Food & Environmental

Simultaneous Analysis of 10 Mycotoxins in Crude Extracts of Different Types of Grains by LC-MS/MS

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Overview

This application note presents a validated LC-MS/MS method to detect 9 Fusarium toxins and Ochratoxin A in diluted crude extracts of grain. The developed sample preparation procedure is quick, easy, robust, and inexpensive. The LC-MS/MS method in Multiple Reaction Monitoring (MRM) detects all compounds in a single run with Limits of Quantitation (LOQ) between 0.3 µg/kg and 10 µg/kg. The complete method was validated for the analysis of wheat, rye, barley, and oat samples.

Introduction

Mycotoxins are known to harm the health of humans and animals. They are known either as carcinogenic or cytotoxic and impair the immune system. Therefore, different countries have set regulations on mycotoxins. In the EU, mycotoxin limits are harmonized in the regulation for contaminants in foodstuffs (EC 1881/2006 of December 19, 2006) and the amended regulation (EC 1126/2007 of September 28, 2007). The focus of the legislation and the European monitoring program is on Fusarium toxins like Deoxynivalenol, Zearalenon, HT-2 and T-2 toxins because of their frequent and increasing occurrence in grain. In addition, the European Commission has decided to set maximum levels for T-2 and HT-2 toxin by July 1, 2008. The expected limit for the sum of both Fusarium toxins could be 50 µg/kg or less.[1-3]

Cereals and grains are often contaminated with Ochratoxin A and Fusarium toxins. Fusarium toxins can be found in all types of grains such as wheat, rye, maize, barley (malt), and oats. The contamination is dependent on climate conditions during growth, harvest, and storage. Because of bad weather conditions in 2007 high contaminations of Deoxynivalenol (DON) were found in wheat and T-2 and HT-2 toxin in wheat and oats.

Due to this, it is necessary to have a reliable, sensitive, robust, and fast method to analyze a high number of mycotoxins in grain. Methods used so far have not shown the required sensitivity. Thus necessary clean up steps with immunoaffinity columns have resulted in time consuming and expensive methods.

A method for the detection of 9 Fusarium toxins: DON, Zearalenon (ZON), 3-Acetyldeoxynivalenol (3- AcDON), 15-Acetyldeoxynivalenol (15-AcDON), HT-2, T-2, Fusarenon X (FUS X), Nivalenol (NIV), Diacetoxyscirpenol (DAS) and Ochratoxin A (OTA) was developed (Figure 1). Diluted crude extract were analyzed using Liquid Chromatography and tandem Mass Spectrometry (LC-MS/MS) in a single run on an API 4000™ LC/MS/MS system. No time consuming and possibly expensive sample preparation is needed. The method was validated for wheat, rye, barley and oat and applied for the analysis approximately 220 grain samples. The LOQ vary between 0.3 µg/kg and 10 µg/kg depending on the compound.

Method Details

Sample Preparation:

1) Mill and homogenize 10 g of grain sample.
2) Add 40 mL of acetonitrile/water (84/16).
3) Extract by mixing for 90 min (220 rpm).
4) Filter through Whatman S&S 1573 ½.
5) Dilute filtrate 1:10 with water + 5mM ammonium acetate.
6) Inject 100 µL into LC/MS/MS.

HPLC Conditions:

A Shimadzu Prominence LC system consisting of system controller, two pumps, degasser, autosampler, and column oven was used. Separation was performed on an Agilent ZORBAX Eclipse XDB C18, 100x4.6 mm (1.8 µm) column. The column oven temperature was set to 40°C. A gradient of eluent A: water + 5 mM ammonium acetate and eluent B: methanol + 5 mM ammonium acetate was used at a flow rate of 500 µL/min. Details of the gradient are given in Table 1. The injection volume was set to 100 µL.

MS/MS Conditions:

An API 4000™ LC/MS/MS system equipped with Turbo V™ source and Electrospray Ionization (ESI) probe was used. The method contained three periods with alternating polarities (0.0-7.6 min negative; 7.6-9.2 min positive; 9.2-16.0 min negative).

