

# Determination of 17 Mycotoxins in Cereals and Cereal Based Food Using Liquid Chromatography–Triple Quadrupole Mass Spectrometry

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## Key Words

Commission Regulation 1881/2006/EC, mycotoxin detection and analysis, cereals, LC-MS/MS, UHPLC, UltiMate 3000, TSQ Endura, TraceFinder

## Goal

To evaluate a dilute-and-shoot approach followed by LC-MS/MS for the analysis of the mycotoxins legislated by the EU as well as some additional mycotoxins of current interest.

## Introduction

Mycotoxins are potentially toxic secondary metabolites produced by different species of fungi. The main relevant species occurring on cereals are *Penicillium sp.*, *Fusarium sp.* and *Aspergillus sp.* The fungal contamination can either occur on the field or during storage. Estimations are given that approximately 25% of all crops are contaminated with mycotoxins. The type and amount of contamination varies with climate conditions. As the contamination can result in serious health issues, mycotoxin analysis is an important part of routine food control.<sup>1,2</sup>

For cereals and baby food, the European Union (EU) legislation currently sets maximum levels (ML) for the following mycotoxins: deoxynivalenol, zearalenone, T-2 toxin, HT-2 toxin, fumonisin B<sub>1</sub>, fumonisin B<sub>2</sub>, aflatoxin B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub> and ochratoxin A.<sup>3-6</sup> The European Food Safety Authority (EFSA) is continuously examining the occurrence and health risks of further mycotoxins. One of the main current topics of interest is the acetylated deoxynivalenol metabolites and deoxynivalenol-3-glucoside.<sup>7</sup> For some mycotoxins, e.g. sterigmatocystin, which is related to aflatoxins, EFSA could not determine the risk due to lack of data.<sup>8</sup>



This application note presents a simple and fast multi-mycotoxin method that covers the legislated mycotoxins as well as 3- and 15-acetyl-deoxynivalenol, deoxynivalenol-3-glucoside, nivalenol, its acetyl derivative fusarenol X and sterigmatocystin. The sample preparation follows a simple dilute-and-shoot approach after extraction of cereals and maize with a mixture of acetonitrile/water. The analysis was carried out on a Thermo Scientific™ UltiMate™ 3000 LC system coupled to a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer. The method was evaluated regarding the LOD/LOQ limits, linearity and matrix effects.

## Experimental

### Overview

The workflow is depicted in Figure 1. Blank samples were homogenized and extracted with an acetonitrile (ACN)/water mixture and diluted prior to injection into the LC-MS/MS system. The mycotoxins were identified based on retention time and ion-ratio confirmation using selected reaction monitoring (SRM) of characteristic transition ions. Variations in the ratio up to 20% were accepted. Matrix-matched standard solutions were prepared as well as calibration solutions in solvent to evaluate the performance criteria of the method.

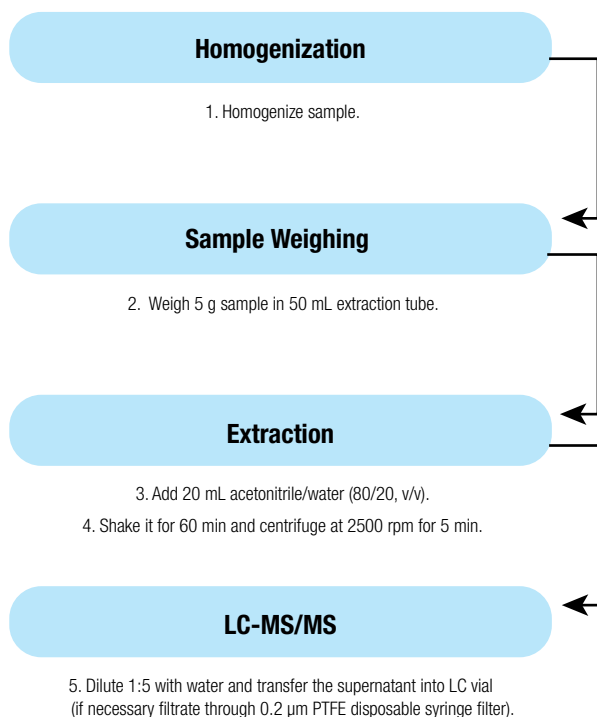


Figure 1. Method overview.

