
EURL-MP-method_001 (version 1)

Determination of deoxynivalenol and related mycotoxins in cereals by LC-MS/MS

Analyte group: Mycotoxins
Analyte(s): deoxynivalenol (DON)
3-acetyl-deoxynivalenol (3-Ac-DON)
15-acetyl-deoxynivalenol (15-Ac-DON)
deoxynivalenol-3-glucoside (DON-3-G)

Commodity group: high starch and/or protein content and low water and fat content
Comm. categories: cereal grain and products thereof
Comm. validated: wheat, maize

Technique: Liquid chromatography / tandem mass spectrometry (LC-MS/MS)

Modifications compared to previous version:

Not applicable.

Method drafted by:

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1 Introduction

Deoxynivalenol (DON), 3-acetyl-DON (3-Ac-DON), 15-acetyl-DON (15-Ac-DON) and DON-3-glucoside (DON-3-G) are the most relevant forms of DON in Europe occurring in food and feed, mainly in cereal grains such as wheat, barley, oats, rye and maize and cereal-based food. While DON, 3-Ac-DON and 15-Ac-DON are produced as toxic secondary metabolites by plant pathogenic fungi of the *Fusarium* genus, DON-3-glucoside is the main plant metabolite of DON and considered as a modified mycotoxin. Currently an EU legal limit is in place for DON and not for the other three related mycotoxins. However, since 3- and 15-Ac-DON are largely deacetylated to DON and DON-3-G is cleaved to DON in the animal and human intestinal tract, they can thus contribute to overall DON exposure [1]. Therefore monitoring of all four forms of DON in food and feed has been recommended by EFSA [1] to acquire knowledge of possible trends, e.g. due to climate change or food and feed processing.

For enforcement in food, maximum limits for DON range from 750 to 1750 µg/kg in cereals intended for direct human consumption and unprocessed cereals, respectively (200 µg/kg in processed cereal-based foods and baby foods for infants and young children) [2]. The other forms of DON, not regulated so far, can co-occur at varying levels but are typically lower (down to a factor of 10 as compared to DON). For feed, guidance values have been set for DON of 8 mg/kg for cereals/cereal products, 12 mg/kg for maize by-products, and 0.9-5 mg/kg for complementary and complete feedingstuffs [3].

Based on a group TDI (tolerable daily intake) for the four forms of DON [1], consumption data, and taking into account the relative abundance of the four forms, the desirable LOQs in cereal grains are approximately 50-100 µg/kg for DON and 10-20 µg/kg for each of the other DON forms [4].

The method described in this document is a modification of a more generic in-house LC-MS/MS-based multi-mycotoxin method. Compared to the generic method, this method includes an evaporative concentration step/solvent switch to achieve better peak shape and lower LOQs, and dedicated LC conditions for chromatographic separation of 3-Ac-DON and 15-Ac-DON.

2 Scope

This document describes a procedure for the determination of deoxynivalenol, 3-acetyl-DON (3-Ac-DON), 15-acetyl-DON (15-Ac-DON) and DON-3-glucoside (DON-3-G) in cereals. The method has been successfully in-house validated with spiked samples of wheat and maize, at 20-100 µg/kg for DON, 3-Ac-DON, and 15-acetyl-DON, and at 50-250 µg/kg for DON-3-G. In-house validation data are provided in Annex A.

3 Principle

DON and related mycotoxins are extracted from the homogenised sample material, after addition of water, by shaking with acidified acetonitrile. After a salt-induced phase partitioning step and centrifugation, an aliquot of the acetonitrile phase is dried with magnesium sulfate, evaporated to dryness, and reconstituted in methanol/water. The extract is analysed by high performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS).

In this method, multiple options for quantification are described. The preferred option is using isotope labelled internal standards (available for all four DON/derivatives), but alternative options without isotope labels are also provided. A more detailed background and explanation of the various options for quantification is given in Annex B.

4 Reagents

Use only reagents of recognized analytical grade. Solvents shall be of quality for LC analysis, unless otherwise specified.

- 4.1 **Water**, deionised (milliQ).
- 4.2 **Water**, LC-MS grade, double distilled (for LC-MS analysis).
- 4.3 **Acetonitrile**, p.a.
- 4.4 **Acetic acid**, purity greater than 98 % (w/w).
- 4.5 **Magnesium sulfate** (MgSO_4) - anhydrous, p.a.
- 4.6 **Methanol**, LC-MS grade.
- 4.7 **Ammonium acetate**, purity greater than 99% (w/w).
- 4.8 **C18 Silica gel**, 40-75 μm (for optional dSPE cleanup).
- 4.9 **Deoxynivalenol (DON)** e.g. crystalline, as a film or as certified standard solution.
- 4.10 **15-Acetyl-deoxynivalenol (15-A-DON)** e.g. crystalline, as a film or as certified standard solution.
- 4.11 **3-Acetyl-deoxynivalenol (3-A-DON)** e.g. crystalline, as a film or as certified standard solution.
- 4.12 **Deoxynivalenol-3-glucoside (DON-3-G)** e.g. solution 50 $\mu\text{g/ml}$, in acetonitrile.
- 4.13 **$^{13}\text{C}_{15}$ -deoxynivalenol ($^{13}\text{C}_{15}$ -DON)** e.g. solution 25 $\mu\text{g/ml}$, in acetonitrile.
- 4.14 **$^{13}\text{C}_{17}$ -3-Acetyl-DON ($^{13}\text{C}_{17}$ -3-Ac-DON)** e.g. solution 25 $\mu\text{g/ml}$ in acetonitrile.
- 4.15 **$^{13}\text{C}_{17}$ -15-Acetyl-DON ($^{13}\text{C}_{17}$ -15-Ac-DON)** e.g. solution 10 $\mu\text{g/ml}$ in acetonitrile.
- 4.16 **$^{13}\text{C}_{21}$ -deoxynivalenol-3-glucoside ($^{13}\text{C}_{21}$ -DON-3-G)** e.g. solution 10 $\mu\text{g/ml}$, in acetonitrile
- 4.17 **Extraction solution**, acetonitrile containing 1 % of acetic acid.
Mix 1 part per volume of acetic acid (4.4) and 99 parts per volume of acetonitrile (4.3) and mix.

4.18 Mixed mycotoxin solution-C

Used for: preparation of calibration standards for evaluation of linearity and quantification based on (multi-level) calibration.

Prepare a mixed mycotoxin solution in acetonitrile (4.3) containing DON, 3-Ac-DON and 15-Ac-DON at 1.0 µg/ml, and DON-3-G at 2.5 µg/ml.

4.19 Mixed mycotoxin-label solution

Used for: quantification using isotope labelled internal standards.

Prepare a mixed mycotoxin-label solution in acetonitrile (4.3) containing ¹³C₁₅-DON at 1.25 µg/ml and ¹³C₁₇-3-Ac-DON, ¹³C₁₇-15-Ac-DON, and ¹³C₂₁-DON-3-G each at 0.625 µg/ml. This solution is added to calibration standards and sample extracts as internal standard.

4.20 Mixed mycotoxin solution-S

Used for: quality control purposes (determination of recovery) and quantification based on standard addition to sample extracts.

Prepare a mixed mycotoxin solution in acetonitrile (4.3) containing DON at 12.5 µg/ml and 3-Ac-DON, 15-Ac-DON, and DON-3-G each at 2.5 µg/ml.

Note: the lower concentrations of the DON related mycotoxins compared to DON reflect the lower relative abundance of the derivatives in cereal grain samples.

4.21 Calibration solutions in solvent

Used for: evaluation of linearity and quantification based on multi-level solvent standards.

