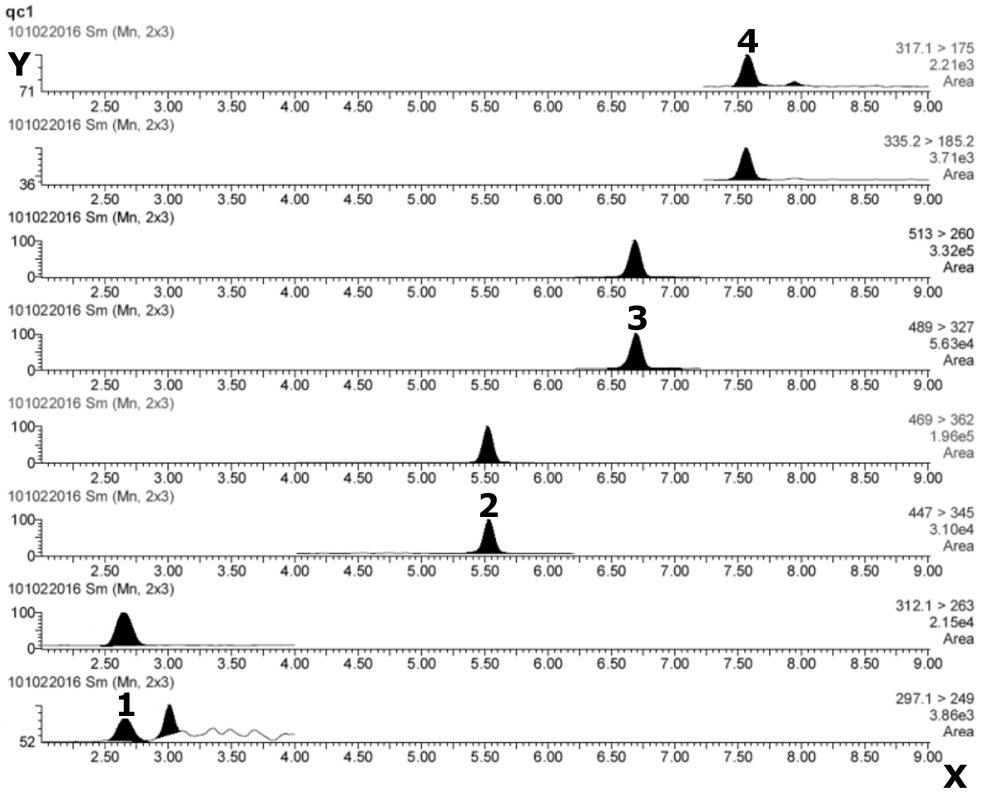
1 90 µg/kg DON RT = 1,65

2 30 µg/kg HT-2 toxin RT = 3,62

3 10 µg/kg T-2 toxin RT = 4,53

4 10 µg/kg ZON RT = 5,51

Figure C.3 — Total Ion Current (TIC) of the same QC sample as in Figure C.1, acquired with the settings of Example 2 in Annex B



Key

X time (min)

Y relative abundance (%)

1 90 µg/kg DON RT = 1,65

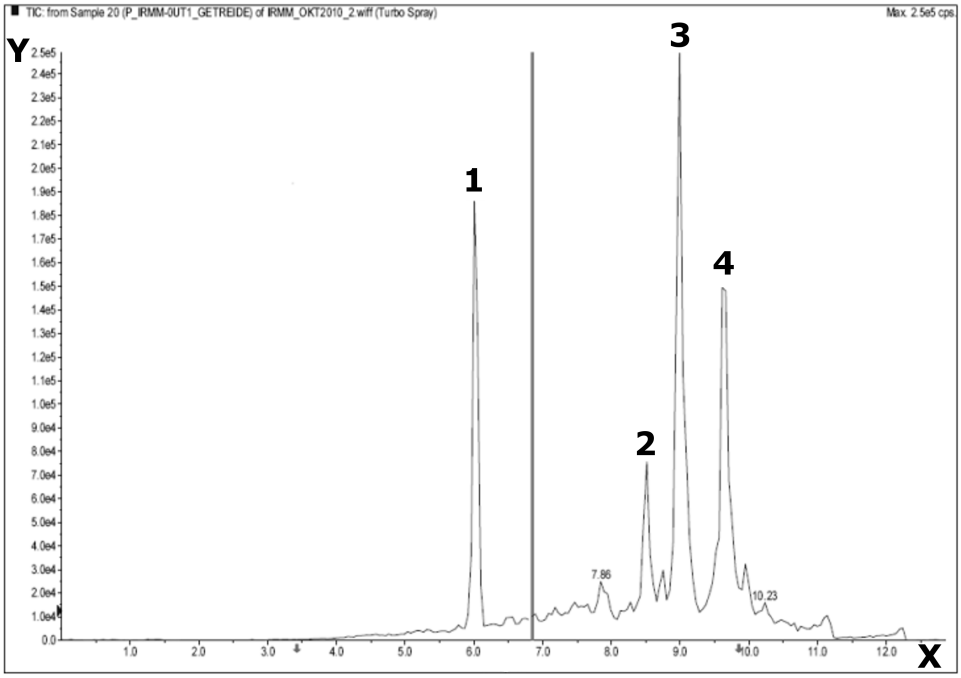
2 30 µg/kg HT-2 toxin RT = 3,62

3 10 µg/kg T-2 toxin RT = 4,53

4 10 µg/kg ZON RT = 5,51

NOTE The traces for the native analyte and the respective labelled analogue are right above each other.

Figure C.4 — Extracted Ion Currents (XIC) of the same QC sample as in Figure C.1, acquired with the settings of Example 2 in Annex B



Key

X time (min)

Y relative abundance (%)

1 90 µg/kg DON RT = 1,65

2 30 µg/kg HT-2 toxin RT = 3,62

3 10 µg/kg T-2 toxin RT = 4,53

4 10 µg/kg ZON RT = 5,51

Figure C.5 — Total Ion Current (TIC) of the same QC sample as in Figure C.1, acquired with the settings of Example 3 in Annex B

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