### Method supplies

In Tables 1A through 1D the reagents, apparatus, consumables and glassware used and their source including Thermo Fisher Scientific part numbers, where appropriate, are listed.

Table 1A. Reagents.

Reagents	Part No. (Source)
Acetonitrile, HPLC Grade	A998 (Fisher Scientific)
Ammonium Acetate, 5 M, BioUltra	09691 (Sigma Aldrich)
Acetic Acid, Glacial, HPLC-Grade	A35-500 (Fisher Scientific)
Methanol, Optima™ LC/MS grade	A456 (Fisher Scientific)
Purified Water	Obtained from Thermo Scientific™ Barnstead™ EASYpure™ II water system

Table 1B. Apparatus/instruments.

Apparatus/Instruments	Part No. (Source)
Fisher precision balance	Fisher Scientific
Sartorius analytical balance	ME235S (Fisher Scientific)
Thermo Scientific Barnstead EASYpure II water	Thermo Scientific
Vortex shaker	Fisher Scientific
Vortex universal cap	Fisher Scientific
Horizontal shaker	Fisher Scientific
Horizontal shaker plate	Fisher Scientific
Centrifuge, Thermo Scientific™ Heraeus™ Multifuge™ ×3	Fisher Scientific
WARING® COMMERCIAL Laboratory Blender	Fisher Scientific
TSQ Endura triple quadrupole mass spectrometer	Thermo Scientific
UltiMate 3000 Basic Automated System	Thermo Scientific

Table 1C. Consumables.

Consumables	Part No. (Fisher Scientific)
Thermo Scientific™ Accucore™ C18 LC column 100 × 2.1 mm, 2.6 µm	17126102130
LC vials (amber)	60180560
LC screw caps	60180514
Pipette Thermo Scientific™ FinnpiPETTE™ Novus 100–1000 µL	46200600
Pipette FinnpiPETTE Novus 30–300 µL	46200500
Pipette FinnpiPETTE Novus 1–10 µL	46200100
Pipette FinnpiPETTE Novus 10–100 µL	46200400
Pipette FinnpiPETTE F2 0.5–5 mL	4642100
Pipette FinnpiPETTE F2 1–10 mL	4642110
Pipette holder 6 manual pipettes	9420290
Pipette holder for FinnpiPETTE Novus	9420360
Pipette tips 0.5–250 µL, 500/box	9400250
Pipette tips 5–300 µL, 10 × 96/rack	9401250
Pipette tips 0.5–5 mL, 75/box	9402050
Pipette tips 100–1000 µL, 200/box	9401070
Pipette tips 1–10 mL, 40/box	9402150
Pipette tips 0.2–10 µL 10 x 96/rack	9400300
Pipette Pasteur soda lime glass 150 mm	
Pipette suction device	
Spatula, 18/8 steel	
Vial rack (2 mL)	
Centrifuge tube rack	
Polypropylene (PP) tubes tubes, 50 mL, 250 pack	
Disposable syringe filters (0.2 µm, PTFE)	

Glassware (Fisher Scientific)
Volumetric flask, 1 mL
Volumetric flask, 2 mL
4 mL screw cap vial
Caps for 4 mL screw cap vial
1000 mL bottle
Volumetric pipette, 20 mL

The following mycotoxin standards were purchased from Fluka:

- Zearalenone, fusarenon X, deoxynivalenol, HT-2 toxin, T-2 toxin: 100 µg/mL in ACN Doxynivalenol-3-glucoside: 50 µg/mL in ACN
- Mixture of fumonisin B<sub>1</sub>+B<sub>2</sub>: 50 µg/mL in ACN/water each, trichothecene B mixture containing deoxynivalenol, nivalenol, 3-acetyldeoxynivalenol, 15-acetyl-deoxynivalenol: 100 µg/mL in ACN each

The following standards were purchased from Romer Labs:

- Nivalenol: 100 µg/mL in ACN
- Sterigmatocystin: 50 µg/mL in ACN

The following standards were purchased from Supelco:

- Ochratoxin A: 50 µg/mL in benzene/acetic acid
- Aflatoxin mix, containing 1 µg/mL aflatoxin B<sub>1</sub> and G<sub>1</sub> and 0.3 µg/mL aflatoxin B<sub>2</sub>, and G<sub>2</sub> in methanol