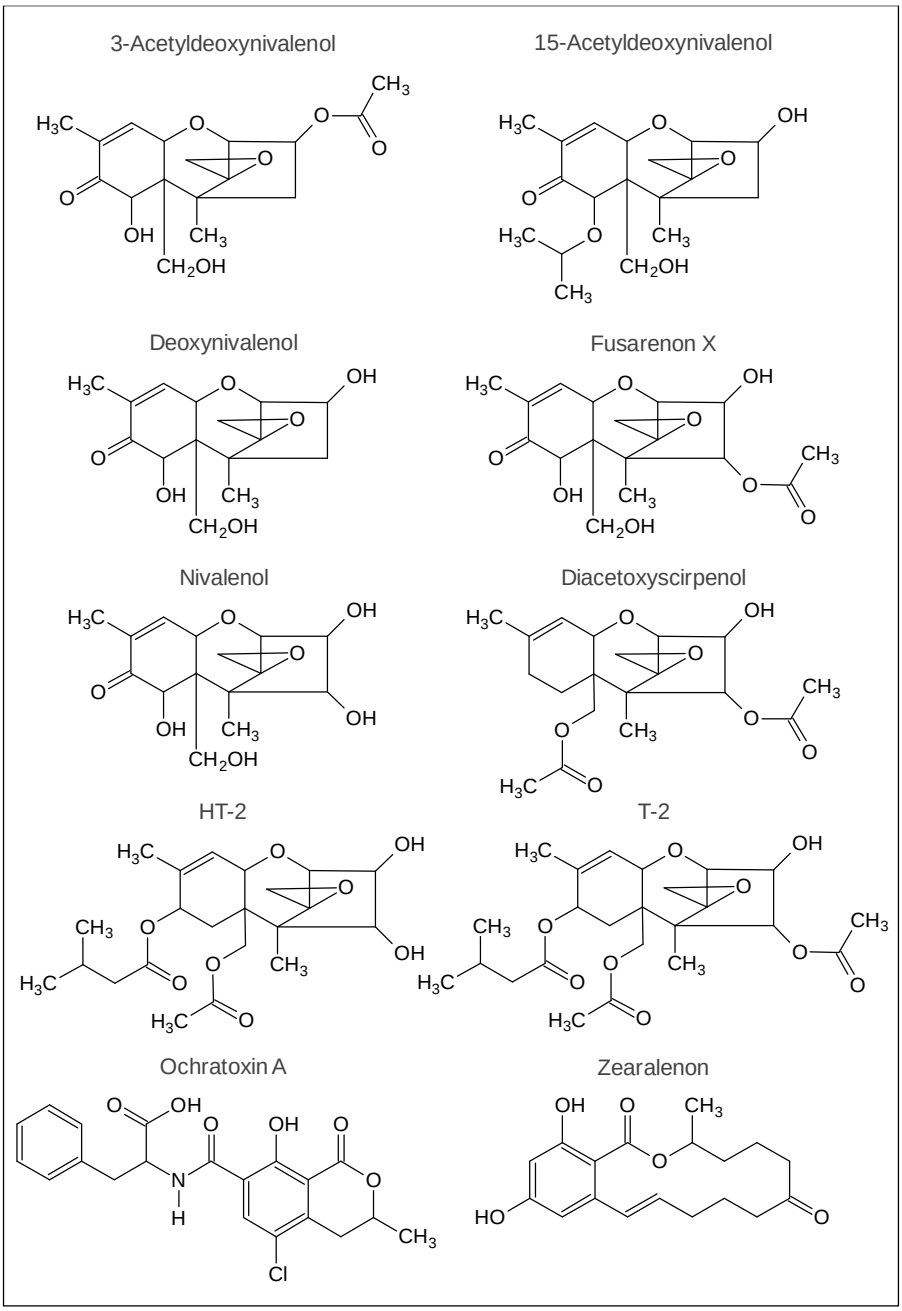

Figure 1. Priority mycotoxins analyzed by LC-MS/MS

The following ion source parameters were used: Temperature 600°C, Curtain Gas 25 psi, Gas1 50 psi, Gas2 70 psi, CAD Gas 6 (positive) or 10 (negative), and IS voltage +5000 V or -4000 V, respectively. All mycotoxins were detected using two MRM transitions in the following order: first period NIV, DON, FUS X, AcDON; second period DAS, OTA, HT-2, T-2; and third period ZON. Since 3-AcDON and 15-AcDON were not separated chromatographically, only compound specific transitions were used for detection. The used MRM transitions are listed in Table 2 and product ion spectra of 3-AcDON and 15-AcDON are shown in Figure 2.