Add the volumes of mixed mycotoxin solution-C (4.18) and mixed mycotoxin-label solution (4.19)* indicated in **Table 1** to a glass tube or autosampler vial.

Evaporate to dryness under a stream of nitrogen at approximately 40°C. Reconstitute in 500 µl methanol/water (20/80 v/v).

Table 1. Example of suitable calibration solutions.

Cal. Sol.	Mixed mycotoxin solution-C (4.18)	Mixed mycotoxin-label solution (4.19)*	DON	3-Ac-DON	15-Ac-DON	DON-3-G	¹³ C ₁₅ -DON*	¹³ C ₁₇ -3-Ac-DON* ¹³ C ₁₇ -15-Ac-DON* ¹³ C ₂₁ -DON-3-G*
No	Add µl	Add µl	Concentration in calibration solution in ng/ml					
0	-	100	0	0	0	0	250	125
1	10	100	20	20	20	50	250	125
2	25	100	50	50	50	125	250	125
3	62.5	100	125	125	125	312.5	250	125
4	125	100	250	250	250	625	250	125
5	250	100	500	500	500	1250	250	125
6	375	100	750	750	750	1875	250	125
7	625	100	1250	1250	1250	3125	250	125

* only needed for quantification using isotope labelled standards, for other quantification methods these columns are not applicable.

4.22 Calibration solutions in matrix

Used for: quantification based on matrix-matched standards and assessment of matrix effects.

Add the volumes of mixed mycotoxin solution-C (4.18) indicated in Table 1 to a glass tube containing V_e ml of blank extract prepared according to 6.3/Annex D.

Evaporate to dryness under a stream of nitrogen at approximately 40°C. Reconstitute in 500 µl methanol/water (20/80 v/v) using a vortex. Transfer 400 µl to a filter vial (5.8), place in the refrigerator for approximately half an hour and press through.

Note: once linearity has been demonstrated, the number of levels may be adjusted to laboratory or application specific requirements. A one-point bracketing calibration at intermediate level has been shown to work well.

5 Apparatus and equipment

Usual laboratory glassware and equipment, in particular, the following:

- 5.1 **Conical polypropylene screw cap centrifuge tubes**, 50 ml with caps.
- 5.2 **Analytical balance**, accuracy: 0.01 mg.
- 5.3 **Laboratory balance**, accuracy: 0.01 g.
- 5.4 **Adjustable mechanical vertical or horizontal shaker or rotary tumbling machine.**
- 5.5 **Laboratory shaker** (vortex).
- 5.6 **Pipets, adjustable**, e.g. 10 µl to 100 µl and 100 µl to 1000 µl, suited for organic solvents (e.g. positive displacement pipets), properly calibrated, with appropriate tips.
- 5.7 **Centrifuge**, capable of generating a relative centrifugal force of 3,000 g.
- 5.8 **Filter vials** (polytetrafluoroethylene (PTFE) 0,45 µm), with crimp cap, e.g. Mini-UniPrep, Whatman, or equivalent.
- 5.9 **Syringe filter or centrifuge filter**, 0,20 µm to 0,45 µm, made of nylon or polytetrafluoroethylene (PTFE), for filtration of extract in case (5.8) is not used.
- 5.10 **Auto sampler vials**, of appropriate size for the auto sampler in use, e.g. glass with insert vials, or filter vials (5.8), with crimp cap or equivalent.
- 5.11 **LC-MS/MS system, with the following components:**
 - 5.11.1 **LC pump**, capable of delivering a binary gradient at flow rates appropriate for the analytical column in use with sufficient accuracy.
 - 5.11.2 **Injection system**, capable of injecting an appropriate volume of injection solution with sufficient accuracy, and cross-contamination below 0.1 %.

5.11.3 LC column, capable of retaining the target mycotoxins, preferably with a retention factor of at least two, and capable of baseline separation of DON and DON-3-G, and baseline separation of 3-Ac-DON and 15-Ac-DON. See 7.1 for example conditions.

Note: the separation of DON and DON-3-G is required when measuring these mycotoxins as positive ions because DON-3-G is prone to in-source fragmentation/loss of the glucose, degrading DON-3-G to DON. Separation of 3-Ac-DON and 15-Ac-DON is desirable because a number of transitions are the same (see 7.2).

5.11.4 Column oven, capable of maintaining a constant temperature.

5.11.5 Tandem mass spectrometer (MS/MS), capable of ionisation of the mycotoxins (either resulting in positive or negative ions), performing Selected Reaction Monitoring (SRM), and with a sufficiently wide dynamic range. See 7.2 for example transitions.

6 Procedures

6.1 Preparation of the test sample

Homogenise the test sample by dry milling to particle size $\emptyset < 1$ mm, or by slurry mixing.

6.2 Test portion

6.2.1 Dry milled test samples of cereals/cereal products

For dry milled samples, 2.5 g is used for extraction. If desirable, higher amounts can be used when proportionally adjusting reagents mentioned under 6.3/Annex C/D/E.

6.2.2 Slurried samples

For samples that have been homogenised by slurry mixing, the equivalent of 2.5 g sample is used. Depending on the sample:water ratio of the slurry, more water is added to achieve a consistent sample:water ratio.

- Weigh the equivalent of 2.5 g of the slurry to the nearest 0.01 g (5.3), into a 50 ml centrifuge tube (5.1). Add water to make up to the equivalent of 7.5 ml of water, according to Table 2:

Table 2. Test portions for slurried samples.

Slurried sample (ratio)	Amount of slurry (g)	Add water (4.1) (ml)
Slurried sample 1+1*	5.0	5.0
Slurried sample 1+2*	7.5	2.5
Slurried sample 1+3*	10.0	None

* Slurried sample x + y means: x g sample slurried with y ml of water

6.3 Extraction, cleanup and preparation of test solutions

The exact procedure for extraction, cleanup, and preparation of the test solutions depends on the method used for quantification and is described in detail in Annexes C-E:

Annex C: sample preparation for quantification with isotope labelled internal standards

Annex D: sample preparation for quantification based on matrix-matched standards

Annex E: sample preparation for quantification based on standard addition to the sample extract

7 LC-MS/MS conditions

7.1 LC conditions

Choose an analytical column, mobile phase and gradient settings such that the requirements laid out in 5.11.3 are met.

Example LC conditions for separation of DON and DON-3-G, and 3-Ac-DON and 15-Ac-DON:

Column: Cortecs C18 2.8 μm , 100 mm x 2 mm ID (Waters)
 Column temperature: 35°C
 Injection volume: 5 μl (standard/extract in methanol/water, 20/80 v/v)
 Flow: 0.30 ml/min
 Mobile phase A: Water, 5 mM ammonium acetate, 0.1% acetic acid
 Mobile phase B: Methanol/water (95/5 v/v), 5 mM ammonium acetate, 0.1% acetic acid

Gradient:	Time (min)	%A	%B
	0	99	1
	4	80	20
	9	70	30
	10	0	100
	13	0	100
	14	99	1
	18	99	1

7.2 MS conditions

There are multiple options for precursor ions for the four mycotoxins, both in positive and in negative mode (for examples see **Table 3**). The precursor ions obtained and their relative responses depend on the mobile phase composition and the MS instrument used. In positive mode, in-source fragmentation of DON-3-G to DON occurs. As long as the two compounds are chromatographically separated, it is an option to measure DON-3-G using the DON transitions. 3-Ac-DON and 15-Ac-DON behave differently in the ion source and hence the optimum precursor is likely to differ. In addition, the product ion spectra are also different (e.g. [5,6]). Depending on the conditions, 3-Ac-DON and 15-Ac-DON can be separated mass spectrometrically to a large extent. However, unless it has been verified that the response for the quantifier and the qualifier transition of one are negligible for the other, a chromatographic separation is desirable (see 5.11.3).