### Sample preparation

Blank matrix samples wheat and popcorn maize used for spiking experiments were purchased in local retail stores. 250 g of the cereals were ground and homogenized using a WARING® COMMERCIAL Laboratory Blender. The extraction was performed using the following steps:

- 1) Weigh 10 g sample into a 50 mL PP tube.
- 2) Add 20 mL acetonitrile/water (80/20, v/v).
- 3) Shake samples for 60 min on a horizontal shaker (300 rpm) then centrifuge at 2500 rpm for 5 min.
- 4) Dilute supernatant 1:5 with water then transfer 1 mL into an LC vial for instrumental analysis. Filtration through a disposable syringe filter (0.2 µm PTFE) is strongly recommended.

The blank matrix extracts were used for the preparation of matrix-matched calibration standards. It was not necessary to use isotopically labeled standards for the quantitation of analytes

### LC-MS/MS analysis

The LC-MS/MS analysis was carried out using an UltiMate 3000 LC system coupled to a TSQ Endura LC-MS/MS triple quadrupole mass spectrometer. TraceFinder software (version 3.2) was used for instrument control, analysis, data review, and reporting. The LC conditions and gradient were as follows.

### LC Conditions

Injection volume	10 µL																								
Column temperature	40 °C																								
Flow rate	400 µL/min																								
Analytical column	Accucore C <sub>18</sub> column, 100 × 2.1 mm, 2.6 µm																								
Run time	12 minutes																								
Tray temperature	10 °C																								
Needle cleaning solvent	50% methanol in water																								
Sample loop	100 µL																								
Mobile phases	A: Water/methanol (98/2) with 5 mM ammonium acetate and 1% acetic acid B: Water/methanol (2/98) with 5 mM ammonium acetate and 1% acetic acid																								
LC gradient	<table border="1"> <thead> <tr> <th>Time [min]</th> <th>Flow [mL/min]</th> <th>A%</th> <th>B%</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>0.400</td> <td>95</td> <td>5</td> </tr> <tr> <td>0.5</td> <td>0.400</td> <td>95</td> <td>5</td> </tr> <tr> <td>3</td> <td>0.400</td> <td>50</td> <td>50</td> </tr> <tr> <td>6</td> <td>0.400</td> <td>100</td> <td>0</td> </tr> <tr> <td>6.1</td> <td>0.600</td> <td>100</td> <td>0</td> </tr> </tbody> </table>	Time [min]	Flow [mL/min]	A%	B%	0	0.400	95	5	0.5	0.400	95	5	3	0.400	50	50	6	0.400	100	0	6.1	0.600	100	0
Time [min]	Flow [mL/min]	A%	B%																						
0	0.400	95	5																						
0.5	0.400	95	5																						
3	0.400	50	50																						
6	0.400	100	0																						
6.1	0.600	100	0																						

The TSQ Endura triple quadrupole mass spectrometer was operated in timed-SRM mode. Therefore, all SRM traces (parent, qualifier, quantifier ion) were individually tuned for each target analyte by direct infusion of a working standard solution (100 ng/mL) into the solvent flow of 50% A using a syringe pump. The mass spectrometer settings were as follows. The optimized SRM-transitions are summarized in Table 2.

### MS Settings

Ionization mode	Heated Electrospray (HESI)
Scan type	Timed-SRM
Polarity	Positive/Negative switching
Spray voltage for positive mode	3500 V
Spray voltage for negative mode	2000 V
Sheath gas pressure	35 arbitrary units (Arb)
Aux gas pressure	11 Arb
Sweep gas pressure	1 Arb
Ion transfer tube temperature	375 °C
Vaporizer temperature	375 °C
CID gas pressure	2 mTorr
Cycle time	0.3 s
Q1 resolution (FWHM)	0.7
Q3 resolution (FWHM)	0.7
Chrom filter	3 s

Table 2. SRM-transitions and ion ratios for different analytes and matrices.

