Analysis of Fusarium Toxins using LC/MS–MS: Application to Various Food and Feed Matrices

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Co-occurrence of several mycotoxins (deoxynivalenol, zearalenone, T-2-toxin, HT-2 toxin) produced by field fungi, such as *Fusarium graminearum* and *Fusarium culmorum*, requires several analysis methods for their characterization. A reliable method for the determination of type A- and B-trichothecenes and zearalenone in cereal-based samples is presented. To achieve optimal mass spectrometric detection, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were compared. Best results were obtained with ESI by implementing a two-period switching for the ionization polarity. The limit of quantification differs for each individual substance within the range 1–10 ppb. Mean recoveries using a standardized clean-up procedure ranged from 54–93%.

Introduction

Fusarium fungi are capable of producing, to a variable degree, two or more toxins. The major Fusarium mycotoxins are fumonisins, A- and B-trichothecenes, and zearalenone (ZON).[1] Trichothecenes are responsible for a wide range of toxicity in animals, including feed refusal, weight loss and vomiting. In particular deoxynivalenol (DON) can inhibit protein biosynthesis and has been reported as an immunosuppressant.[2] In order to reduce the levels of biogenic toxins, European authorities are currently discussing further regulations on mycotoxins. Within the European Union (EU), harmonized legislation is setting maximum limits for aflatoxins and ochratoxin A in cereals and cereal products. Limits for

Fusarium toxins (DON, ZEA, HT2, T2) are currently being drafted in EU member states; for example, maximum limits for Fusarium toxins (DON 100–500 µg/kg, ZON 20–50 µg/kg) were established in February 2004.[3]

The most common hyphenated methods for the determination of A- and B-trichothecenes include gas chromatography–electron capture detection, gas chromatography–mass spectrometry (MS) or liquid chromatography (LC)–postcolumn derivatization and fluorescence detection.[4] Increasingly, LC–tandem MS (MS–MS) has been applied to mycotoxin analysis despite higher costs and the need for experienced personnel. The main advantages of the technique include its general applicability to a broad range of compounds, high sensitivity and outstanding selectivity. Several methods have already been reported for the simultaneous determination of mycotoxins, which offer significant advantages over conventional techniques.[5–9]

Here, we present a new method for the analysis of mycotoxins in cereal-based samples using a triple quadrupole LC–MS–MS system (API 2000, Applied Biosystems, Foster City, California, USA). The method analyses the mycotoxins deoxynivalenol (DON), nivalenol (NIV), fusarenone X (FX), verrucarol (VOL), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), diacetoxyscirpenol (DAS), HT-2 toxin (HT2), T-2 toxin (T2), zeralanone (ZAN) and zearalenone (ZON). For additional verification purposes ochratoxin A and aflatoxins were included in the study. We will briefly discuss our experiences regarding the choice of solvents used, solvent flow, split of LC eluents, negative or positive ionization and a comparison of APCI and ESI interfaces.

Development of the Mass Spectrometric Method

First, an LC–MS/MS method was developed that achieved good separation and sensitivity for the detection of 18 mycotoxins under the same instrumental conditions. The MS data for individual substances were optimized by flow-injection with a syringe pump (flow 4–8 µL/min, HPLC 125 µL/min). The optimized instrumental conditions are summarized in Table 1. A standard chromatogram of the Fusarium mycotoxins of major interest is shown in Figure 1.

In total, MS data were recorded for a total of 18 mycotoxins, details of which are summarized in Table 2. Simultaneous

Table 1: Instrumental conditions.