Table 3. Example MS/MS transitions (not exhaustive).

Mycotoxin	ESI	precursor ion (m/z) ^{a)}	product ion 1 ^{a)}	product ion 2 ^{a)}	product ion 3 ^{a)}	
DON	pos	M+H	297	249	231	203
	neg	M+acetate ^{b)}	355	265	295	59 ^{c)}
¹³ C ₁₅ -DON	pos	M+H	312	263	245	216
	neg	M+acetate ^{b)}	370	279	310	59 ^{c)}
3-Ac-DON	neg	M+acetate ^{b)}	397	337	307	59 ^{c)}
	pos	M+H	339	231	213	203
¹³ C ₁₇ -3-Ac-DON	neg	M+acetate ^{b)}	414	354	323	59 ^{c)}
	pos	M+H	356	245	227	216

15-Ac-DON	pos	M+NH ₄	356	321	137	261
	pos	M+H	339	321	137	261
¹³ C ₁₇ -15-Ac-DON	pos	M+NH ₄	373	338	145	276
	pos	M+H	356	338	145	276
DON-3G	neg	M+acetate ^{b)}	517	457	247	427
	pos ^{d)}	M+H	297	249	231	203
¹³ C ₂₁ -DON-3-G	neg	M+acetate ^{b)}	538	478	261	447
	pos ^{d)}	M+H	312	263	245	216

a) the relative abundance or optimum S/N for the transitions depend on the instrument and matrix, and needs to be experimentally optimized/verified.

b) when formate and/or formic acid is used in the mobile phase, formate adducts instead of acetate adducts can be formed.

c) measurement of acetate as product ion can be rather non-specific and is therefore not recommended.

d) transition obtained for DON-3-G when in-source fragmentation of DON-3-G to DON occurs.

7.3 Injection sequence

Start a batch of measurements with an injection of reconstitution solvent (methanol/water (20/80 v/v) to check non-contamination of the system. Then inject the calibration standards, followed by methanol/water (20/80 v/v) to check for possible carry-over. Next, the test solutions of the (QC) samples are measured. For larger batches of samples, inject calibration standard number 4 (4.21/4.22) after every 5-10 samples. Always end the sequence with this calibrant. Example sequences are included in Annexes C-E.

8 Evaluation and calculations

Peak areas are used for all subsequent calculations. For each injection, check peak assignment and integration for all measured transitions and adjust if needed.

For quantification with isotope labelled internal standards, calculate the ratio of the quantifier peak area of each mycotoxin to the quantifier peak area of the respective labelled analogue.

8.1 Verification of linearity of LC-MS/MS measurement

Plot the response of the quantifier of all individual calibration solutions (4.21 or 4.22, calibration solutions 0 to 7) against the corresponding concentrations in ng/ml. Construct a calibration curve using (weighted) least-square regression with all individual data points obtained. Linearity has been demonstrated and the calibration curve is fit-for-purpose when the deviation of the back-calculated concentrations of the calibration standards from the true concentrations, using the calibration equation, do not exceed 20%.

8.2 Identification of mycotoxins in the samples

Identify the mycotoxins in the samples by comparing retention time and ion ratio with that of the calibration standards according to SANTE/12089/2016 [8]. A mycotoxin is considered present and identified when:

a) the blank sample (QC_{bl}) does not show a peak for the quantifier ion at the retention time of the mycotoxin that exceeds 30% of the reporting limit;

b) the retention time of the peak observed for the test solution differs less than 0.1 min from the average retention time as calculated from the calibration solutions;

c) the ratio of the area of the quantifier and qualifier transition (lowest area/highest area) for the mycotoxins in the sample extracts deviates less than 30% (relative) from the average ion ratio of the calibration standards (=reference ion ratio).

Note: for calculation of the reference ion ratio use only responses with an S/N > 10. For the higher concentrations, exclude peak areas exceeding the linear range from calculation of the reference ion ratio.

8.3 Quantification of mycotoxins in the samples

8.3.1 Quantification with isotope labelled internal standards

When the response is within the linear range, the concentration of the mycotoxin in the sample is calculated based on multi-level calibration, according to Equation 1:

$$C_s = \frac{R-I}{S} \times \frac{V_{es}}{m_s} \times \frac{V_r}{V_e} \quad (1)$$

where

- C_s is the concentration of the mycotoxin in the sample in $\mu\text{g}/\text{kg}$;
- R is the ratio of the quantifier peak area of the mycotoxin and the quantifier peak area of its isotope labelled internal standard;
- I is the intercept, obtained with (weighted) least square regression from calibration data with concentrations in ng/ml ;
- S is the slope, obtained with (weighted) least-square regression from calibration data with concentration in ng/ml ;
- V_{es} is the volume of the extraction solvent, in ml (here: 10.0 ml);
- m_s is the mass of the test portion, in g (here 2.5);
- V_r is the volume used for reconstitution after evaporation to dryness, in ml (here: 0.5 ml);
- V_e is the volume of the aliquot of supernatant taken to evaporate to dryness, in ml (here: default is 5.0 ml).

If the response obtained for the sample is exceeding the linear range, a smaller aliquot of the supernatant (V_e) needs to be taken during preparation of the test solution (see Annex C), and the analysis repeated.

8.3.2 Quantification based on matrix-matched standards

When the peak area of the quantifier is within the linear range, the concentration of the mycotoxin in the sample is calculated based on 1-point bracketing calibration, according to Equation 2:

$$C_s = \frac{A_s}{A_{mms(av)}} \times C_{mms} \times \frac{V_{es}}{m_s} \times \frac{V_r}{V_e} \quad (2)$$

where

- C_s is the concentration of the mycotoxin in the sample in $\mu\text{g}/\text{kg}$;
- A_s is the area of the quantifier in the sample extract;
- $A_{mms(av)}$ is the average of the areas of the quantifier in the matrix-matched calibration standard injected before and after the sample;
- C_{mms} is the concentration of the mycotoxin in the matrix-matched standard in ng/ml ;
- V_{es} is the volume of the extraction solvent, in ml (here: 10.0 ml);

- m_s is the mass of the test portion, in g (here 2.5 g);
 V_r is the volume used for reconstitution after evaporation to dryness, in ml (here: 0.5 ml);
 V_e is the volume of the aliquot of supernatant taken to evaporate to dryness, in ml (here: default is 5.0 ml).

If the peak area obtained for the sample is exceeding the linear range, a smaller aliquot of the supernatant (V_e) needs to be taken during preparation of the test solution (see Annex D), and the analysis repeated.

8.3.3 Quantification based on standard addition to the sample extract

When the peak area of the quantifier is within the linear range, and A_{s+} is at least 2 times A_s , the concentration of the mycotoxin in the sample is calculated based on 1-point standard addition to the extract, according to Equation 3:

$$C_s = \frac{A_s}{A_{s+} - A_s} \times C_{st.add} \times \frac{V_{es}}{m_s} \times \frac{V_r}{V_e} \quad (3)$$

where

- C_s is the concentration of the mycotoxin in the sample in $\mu\text{g}/\text{kg}$;
 A_s is the area of the quantifier in the sample extract;
 A_{s+} is the area of the quantifier in the sample extract to which the standard has been added;
 $C_{st.add}$ is the added concentration of the mycotoxin in sample extract plus standard addition in ng/ml (here: concentration mycotoxin in 'mixed mycotoxin solution-S' (4.20) in $\text{mg}/\text{ml} * 1000 * 25 \mu\text{l}/500 \mu\text{l}$);
 V_{es} is the volume of the extraction solvent, in ml (here: 10.0 ml);
 m_s is the mass of the test portion, in g (here 2.5 g);
 V_r is the volume used for reconstitution after evaporation to dryness, in ml (here: 0.5 ml);
 V_e is the volume of the aliquot of supernatant taken to evaporate to dryness, in ml (here: default is 2.5 ml).

