ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Journal of Chromatography B, 796 (2003) 21–33

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Simultaneous determination of major B-trichothecenes and the de-epoxy-metabolite of deoxynivalenol in pig urine and maize using high-performance liquid chromatography–mass spectrometry

E. Razzazi-Fazeli*, J. Böhm, K. Jarukamjorn, J. Zentek

Institute of Nutrition, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria

Received 5 June 2003; received in revised form 24 July 2003; accepted 25 July 2003

Abstract

A selective analytical method based on high-performance liquid chromatography (HPLC), combined with atmospheric pressure chemical ionisation ($APCI^-$) mass spectrometry (MS), has been developed for simultaneous determination of B-trichothecenes and the major metabolites of deoxynivalenol. The method allows simultaneous analysis of nivalenol (NIV), deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-AcDON), 3-acetyldeoxynivalenol (3-AcDON), fusarenon X (Fus-X) and de-epoxydeoxynivalenol (DOM-1). The method is based on one-step sample clean-up using a multifunctional MycoSep column. A linear gradient mobile phase system, consisting of water:acetonitrile:methanol (H_2O:ACN:MeOH) at a flow-rate of 1 ml/min, and a Polar-RP C18 column, were utilised to obtain the best resolution of all tested compounds along with column and equilibrating within 30 min. Dexamethasone (Dex) was used as internal standard. The developed method shows good repeatability for inter- and intra-day precisions as well as good linearity of calibration curves (r^2 ranged from 0.9936 to 0.9998). Average recoveries for tested compounds in both matrices have been determined ranging from 63.7 to 102.3% and limit of quantification (LOQ) ranged from 25 to 150 ng/g. The utility and practical impact of the method is demonstrated using contaminated pig urine and maize samples.
© 2003 Elsevier B.V. All rights reserved.

Keywords: B-Trichothecenes; Deoxynivalenol

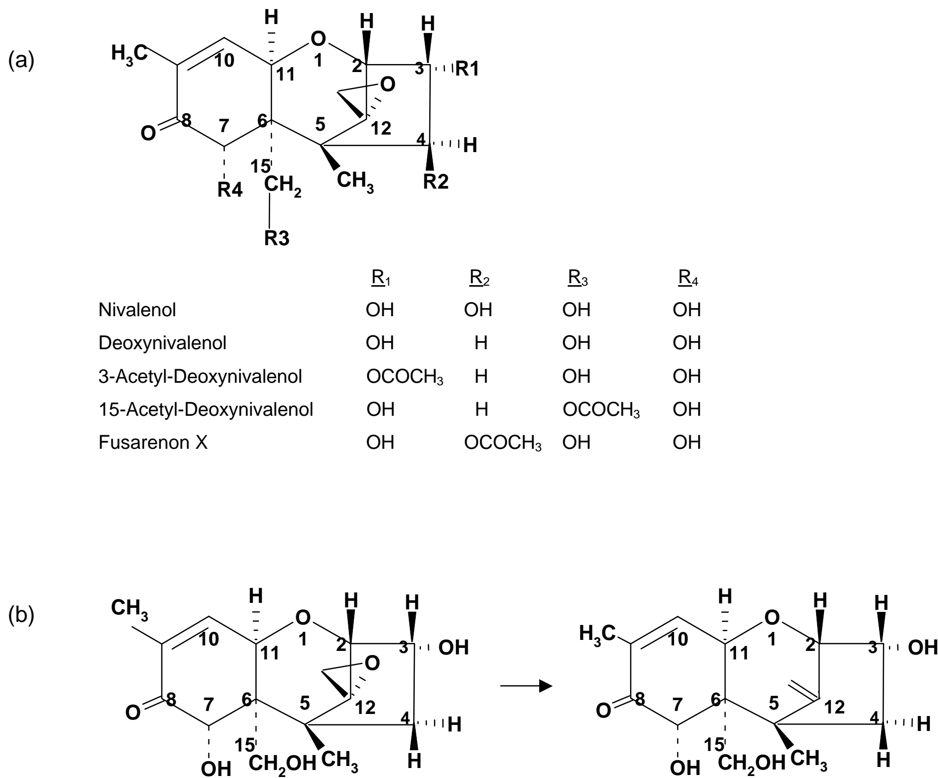
1. Introduction

The presence of mycotoxins in food and feed has long been recognised as a potential human and animal health hazard [1,2]. Trichothecene mycotoxins are a large group of toxins produced in particular by moulds belonging to the genus *Fusarium*. Trichothecenes contain sesquiterpene rings with different constituents on positions 3, 4, 7, 8, and most of which have a double bond at positions C-9 and C-10. The 12,13-epoxide ring is responsible for their toxicity [1]. According to their structure, trichothecenes have been classified into four groups, named A–D. Type

* Corresponding author. Tel.: +43-1-25077-3216; fax: +43-1-25077-3290.

E-mail address: ebrahim.razzazi@vu-wien.ac.at (E. Razzazi-Fazeli).

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S1570-0232(03)00604-4



	R_1	R_2	R_3	R_4
Nivalenol	OH	OH	OH	OH
Deoxynivalenol	OH	H	OH	OH
3-Acetyl-Deoxynivalenol	$OCOCH_3$	H	OH	OH
15-Acetyl-Deoxynivalenol	OH	H	$OCOCH_3$	OH
Fusarenon X	OH	$OCOCH_3$	OH	OH

Fig. 1. Chemical structures of investigated B-trichothecenes. (a) Structures of nivalenol (NIV), deoxynivalenol (DON), 15-acetyldeoxynivalenol (15-AcDON), and 3-acetyldeoxynivalenol (3-AcDON). (b) De-epoxidation of deoxynivalenol (DON) to DOM-1 and structure of DOM-1.