Analyte	RT (Window 1 min)	Precursor Ion	Quantifier/ Qualifier Ion	CE (V)	RF (V)	Ion Ratio		
						Solvent	Wheat	Maize
Nivalenol [M+Ac] <sup>-</sup>	1.46	371.1	281.2/311.1	16/10	86	0.80	0.67	0.61
Deoxynivalenol [M+Ac] <sup>-</sup>	2.35	355.2	295.2/265.1	11/16	91	0.40	0.37	0.53
Deoxynivalenol-3-glucoside [M+Ac] <sup>-</sup>	2.47	517.2	427.2/457.1	24/16	145	1	0.93	0.98
Fusarenon X [M+Ac] <sup>-</sup>	3.01	413.1	352.8/263.1	11/20	92	0.30	0.28	0.30
3-Acetyl-deoxynivalenol [M+Ac] <sup>-</sup>	3.57	397.1	307.1/337.5	16/8	84	0.68	0.66	0.75
15-Acetyl-deoxynivalenol [M+H] <sup>+</sup>	3.57	339.1	321.1/137.2	11/25	101	0.19	0.19	0.19
Aflatoxin B <sub>1</sub> [M+H] <sup>+</sup>	4.25	313.0	285.0/241.1	26/41	143	0.78	0.86	0.79
Aflatoxin B <sub>2</sub> [M+H] <sup>+</sup>	4.11	315.1	287.1/259.1	24/33	143	0.61	0.66	0.72
Aflatoxin G <sub>1</sub> [M+H] <sup>+</sup>	3.96	329.1	243.1/283.1/ 200.0	28/26/44	130	0.49/0.52	0.51/0.65	0.49/0.63
Aflatoxin G <sub>2</sub> [M+H] <sup>+</sup>	3.80	331.1	245.1/189.1/ 275.1	32/51/29	186	0.33/0.39	0.38/0.40	0.35/0.38
Ochratoxin A [M+H] <sup>+</sup>	5.38	404.1	238.9/357.9/ 341.1	24/15/20	145	0.73/0.25	0.77/0.28	0.81/0.27
Zearalenone [M+H] <sup>+</sup>	5.45	317.1	175.1/272.9	24/20	165	0.78	0.87	0.94
Sterigmatocystin [M+H] <sup>+</sup>	5.59	325.1	281.0/310.0/ 282.1	37/26/30	167	0.85/0.11	0.85/0.11	0.89/0.12
Fumonisin B <sub>1</sub> [M+H] <sup>+</sup>	5.02	722.4	334.2/352.3/ 704.4	38/35/28	229	0.98/0.72	0.90/1	0.98/0.85
Fumonisin B <sub>2</sub> [M+H] <sup>+</sup>	5.62	706.4	336.3/318.4	41/38	237	0.49	0.46	0.47
HT-2 toxin [M+Na] <sup>+</sup>	4.93	447.2	345.1/285.1	18/19	149	0.70	0.64	0.68
T-2 toxin [M+Na] <sup>+</sup>	5.26	489.2	387.1/327.1	21/23	178	1	0.92	0.95

## Results and Discussion

For the evaluation of the method performance, wheat and maize matrices were chosen. Figure 2 shows a typical chromatogram of spiked wheat matrix. Except for nivalenol, all analytes show symmetrical peaks.

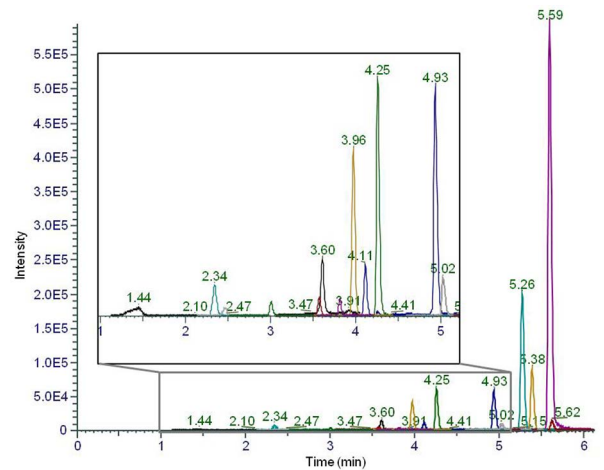


Figure 2. Example chromatogram of wheat matrix spiked with 1 mg/kg deoxynivalenol and nivalenol, 200 µg/kg aflatoxin B<sub>1</sub> and G<sub>1</sub>, 60 µg/kg aflatoxin B<sub>2</sub> and G<sub>2</sub>, and 500 µg/kg of all other analytes. (See Table 2 for analyte list). Chromatogram from Thermo Scientific™ FreeStyle™ software version 1.0.

The method parameters that were characterized were linearity, LOD/LOQ, injection precision and matrix effects.

The LOD/LOQ values that were determined in solvent, wheat and maize matrix were compared to the EU legislated values and are suitable for all analytes to monitor the contamination level in cereals.<sup>3-6</sup> In case of baby food, an improvement of the method sensitivity using sample clean up is recommended to achieve more sensitive LOQs for aflatoxins and ochratoxin A (Table 3).

Matrix effects can severely influence the accurate quantitation in LC-MS/MS and to address this issue several options exist.

- (I) Calibration using matrix matched standards
- (II) Isotope dilution using isotopically labeled analogues as internal standards
- (III) Standard addition
- (IV) Dilution of sample before injection to minimize matrix influence on the signal

Table 3. Method performance: LODs and LOQs ( $\mu\text{g}/\text{kg}$ ) in solvent (calculated as  $\mu\text{g}/\text{kg}$ ) and wheat and maize matrix compared to ML from EU legislation.<sup>3-6</sup> Blank contamination: blank matrix was already contaminated with this mycotoxin, thus no LOD/LOQ could be determined.

Analyte	LOD/LOQ			Maximum Limit Defined by EC 1881/2006
	Solvent ( $\mu\text{g}/\text{kg}$ )	Wheat ( $\mu\text{g}/\text{kg}$ )	Maize ( $\mu\text{g}/\text{kg}$ )	Cereals ( $\mu\text{g}/\text{kg}$ )
Nivalenol	10/20	200/400	200/400	No limit
Deoxynivalenol	10/20	10/20	10/20	750
Deoxynivalenol-3-glucoside	5/10	20/100	100/200	No limit
Fusarenon X	15/20	20/100	20/100	No limit
3-Acetyl-deoxynivalenol	15/20	15/20	10/20	No limit
15-Acetyl-deoxynivalenol	15/20	20/100	20/100	No limit
Aflatoxin B <sub>1</sub>	0.04/0.2	0.1/0.4	0.2/0.4	2
Aflatoxin B <sub>2</sub>	0.09/0.3	0.6/0.6	0.3/0.6	4 (sum of 4)
Aflatoxin G <sub>1</sub>	0.1/0.2	0.2/0.4	0.4/1	4 (sum of 4)
Aflatoxin G <sub>2</sub>	0.6/0.9	0.9/1.2	1.2/6	4 (sum of 4)
Ochratoxin A	1/1.5	0.5/1	0.5/1	3
Zearalenone	1/2	1/1.5	1/1.5	75
Sterigmatocystin	0.2/0.5	Blank contam.	0.15/0.5	No limit
Fumonisin B <sub>1</sub>	1.5/5	2/5	Blank contam.	1000 (sum)
Fumonisin B <sub>2</sub>	1.5/5	1/1.5	Blank contam.	1000 (sum)
HT-2 toxin	0.5/1	1/1.5	0.5/1	50 (sum + T-2)
T-2 toxin	0.5/1	0.5/1	0.5/1	50 (sum + HT-2)

In our measurements, matrix effects were estimated by comparing the slope of the calibration curve in solvent and matrix (see Table 4). Even after dilution of extract, most analytes showed medium to high matrix effects. The matrix effects in maize are generally higher than in wheat. Thus, for this approach a matrix matched calibration for quantitation is still highly recommended. The linearity was reviewed for solvent as well as for the two matrices.

The values are summarized in Table 4 and show good linearity ( $>0.99$ ) for most analytes in solvent and matrix.

The injection precision was determined by six replicate injections of a standard in solvent, wheat and maize. The relative standard deviation (RSD) in % is summarized in Table 5.

Table 4. Linear range, correlation coefficient, and matrix effect.