If the peak area obtained for the sample extract+standard addition is exceeding the linear range, and in case $A_{s+} < 2 * A_s$, then a smaller aliquot of the supernatant (V_e) needs to be taken during preparation of the test solution (see Annex E), and the analysis repeated.

8.4 Correction for recovery

The analytical result must be reported corrected for recovery when the recovery is not between 90-110% [7].

Note: in general, for correction it is recommended to use the average recovery obtained from QC samples from the applicable commodity group which have been concurrently analysed with the samples in multiple (>6) batches over a longer period of time, e.g. one (preceding) year, rather than a single recovery obtained for a particular batch of samples.

9 References

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10 Annex A. In-house validation data.

The method described in this document is a modification of a more generic in-house LC-MS/MS-based multi-mycotoxin method. Compared to the generic methods, this method includes an evaporative concentration step/solvent switch to achieve better peak shape and lower LOQs, and LC conditions that not only separate DON from DON-3-G, but also 3-Ac-DON from 15-Ac-DON. A limited validation of the modified method was done in February 2018 for two cereal matrices (wheat and maize). Emphasis was on the lower concentration range: 20-100 µg/kg for DON, 3-Ac-DON, and 15-acetyl-DON, and at 50-250 µg/kg for DON-3-G. Spiking was done in triplicate for each matrix/level.

For DON and 3-Ac-DON, quantification with isotope labelled internal standards was done (Annex C). For 15-Ac-DON and DON-3-G, quantification based on matrix-matched standards was done (Annex D, bracketing 1-point matrix-matched calibration). For LC-MS/MS analysis, a Waters Acquity® LC-system combined with a Sciex QTRAP® 6500 was used.

The LC-conditions were as described in the example conditions in 7.1. The retention times and transitions used for validation are summarized in Table A-1.

Table A-1. Retention times and MS/MS transitions used during validation.

	t _r (min)	Quan	qual-1	qual-2
DON	4.42	297.0 > 231.0	297.0 > 249.0	
¹³ C ₁₅ -DON	4.42	312.2 > 263.2		
3-Ac-DON	8.84	397.1 > 337.1	397.1 > 307.1	
¹³ C ₁₇ -3-Ac-DON	8.84	414.2 > 323.1		
15-Ac-DON	9.11	356.1 > 321.1	356.1 > 137.1	356.1 > 261.0
DON-3-G	4.80	517.1 > 457.2	517.1 > 427.1	517.1 > 247.1

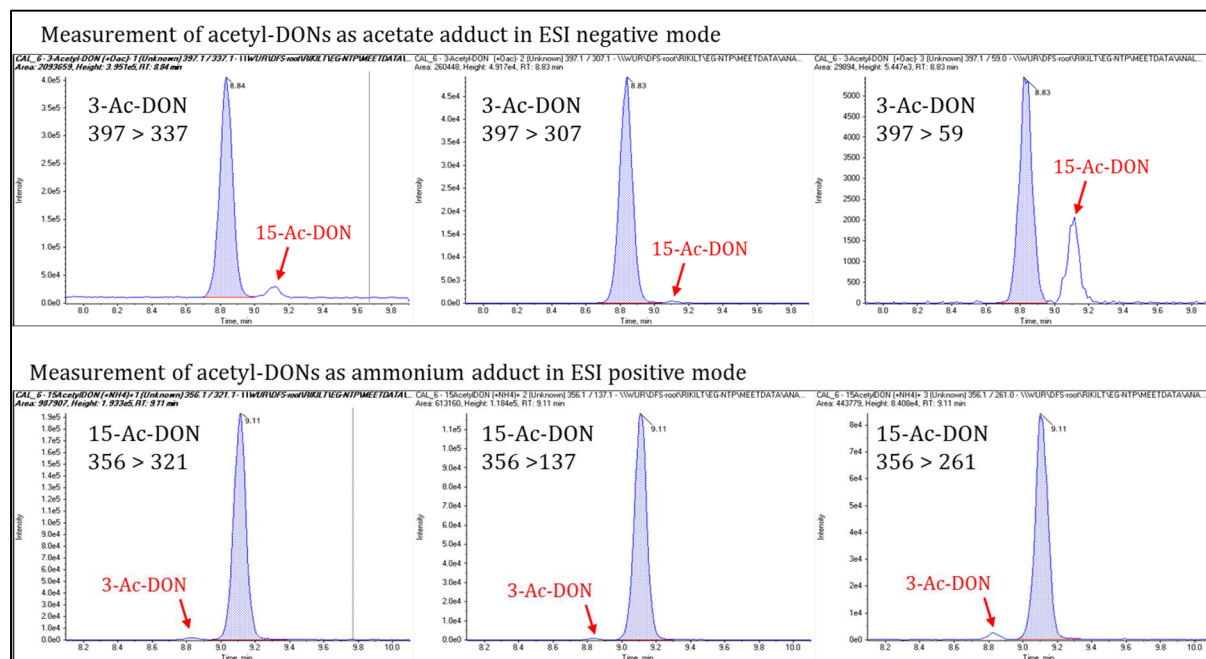


Figure A-1. Illustration of chromatographic and mass spectrometric separation of 3-Ac-DON and 15-Ac-DON. Solvent standard 500 ng/ml. LC-MS/MS conditions see text above.

As can be seen from figure A-1, baseline separation of 3-Ac-DON and 15-Ac-DON is obtained under the LC-conditions applied. Furthermore, the acetyl-DONs are also mass spectrometrically separated to a large extent by measuring 3-Ac-DON as acetate adduct (ESI negative mode, when not using the acetate product ion m/z 59) and 15-Ac-DON as ammonium adduct in ESI positive mode.

Figure A-2 shows that a signal is obtained for DON-3-G in ESI positive mode using the DON transitions, due to in-source fragmentation/loss of the glucose, degrading DON-3-G to DON.

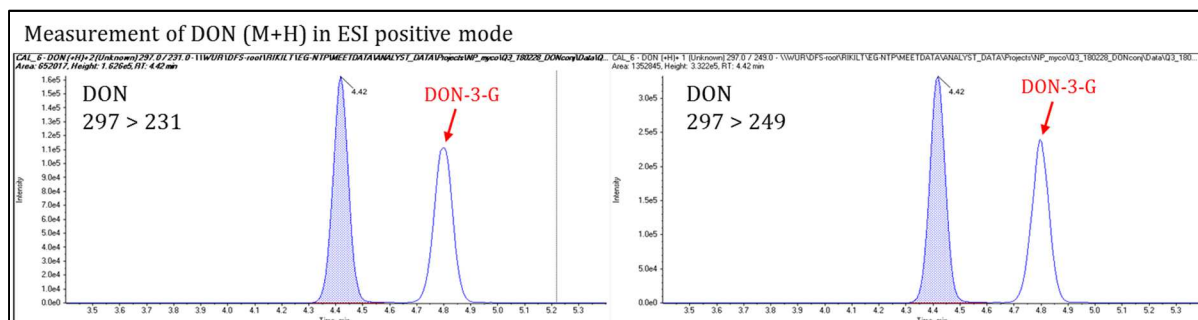


Figure A-2. XICs for DON transitions in ESI positive mode, obtained for injection of a solvent standard of DON 500 ng/ml and DON-3-G 1250 ng/ml. The signal of DON-3-G results from in-source fragmentation loss of the glucose, degrading DON-3-G to DON. LC-MS/MS conditions see text above.