Standard Chromatographic Conditions

LC Column:	a) Agilent Zorbax 150 × 3 mm; 3.5 µm b) Thermo Aquasil 150 × 4.6 mm; 3 µm
Packing:	RP 18
Flow-rate:	a) 250 µL/min b) 1000 µL/min
Mobile phase:	A: MeOH/H_2O (10/90) B: MeOH
Gradient:	a) 30/70 (MeOH/$H_2$2O) -> 100% B (10 min) b) 30/70 (MeOH/H_2O) -> 100% B (4.5 min)
Injection volume:	a) 10–30 µL b) 10–70 µL
Column temperature:	a) 30 °C b) 50 °C

Mass Spectrometer Conditions

Instrument:	API 200 (Applied Biosystems)
Ionization:	TSIP (ESI)
Polarity:	negative/positive/negative
Dwell times:	70 to 100 mms
Source temperature:	400 °C
IS-Voltage:	4500 V

Table 2: Mass spectrometric data for 12 mycotoxins of the multicomponent method.

RT min.	Component	Parent *m/z*	Daughter (*m/z*) primary	Daughter (*m/z*) secondary	Ratio Prim/sec.	Polarity	Linearity* (R_)	Range (ng/mL)	LOQ**
3.80	Nivalenol (NIV)	311	281	205	10	–	0998	10–500	10
5.25	Deoxynivalenol (DON)	295	265	138	1.4	–	0.998	10–500	10
9.19	Verrucarol (VOL-ISTD)	265	125	143	1.6	–	0995	10–500	10
7.49	Fusarenon X (FX)	353	187	205	1.4	–	0997	10–500	10
10.00	15-Acetyldeoxy-nivalenol (ADON)	337	219	150	2.1	–	0999	1–200	10
10.00	3-Acetyldeoxy-nivalenol (ADON)	337	307	173	1.0	–	0.999	1–200	10
11.98	Diacetoxyscirpenol (DAS)	389	247	329		+	0.993	1–200	10
13.01	HT2-Toxin (HT2)	447	345	285	2.4	+	0.993	1–200	10
13.61	T2-Toxin(T2)	489	245	387	1.5	+	0.991	1–200	10
13.95	Zearalanon(ZAN-ISTD)	321	187	136	1.3	–	0.991	1–200	10
14.09	Zearalenon (ZON)	319	185	187	1.2	–	0.997	1–200	10

* Linear regression (1/x weighting) ISTD = internal standard **LOQ was determined for the entire method using MycoSep clean up columns 226.

determination of a wide variety of different mycotoxins requires incorporation of two periods of polarity switching into the MS/MS method. In conformity with the results reported by Razzazi et al.[7] the best sensitivity was found for type A-trichothecenes (DAS/HT2/T2) with positive ionization in ESI mode, while for type B-trichothecenes, ZAN and ZON, negative ionization is preferred. As is shown in Figure 2 the response for nivalenol is below the limit of detection (at a level

> **While identification of compounds is always possible, reliable and correct quantification remains critical.**

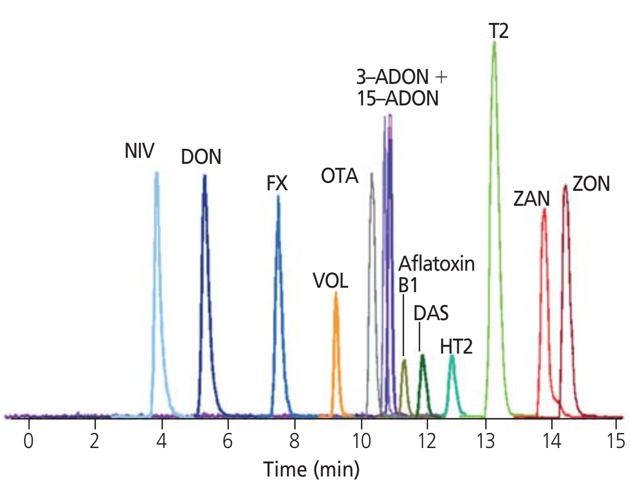


Figure 1: Standard chromatogram of the Fusarium mycotoxins of major interest.