Analyte	Solvent		Wheat			Maize		
	Linear range (µg/kg)	R <sup>2</sup>	Linear range (µg/kg)	R <sup>2</sup>	Matrix effect (%)	Linear range (µg/kg)	R <sup>2</sup>	Matrix effect (%)
Nivalenol	20–2000	0.9993	400–4000	0.9920	51	400–4000	0.9887	118
Deoxynivalenol	20–4000	0.9813	20–2000	0.9982	17*	20–4000	0.9981	24*
Deoxynivalenol-3-glucoside	10–1000	0.9980	100–1000	0.9687	28	200–2000	0.9951	52
Fusarenol X	20–2000	0.9957	100–2000	0.9987	5	100–2000	0.9988	5
3-Acetyl-deoxynivalenol	20–2000	0.9966	20–2000	0.9964	14	20–2000	0.9985	30
15-Acetyl-deoxynivalenol	20–2000	0.9968	100–2000	0.9985	13	100–2000	0.9989	33
Aflatoxin B <sub>1</sub>	0.2–40	0.9994	0.4–40	0.9956	24	0.4–200	0.9982	64
Aflatoxin B <sub>2</sub>	0.3–60	0.9987	0.6–30	0.9918	26	0.6–60	0.9890	62
Aflatoxin G <sub>1</sub>	0.2–40	0.9970	0.4–40	0.9956	27	1–200	0.9977	55
Aflatoxin G <sub>2</sub>	0.9–30	0.9917	1.2–60	0.9911	22	6–120	0.9919	44
Ochratoxin A	1.5–200	0.9978	1–200	0.9964	5	1–500	0.9988	2
Zearalenone	1.5–200	0.9985	1.5–200	0.9937	14	1.5–2000	0.9992	4*
Sterigmatocystin	0.5–200	0.9993	0.5–1000	0.9980	4*	0.5–1000	0.9973	5
Fumonisin B <sub>1</sub>	5–1000	0.9937	5–500	0.9843	5*	2–2000	0.9977	55*
Fumonisin B <sub>2</sub>	2–2000	0.9919	1.5–200	0.9943	29*	2–2000	0.9975	53*
HT-2 toxin	1–200	0.9984	1.5–1000	0.9978	17	1–200	0.9993	80*
T-2 toxin	1–500	0.9983	1–100	0.9964	19*	1–200	0.9993	60*

Table 5. Relative standard deviation (RSD) in % from six replicate injections of a standard in solvent, wheat and maize. Level 1/Level 2 contain 5/200 µg/kg of the analyte (except deoxynivalenol, nivalenol: 10/400 µg/kg, aflatoxin B<sub>1</sub>, G<sub>1</sub>: 1/40 µg/kg, aflatoxin B<sub>2</sub>, G<sub>2</sub>: 0.3/12 µg/kg).

Analyte	Solvent		Wheat		Maize	
	Level 1 RSD (%)	Level 2 RSD (%)	Level 1 RSD (%)	Level 2 RSD (%)	Level 1 RSD (%)	Level 2 RSD (%)
Nivalenol	*	3.6	*	7.3	*	19.7
Deoxynivalenol	*	9.5	*	2.6	*	3.6
Deoxynivalenol-3-glucoside	*	4.4	*	6.8	*	17.1
Fusarenol X	*	7.1	*	7.5	*	7.2
3-Acetyl-deoxynivalenol	*	9.7	*	5.5	*	11.9
15-Acetyl-deoxynivalenol	*	4.6	*	7.5	*	5.5
Aflatoxin B <sub>1</sub>	11.2	1.8	15.7	2	17.9	3.5
Aflatoxin B <sub>2</sub>	15.3	2.4	*	5.2	*	6
Aflatoxin G <sub>1</sub>	12.8	4.5	22.6	4.7	31.4	4
Aflatoxin G <sub>2</sub>	*	9.4	*	16.1	*	11.4
Ochratoxin A	15.6	4	14.4	2.6	22.6	3.6
Zearalenone	7.4	2.5	11.6	2.5	8.5	2.5
Sterigmatocystin	9.7	1.6	4.7	3.9	7.1	6.2
Fumonisin B <sub>1</sub>	15.1	7.6	21.2	12.3	22.3	7.6
Fumonisin B <sub>2</sub>	15.4	6.1	29.7	2.5	22.7	4.1
HT-2 toxin	15.5	3.6	20.8	2.8	16.3	2.2
T-2 toxin	14.2	4.7	6.5	4.6	18.2	0.7

\*Level below LOD/LOQ.