The samples used for matrix-matched calibration were different wheat and maize samples than the ones used for recovery determinations. The wheat sample used for recovery determination contained a low concentration of DON (10.7 $\mu\text{g}/\text{kg}$). This amount was subtracted before calculation of the recovery. Linearity for all four analytes was excellent with deviations of back-calculated concentrations within 10% in most cases and always below 20%. For the wheat and maize samples used, and under the LC-MS/MS conditions applied, matrix effects were not very pronounced (<15% for DON, 3-Ac-DON and 15-Ac-DON, 18-27% for DON-3-G), despite the concentration step in the sample preparation. The average recoveries and repeatabilities obtained during validation are summarized in Table A-2. Figure A-3 shows example extracted ion chromatograms (XICs) for wheat and maize at the lowest spike levels.

Table A-2. Average recovery and repeatability as obtained during validation.

Deoxynivalenol*			
matrix	level $\mu\text{g}/\text{kg}$	recovery average (n=3)	repeatability RSD% (n=3)
wheat	20	75%	9%
	100	85%	1%
maize	20	91%	2%
	100	88%	3%
3-Acetyl-deoxynivalenol*			
matrix	level $\mu\text{g}/\text{kg}$	recovery average (n=3)	repeatability RSD% (n=3)
wheat	20	92%	3%
	100	90%	3%
maize	20	86%	4%

	100	89%	2%
15-Acetyl-deoxynivalenol**			
matrix	level µg/kg	recovery average (n=3)	repeatability RSD% (n=3)
wheat	20	90%	5%
	100	90%	1%
maize	20	91%	3%
	100	85%	0.4%
Deoxynivalenol-3-glucoside**			
matrix	level µg/kg	recovery average (n=3)	repeatability RSD% (n=3)
wheat	50	75%	1%
	250	72%	2%
maize	50	79%	1%
	250	76%	1%

* quantification with isotope labelled standards

** quantification based on matrix-matched standards

In Regulation (EC) No 401/2006 [7], performance criteria are given for DON at levels >100-≤500 µg/kg (recovery 60-110%, RSD_r ≤20%) and >500 µg/kg (recovery 70-120%, RSD_r ≤20%). For lower levels and for the acetyl-DONs and DON-3-G, no criteria have been set. The recovery and repeatability obtained during validation meet the criteria for DON, and therefore the method is considered suited for quantitative determination of all four DON forms down to 20-50 µg/kg.

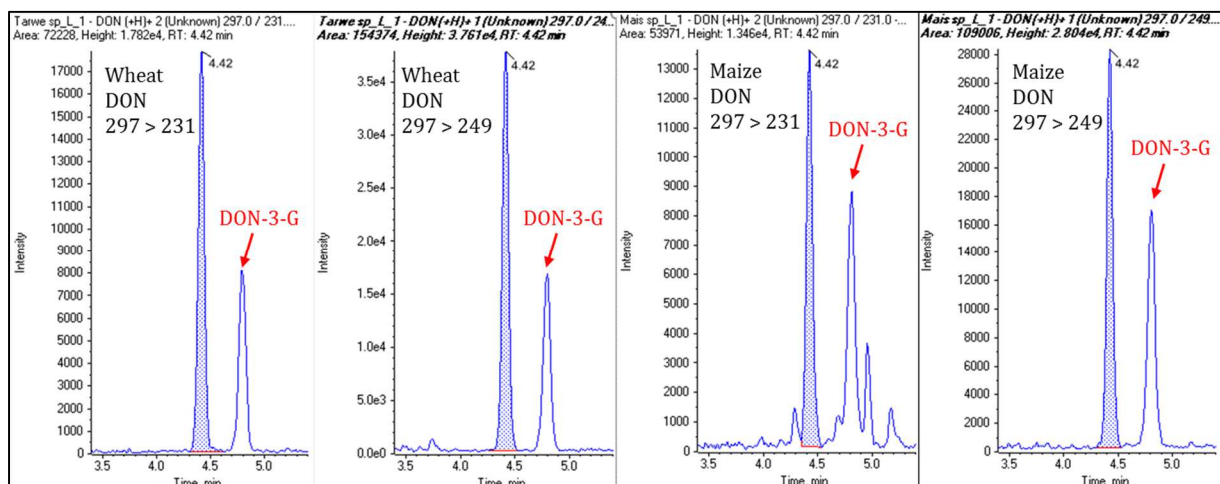


Figure A-3a. XICs for DON spiked to wheat and maize at 20 µg/kg. In these XICs, DON-3-G (spiked at 50 µg/kg) is also observed. Note: in wheat, DON was present in the sample used for validation, the signal therefore corresponds to approx. 30 µg/kg.

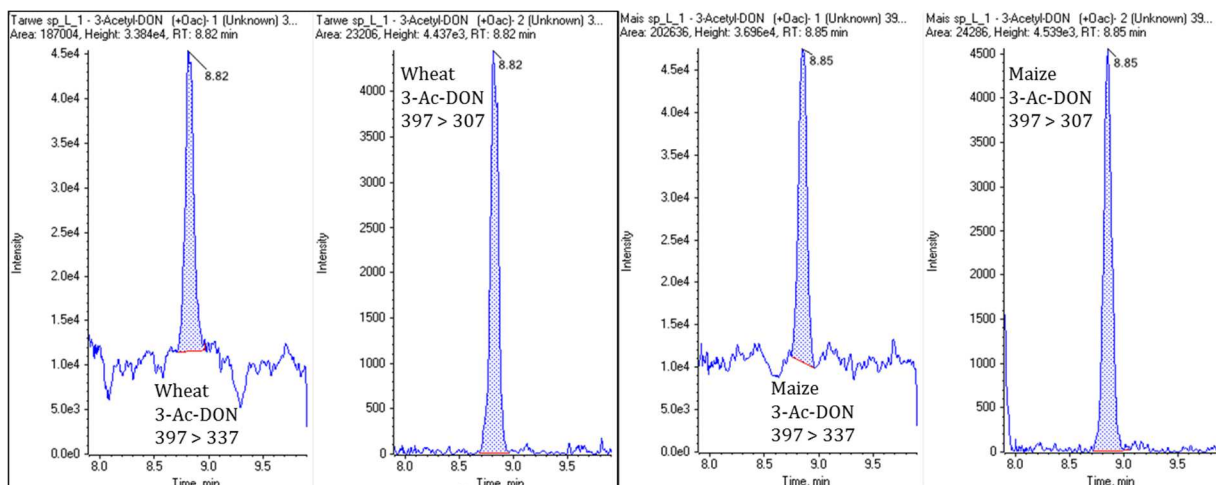


Figure A-3b. XICs for 3-Ac-DON spiked to wheat and maize at 20 µg/kg.

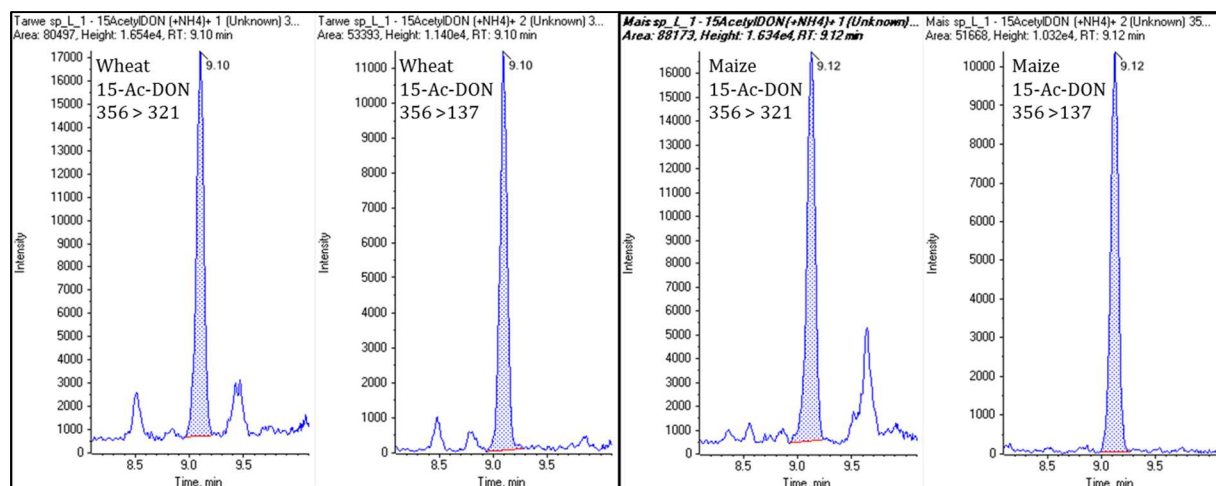


Figure A-3b. XICs for 15-Ac-DON spiked to wheat and maize at 20 µg/kg.

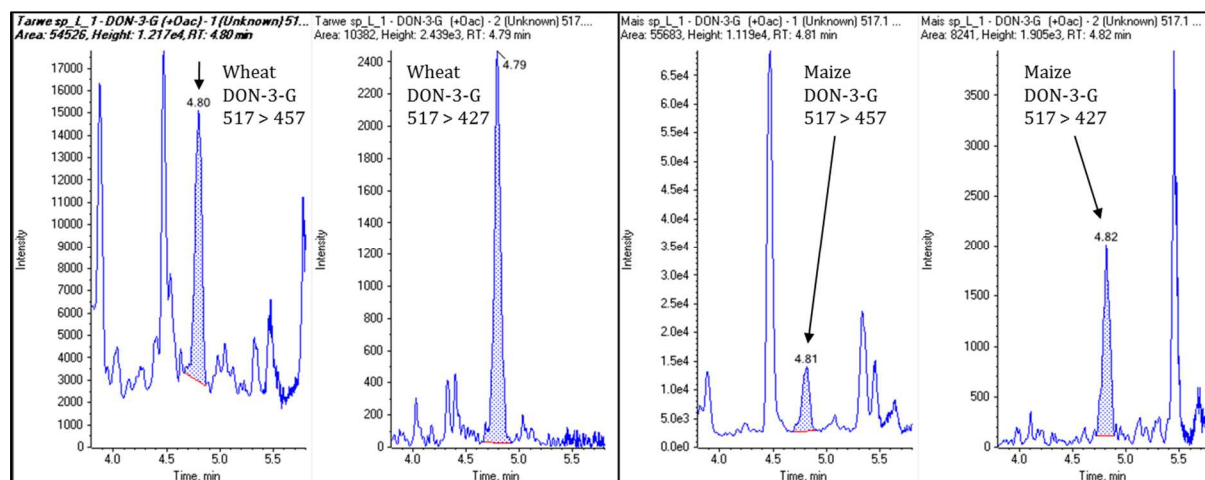


Figure A-3d. XICs for DON-3-G spiked to wheat and maize at 50 µg/kg.

11 Annex B. Options for quantification.

Background

In LC-MS(/MS) analysis, the response of an analyte can be affected by matrix compounds in the final extract. The effect is that the response of an analyte in extract can be lower (or sometimes higher) compared to the same concentration of analyte in a solvent standard. The reason for this lies in the ionisation of compounds in the ion source, where matrix compounds may cause suppression (or sometimes enhancement) of the ionisation of the analyte. These **matrix effects** need to be addressed in quantification when using LC-MS/MS.

Matrix effects depend on many parameters, including the sample, the amount of matrix introduced into the LC-MS, the LC-MS/MS system and conditions used, and the analyte. The more complex the extract (many co-extractants, little or no cleanup, concentrated extracts) the more pronounced matrix effects will be. For a particular analyte in a particular matrix, matrix effects are normally independent on the concentration of the analyte, as long as the response is within the linear range of the detector. In other words: a calibration curve prepared in clean solvent or in a blank sample extract will both be linear, but the slopes may be different.

Matrix effects can be examined by comparing the responses obtained for standards prepared in solvent with that of standards prepared in final extracts of a blank sample at the same concentrations. **When the difference in response (or slope) exceeds 20%, matrix effects are considered significant and need to be compensated for in quantification of the samples.** Since matrix effects are matrix-dependent, this assessment needs to be done for each matrix, e.g. for wheat, maize, rye, etc. Also *within* one type of matrix, e.g. different wheat samples, matrix effects may differ, however, these differences are typically minor compared to differences between matrices.

Options for quantification:

1. Multi-level solvent standards

When to use: in case matrix effects are not significant.

This option is only possible when it has been demonstrated that matrix-effects are not significant (less than 20%). For mycotoxins in cereals, matrix effects exceeding 20% often occur, especially in concentrated extracts, so generally one of the other options described below are needed for quantification.

2. Multi-level solvent standards with the use of isotope labelled internal standards

When to use: whenever possible.

Fully ¹³C-isotope labelled internal standards are commercially available for most regulated mycotoxins and a number of others. These are added to the calibration standards in solvent and to the sample extracts. For quantification, the responses of the mycotoxins relative to their respective internal standards are used. Since matrix effects for the native and the isotope labelled mycotoxin are the same, this will fully compensate for matrix effects on an individual sample basis. In addition, any drift in MS/MS response is also compensated for. Therefore, **this is the preferred option for quantification.**

Note: isotope labels are relatively expensive, but they can be used in a cost-effective way by adding them to a small volume of the final extract. This way, the amount of isotope label needed per sample is small and the additional cost per sample is often worth the improved performance and lower demand on instrument time compared to the options 3 and 4.

Note: matrix effects depend on the matrix compounds that co-elute from the LC column with the analyte of interest. The use of internal standards other than their isotope labels may not be a reliable alternative because a) they do not exactly co-elute with the analyte and experience a different matrix effect, b) matrix effects can be compound dependent.

When no isotope labels are available and matrix effects are significant, one of the options below needs to be applied:

3. Matrix-matched calibration

When to use: sample batches of the same (well defined) matrix

Here calibration standards are prepared in blank extracts of the same matrix and prepared in the same way as the samples. Since matrix effects can differ for different matrices, separate calibration standards need to be prepared for wheat, maize etc. Within one matrix, e.g. wheat, it is assumed that for different samples the matrix effects are similar (do not differ more than 20%). For this option, samples free of the target mycotoxin are needed which can be troublesome to find for certain mycotoxins especially when analysing down to lower levels.

Note: for multi-level calibration a substantial volume of blank final extract is required, which may be laborious to prepare in case the amount of extract obtained after sample preparation is small. Since matrix effects are independent of the analyte concentration, a one-point bracketing calibration using a matrix-matched standard at an intermediate level can be a more efficient alternative. The requirement here is that the response of samples and matrix-matched standard must be within the linear range of the detection. This can be verified through a calibration curve in solvent.

Note: this option is less convenient for sample batches containing a variety of matrices, because many matrix-matched standards need to be prepared. For matrices that are not well defined, such as 'breakfast cereal', 'complete feed', it might be difficult or impossible to find an equivalent blank matrix for preparation of matrix-matched standards. In the latter case, option 2 or 4 are better alternatives.

4. Standard addition to final extract

When to use: sample batches with variable or non-defined matrices

In this option matrix effects are compensated for on an individual sample basis. The final extract is split into multiple aliquots. One aliquot is analysed as such. To the other aliquots, a small volume of solvent standard of increasing concentration is added. The concentration in the sample extract is calculated from the resulting calibration curve. A disadvantage of this option as described here is that each sample requires typically 3 or more injections.

Note: a one-point standard addition has been shown to give comparable results as multi-level standard addition when: a) the response obtained after standard addition is at least two times the response of the peak in the extract without addition, b) the response of extracts with and without addition are within the linear range of the detection. The latter can be verified through a calibration curve in solvent.

12 Annex C. Sample preparation for quantification with isotope labelled internal standards.

Quality control (QC) samples:

With each sample batch, include at least one blank sample (QC_{bl}, verification cross-contamination/false positives), and one spiked blank sample (QC_{rec}, determination of recovery).

Samples:

For dry milled samples:

- Weigh 2.5 g of dry milled sample to the nearest 0.01 g (5.3), into a 50 ml centrifuge tube (5.1).

For the QC_{rec} sample only: spike a blank sample at this point, add 100 µl mixed mycotoxin solution-S (4.20), this corresponds to 500 µg/kg for DON, and 100 µg/kg for each of the other DON forms.

- Add 7.5 ml of water (4.1) and shake/vortex, ensure that the dry sample is suspended into the water. Proceed to Extraction.

For slurry-mixed samples:

- Use slurry with additional water as indicated in 6.2.2 (Table 2).

For the QC_{rec} sample only: spike a blank sample at this point, add 100 µl mixed mycotoxin solution-S (4.20), this corresponds to 500 µg/kg for DON, and 100 µg/kg each for the other DON forms.

- Proceed to Extraction

Extraction:

- Add 10.0 ml extraction solution 4.17, shake by hand such that no sample material is clogged at the bottom of the centrifuge tube.

- Place the tubes in a mechanical shaker (5.4) and shake for 30 min.

- Open the tube, add 4.0 g of magnesium sulfate (4.5), close the tube, immediately shake for approximately 5 s to avoid formation of lumps of magnesium sulfate. Shake the tube vigorously for approximately 30 s by hand or vortex (5.5).

- Centrifuge the tubes at approximately 3,000 g (5.7) for at least 5 min to aid settlement of particulate matter and phase separation

Preparation of test solution

The acetonitrile extract obtained after extraction is dried with 150 mg MgSO₄/ml extract. Co-extracted fat can be removed by addition of 25 mg C18/ml extract.

- Transfer 7.0 ml of the acetonitrile phase (upper layer) into a clean tube containing 1050 mg magnesium sulfate (4.5) and optionally 175 mg of C18 material (4.8). Shake for approximately 30 s by hand or vortex (5.5). Let settle or centrifuge if necessary to obtain a clear supernatant.

- Transfer an aliquot (V_e) of the supernatant into a clean tube. By default, V_e = 5.0 ml

Note: for higher levels of mycotoxins (e.g. >500 µg/kg), a smaller aliquot V_e may need to be taken in order to keep the response of the analytes within the linear range. This depends on the sensitivity and linearity obtained in the LC-MS/MS analysis.

It is recommended to transfer an additional aliquot from the remaining supernatant (at least 0.5 ml) into a separate tube or vial and store this in the refrigerator to allow for repeat analysis with a smaller V_e if needed.

- Add 100 µl of mixed mycotoxin-label solution (4.19).
- Evaporate to dryness under a stream of nitrogen at approximately 40 °C.
- Reconstitute in 500 µl of a mixture of methanol/water (20/80 v/v), using vortex mixing.

Note: evaporation of 5.0 ml raw extract and subsequent reconstitution in 500 µl methanol/water can result in a (very) turbid extract. Transfer into an eppendorf tube for high speed centrifugation may aid in obtaining a clearer extract.

- Transfer the extract into a filter vial (5.8) and place in the refrigerator for approx. 30 min.
- Press down the filter vial while the extract is still cold.

Note: when $V_e = 5.0$ ml, the final extract contains the equivalent of 2.5 g sample/ml extract.

LC-MS/MS analysis, sequence

- Analyse the sample by LC-MS/MS (see 5.11). Together with the samples, the QC samples and calibration standards in solvent (4.21) are analysed. An example sequence is provided below:

- methanol/water 20/80 v/v
- cal 0 in solvent (4.21)
- cal 1 in solvent (4.21)
- cal 2 in solvent (4.21)
- cal 3 in solvent (4.21)
- cal 4 in solvent (4.21)
- cal 5 in solvent (4.21)
- cal 6 in solvent (4.21)
- cal 7 in solvent (4.21)
- methanol/water 20/80 v/v
- QC_{bl}
- QC_{rec}
- sample_01
- sample_02
-
- Sample_xx
- cal 4

cal = calibration solution

Repeat injection of cal 4 after every 10 injections and end the sequence with injection of cal 4.

13 Annex D. Sample preparation for quantification based on matrix-match standards

Matrix-matched standards:

For preparation of matrix-matched standards, blank sample material of the same matrix as the samples to be analysed is required. The blank extract must be prepared in the same way as the samples, i.e. the amount of matrix equivalent/ml final extract must be the same.

Multiple portions of blank samples may need to be extracted to generate a sufficient volume of blank extract. This depends on the number of matrix-matched calibration standards to be prepared and on the aliquot of supernatant, V_e , used for preparation of the matrix-matched standard (details see below under 'Preparation of test solution' below).

Quality control (QC) samples:

With each sample batch, include at least one blank sample (QC_{bl} , verification cross-contamination/false positives), and one spiked blank sample (QC_{rec} , determination of recovery).

Samples:

For dry milled samples:

- Weigh 2.5 g of dry milled sample to the nearest 0.01 g (5.3), into a 50 ml centrifuge tube (5.1).

For the QC_{rec} sample only: spike a blank sample at this point, add 100 μ l mixed mycotoxin solution-S (4.20), this corresponds to 500 μ g/kg for DON, and 100 μ g/kg for each of the other DON forms.

- Add 7.5 ml of water (4.1) and shake/vortex, ensure that the dry sample is suspended into the water. Proceed to Extraction.

For slurry-mixed samples:

- Use slurry with additional water as indicated in 6.2.2 (Table 2).

For the QC_{rec} sample only: spike a blank sample at this point, add 100 μ l mixed mycotoxin solution-S (4.20), this corresponds to 500 μ g/kg for DON, and 100 μ g/kg each for the other DON forms.

- Proceed to Extraction

Extraction:

- Add 10.0 ml extraction solution 4.17, shake by hand such that no sample material is clogged at the bottom of the centrifuge tube.

- Place the tubes in a mechanical shaker (5.4) and shake for 30 min.

- Open the tube, add 4.0 g of magnesium sulfate (4.5), close the tube, immediately shake for approximately 5 s to avoid formation of lumps of magnesium sulfate. Shake the tube vigorously for approximately 30 s by hand or vortex (5.5).

- Centrifuge the tubes at approximately 3,000 g (5.7) for at least 5 min to aid settlement of particulate matter and phase separation

Preparation of test solution

The acetonitrile extract obtained after extraction is dried with 150 mg MgSO₄/ml extract. Co-extracted fat can be removed by addition of 25 mg C18/ml extract.

- Transfer 7.0 ml of the acetonitrile phase (upper layer) into a clean tube containing 1050 mg magnesium sulfate (4.5) and optionally 175 mg of C18 material (4.8). Shake for approximately 30 s by hand or vortex (5.5). Let settle or centrifuge if necessary to obtain a clear supernatant.

- Transfer an aliquot (V_e) of the supernatant into a clean tube. By default, $V_e = 5.0$ ml

Note: for higher levels of mycotoxins (e.g. >500 µg/kg), a smaller aliquot V_e may need to be taken in order to keep the response of the analytes within the linear range. This depends on the sensitivity and linearity obtained in the LC-MS/MS analysis.