Table 1. LC gradient to separate mycotoxins

Step	Time (min)	A (%)	B (%)
0	0.0	80	20
1	0.5	80	20
2	5.5	10	90
3	15	10	90
4	16	80	20

Table 2. MRM transitions to detect mycotoxins

Mycotoxin	Precursor Ion	MRM 1	MRM 2
3-AcDON	$[M-H]^-$	337/307	337/173
15-AcDON	$[M-H]^-$	337/219	337/150
DON	$[M-H]^-$	295/265	295/138
FUS X	$[M+CH_3COOH]^-$	413/353	413/263
NIV	$M+CH_3COOH]^-/[M-H]^-$	371/281	311/281
DAS	$M+H]^+$	384/307	384/105
OTA	$M+H]^+$	404/239	404/358
HT-2	$M+Na]^+$	447/345	447/285
T-2	$M+NH_4]^+$	484/215	484/185
ZON	$[M-H]^-$	317/131	317/175
3-AcDON	$[M-H]^-$	337/307	337/173

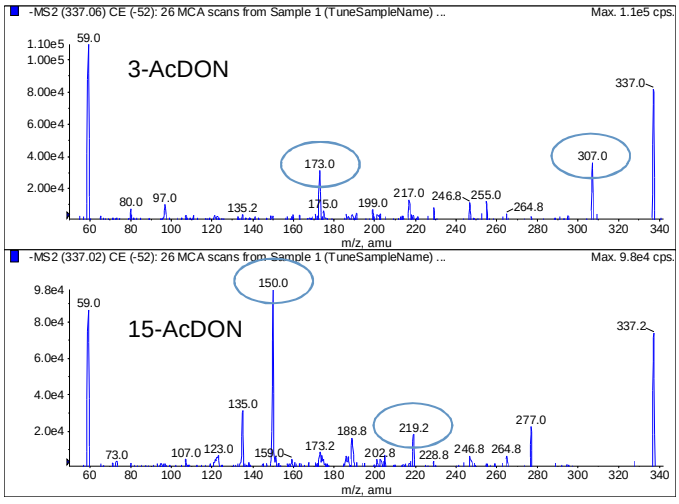

Figure 2. Product ion spectra of 3-AcDON and 15-AcDON (the in MRM detected product ions are highlighted)

Results and Discussion

Figure 3 shows a standard chromatogram of 9 Fusarium toxins at 50 µg/kg and Ochratoxin A at 10 µg/kg. During the evaluation it was shown that the sensitivity of the MRM transitions depends on the quality of the used solvents as well as on the analyzed matrices.

AcDON, DON, FUS X and NIV generally show good sensitivity for both the $[M+CH_3COO]^-$ and the $[M-H]^-$. However, in matrix samples the in Table 2 listed MRM transitions were used for better S/N, reproducibility, and recovery.