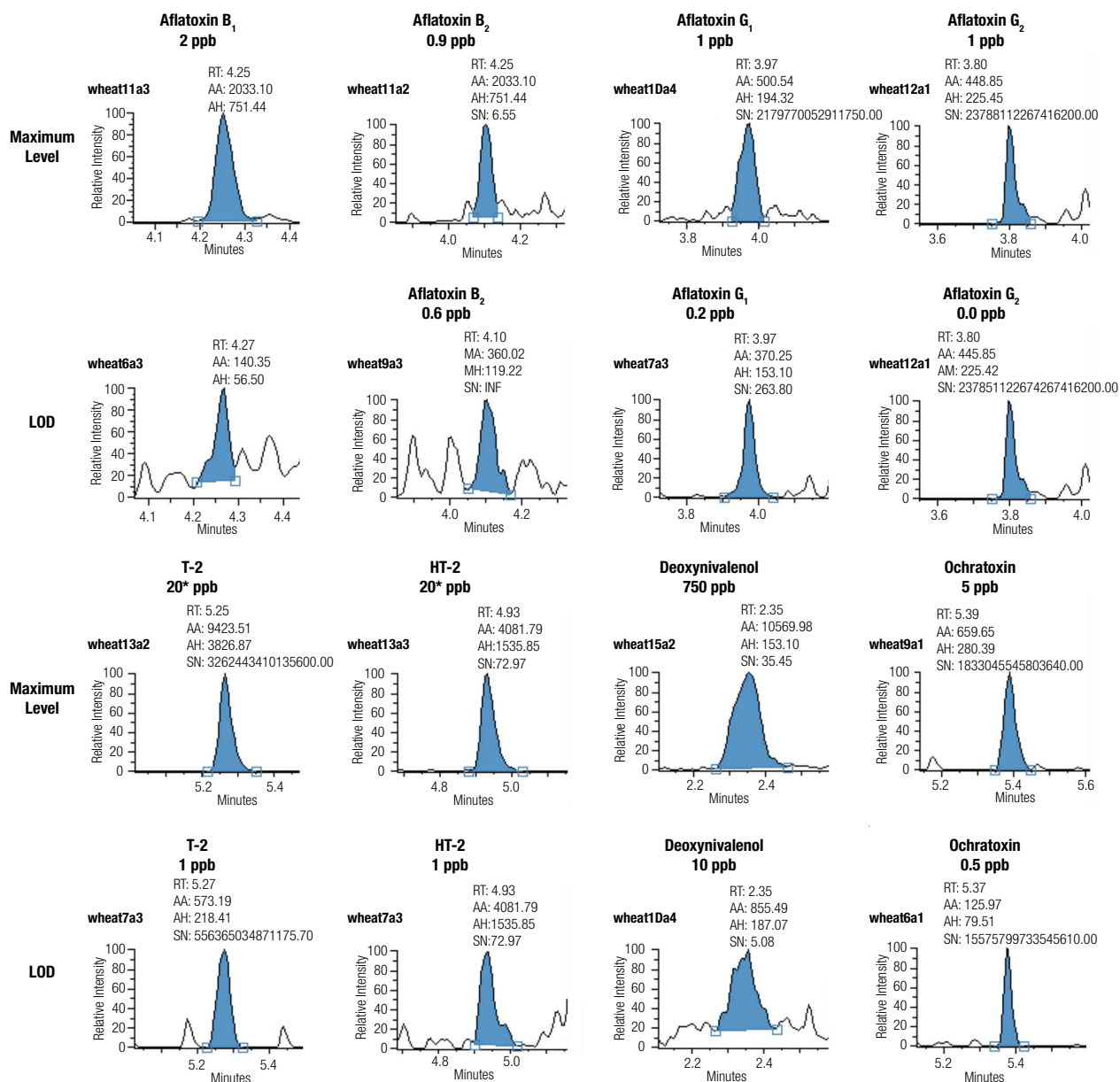


Figure 3. Examples of extracted ion chromatograms for main mycotoxins at LOD and maximum level concentrations in wheat extracts.

## Conclusion

The method is suitable and recommended for the analysis of 17 mycotoxins in cereals and cereal based foods. It enables quantitation that is compliant with the ML set by the EU for mycotoxins in cereals. With exception of aflatoxin B<sub>1</sub> the same method could be applied for analysis of baby food samples.

If compliance with baby food regulations is required, additional sample cleanup using solid phase extraction (SPE) and/or the use of a more sensitive mass spectrometer such as the Thermo Scientific™ TSQ Quantiva™ MS is recommended to obtain lower LODs.

Matrix dilution prior to the injection improves overall system robustness and significantly extends the intervals between necessary system maintenance.

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