It is recommended to transfer an additional aliquot from the remaining supernatant (at least 0.5 ml) into a separate tube or vial and store this in the refrigerator to allow for repeat analysis with a smaller V_e if needed.

[For preparation of matrix-matched standards: add mixed mycotoxins solution-C (4.18) to extract of blank sample at this point, details see 4.22. In case of 1-point calibration, prepare calibration solution number 4.]

- Evaporate to dryness under a stream of nitrogen at approximately 40 °C.

- Reconstitute in 500 µl of a mixture of methanol/water (20/80 v/v), using vortex mixing.

Note: evaporation of 5.0 ml raw extract and subsequent reconstitution in 500 µl methanol/water can result in a (very) turbid extract. Transfer into an eppendorf tube for high speed centrifugation may aid in obtaining a clearer extract.

- Transfer the extract into a filter vial (5.8) and place in the refrigerator for approx. 30 min.

- Press down the filter vial while the extract is still cold.

Note: when $V_e = 5.0$ ml, the final extract contains the equivalent of 2.5 g sample/ml extract.

LC-MS/MS analysis, sequence

- Analyse the sample by LC-MS/MS (see 5.11). Together with the samples, the QC samples and the calibration standards in matrix (4.21) are analysed. Since matrix effects are normally independent of mycotoxin concentration, it is an option to use solvent standards to assess the linear range of MS/MS detection, and quantify the samples by bracketing 1-point calibration using one matrix-matched standard of intermediate concentration.

Sequence (example)	Alternative option
- methanol/water 20/80 v/v	- methanol/water 20/80 v/v
- cal 0 in matrix (4.22)	- <i>cal 0 in solvent (4.21)</i>
- cal 1 in matrix (4.22)	- <i>cal 1 in solvent (4.21)</i>
- cal 2 in matrix (4.22)	- <i>cal 2 in solvent (4.21)</i>
- cal 3 in matrix (4.22)	- <i>cal 3 in solvent (4.21)</i>
- cal 4 in matrix (4.22)	- <i>cal 4 in solvent (4.21)</i>
- cal 5 in matrix (4.22)	- <i>cal 5 in solvent (4.21)</i>
- cal 6 in matrix (4.22)	- <i>cal 6 in solvent (4.21)</i>
- cal 7 in matrix (4.22)	- <i>cal 7 in solvent (4.21)</i>
- methanol/water 20/80 v/v	- methanol/water 20/80 v/v
- QC _{bl}	- cal 4 in matrix (4.22)
- QC _{rec}	- QC _{bl}
- sample_01	- QC _{rec}
- sample_02	- sample_01
.....	- sample_02
Sample_xx
- cal 4 in matrix (4.22)	Sample_xx
	- cal 4 in matrix (4.22)

Repeat injection of cal 4 in matrix (4.22) after every 5 to 10 injections (depending on stability of the LC-MS/MS response) and end the sequence with injection of cal 4 in matrix (4.22).

14 Annex E. Sample preparation for quantification based on standard addition to the sample extract

Quality control (QC) samples:

With each sample batch, include at least one blank sample (QC_{bl} , verification cross-contamination/false positives), and one spiked blank sample (QC_{rec} , determination of recovery).

Samples:

For dry milled samples:

- Weigh 2.5 g of dry milled sample to the nearest 0.01 g (5.3), into a 50 ml centrifuge tube (5.1).

For the QC_{rec} sample only: spike a blank sample at this point, add 100 μ l mixed mycotoxin solution-S (4.20), this corresponds to 500 μ g/kg for DON, and 100 μ g/kg for each of the other DON forms.

- Add 7.5 ml of water (4.1) and shake/vortex, ensure that the dry sample is suspended into the water. Proceed to Extraction.

For slurry-mixed samples:

- Use slurry with additional water as indicated in 6.2.2 (Table 2).

For the QC_{rec} sample only: spike a blank sample at this point, add 100 μ l mixed mycotoxin solution-S (4.20), this corresponds to 500 μ g/kg for DON, and 100 μ g/kg each for the other DON forms.

- Proceed to Extraction.

Extraction:

- Add 10.0 ml extraction solution 4.17, shake by hand such that no sample material is clogged at the bottom of the centrifuge tube.

- Place the tubes in a mechanical shaker (5.4) and shake for 30 min.

- Open the tube, add 4.0 g of magnesium sulfate (4.5), close the tube, immediately shake for approximately 5 s to avoid formation of lumps of magnesium sulfate. Shake the tube vigorously for approximately 30 s by hand or vortex (5.5).

- Centrifuge the tubes at approximately 3,000 g (5.7) for at least 5 min to aid settlement of particulate matter and phase separation

Preparation of test solution

The acetonitrile extract obtained after extraction is dried with 150 mg $MgSO_4$ /ml extract. Co-extracted fat can be removed by addition of 25 mg C18/ml extract.

- Transfer 7.0 ml of the acetonitrile phase (upper layer) into a clean tube containing 1050 mg magnesium sulfate (4.5) and optionally 175 mg of C18 material (4.8). Shake for approximately 30 s by hand or vortex (5.5). Let settle or centrifuge if necessary to obtain a clear supernatant.

- Transfer two* aliquots (V_e) of the supernatant into two clean tubes, labelled <sample code> and sample code +> . By default, $V_e = 2.5$ ml

** ideally, standard addition is performed with at least two additional levels (i.e. ≥ 3 -point calibration curve). To reduce the volume of extract needed and to reduce the number injections per sample, here a compromise is made and addition is done at only one level.*

Note: the 2.5 ml aliquots taken here (rather than 5.0 ml as done in Annex B and C) is to allow two aliquots to be taken from the same tube. It reduces sensitivity by a factor of 2.

Note: for higher levels of mycotoxins (e.g. $>1000 \mu\text{g}/\text{kg}$), a smaller aliquot V_e may need to be taken in order to keep the response of the analytes within the linear range. This depends on the sensitivity and linearity obtained in the LC-MS/MS analysis. It is recommended to transfer an additional aliquot from the remaining supernatant (at least 1.0 ml) into a separate tube or vial and store this in the refrigerator to allow for repeat analysis with a smaller V_e if needed.

- To the tubes <sample code +>, add 25 μl of mixed mycotoxin solution-S (4.20)
- Evaporate to dryness under a stream of nitrogen at approximately 40 °C.
- Reconstitute in 500 μl of a mixture of methanol/water (20/80 v/v), using vortex mixing.
- Transfer the extract into a filter vial (5.8) and place in the refrigerator for approx. 30 min.
- Press down the filter vial while the extract is still cold.

Note: when $V_e = 2.5 \text{ ml}$, the final extract contains the equivalent of 1.25 g sample/ml extract.

LC-MS/MS analysis, sequence

- Analyse the sample by LC-MS/MS (see 5.11). Together with the samples, the QC samples and calibration standards in solvent (4.21) are analysed. The calibration standards in solvent are included here to (re)establish the linear range of the MS/MS response.

An example sequence is provided below ('+' indicates the extract with standard addition):

- methanol/water 20/80 v/v
- cal 0 in solvent (4.21)
- cal 1 in solvent (4.21)
- cal 2 in solvent (4.21)
- cal 3 in solvent (4.21)
- cal 4 in solvent (4.21)
- cal 5 in solvent (4.21)
- cal 6 in solvent (4.21)
- cal 7 in solvent (4.21)
- methanol/water 20/80 v/v
- QC_{bl}
- QC_{bl} +
- QC_{rec}
- QC_{rec} +
- sample_01
- sample_01+
-
- Sample_xx
- Sample_xx+