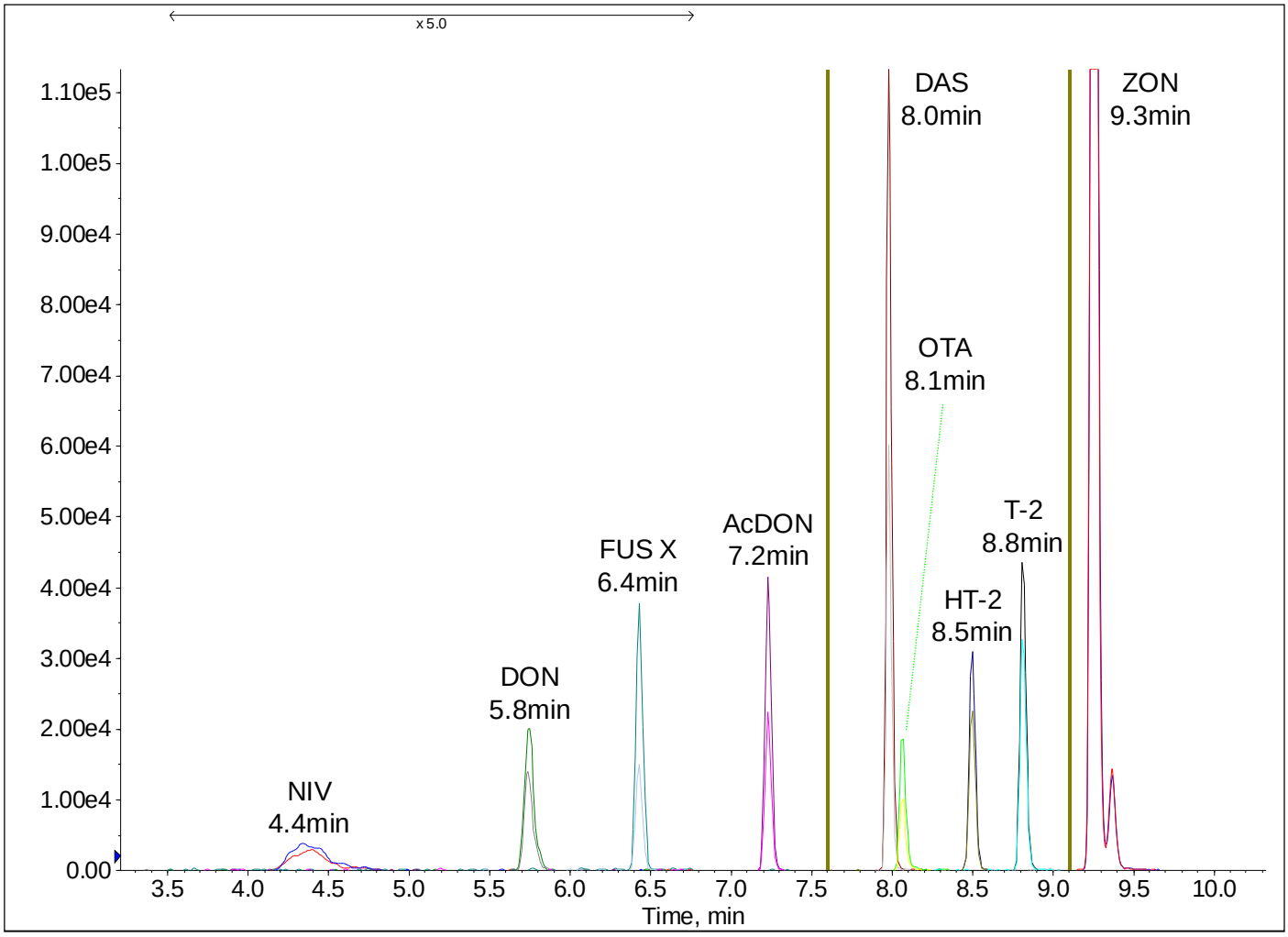

Figure 3. Mycotoxin standard analyzed using LC-MS/MS in MRM

A special characteristic of ionization was found for HT-2. The sensitivity of the MRMs either $[M+NH_4]^+$ or $[M+Na]^+$ differ in accordance to the analyzed matrices (Figure 4).

The LOQ were validated as 0.3 µg/kg for OTA, 5 µg/kg for HT-2, T-2 and ZON, and as 10µg/kg for AcDON, DON, FUS X, DAS and NIV.

The injection volume of 100 µL with a ten times diluted sample showed much better LOQ than 25 µL of a direct injected or 50 µL of a 1/5 diluted sample. Responsible for this finding are the specific initial chromatographic conditions needed for NIV. The acetonitrile in the sample, at the end of the extraction procedure, caused peak broadening for NIV. This could only be eliminated by diluting the sample in 100% water +5 mM ammonium acetate by a factor of 10.

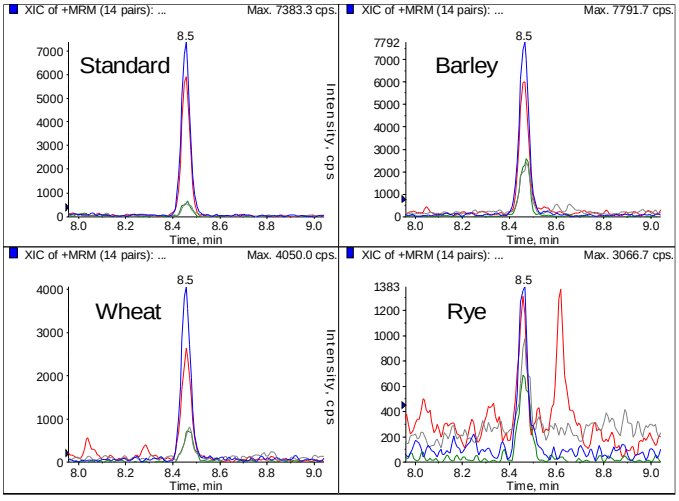

Figure 4. Comparison of MRM intensities of HT-2 in different matrices ($[M+Na]^+$: 447/345 blue, 447/285 red, $[M+NH_4]^+$: 442/263 green, 442/105 grey)

Table 3. Limits of Quantitation (LOQ) and linear dynamic range of detected mycotoxins

Mycotoxin	LOQ (µg/kg)	Linear Range (µg/kg)	EU MRL#
3-AcDON	10	400	(1)
15-AcDON	10	150	(1)
DON	10	10000	1750*; 1250** (2)
FUS X	10	2000	(1)
NIV	10	4000	(1)
DAS	10	400	(1)
OTA	0.3	> 10	5***
HT-2	5	200	(2)
T-2	5	1000	(2)
ZON	5	80	100*** (2)

Footnotes to Table 3:

\# EC 1881/2006 and the amended EC 1126/2007

* Unprocessed durum wheat and oats

** Unprocessed cereals other than durum wheat and oats

*** Unprocessed cereals

(1) Due to co-occurrences and as “generally low” considered levels no MRL was estimated

(2) Appropriateness of setting a maximum level should be considered by 1 July 2008

The calibration curves of all compounds were linear ranges differ highly. LOQ and upper ends of the linear dynamic range of all detected mycotoxins are shown in Table 3.

The recoveries were determined for ach mycotoxin in each matrix compared to the calibration curves without matrix (Table 4-6).

It was shown that the solutions of the extracted grains are stable over 36 hours under cool conditions (4°C).

A large carryover of OTA in the injection port was observed when injecting high standard concentrations, thus solvent blanks were injected after standard injections.

With the here presented validated method about 220 grain samples of the new harvest have been analyzed since July 2007.

The presented data are based on the European Grain Monitoring Program (EGM) and selected data are shown in Tables 4 to 6.

Table 4. Results in wheat / durum

Mycotoxin	n	LOQ (µg/kg)	Below LOQ	Above LOQ	Above EU limit	Recovery (%)
DON	175	10	33	13	66	100
ZON	169	5	138	29	2	60
NIV	168	10	101	67	-	100
T-2	168	5	166	2	-	85
HT-2	163	5	130	33	-	25
OTA	165	0.3	159	6	0	90

Table 5. Results in barley

Mycotoxin	n	LOQ (µg/kg)	Below LOQ	Above LOQ	Above EU limit	Recovery (%)
DON	21	10	5	16	0	82
ZON	20	5	16	4	0	40
NIV	21	10	7	14	-	100
T-2	21	5	10	11	-	100
HT-2	21	5	4	17	-	21
OTA	22	0.3	2	0	0	93

Table 6. Results in rye

Mycotoxin	n	LOQ (µg/kg)	Below LOQ	Above LOQ	Above EU limit	Recovery (%)
DON	25	10	12	12	1	98
ZON	25	5	22	2	1	45
NIV	24	10	22	2	-	100
T-2	24	5	24	0	-	88
HT-2	25	5	24	1	-	85
OTA	25	0.3	23	2	0	100

Summary

The developed method is appropriate for the analysis of 9 Fusarium toxins and OTA in one single LC-MS/MS run without time consuming sample preparation/enrichment. The LOQ were found at 0.3 µg/kg for OTA, 5 µg/kg for HT-2, T-2 and ZON and at 10 µg/kg for AcDON, DON, FUS X, DAS and NIV and meet the National and European law required detection limits. Recoveries were determined in the range of 21 to 100%.

References

1 EC 1881/2006 of December 19, 2006
2 Amended regulation EC 1126/2007 of September 28, 2007
3 http://www.mykotoxin.de/Gesetzgebung.htm

Publication number: 1121410-01

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