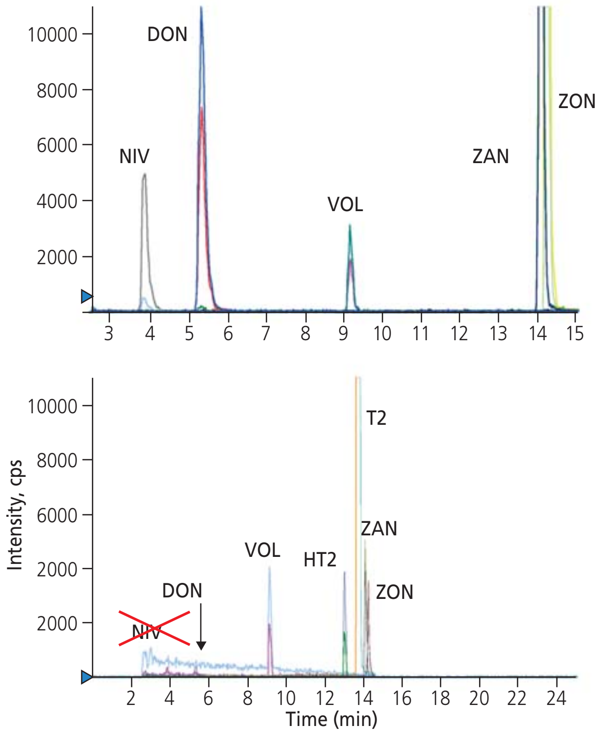


Figure 2: Response of selected mycotoxins in the negative mode compared with the response in the positive ion mode.

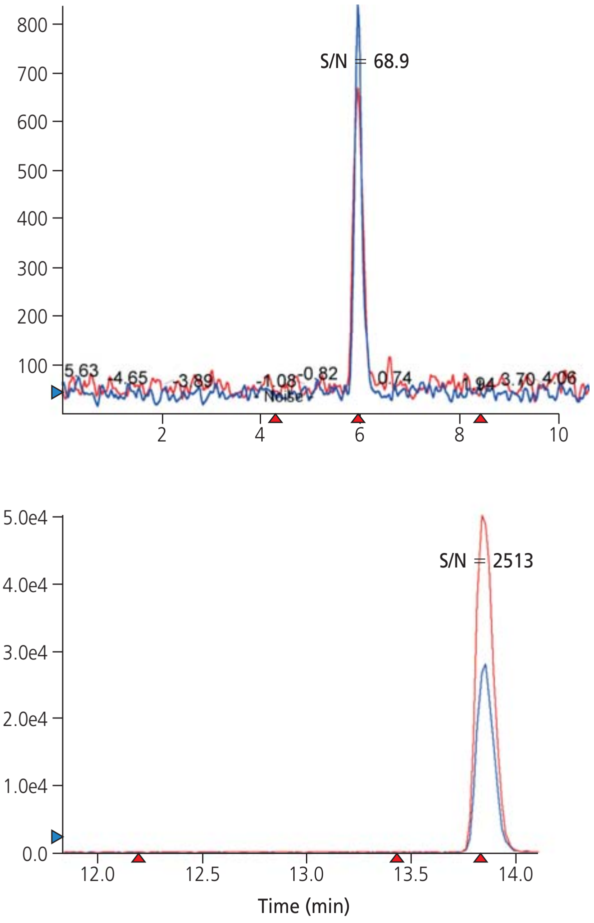


Figure 3: MRM of DON at a level of 20 ng/mL (S/N = 70) compared with MRM of T-2 toxin at a level of 10 ng/mL (S/N 2500).

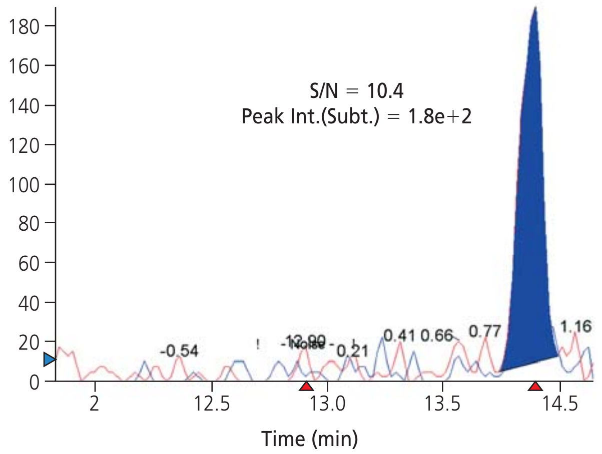


Figure 4: T-2 toxin at a level of 0.2 ng/mL in a babyfood sample extract.

of 200 ng/mL) when positive ionization is used. For quantification, two specific mass transitions for each compound were monitored. For all toxins investigated the molecular ions (M+/M−) were used for fragmentation, except for the type A trichothecenes, which predominantly formed sodium adducts. Because of their stability the sodium adducts proved to be suitable as parent ions for further fragmentation. The consequent benefit of using these pseudo parent ions is much higher sensitivity. For example, T-2 toxin produces a 40-times higher response at a 10 ng/mL level compared with the response of DON at 20 ng/mL (Figure 3). Detection limits in sample extracts of 0.2 µg/kg were possible using the signal-to-noise approach with an S/N ratio >10 (Figure 4). Mycotoxins that were detectable under the same instrumental conditions but not integrated in the multimethod are shown in Table 3.

Solvent Influence on the MS Response

The comparison of different solvents demonstrated that for ESI ionization a mixture of methanol/water improved the detector response, while the use of acetonitrile led to much lower signals.

The use of buffers is very often recommended. However, the use of ammonium acetate (5 mM) for instance, with type-B trichothecenes resulted in the formation of adduct ions which sometimes lead to fewer or less significant fragmentation reactions. Here, often only transition of the acetate ion showed a high response. In our situation, as a business laboratory we should have the ability to change LC methods without losing much time during routine analysis. In practice, however, it proved difficult to change, for example, from one solvent containing ammonium acetate to another method using a lower concentrated formate mixture. Consequently, we decided to use, on the one hand, the molecular ions for fragmentation, because of more specific mass transitions and, on the other hand, to have the ability to share the instrument with other methods not using ammonium acetate buffer.

APCI versus ESI

APCI ionization is most frequently cited in the literature with respect to mycotoxin analysis. In our laboratory, better responses were achieved by using TurboIonSpray© (ESI) compared with Heated Nebulizer© (APCI), which is demonstrated in Table 4. Lagana et al. reported similar results for an older type of the API instrument (API 365).[6]

In summary, we achieved the best MS response for B-trichothecenes, OTA, ZAN and ZON using the ESI interface and negative ionization, an eluent of methanol/water, and a flow-rate of 250 µL with a split ratio of 1:1.

Optimum Chromatography Conditions

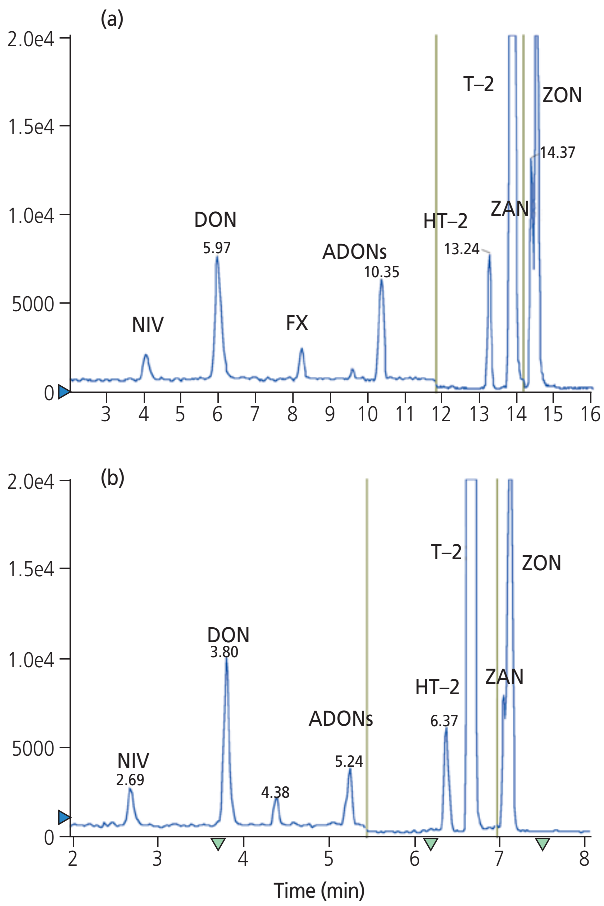
For every commercial analytical laboratory time is limited and the amount of samples requiring analysis on any one instrument increases steadily. Therefore, a fast chromatography method is desirable to realize maximum sample throughput. To begin with we used an RP18 column (150 × 3 mm) with a particle size of 3.5 µm, and a flow-rate of 250 µL/min. One run required 22 min and good separation was obtained. To increase elution times the flow-rate was increased up to 1 mL/min. To maintain this throughput level an RP 18 column with a larger diameter (150 × 4.6 mm, 3 µm) was required to prevent elevated pressures. As can be seen in Figure 5, it was possible to achieve separation of all relevant mycotoxins in a 15 min run, in which the final substance eluted at 7.38 min. Additionally, the toxins T-2 and zearalanon (ZAN) showed better separation on the wider column.

Validating the Method

For the analysis of food and feed samples an existing clean-up procedure (MycoSep® method) was checked for its applicability to MS detection. The sample preparation consists of an extraction of analytes from the sample matrix by shaking 20 g of homogenized ground sample for 1 h with 100 mL of an acetonitrile/water solution (85/15, v/v). Prior to the extraction step, 0.5 mL of a standard solution containing VOL and ZAN (10 and 5 µg/mL, respectively) in methanol were added. After filtration aliquots of the extract (corr. 1 g dry weight) were used for purification on MycoSep® columns 226 (Romer Labs, Austria). Validation of the entire method was performed with wheat flour and obtained recoveries are depicted in Table 5.

The main task required for the method, which is used for routine food control, is its general applicability and robustness for several different food and feed matrices. Any reduction of sample

Figure 5: Comparison of two versions of RP18 chromatography; (a): 3 mm i.d. at a flow of 250 µL/min and a split of 1:1, oven temperature: 30 °C, (b): 4.6 mm i.d., flow 1 mL/min, split 5:1.



preparation, as described, resulted in unacceptable high matrix effects as shown in Figure 6 for DON and VOL. As a result of these matrix effects, an effective sample preparation as described is mandatory to achieve a reliable and sensitive determination.

Because validation for each product matrix is normally not feasible, various recovery trials for the different test materials were performed to cover potential matrix effects. For most cereal commodities including fine ground wheat flour, corn, pasta, bread and pastries virtually no matrix effects were observed. In summary we noticed, that even with MycoSep clean-up strong signal suppression up to 40% were obtained for soy beans or for instance rape-seed (see Figure 3). To take specific matrix effects into account, matrix matched calibration must be performed. Alternatively, a dilution of the sample extract sometimes compensates most effects successfully.

The obtained recoveries for T-2 toxin in the different food and feed commodities were in the range from 60 to 95%. When the clean-up column is additionally rinsed afterwards with the extraction solvent, up to 20% higher recoveries for HT-2 and T-2 were possible.

Therefore, sample extract dilution or alternatively quantification by the use of matrix matched standard calibrations is recommended (see Figure 7).

For most cereal commodities including fine ground wheat flour, corn, pasta, bread and pastries, virtually no matrix effects were observed. A stronger effect was observed when soy beans or rape-seed were analysed with signal suppression up to 40% being possible. To take specific matrix effects into account, matrix matched calibration must be performed. A higher difference of the slope of the linear regressions indicates generally effects such as suppression or enhancement of the signal Usually, sample extract dilution compensates most effects successfully; nevertheless for samples close to the limit of quantification only standard addition could verify the quantified concentration.

The obtained recoveries for T-2 toxin in the different food and feed commodities were in the range from 60 to 95%. When the clean-up column was subsequently rinsed with the extraction solvent, up to 20% higher recoveries for HT-2 and T-2 were possible.

Table 3: Mycotoxins which were detectable under the same instrumental conditions but not integrated in the mulitmethod; method only for verification purposes.

RT min.	Component	Parent *m/z*	Daughter (*m/z*) primary	secondary	Polarity	LOQ ppb*
10.08	Aflatoxin M2	331	273	229	+	1
10.63	Aflatoxin M1	329	273	229	+	1
10.63	Aflatoxin G2	331	245	275	+	1
11.03	Aflatoxin G1	329	243	283	+	1
11.54	Aflatoxin B2	315	259	287	+	1
11.84	Aflatoxin B1	313	241	285	+	1
9.70	Ochratoxin A (OTA)	402		358 167	–	1

*LOQ was determined using the signal-to-noise approach for a matrix matched sample (C = 1 ng/mL).

Figure 6: Comparison of sample extract, before and after clean-up with MycoSep 226.

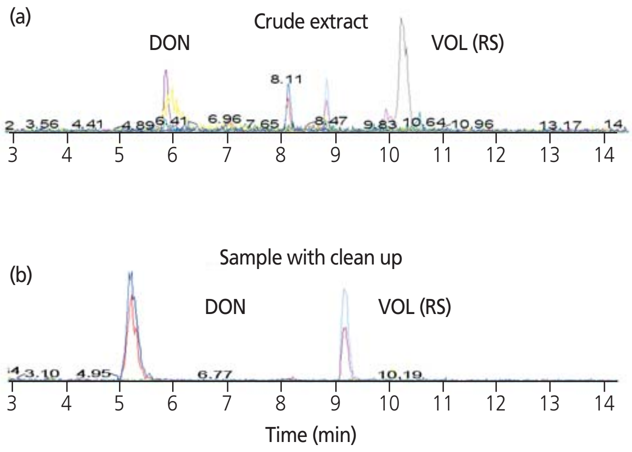


Figure 7: Results of different recovery trials for DON and its internal standards VOL in different commodities. Results of every sample matrix based on average values of various fortified samples (flour = 29, bread/pastries n = 13, cereals n= 23, pasta n= 6, corn n= 14, feed n=5, soy products n = 8, molasses n = 5).

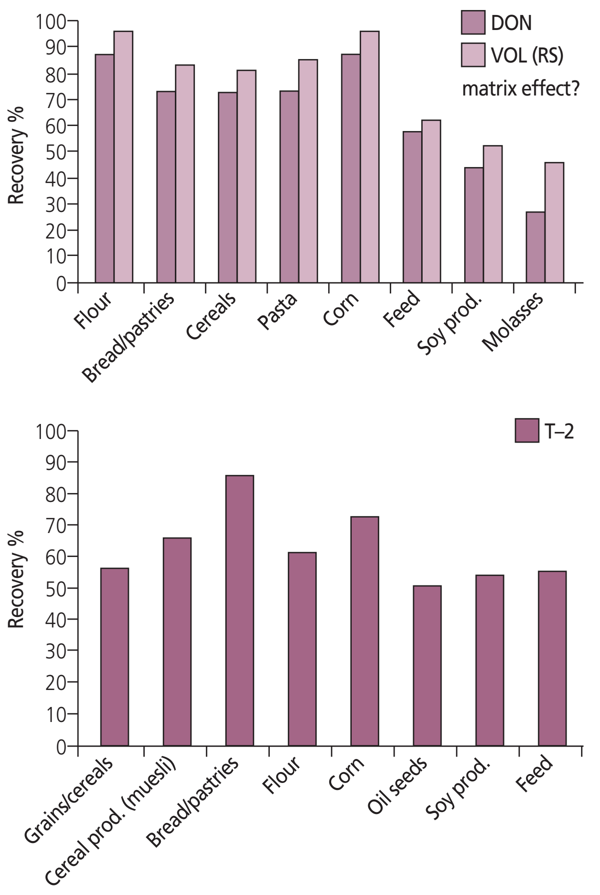


Table 4: Influences of different solvents and ionization interfaces on the MS response.

Solvent	Chromatography	Mass Spectrometer	
Acetonitrile/water	+ + +	ESI: +	APCI: +
Methanol/water	+ +	ESI: +++	APCI: +
Acetonitrile/water/formate		ESI: −	APCI: −
Acetonitrile/water/ammonium acetate		ESI: −	APCI: ++
Flow-rate	i.d. 3 mm; 250 μL/min	ESI: ++	APCI: +
	Split (1:1)	ESI: +++	APCI: +
	i.d. 4.6 mm; 1 mL/min	ESI: −−	APCI: ++
	Split (5:1)	ESI: +++	

Table 5: Comparison of different detector response for selected mycotoxins by using the APCI and ESI ion source in negative ionization mode.

Compound	MRM (neg)	Peak height TISP	Peak height APCI
NIV	311/281	5 320	890
DON	295/265	16 000	1 680
VOL (IS)	265/125	23 450	310
OTA	402/358	39 000	
ZAN	319/161	52 000	3 810
ZON	317/175	40 000	2 350

Table 6: Average recoveries in % of the validation study with MycoSep® material 226.

Substance	Range [ppb]*	MycoSep 226	RSD %
NIV	25–500	89	5
DON	25–500	93	17
FX	10–200	89	15
VOL (RS)	125	86	4
3ADON	10–200	70	9
15ADON	10–200	73	9
HT2	10–200	54	3
T2	10–200	71	3
ZON (IS)**	10–200	92	4

* Triplicate analysis of 4 fortification level.
** Quantified by the internal standard ZAN
VOL (RS) = verrucarol is used as recovery standard

Conclusions

The presented study underlines the high potential of tandem mass spectrometry coupled to liquid chromatography. Multiresidue methods for the determination of more or less polar compounds must be based on LC–MS techniques. Such methods are applicable to a wide variety of compounds in various matrices.

While identification of compounds is always possible, reliable and correct quantification remains critical. However, by application of suitable sample preparation procedures prior to LC–MS and through the use of internal standards these critical points can be handled satisfactory. Although the method was not primary developed for its application to the aflatoxin family, it does offer a suitable second method to verify results obtained with classic methods based on fluorescence detection.

Upcoming EU regulations have increased the pressure to develop and validate chemical methods for the simultaneous detection of different Fusarium toxins. The method presented herein, shows a high response for T-2 toxin, thereby permitting the analysis of these biogenic contaminants down to low ppb levels in broad range of commodities. The comparison of various method parameters, including the solvents used, the chosen interface and polarity mode has demonstrated that there are several variables requiring careful evaluation during development of an LC–MS/MS method to achieve optimal chromatographic and mass spectrometric results.

References

1. J.W. Bennett, M. Klich, *Clinical Microbiology Reviews*, 497–516 (2003).
2. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to Deoxynivalenol (DON) as undesirable substance in animal feed. *The EFSA Journal*, **73**, 1–41 (2004).
3. Myktoxin Höchstmengen Verordnung, BGBl, 1248; last rev. 04.02.2004 (1999).
4. R. Krska and J. Fresenius, *Anal. Chem.*, **371**, 285 (2001).
5. U. Berger, *J. Agr. Food Chem.*, **47**, 4240–45 (1999).
6. A. Lagana et al., *Rapid Commun. Mass Spectrom.*, **17**, 1037–1043 (2003).
7. E. Razzazi-Fazeli et al., *J. Chrom. A*, **968**, 129–142 (2002).
8. W. Langseth, *J. Chrom A*, **815**, 103–121 (1998).
9. T. Rundberget, *J. Chrom. A*, **964**, 189-197 (2001).

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