B-trichothecenes (Fig. 1a) differ from type A by the presence of a carbonyl group at the C-8 position and are synthesised mainly by *F. graminearum* and *F. culmorum*. Several surveys suggest that the most prevalent B-trichothecenes are deoxynivalenol (DON), nivalenol (NIV), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON) and fusarenon X (Fus-X). Type B-trichothecenes have been found mainly on maize, oats, barley, and wheat [3–5]. Within the trichothecene group, DON is most commonly associated with toxicity. The co-occurrence of DON with NIV, 15-AcDON or 3-AcDON and Fus-X has been observed world-wide [3–5].

B-Trichothecenes are responsible for a wide range of toxicity symptoms in animals, including feed refusal, weight loss and vomiting [1,2,6,7]. In particular, DON can inhibit protein biosynthesis and has been reported to suppress immune responses [6–8]. Swines are among the most sensitive animal species to DON, while trichothecenes are detoxified in ruminants [8,9]. The trichothecenes, especially NIV and DON, are soluble in polar solvents and are therefore rapidly distributed and metabolised after ingestion [10]. They are excreted mainly in the urine and to a lesser extent in bile [9]. DOM-1 is formed from DON by gut and rumen bacteria through reduction of the epoxide group of DON (Fig. 1b). De-epoxydation leads to significant reduction of toxicity. DOM-1 was first characterised by Yoshizawa et al. and demonstrated in rats [11], pigs [9,12,13] and ruminants [14]. DOM-1 can also be produced in vitro [15]. Gas chromatography (GC)–either electron-capture (ECD) [16] and GC–MS [17,18] have been used for quantification of DOM-1 in bovine urine and faeces.

Accurate and reliable methods for the determination of these compounds and metabolites down to the

ng/g level are required for studying toxicodynamics or levels in naturally contaminated samples.

A variety of chromatographic methods have been described for determination of B-trichothecenes. Methods based on thin-layer chromatography (TLC) and enzyme-linked immunosorbent assays (ELISA) have been developed for rapid screening purposes [19–23]. However, precise quantification by ELISA is often difficult, due to cross-reactivities of antibodies with 3-AcDON and 15-AcDON. In contrast, GC with either ECD or mass spectrometric (MS) detection has been established as the method of choice in trichothecene analysis [23,24]. Additionally, tandem mass spectrometric (MS/MS) analysis has been performed recently by Nielsen and Thrane [25]. Generally, derivatisation steps are performed prior to GC analysis and B-trichothecenes can be determined as their trimethylsilyl, pentafluoropropionyl, trifluoroacetyl (TFAA) or Tri-Sil-TBT derivatives [23–28]. The use of pentafluoropropionyl ester provides highest sensitivity when using MS [24].

Onji et al. have published a GC–MS method with an improved “cold on-column injection” without derivatisation for some typical A- and B-trichothecenes [29].

High-performance liquid chromatography (HPLC) with ultraviolet (UV) or diode-array detectors have been applied [23,24,30–35]. Due to poor specific UV absorption, derivatisation of B-trichothecenes with cumarin-3-carbonyl followed by fluorescent detection were performed by Mateo et al. [33]. However, cumarin derivatives are more suitable for type A- and not type B-trichothecenes to enhance the detection limit [33,34]. Prelusky and Trenholm have analysed DON and DOM-1 in urine by using thermospray LC–MS [36]. Recently, LC–MS instruments, particularly atmospheric pressure chemical ionisation (APCI) interfaces, have been employed for the determination and identification of trichothecenes at trace levels [37]. An isocratic HPLC method combined with negative APCI–MS for determination of NIV and DON in wheat was published by our group [38]. Additionally, Berger et al. have published a method for determining various trichothecenes in wheat using LC–APCI–MS in positive ionisation mode [39].

This paper describes a new HPLC analytical method for simultaneous determination of five B-trichothecenes and the major metabolites of DON by using APCI–LC–MS with the advantage of having no derivatisation step. In addition, the method was validated for all tested substances.

2. Materials and methods

2.1. Chemicals and reagents

B-Trichothecene standards, including NIV, DON, 15-AcDON, 3-AcDON and Fus-X as well as dexamethasone (Dex) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The de-epoxy-metabolite of DON (DOM-1; 3α,7α,15-trihydroxytrichothec-9,12-dien-8-one) was kindly provided by Biomin GmbH (Herzogenburg, Austria). The standards were individually dissolved in methanol to give stock concentrations of 1 mg/ml which were stored at −20 °C until use. Standard working solutions were prepared by diluting each stock solution with the mobile phase consisting of water:acetonitrile:methanol (H_2O:ACN:MeOH) (82:9:9, v/v/v). The MycoSep 227 column was a product of Romer Labs, Inc. (Washington, MO, USA). Acetonitrile (ACN) and methanol, both HPLC gradient grade (LiChrosolv) and analytical grade, were obtained from Merck (Darmstadt, Germany). Water was purified in a UPW2 system (F&L, Vienna, Austria). All solvents were degassed using a Waters in-line degasser (Milford, MA, USA).

2.2. HPLC–MS equipment

Chromatographic separation was performed on the HPLC–MS system, comprising a Waters 626-LC pump and Waters 717plus autosampler (Milford, MA, USA). Detection was performed on a Platform II mass spectrometer using an APCI interface equipped with a Pepperpot counter electrode (Micromass, Manchester, UK).

The B-trichothecenes and the internal standard were separated on a Synergi 4 μm Polar-RP 80A analytical column (150 mm length × 4.6 mm i.d., 4 μm, Phenomenex, Cheshire, England) using a 4 μm Polar-RP guard column (4 mm length × 3.0 mm i.d., 4 μm, Phenomenex, Cheshire, England). The column was maintained at a temperature of 30 °C (W.O. Electronics, Vienna, Austria).

The HPLC was operated with a linear gradient mobile phase system consisting of H_2O:ACN:MeOH at

Table 1
Molecular ions and relative molecular weight (M_r) used for quantification and the main fragments used for verification of tested compounds

Compounds	M_r	Fragment ions monitored (*m/z*)
NIV	312	311, 281, 205
DON	296	295, 265, 249
DOM-1	280	279, 233, 214
15-AcDON	338	337, 295, 265
3-AcDON	338	337, 291, 249
Fus-X	354	353, 263, 205
Dex	392	391, 434

a flow-rate of 1 ml/min without splitting. The HPLC pump was programmed as follows: start H_2O:ACN: MeOH (82:9:9, v/v/v), increase to H_2O:ACN:MeOH (40:60:0, v/v/v) over 25 min, followed by a return to H_2O:ACN:MeOH (82:9:9, v/v/v), and finally held for 5 min. Injection volume was 50 μl.

MS measurements were performed in negative mode using an APCI interface. The optimisation processes were carried out in scan mode (*m/z* 150–500). Pure nitrogen as nebulising and carrier gas was produced in a Parker Balston generator (Tewksbury, MA, USA). Drying gas flow-rate was set at 300 l/h and sheath gas flow was held at 150 l/h. The source and APCI vaporising temperature were maintained at 100 and 400 °C, respectively, and the cone voltage was set at 20 V. Quantitative determination of all compounds was applied in the single ion monitoring (SIM) mode for corresponding deprotonated molecules, which were analysed with a dwell time of 0.3 s and a span of 0.2 u. The molecular ions were used for quantification and main fragments were used for verification (Table 1). For internal standard the molecular ion adduct as *m/z* 434 was used for quantification process in negative ionisation mode.

2.3. Analytical procedures

2.3.1. Sample preparation

Sample clean-up steps were carried out using multifunctional MycoSep 227 columns (Romer Labs, Inc., Washington, MO, USA). The extraction was performed by stirring mixtures of pig urine (4 ml) with 21 ml of acetonitrile for 1 h. The extract was filtered and a 10 ml aliquot of filtrate transferred into a culture tube of the MycoSep 227 column. The purification step was executed by rapidly pushing the flange end of the column into the culture tube. The clean-up extract (6 ml) was then evaporated under a nitrogen stream at 40 °C. Consequently, the residue was reconstituted in 600 μl of the mobile phase (H_2O:ACN:MeOH, 82:9:9, v/v/v) and a 50 μl-aliquot was subsequently applied to the LC–MS system.

Grain samples (25 g) were extracted with 100 ml of ACN:H_2O (84:16, v/v) by stirring the mixture for 90 min. The resulting extract was filtered through a cellulose filter. An aliquot of 10 ml of crude extract was then transferred to the MycoSep 227 column. The clean-up extracts were then treated as described above.

2.3.2. Recovery experiments

The recovery experiments were carried out on maize extracts as well as pig urine spiked with different concentrations of B-trichothecenes. A 4 ml-blank urine sample was transferred to a round-bottom Erlenmeyer flask and then spiked with standard working solutions of DON, DOM-1 and the internal standard. The flask was then shaken manually. For spike experiments, a pool of maize extracts was used. Samples were analysed according to the described procedure. The percentage of recovery was calculated from peak areas of spiked crude extracts before and after the clean-up procedure.

2.3.3. Calibration curves on blank urine and maize

Calibration curves were performed by spiking standard solutions of different concentrations and the internal standard solution (250 ng/ml) to an extract of blank maize. Additionally, for DON and DOM-1, standard calibration curves were performed in blank pig urine. The resulting mixtures were subjected to the MycoSep 227 column. The peak area ratios of each B-trichothecene to the internal standard were plotted against the respective concentrations and the calibration curves were obtained by the linear regression analysis.

3. Results and discussion

As with our earlier investigations, negative ion mode was used in this study for analysis, since the

signals of tested compounds in positive ion mode were 10–20 times lower than those in negative ion mode. The optimised LC–APCI–MS parameters, including cone voltage at 20 V, as well as source and APCI vaporising temperature at 100 and 400 °C, respectively, were consistently employed as previously described [38]. The confirmation of B-trichothecenes was determined with regard to retention time and fragmentation pattern. Intensive fragmentation in APCI, especially in our instrument, is advantageous as a diagnostic fingerprint and, thus, identifies compounds in the matrices. The quantification could be carried out using the mass chromatograms of the molecular ions as described.

Mass spectra of 3-AcDON, 15-AcDON, Fus-X and de-epoxy-metabolite are illustrated in Fig. 2. Generally, similar fragmentation patterns and intensive fragmentation could be registered in all cases. Fragment *m/z* 249 could be observed in spectra of all DON derivatives indicating the fragments $[M - \text{H-CH}_2\text{O}]^-$ for DOM-1, $[M - \text{H-CH}_2\text{O-CH}_4]^-$ for DON and $[M - \text{H-88}]^-$ for acetyl derivates respectively. In the DOM-1 spectrum the molecular ion *m/z* 279 as $[M - \text{H}]^-$ and the fragment *m/z* 233 as $[M - \text{H-CH}_2\text{O-CH}_4]^-$ could be noted. However, the resolution of our instrument is not sufficient enough to distinguish between the two possible interpretations of either $[M - \text{H-CH}_2\text{O-CH}_4]^-$ or $[M - \text{H-CH}_2\text{O-O}]^-$. Additionally, spectra of DOM-1 in urine were verified by comparison with those of the standard solution. For Fus-X an intensive *m/z* 353 base peak as $[M - \text{H}]^-$ was observed, additionally two fragments *m/z* 263 and 205 were noted, which could not be interpreted.

Generally, similar fragmentation patterns but with very different abundances for 3-AcDON and 15-AcDON were observed. For both the molecular ion *m/z* 337 was noted. The *m/z* 291 fragment was observed in the 3-AcDON spectrum and showed higher intensity. On the other hand, in the case of 15-AcDON relatively intensive fragment of *m/z* 277 as $[M - \text{H-CH}_3\text{COOH}]^-$ and *m/z* 295 as $[M - \text{H-42}]^-$ were noted. However, none of these were observed of an appropriate intensity in the spectrum of 3-AcDON. A similar fragmentation pattern for 3-AcDON, but with different abundances, was reported by Berger et al. by using MS^2 in positive ion mode [39]. In our previous publication [38] we suggested the fragment $[M - \text{H-CH}_2\text{O}]^-$ as the cleavage of epoxy group for NIV and DON. However, as suggested by Berthiller et al. [40] the loss of 30 amu indicates the cleavage of C15 group and not the epoxy group. As can be seen in Fig. 2, the cleavage of 30 amu could be noted in the spectrum of DOM (*m/z* 307), which is de-epoxide form and in spectrum of 3-AcDON (*m/z* 291). In contrast for 15-AcDON, no $[M - \text{H-CH}_2\text{O}]^-$ fragment could be observed.

3.1. Development and validation of the HPLC method

The only published simultaneous HPLC separation method for the mentioned compounds was described by Walker and Meier [32]. In contrast, all other described methods including those of Berger et al. [39] and Jiménez and Mateo [31] could not perform a separation of 3-AcDON and 15-AcDON by using either gradient or isocratic HPLC. In addition the method described by Walker et al. performs changes of flow-rate and a much more complex gradient system [32].

In our experiments, a number of analytical columns, mainly based on reversed-phase C18, were used to separate the tested compounds. However, it was not possible to separate 3-AcDON and 15-AcDON. An adequate resolution and base line separation of 3-AcDON (RT = 12.05 min) and 15-AcDON (RT = 11.58 min) could be successfully obtained by employing a column with more selectivity for polar components, such as Polar-RP. Additionally, a gradient system had to be applied for separation even after using a selective column. The linear gradient system was used in accordance with simultaneous determination of all tested B-trichothecenes and internal standards within 30 min (Fig. 3a and b).

We could not use hydrocortisone as internal standard suggested by Berger et al. [39], since this component occurs naturally in urine of animals. Consequently, Dex, a synthetic glucocorticoid was employed as an internal standard to compensate the variability during the HPLC–APCI–MS analysis. Although the retention time of the internal standard is far from the tested B-trichothecenes, by using this internal standard we could at least reach a compensation of the drift of LC–MS response due to uncleanness of the APCI source, cone and the focusing hexapoles at the front of the instrument. This is especially a

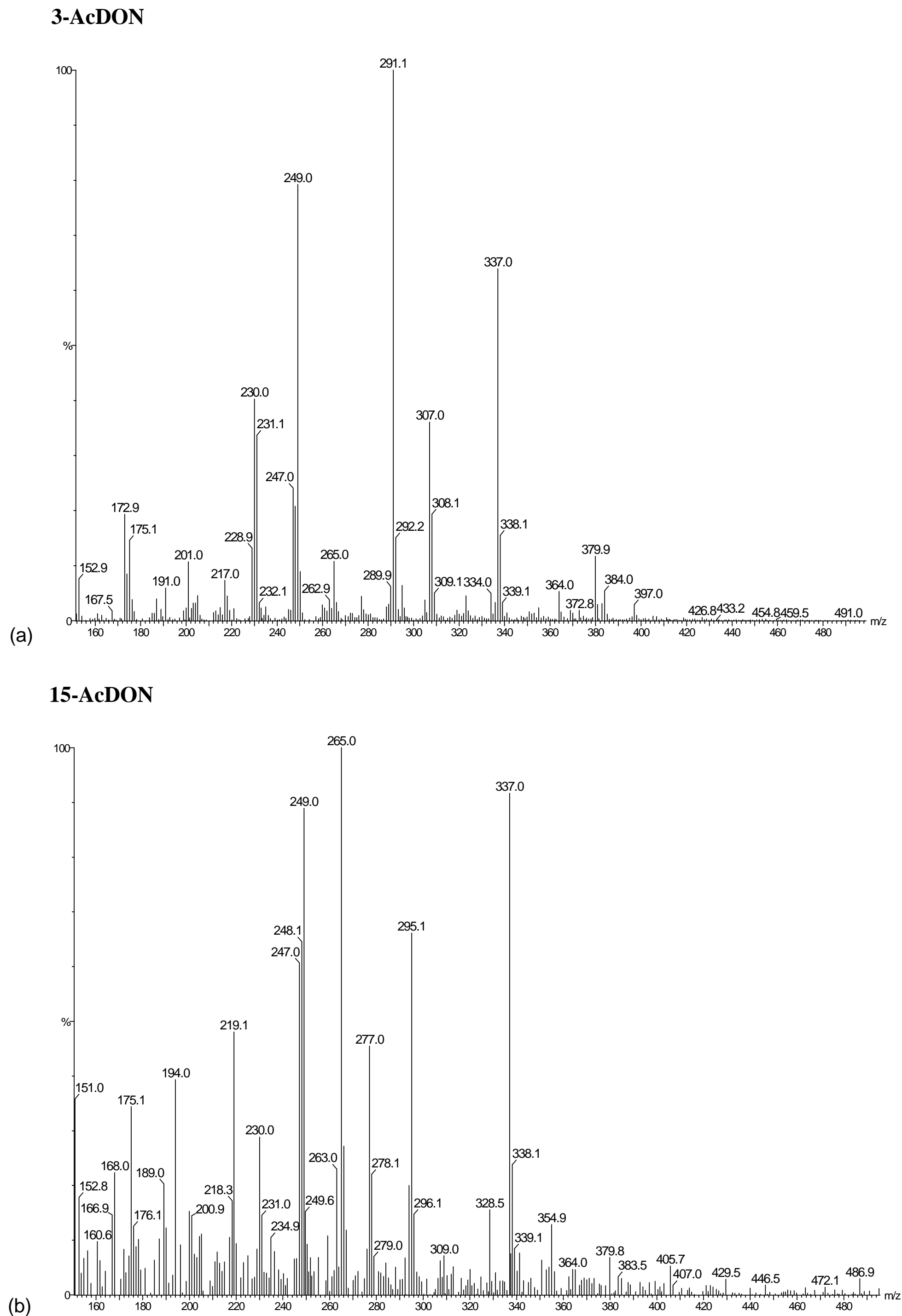
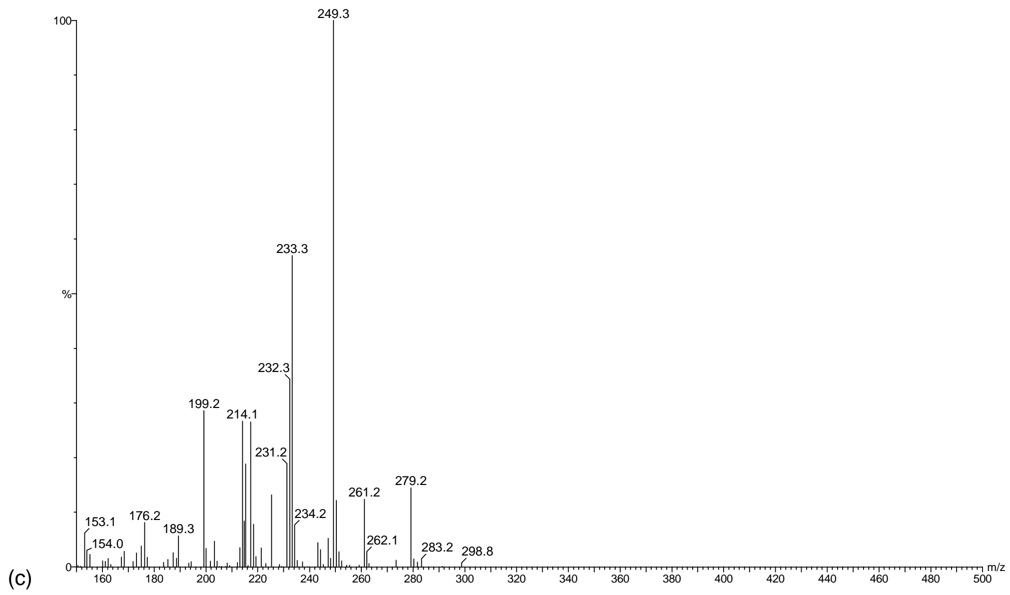


Fig. 2. Mass spectra of investigated B-trichothecenesin negative ionisation mode (LC–APCI/MS).

DOM-1



Fus-X

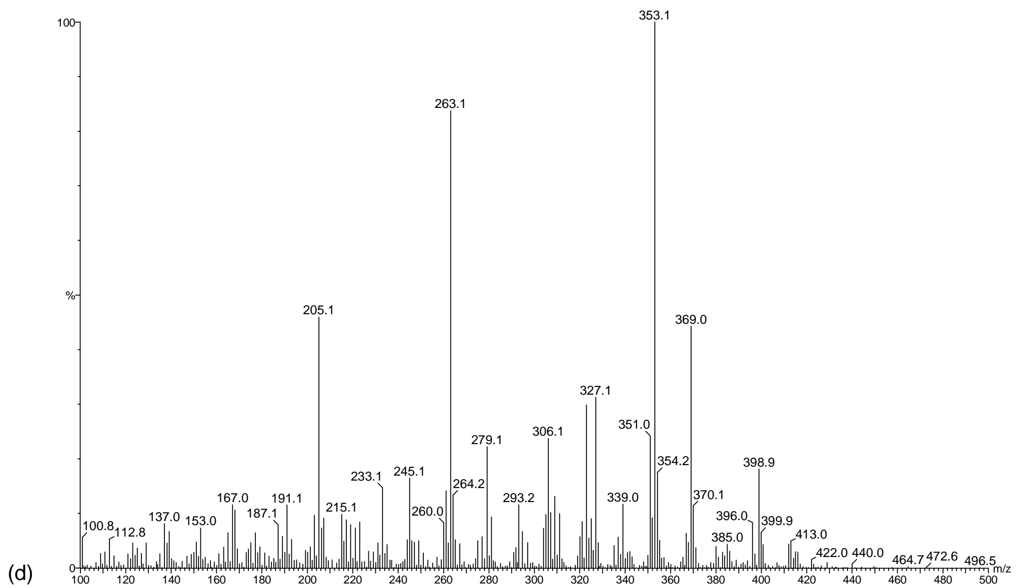


Fig. 2. (*Continued*).

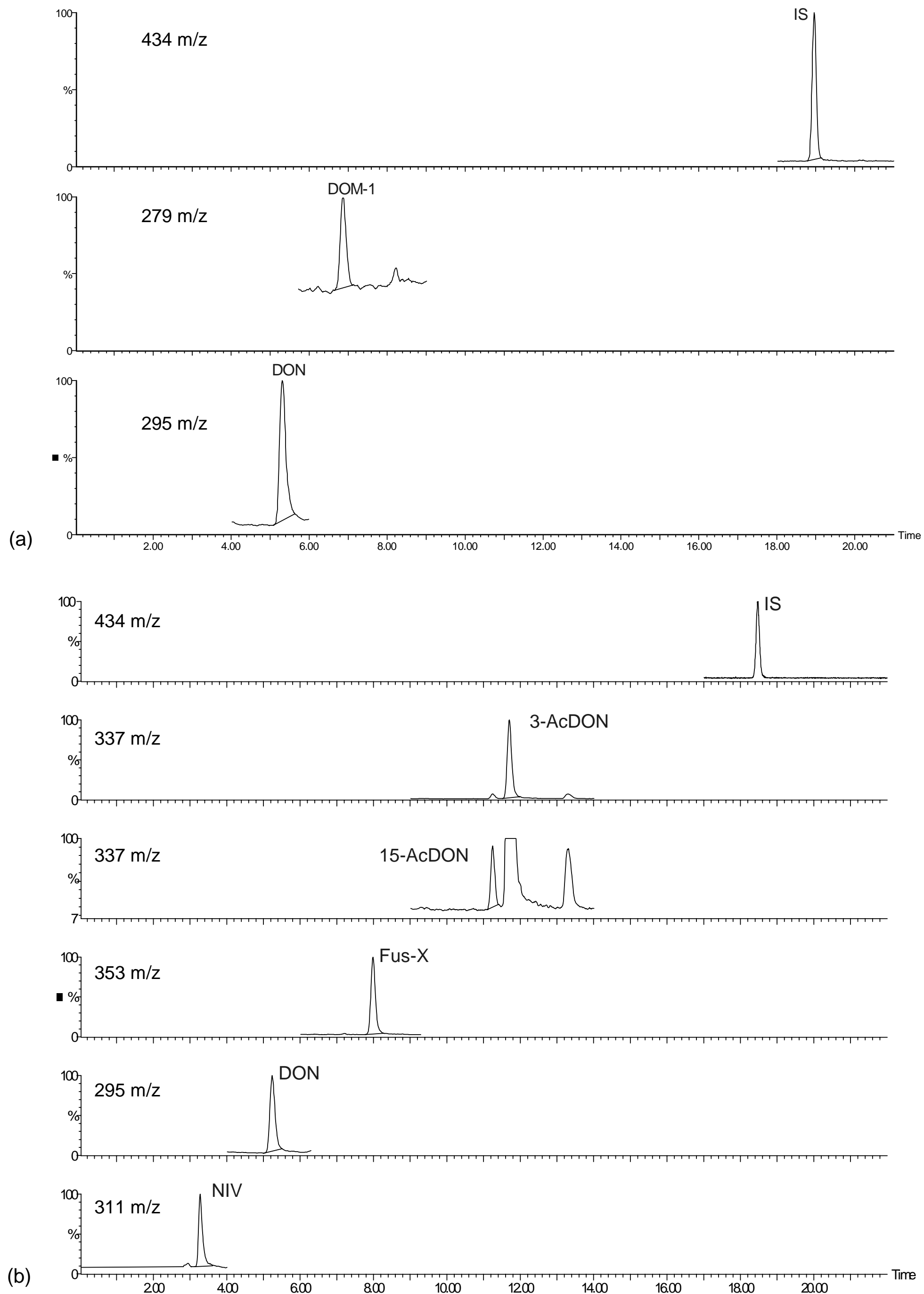


Fig. 3. LC–APCI/MS chromatogram of a contaminated pig urine sample containing 185 ng/ml DON and 80 ng/ml DOM-1 (a) and spiked maize sample with 250 ng/g of each toxin (b) in single ion monitoring mode with the internal standard (dexamethasone 250 ng/ml).

problem when employing LC–MS in the analysis of complex matrices.

3.1.1. Implementation of the MycoSep 227 column with pig urine

The mixture of ACN:H_2O (84:16, v/v) provided the best extracting efficiency for B-trichothecenes [28]. Therefore, an aliquot of urine was simply mixed with acetonitrile to reach the appropriate ratio. Besides the optimal extraction efficiency, the extract could be directly followed by the MycoSep 227 column, which provides a simple and rapid clean-up process. In contrast to our previous publication [38], we decided not to use an additional MycoSep 216 column due to the time consuming procedure for maize samples and the relatively less endogenous interference with pig urine.

The clean-up extract of urine was clear and conceivable enough to be directly applied after reconstitution without additional modification. However, the clean-up extract of grain samples generally had to be centrifuged prior to injection into LC–MS. The advanced percentage recoveries of all tested compounds across the linear range obtained are displayed in Table 2. An exception was noted for DOM-1, due to the lack of a commercially-available DOM-1 standard. Therefore, recovery of DOM-1 with 96.6% was evaluated at a concentration of 100 ng/ml. Our results were comparable to those reported by Dahlem et al. with 86% for DON and 93.6% for DOM-1, when using GC–ECD for analysis of DOM-1 in pig urine and faeces [16]. Also, the recoveries for DON in urine matrix (83.8%) are comparable with the GC–ECD methods, but higher than those in maize matrix in our study (81.5%). Prelusky and Trenholm have also reported similar values of DON recovery (93.8%) from spiked pig urine at a level of 100 ng/ml by using thermospray LC–MS [36].

The use of β-glucuronidase in analysis of metabolites is normally performed; when the degree of glucuronidation in the biological samples is quite high. The first indication on the degree of glucuronidation of DON in swine was reported by Prelusky et al. [9]. The authors found out that only small amount (<5%) of glucuronide conjugate metabolite was present in urine of the trial animals after DON was administered intra-gastric or intravenously. Therefore, in our experiments, we did not perform an enzymatic deglucuronidation step prior to sample clean up.

Table 2
Average recoveries of tested compounds

Compounds	Concentration (ng/g)	Recovery (%) ($n = 4$)
(a) Spiked maize extract		
NIV	100	62.3
	200	68.5
	500	50.9
	1000	73.1
	Overall	63.7
DON	100	70.2
	200	85.9
	500	81.3
	1000	88.0
	Overall	81.5
15-AcDON	200	98.5
	500	102.4
	1000	106.1
	Overall	102.3
3-AcDON	100	83.8
	200	75.9
	500	70.3
	1000	89.1
	Overall	79.7
Fus-X	100	97.1
	200	79.7
	500	88.9
	Overall	88.6
	Concentration (ng/ml urine)	Recovery (%) ($n = 3$)
(b) Pig urine		
DON in urine	100	78.4
	200	85.7
	500	86.6
	1000	84.4
	Overall	83.8
DOM-1 in urine	100	96.6

Fig. 3b shows selected ion chromatogram of a spiked maize sample. Generally, recoveries in maize matrix for DON (81.5%), 3-AcDON (79.7%) and 15-AcDON (102.3%) as well as for Fus-X (88.6%) are comparable to those reported by Berger et al. [39] or our previous report with LC–MS [38] and those recorded by GC–MS and GC–ECD methods [27,28,33]. Similarly, as reported by Weingärtner and co-workers [23,27], for NIV (63.7%) significantly lower recoveries have been obtained.

Table 3
Limit of quantification (LOQ) and linearity for tested B-trichothecenes

Compounds	LOQ (ng/g)	Linearity		
		Range (ng/g)	r^2	$y = ax + b$
(a) Spiked maize				
NIV	70	70–1000	0.9998	$y = 0.3006x - 0.0399$
DON	50	50–1000	0.9986	$y = 0.7882x - 0.1448$
15-AcDON	150	150–1000	0.9997	$y = 0.0326x - 0.0013$
3-AcDON	50	50–1000	0.9936	$y = 0.5346x + 0.0123$
Fus-X	50	50–1000	0.9931	$y = 0.3961x - 0.0363$
	LOQ (ng/ml)	Linearity		
		Range (ng/ml)	r^2	$y = ax + b$
(b) Metabolite in pig urine				
DON	25	25–1000	0.9965	$y = 0.0629x + 1.6259$
DOM-1	25	25–100	0.9990	$y = 0.0913x + 1.098$

Table 4
Precision of the developed method for tested trichothecenes and the de-epoxy-metabolite

Compounds	Concentration (ng/g maize)	Inter-day precision R.S.D. ($n = 4$)	Intra-day precision R.S.D. ($n = 4$)
(a) Spiked maize extract			
NIV	100	14.0	10.3
	200	10.4	9.6
	500	12.5	10.2
	1000	9.2	8.6
	Overall	11.5	9.6
DON	100	11.1	12.1
	200	14.6	9.8
	500	10.5	4.9
	1000	7.1	3.5
	Overall	10.8	7.5
15-AcDON	200	15.1	12.3
	500	10.7	11.9
	1000	9.1	9.2
	Overall	11.6	11.1
3-AcDON	100	9.8	9.3
	200	5.9	4.5
	500	3.5	8.6
	1000	4.1	3.1
	Overall	5.8	6.4
Fus-X	100	11.5	9.7
	200	9.1	4.8
	500	3.6	5.2
	Overall	8.0	6.5

Table 4 (*Continued*)

	Concentration (ng/ml urine)	Inter-day precision R.S.D. ($n = 3$)	Intra-day precision R.S.D. ($n = 3$)
(b) Pig urine			
DON in urine	100	9.5	5.4
	200	15.3	11.5
	500	11.1	11.9
	1000	9.6	6.0
	Overall	11.4	8.7
DOM-1 in urine	100	9.4	8.8

3.1.2. Linearity and sensitivity for tested B-trichothecenes and the de-epoxy-metabolite

Calibration curves were obtained by linear regression analysis and revealed good linearity and repeatability within a concentration range of 100–1000 ng/ml in urine and ng/g maize, respectively. The limit of quantification (LOQ) for the method in SIM mode was determined by S/N to be more than 10/1 [41], for triplicates in spiked pig urine and maize extract. The quantification limits in pig urine and maize are illustrated in Table 3. As can be seen the developed method shows excellent sensitivity for DON and DOM-1 in urine. Moreover, the detection limit in our LC–MS system seems to be matrix dependent, if the DON values in urine (25 ng/ml) and maize (50 ng/g)

are compared. In maize, detection limits for all tested substances are about two times higher than in urine (data not shown). In urine, similar detection limits of 50 ng/ml for DON and DOM-1 were reported by Dahlem et al. [16] by applying GC–ECD. Prelusky and Trenholm have performed thermospray LC–MS for determination and confirmation of DON and DOM-1 with a detection limit of 1 ng/ml [36]. In contrast, our LC–APCI–MS method shows a quantification limit of 25 ng/ml for DON and DOM-1 in pig urine. The LOQ in maize matrix for NIV and DON as well as 15-AcDON, 3-AcDON and Fus-X were comparable to those reported by Berger et al. [39]. An interesting observation was the outsized difference in sensitivity for 3-AcDON (50 ng/g) and 15-AcDON (150 ng/g), if it is taken into account that these two molecules differ only in the position of the acetate substitute. This observation reveals once more the substance specificity of LC–MS interface. Overall, we can report that the LC–MS method does not seem to be as sensitive as the GC–ECD or GC–MS methods [23]. However, it can be seen as a less time consuming powerful technique for the analysis of the tested compounds. Subject to error-prone derivatisation processes and problems reported by Pettersson [42] and Krska et al. [23], LC–MS could be seen as an alternative method.

3.1.3. Precision of the developed method

Precision assessments were carried out with corresponding concentrations and internal standard in pig urine and maize extract, except DOM-1, which was performed at 100 ng/ml due to the limitation of DOM-1 standard as described (Table 4). The overall intra-day precessions for NIV with 9.6% and for DON with 7.5% as well as for 3-AcDON with 6.4% and Fus-X with 6.5% in maize matrix were satisfactory. In the case of 15-AcDON, a higher relative standard deviation (R.S.D.) 11.1% was observed. The inter-day precisions for tested compounds (Table 4) and the overall inter-day R.S.D. for all tested compounds are generally comparable to our previous report and those obtained by GC–MS and GC–ECD [23–27,38].

3.2. Application of the method to contaminated pig urine and maize samples

The developed method has been applied to analyse a number of contaminated pig urine and grain samples after the validation to verify practicality. The contaminated urine samples were provided by the Medical Clinic for Ruminants and Swine at the University of Veterinary Medicine in Vienna after pigs were fed with deoxynivalenol contaminated feed at a level of 0.23 mg/kg body mass per day. The parallel control group were fed with DON-free feed. Urine samples were taken and the content of DON and DOM-1 in urine was determined by the developed LC–APCI–MS method. The mean concentration of DON in urine of animals in the trial group was 580 μg/l, whereas DOM-1 was 32 μg/l. Fig. 3a shows an obtained selected ion chromatogram of contaminated pig urine. The urine samples of those animals in the control group were negative throughout and no DON and DOM-1 could be detected. Those urine samples of animals fed with naturally contaminated feed were positive.

3.2.1. Inter-laboratory comparison

In an inter-laboratory comparison we compared and verified our results with a validated GC–MS method. Different maize and oats samples were analysed by the developed LC–MS method after being analysed at the Federal Institute of Agrobiology (Linz, Austria). The LC–MS analysis of two negative samples showed no

Table 5
Comparison of LC–MS data vs. GC–MS

Sample	NIV (ng/g)		DON (ng/g)		15-AcDON (ng/g)		3-AcDON (ng/g)		Fux-X (ng/g)	
	LC–MS	GC–MS	LC–MS	GC–MS	LC–MS	GC–MS	LC–MS	GC–MS	LC–MS	GC–MS
Maize (a)	<70	<50	<50	<50	<150	<50	<50	<50	<50	<50
Maize (b)	<70	<50	1300	984	330	250	<50	<50	<50	<50
Oats (a)	<70	<50	<50	<50	<150	<50	<50	<50	<50	<50
Oats (b)	300	219	1890	2389	<50	<50	710	960	<50	<50

peaks of any tested compounds. Two additional samples with different contaminations of B-trichothecenes were analysed to ensure the selectivity and accuracy of the LC–MS method (Table 5). Those samples, which were contaminated with trichothecenes, could be verified. Moreover, the quantitative analysis of LC–MS differed 27% or less from those obtained by a validated GC–MS method.

4. Conclusions

The presented LC–APCI–MS method with the internal standard allows for the first time separation, identification, and simultaneous quantification of major B-trichothecenes and the important metabolite DOM-1 of DON at trace levels in maize and pig urine. In particular, DOM-1 was alternatively evaluated by this method and it could be resolved from other B-trichothecenes. The rapid one-step sample clean-up by using the selective RP C18 column for polar substances allows for a satisfactory separation, especially for 15-AcDON and 3-AcDON. The performed simple gradient system delivers highly reproducible retention times of tested toxins within 30 min without changes of flow-rates as described by Walker and Meier [32]. The linearity and repeatability of the method have precisely accessed and demonstrated their impact and feasibility. Due to the selectivity and simplicity of the method, it is ideally suited for determination of B-trichothecenes in biological matrices. Although the sensitivity of LC–MS is generally poorer than those of GC–ECD or GC–MS, the latter method necessitates time-consuming derivatisation. The presented LC–APCI–MS method can be employed for the measurement of B-trichothecenes in grains or for diagnostic purposes in cases of suspected toxicity.

Acknowledgements

The authors express special thanks to Dr. Elisabeth Fuchs, Biomin, Vienna, Austria, for providing the DOM-1 standard. Dr. Hans Lew (Federal Institute of Agrobiology, Linz, Austria) is thanked for providing the blank and control samples and Ms. Anita Dockner for her laboratory assistance.

References

[1] S.H. Hussein, J.M. Brasel, Toxicology 167 (2001) 101.
[2] J.P.F. D'Mello, C.M. Placinta, A.M.C. Macdonald, Anim. Feed Sci. Technol. 80 (1999) 183.
[3] E.E. Creppy, Toxicol. Lett. 127 (2002) 19.
[4] H.M. Müller, J. Reimann, U. Schumacher, K. Schwadorf, Food Add. Contam. 15 (1997) 801.
[5] H. Lew, A. