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Rapid simultaneous determination of major type A- and B-trichothecenes as well as zearalenone in maize by high performance liquid chromatography–tandem mass spectrometry

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Abstract

A novel method for the simultaneous determination of the *Fusarium* mycotoxins nivalenol, deoxynivalenol, fusarenon-X, 3-acetyl-deoxynivalenol, the sum of 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol, diacetoxy-scirpenol, HT-2 toxin, T-2 toxin and zearalenone in maize has been developed using gradient RP-LC with atmospheric pressure chemical ionization triple quadrupole mass spectrometry (LC–APCI–MS/MS). Swift clean-up of maize samples was performed with MycoSep® #226 columns. Quantification of zearalenone was performed with zearalanone as internal standard (IS), while no IS was used for the trichothecenes. Detection of the mycotoxins was carried out in the multiple reaction monitoring (MRM) mode. Method performance characteristics were estimated after analysis of spiked blank maize samples. Calibration curves were linear between 10 and 1000 μg/kg and the limits of detection ranged from 0.3 to 3.8 μg/kg depending on the mycotoxin. Moreover, the accuracy of the method was confirmed by comparing analytical data to certified values from reference materials for deoxynivalenol and zearalenone.

Keywords: Mycotoxins; Trichothecenes; Zearalenone; Tandem mass spectrometry; Maize

1. Introduction

All mycotoxins are low-molecular weight substances produced as secondary metabolites by various molds [1]. By definition, mycotoxins are poisonous to vertebrates in low concentrations. Numerous *Fusarium* species, such as *Fusarium culmorum*, *Fusarium equiseti*, *Fusarium graminearum*, *Fusarium moniliforme*, *Fusarium proliferatum*, *Fusarium poae*, *Fusarium sporotrichioides* and *Fusarium verticillioides* belong to the most prevalent molds in temperate climatic regions [2]. *Fusarium* species can occur on a broad range of hosts, including barley, maize, millet, oat, rice, rye and wheat [3]. Trichothecenes and zearalenone are major *Fusarium* mycotoxins [4]. The trichothecenes are polycyclic sesquiterpenoids, possessing a C-9,10 double bond, a C-12,13-epoxide ring, and various hydroxyl and acetoxy groups. According to their characteristic functional groups, trichothecenes can be grouped into four types (A–D). Type-A trichothecenes have an oxygen function at C-8, which is different from a keto-group. Members of this group include the highly toxic HT-2 toxin (HT-2), T-2 toxin (T-2) and diacetoxyscirpenol (DAS). The type-B trichothecenes, such as nivalenol (NIV), deoxynivalenol (DON), fusarenon X (FUS-X), 3-acetyl-deoxynivalneol (3ADON) and 15-acetyldeoxynivalenol (15ADON), possess a C-8 keto-group. Type C trichothecenes are characterized by a second epoxide-group, while type D trichothecenes are macrocyclic compounds. Chemical structures of the *Fusarium* mycotoxins investigated in the present paper are shown in Fig. 1. Acute and chronic ingestion by humans and animals can elicit a variety

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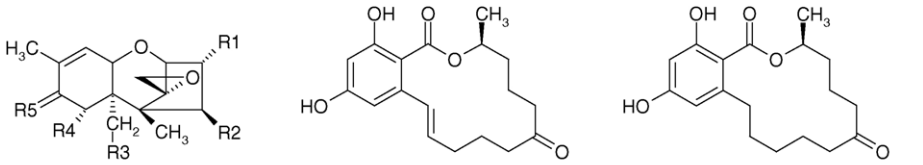

Trichothecene	MW	R1	R2	R3	R4	R5
Nivalenol	312	-OH	-OH	-OH	-OH	=O
Deoxynivalenol	296	-OH	-H	-OH	-OH	=O
Fusarenon-X	354	-OH	-OAc	-OH	-OH	=O
3-Acetyl-DON	338	-OAc	-H	-OH	-OH	=O
15-Acetyl-DON	338	-OH	-H	-OAc	-OH	=O
Diacetoxyscirpenol	366	-OH	-OAc	-OAc	-H	-H
HT-2 Toxin	424	-OH	-OH	-OAc	-H	$-OCOCH_2CH(CH_3)_2$
T-2 Toxin	466	-OH	-OAc	-OAc	-H	$-OCOCH_2CH(CH_3)_2$

Fig. 1. Chemical structures of the *Fusarium* mycotoxins and the internal standard.

of toxic effects, including fever, diarrhea, vomiting, necrosis, hemorrhage and depletion of bone marrow [5]. Maximum tolerated levels for DON, the most commonly detected type-B trichothecene, in food commodities are usually in the range from 500 to 1000 μg/kg [6].

Analytical methods for the determination of trichothecenes have been reviewed [3,5,7–9]. For the determination of type A-trichothecenes analytical methods using GC with electron capture detection (ECD), GC–MS and LC–MS [10,11] for the chromatographic separation and detection are described. The most common methods for the determination of type B-trichothecenes include GC–ECD, GC–MS, LC–UV, LC with either pre or post column derivatisation and fluorescence detection (FLD), LC–MS [12,13] and recently also LC–MS/MS [14]. There are also methods capable of determining both type A- and B-trichothecenes simultaneously, using GC–ECD, GC–MS, LC–MS [15,16] or LC–MS/MS [17,18]. Clean-up of sample extracts for the determination of trichothecenes in cereals is frequently done by MycoSep® #227 columns, which have also been successfully applied in several LC–MS methods [10–13,17].

Zearalenone, 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcyclic acid μ-lactone, is a *Fusarium* metabolite with potent estrogenic activity [1]. Maximum tolerated or guideline levels for ZON in food are ranging from 30 to 1000 μg/kg [19]. Analytical methods for the determination of ZON have also been reviewed [20]. TLC and GC methods are available, but ZON is usually determined either by LC–FLD, by LC–MS [21,22] or by LC–MS/MS [23–26]. MycoSep® #226 columns were used for LC–MS analysis of ZON in a method by Rosenberg et al. [21].

The development of multimycotoxin methods, allowing to detect and determine several groups of co-occurring mycotoxins with single chromatographic runs, is highly desirable. Frisvad and Thrane did pioneer work in this field as early as 1987 using LC–UV [27,28]. The complex composition of food and feed matrices as well as the wide range of physical and chemical properties of mycotoxins require selective and sensitive detection techniques, such as mass spectrometry, especially for multitoxin methods. In the field of *Fusarium* mycotoxins there are some GC–MS methods for the simultaneous determination of trichothecenes and ZON [29,30]. However, the need to derivatise the samples prior to GC analysis and the huge technical improvements in the field of LC–MS have increased the number of methods using LC–MS. Nielsen and Smedsgaard used electrospray (ESI) time-of-flight (TOF) mass spectrometry after LC separation to compile a database of 474 fungal metabolites [31]. Still, the number of LC–MS or LC–MS/MS methods for the simultaneous determination of more than one family of mycotoxins is very limited. Rundberget and Wilkins developed and validated such a method for the determination of *Penicillium* mycotoxins in food and feed [32]. Driffield et al. [33] published an ESI-MS/MS method, capable to determine NIV, DON, ZON, ochratoxin A and aflatoxin B1 in pig livers. Just recently, Feldmann et al. [18] put forward another ESI–MS/MS method for the detection of NIV, DON, T-2, DAS, trichothecin and trichothecolon in apple products. Berger et al. [17] introduced an LC-ion trap

method for the quantitative determination and structure elucidation of nine type A- and B-trichothecenes.

The aim of this paper is to present a fast, accurate and reliable LC—triple quadrupole mass spectrometric method for the simultaneous determination of the *Fusarium* mycotoxins NIV, DON, FUS-X, 3ADON, 15ADON, DAS, HT-2, T-2 and ZON in maize. During method development, two different commercially available clean-up columns, MycoSep® #226 and #227 from Romer Labs®, were tested for their suitability to be combined with LC–MS/MS. Method performance characteristics, such as recoveries, linearities over the working ranges, limits of detection, limits of quantification, trueness and precision have been evaluated and are presented.

2. Experimental

2.1. Chemicals and materials

The mycotoxins NIV, DON, FUS-X, 3ADON, 15ADON, DAS, HT-2, T-2 and ZON as well as the internal standard zearalanone (ZAN) were purchased from biopure Referenzsubstanzen GmbH (Tulln, Austria) as liquid calibrants in acetonitrile and stored at 4 °C in the dark. Before use, the solutions were brought to room temperature. Ammonium acetate (MS grade), ammonium formiate (MS grade), acetic acid (p.a.) and formic acid (p.a.) were bought from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile were LC grade and obtained from J.T. Baker (Deventer, The Netherlands). Water for LC mobile phase was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France). MycoSep® #226 and #227 clean-up columns were provided by Romer Labs® Diagnostic GmbH (Herzogenburg, Austria). A NM20ZA nitrogen generator from Peak Scientific Instruments (Inchinnan, Scotland) was used for the gas supply of the mass spectrometer. BCR 378 and BCR 717 are certified reference materials and were purchased from IRMM (Geel, Belgium).

2.2. Instrumentation

LC–MS/MS analysis was performed on a QTrap-LC–MS/MS system from Applied Biosystems (Foster City, CA, USA) equipped with either an APCI or an ESI interface and a 1100 Series LC system from Agilent Technologies (Waldbronn, Germany). The mass spectrometer was used exclusively in the triple quadrupole mode.

2.3. LC parameters

Chromatographic separation was achieved on an Aquasil® RP-18 column (3 μm, 100 mm × 4.6 mm) from Thermo Electron (Woburn, MA, USA), equipped with a C-18 guard column, at 25 °C using gradient elution. Mobile phase A consisted of methanol/water, 20/80 (v/v), containing 5 mM ammonium acetate, while mobile phase B consisted of methanol/water, 90/10 (v/v), also containing 5 mM ammonium acetate. Elution with mobile phase A was maintained for 0.5 min, afterwards a linear gradient was applied, reaching 100% mobile phase B after 4.5 min (holding time: 2.5 min), and than switched back (7.1 min) to mobile phase A (holding time: 2.9 min), which was maintained till the end of the run at 10.0 min. In order to prevent highly polar matrix compounds from entering the MS ion source, a switching valve was used to discard the LC eluent, before transferring it into the MS interface 2 min after LC injection. The flow rate was set to 1.0 ml/min, while the injection volume was 25 μl.

2.4. MS parameters

The APCI interface was used in both negative and positive ion modes at 450 °C with the following settings: curtain gas (CUR) 35 psi (241 kPa of max. 99.5% nitrogen), nebulizer gas (GS1) 60 psi (414 kPa of zero grade air), auxiliary gas (GS2) 15 psi (103 kPa of zero grade air), corona discharge needle current (NC), −2 or +2 μA, respectively, collision-activated dissociation gas (CAD) 6 (arbitrary units, corresponding to the pressure of collision gas (nitrogen) in Q2), MRM dwell time 25 ms, pause between mass ranges 5 ms, settling time (polarity switch only) 700 ms. The ESI interface was solely used for optimization of the MS/MS parameters—experimental settings were as follows: temperature 400 °C, CUR 20 psi (138 kPa), GS1 30 psi (207 kPa), GS2 75 psi (517 kPa), ionization voltage −4200 or +5000 V, respectively, CAD 6.

2.5. Spiking experiments

For spiking experiments, a mixed stock solution, containing NIV, DON, FUS-X, 3ADON, 15ADON, DAS, HT-2, T-2 and ZON in acetonitrile, was added to blank maize. The spiked samples were left overnight, to allow solvent evaporation prior to the extraction. Five replicates of eight levels of 0, 10, 30, 50, 100, 300, 500 and 1000 μg/kg were prepared.

2.6. Sample preparation and clean-up

Ground maize samples (10.00 ± 0.01 g) were spiked with 5 μg ZAN (50 μl of a 100.0 μg/ml solution in acetonitrile). After solvent evaporation the samples were extracted for 90 min at 180 rpm on an automatic shaker (GFL, Burgwedel, Germany) with 40.0 ml acetonitrile/water (84/16, v/v). The extracts were filtered (S&S folded filters, $595\frac{1}{2}$, Schleicher & Schuell, Dassel, Germany) and 8.0 ml thereof were transferred into sample tubes. Afterwards the MycoSep® #226 (or #227) columns were pushed into the tubes. Four milliliter of the purified extracts were withdrawn using a 1 ml pipette, transferred into 4 ml glass vials and evaporated to dryness at 50 °C under a constant stream of nitrogen. The residues were then dissolved in 1.0 ml of the starting gradient LC mobile phase A (methanol/water, 20/80 (v/v), containing 5 mM ammonium acetate) by vortexing vigorously for 1 min. Be-

fore LC analysis, the solutions were pressed through 0.22 μm membrane filters (Millex-GV, Millipore, Molsheim, France).

2.7. Quantitative analysis

Mixed standard solutions of 0, 10, 30, 50, 100, 300, 500 and 1000 μg/l were prepared. Therefore, 0, 10, 30, 50, 100, 300, 500 or 1000 μl of a mixed stock solution containing 1000 μg/l of each NIV, DON, FUS-X, 3ADON, 15ADON, DAS, HT-2, T-2 and ZON in acetonitrile were mixed with 50 μl of ZAN solution (10.0 μg/ml in acetonitrile). The liquid was evaporated to dryness at 50 °C under a constant stream of nitrogen. Residues were then dissolved in 1.0 ml methanol/water, 20/80 (v/v), containing 5 mM ammonium acetate, by vortexing vigorously for 1 min.

2.8. Data evaluation

Calibration curves for each analyte were constructed by plotting the analyte concentration versus the signal intensity (area) of the analyte using the Analyst® software Version 1.4 [34]. For ZON, the analyte concentration divided by the concentration of the internal standard was plotted versus the area of the analyte divided by the area of the internal standard. Recoveries and R.S.D. were calculated from linear regression curves using Validata, a Microsoft Excel macro by Wegscheider et al. [35]. Briefly, the MS signal intensities obtained from spiking experiments were used for the calculation of analyte concentrations. These concentrations were plotted against the actual spiking levels and recoveries were taken from the slopes of the regression lines. Limits of detection and limits of quantification were calculated, based on signal to noise ratios (S/N) of 3/1 and 10/1, respectively, using the Analyst® software from Applied Biosystems [34].

3. Results and discussion

3.1. LC–MS optimization

Method development started with full scan experiments (Q1 scan mode, scan range 250–550 amu, scan time 0.25 s) of 5 μg/ml solutions of the intended mycotoxins in both positive and negative modes using flow injections. 10 μl of the mycotoxin standards in acetonitrile were injected into a stream 0.5 ml/min of either acetonitrile/water, 50/50 (v/v) or methanol/water, 50/50 (v/v) and analyzed using the ESI interface or the APCI interface. No analytical column was used with the flow injections, while standard settings were used for the interfaces. The use of methanol instead of acetonitrile in the solvent mixtures resulted in higher intensities for all analytes, but especially for the type B-trichothecenes. This is in agreement with other LC–MS methods [12–18]. While the type B-trichothecenes and ZON showed higher signal intensities in the negative ionization mode, type A-trichothecenes were more easily transferred to positive ions. With the exception of ZON, which was almost exclusively ionized to $[ZON - H]^-$ ions in the negative ionization modes with both interfaces, adduct formation was detected. Depending on the structure of the trichothecene $[M - H]^-$, $[M + HCOO]^-$ and $[M + CH_3COO]^-$ ions in negative and $[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$ as well as $[M + K]^+$ ions in positive ionization mode are formed. The multitude of trichothecene adduct ions did not decrease using the APCI interface.

We continued to verify different settings, as we repeated the above experiments with new solvent mixtures, consisting of methanol/water, containing either 5 mM ammonium formiate or 5 mM ammonium acetate. The ratio of methanol in the eluent was estimated according to the retention of the target analyte on an Aquasil® C-18 RP column. Again, flow injections were used for APCI and ESI, but this time an eluent split of 1:50 before the ESI interface was applied to reduce the flow into the MS ion source. Using buffers, the adduct ion formation could be shifted towards $[M + NH_4]^+$ ions in positive mode for the type A-trichothecenes and to either $[M + HCOO]^-$ or $[M + CH_3COO]^-$ ions in negative mode for the type B-trichothecenes depending on the buffer added to the solvent. As a result of these experiments ammonium acetate was used as buffer for all further experiments. Since the use of the buffer lead to the formation of single trichothecene-adduct ion species, the intensities of these ions were much higher compared to experiments without buffers. Using ammonium acetate, the peak areas of both $[M + NH_4]^+$ ions for type A-trichothecenes in the positive ionization mode and $[M + CH_3COO]^-$ ions for type B-trichothecenes in the negative ionization mode roughly equaled the sum of the different analyte adduct ions when using no buffer at all.

For ZON signal intensities of the quasi molecular ions and the analyte adduct ions were in the same range for both interfaces, but the APCI interface performed slightly better for type A-trichothecenes (particularly for HT-2) and significantly better for type B-trichothecenes with our settings (Fig. 2). The latter is in contrary to the results of Lagana et al. [14], who opted for the ESI interface in their LC–MS/MS method for type B-trichothecenes and to Feldmann et al. [18] in their multitoxin method. Like Berger et al. [17] in their ion-trap method and Razzazi-Fazeli et al. in their LC–MS methods [10,12,13] we decided to use the APCI interface for our method.

Chromatographic separation on an Thermo Electron Aquasil® RP-18 column using a methanol/water gradient containing 5 mM ammonium acetate revealed that all five type B-trichothecenes are eluting before the three type A-trichothecenes, which are eluting before zearalenone. This way, it was possible to switch ionization polarities from negative for the type B-trichothecenes to positive for the type A-trichothecenes and back to negative for ZON in the same LC run, resulting in maximum intensities. The use of a 100 mm × 4.6 mm column, combined with the ability of the APCI interface to handle large solvent flows, resulted in an analysis time of only 10 min for the nine mycotoxins. This is

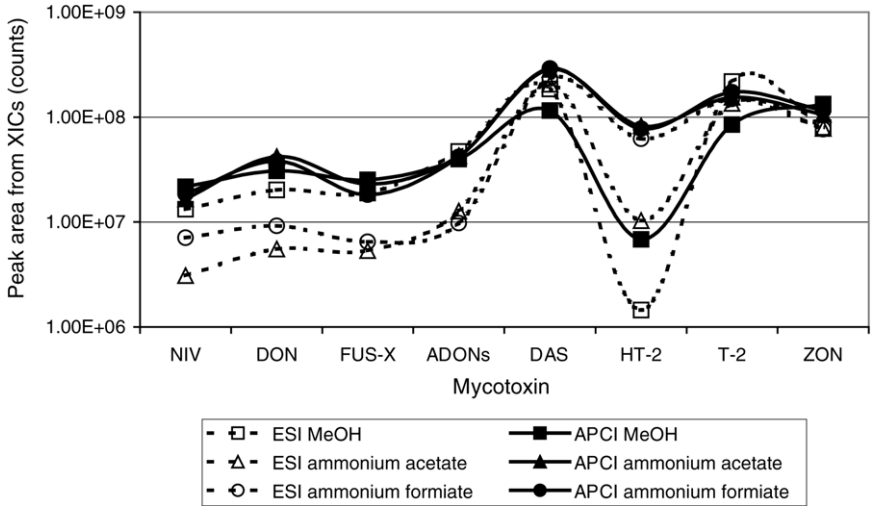

Fig. 2. Flow injection intensities of mycotoxin standards (Q1 MS-Scans). Shown are the intensities (peak areas, XICs of base peak ions) of Q1 scans (*m/z* 250–550) after flow injection of mycotoxin standards in methanol/water either containing 5 mM ammonium acetate (triangles), 5 mM ammonium formiate (dots), or no buffers (squares). Mycotoxins were analyzed using APCI (solid lines) or ESI (dashed lines) sources. Data points are connected with lines just for better visualization.

quicker than any of the LC–MS methods mentioned above, which have LC run times ranging from 12 to 30 min.

3.2. LC–MS/MS optimization

The ESI interface was used in conjunction with a syringe pump (direct infusion of 5 μg/ml solutions of the individual mycotoxins into the LC eluent, using a T-piece) for optimization of the MS/MS parameters. The quantitative optimization tool of the instrument software was used for this purpose. Resulting MRM transitions, declustering potentials (DP) and collision energies (CE) are summarized in Table 1. For the type B-trichothecenes the highest abundances after fragmentation of the $[M + CH_3COO]^-$ ions was monitored with either $[CH_3COO]^-$ ($m/z = 59$) or $[M - H]^-$ ions, showing the tendency of the adduct ions to preferentially form acetate in the collision cell. Since structural information for the secure identification of analytes is lost when non-analyte-specific fragments, such as acetate, are used for detection, fragments of the parent toxins have been monitored as qualifiers in addition to the acetate ions to confirm the identities of the toxins and to monitor the ratios of acetate to "qualifier". Although these qualifiers showed higher detection limits, they were additionally used in quantification to confirm constant ion

Table 1
MS/MS parameters for detection of the measured mycotoxins and the internal standard in the multiple reaction mode (MRM)

Analyte	Measured ion	Q1 (*m/z*)	Q3 (*m/z*)	DP (V)	CE (eV)
NIV	$[NIV + CH_3COO]^-$	371.1	59.1	−26	−48
NIV, qualifier A	$[NIV + CH_3COO]^-$	371.1	281.1	−26	−32
NIV, qualifier B	$[NIV + CH_3COO]^-$	371.1	311.1	−26	−10
DON	$[DON + CH_3COO]^-$	355.1	59.1	−16	−30
DON, qualifier A	$[DON + CH_3COO]^-$	355.1	265.0	−16	−12
DON, qualifier B	$[DON + CH_3COO]^-$	355.1	295.1	−16	−18
FUS-X	$[FUS\text{-}X + CH_3COO]^-$	413.1	59.1	−16	−40
FUS-X, qualifier A	$[FUS\text{-}X + CH_3COO]^-$	413.1	187.0	−16	−34
FUS-X, qualifier B	$[FUS\text{-}X + CH_3COO]^-$	413.1	353.1	−16	−10
ADONs	$[ADONs + CH_3COO]^-$	397.1	59.1	−11	−34
ADONs, qualifier A	$[ADONs + CH_3COO]^-$	397.1	337.1	−11	−10
ADONs, qualifier B	$[ADONs + CH_3COO]^-$	397.1	173.1	−11	−20
3ADON	$[3ADON + CH_3COO]^-$	397.1	307.1	−11	−14
DAS	$[DAS + NH_4]^+$	384.2	307.2	21	15
DAS, qualifier	$[DAS + NH_4]^+$	384.2	105.1	21	49
HT-2	$[HT\text{-}2 + NH_4]^+$	442.2	263.1	16	17
HT-2, qualifier	$[HT\text{-}2 + NH_4]^+$	442.2	215.0	16	17
T-2	$[T\text{-}2 + NH_4]^+$	484.2	185.2	21	25
T-2, qualifier	$[T\text{-}2 + NH_4]^+$	484.2	305.1	21	19
ZON	$[ZON - H]^-$	317.2	131.2	−51	−38
ZON, qualifier	$[ZON - H]^-$	317.2	175.1	−51	−32
ZAN (IS)	$[ZAN - H]^-$	319.2	275.2	−56	−16

abundance ratios and therefore the presence of the respective analytes.

Due to the fast LC gradient, 3ADON and 15ADON could not be separated chromatographically. While we obtained a single LC peak for the sum of both 3- and 15-ADON (ADONs), different fragmentation behavior also allows the separate determination and quantification of 3ADON, albeit at a lower sensitivity. In the case of 3ADON, $[M-\text{H-30}]^-$ ions with *m/z* 307 are formed in the collision cell of the mass spectrometer by cleavage of the C-15 group. As an acetate group is present in the case of 15ADON, this cleavage of $[CH_2O]$ could not be seen there. The specific transition for 3ADON was first considered by Berger et al. [17], proven by the authors [36] and confirmed by Razzazi-Fazeli et al. [13].

3.3. Clean-up and matrix effects on the MS response

With the exception of NIV, the recoveries for the MycoSep® #227 column, were excellent (82–98%) for both type A- and B-trichothecenes in our LC–MS/MS method (Table 2). ZON, however, is retarded almost completely on these columns, making the MycoSep® #227 columns unsuitable for this mycotoxin. Nonetheless, these columns can be used effectively for clean-up in the determination of trichothecenes.

The MycoSep® #226 columns allow compounds to pass through over a wider range of polarities. Therefore, more analytes—including ZON—but also more matrix compounds could be found. Recoveries with this column ranged from about 73% (T-2) to about 89% (FUS-X) for all trichothecenes but for NIV, and therefore it can be regarded as acceptable for the method described (Table 2). About 50% recovery was found for NIV, which is in accordance to the manufacturer's specifications. In case of ZON, however, just 30% recovery could be obtained, although about 100% recovery were specified. We suspected that a strong matrix effect, resulting in ion suppression, could be the cause for that. Therefore, we tested the suitability of ZAN as internal standard compound by adding it to the extracts immediately after sample clean-up. ZAN, a compound closely related to ZON is possessing a single bond between C-11 and C-12 instead of a double bond. ZAN is partly co-eluting with ZON under our LC conditions. Using ZAN as internal standard, the recovery for ZON was 99%, so ZAN emended well for matrix effects. Further experiments revealed that both ZON and ZAN are behaving identically with regard to the MycoSep® #226 clean-up, so we opted to apply ZAN before clean-up. Furthermore, it is recommended to wash the LC column with solvent B after every four to five injections for 10 min, in order to wash out non-polar components, when clean-up with the #226 column is performed.

Table 2
Recoveries and R.S.D. obtained for A- and B-trichothecenes as well as ZON from spiked maize, after clean-up with MycoSep® #227 (left) and #226 (right) columns (spiking levels 10, 30, 50, 100, 300, 500, 1000 μg/kg; $n = 5$ each)

Analyte	MycoSep® #227		MycoSep® #226	
	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
NIV	50	10.3	50	8.6
DON	82	7.2	79	7.2
FUS-X	96	7.3	89	7.4
ADONs	94	13.5	84	16.0
3ADON	94	6.4	85	10.3
DAS	94	9.4	80	10.8
HT-2	98	5.9	77	12.7
T-2	94	4.5	73	14.2
ZON	–	–	30	23.0
ZON/ZAN	–	–	99	9.6

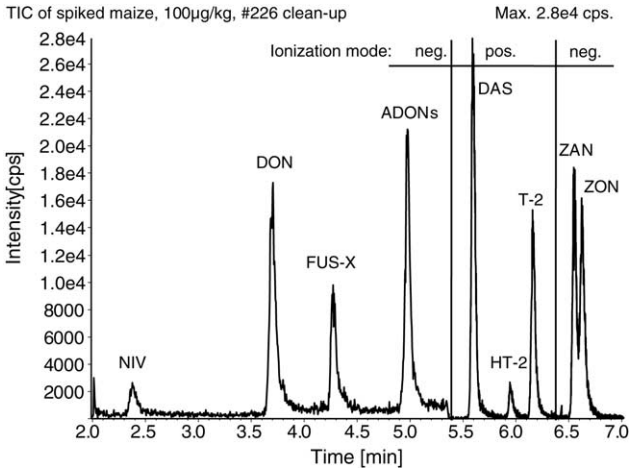

Fig. 3. Total ion chromatogram obtained after clean-up with MycoSep® #226 columns and LC–MS/MS analysis of a spiked maize sample containing 100 μg/kg of each mycotoxin. Vertical lines illustrate change of ionization polarities from negative to positive (5.4 min) and back to negative (6.4 min).

3.4. Performance characteristics

Calibration curves for all analytes are linear over the working range of 30–1000 μg/kg, respectively. Thirty to one thousand μg/kg for NIV and 3ADON, according to the Validata software program [37]. Squared correlation coefficients (R^2) were in the range of 0.994–0.999 for the eight point calibration curves, depending on the mycotoxin. Limits of detection ranged from 0.3 μg/kg for T-2 to 3.8 μg/kg for 3ADON using #226 clean-up. Analysis of the type B-trichothecenes, using the unusual $[M + CH_3COO]^-$ to $[CH_3COO]^-$ MRM transitions worked well. No matrix influences, differences in linearity or changes in signal intensity ratios could be detected in comparison to the "qualifiers". Limits of detection (S/N = 3/1) and quantification (S/N = 10/1) were, however, about four to five times lower than for the qualifying transitions. Figs. 3 and 4 show total (TIC) and extracted (XIC) ion chromatograms of maize which was spiked with 100 μg/kg per mycotoxin, while Table 3 comprises limits of detection, limits of quantification and linear working ranges for the method.

Certified reference materials (CRM) for both DON (CRM 378) [37] and ZON (CRM 717) [38] were available from

Table 3
Limits of detection (LOD), limits of quantification (LOQ) and linear working ranges for standard solutions (μg/l) ($n = 24$) as well as complete method LODs, LOQs and linearity ranges for spiked maize, cleaned-up with MycoSep® #226 columns (μg/kg) ($n = 40$)

Analyte	Standard solutions			MycoSep® #226 clean-up		
	LOD	LOQ	Range	LOD	LOQ	Range
NIV	3.1	10.0	10–1000	3.7	18.3	30–1000
DON	0.5	1.5	10–1000	0.8	2.7	10–1000
FUS-X	0.5	2.3	10–1000	1.6	5.6	10–1000
ADONs	0.4	1.4	10–1000	0.9	3.5	10–1000
3ADON	1.9	8.3	10–1000	3.8	13.4	30–1000
DAS	0.2	0.5	10–1000	0.3	1.1	10–1000
HT-2	0.2	0.7	10–1000	1.0	3.5	10–1000
T-2	0.1	0.3	10–1000	0.3	0.8	10–1000
ZON/ZAN	0.3	1.1	10–1000	0.9	3.2	10–1000

the Community Bureau of Reference (BCR) of the European Commission. In order to confirm the applicability to real world samples and the accuracy of our method, we analyzed these CRMs and compared our results to the certified values. The value for DON was corrected for its recovery, while this was not necessary for ZON, as a suitable internal standard was found for this mycotoxin. Results matched nicely, as we found 447 ± 32 μg DON/kg compared to the certified value of 430 ± 40 μg DON/kg and 78.4 ± 1.3 μg ZON/kg compared to the certified value of 83.0 ± 9.0 μg ZON/kg. Evaluation of the results according to ISO guide 33 [39] showed no significant bias for both mycotoxins.

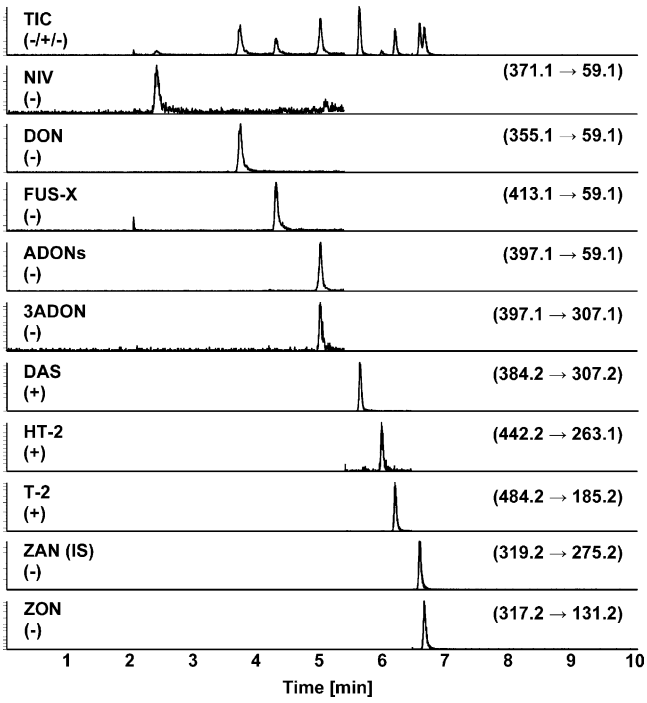

Fig. 4. Total (TIC) and extracted ion chromatograms (XICs) of NIV, DON, FUS-X, ADONs, 3ADON, DAS, HT-2, T-2, ZAN and ZON obtained after clean-up with MycoSep® #226 columns and LC–MS/MS analysis of a spiked maize sample containing 100 μg/kg of each mycotoxin. The signs in brackets underneath the analyte names are corresponding to the chosen polarization polarity, while the respective MRM transitions are shown to the right.

3.5. Comparison of current LC–MS methods

Although a variety of different LC–MS and LC–MS/MS methods exists for the determination of trichothecenes and for zearalenone, this paper describes the first method for the simultaneous determination of all the major A- and B-trichothecenes and ZON in a single run. Moreover, the use of a triple quadrupole mass spectrometer yields better detection limits than single quadrupole instruments. This is caused by improved signal to noise ratios due to the additional selectiv-

Table 4
Comparison of LC-MS methods for the determination of DON, T-2 and ZON in various matrices, sorted by on-column limits of detection

Reference	Analyte	Matrix	LC solvent system	MS instrumentation	LOD (1) (ng/g)	LOD (2) (pg)
This paper	DON	Maize	MeOH/NH_4OAc (5 mM)	APCI negative triple quad	0.8	20
Lagana [14]	DON	Maize	MeOH/ACN/water	ESI negative triple quad	1.5	60
Berger [17]	DON	Wheat	MeOH/water	APCI positive ion-trap	6	60
Feldmann [18]	DON	Apples	MeOH/NH_4OAc (5 mM)	ESI negative triple quad	5	100
Huopalahti [15]	DON	Grains	MeOH/ACN/NH_4OAc (3 mM)	APCI positive triple quad	“Low”	–
Razzazi-Fazeli [12]	DON	Wheat	MeOH/ACN/water	APCI negative single quad	40 (LOQ)	12500 (LOQ)
This paper	T-2	Maize	MeOH/NH_4OAc (5 mM)	APCI positive triple quad	0.3	7.5
Feldmann [18]	T-2	Apples	MeOH/NH_4OAc (5 mM)	ESI positive triple quad	1	20
Berger [17]	T-2	Wheat	MeOH/water	APCI positive ion-trap	3	30
Fuchs [11]	T-2	Grains	ACN/ammonium formiate (10 mM)	ESI positive single quad	9	225
Huopalahti [15]	T-2	Grains	MeOH/ACN/NH_4OAc (3 mM)	APCI positive triple quad	“Low”	–
Razzazi-Fazeli [10]	T-2	Grains	ACN/NH_4OAc (1 mM)	APCI positive single quad	50 (LOQ)	6250 (LOQ)
Pallaroni [22]	ZON	Grains	MeOH/acetic acid (0.2%)	ESI negative ion-trap	4	7.5
This paper	ZON	Maize	MeOH/NH_4OAc (5 mM)	APCI negative triple quad	0.9	22.5
Zöllner [25]	ZON	Grains	MeOH/NH_4OAc (15 mM)	APCI negative triple quad	0.5	25
Rosenberg [21]	ZON	Various	ACN/water	APCI positive single quad	0.12	48
Zöllner [24]	ZON	Beer	MeOH/NH_4OAc (15 mM)	APCI negative triple quad	0.03	54
Van Bennekom [23]	ZON	Urine	ACN/water	ESI negative triple quad	<1.0	<665

LOD(1): method detection limits in ng/g sample as stated in the respective publication. Note that evaluation of LODs has been different for the publications cited. LOD(2): LODs expressed as absolute amount of toxin (in pg), injected to the LC column.

ity of the second MS step. LODs gained with ion-traps were comparably low for the determination of trichothecenes and zearalenone. The drawback of 3-dimensional ion-trap instruments for the quantification of trace contaminants, such as mycotoxins, has been described in literature and comprise poor calibration linearity and lower measurement repeatability, compared to triple quadrupole instruments. For DON, T-2 and ZON, which are the most frequently monitored compounds of the mycotoxins investigated in this study, selected characteristics of different LC/MS methods have been illustrated in Table 4.

4. Conclusion

We developed a rapid quantitative method for the simultaneous determination of the major *Fusarium* mycotoxins NIV, DON, FUS-X, 3ADON, 15ADON, DAS, HT-2, T-2 and ZON in maize using LC-APCI-MS/MS detection. Simple clean-up using MycoSep® columns combined with fast LC separation greatly reduce the analysis time and make this method suitable for routine analysis. The use of a triple quadrupole mass spectrometer allows the unambiguous identification of these nine mycotoxins and their concurrent quantification in the low μg/kg-range. The increased analytical throughput and the simultaneous determination of different groups of mycotoxins hold great promises for this and other LC–MS/MS analyses in the near future. Nonetheless, suitable internal standards for the quantification of trichothecenes by LC–MS methods would be highly desirable, as the accuracy of such methods would be even better.

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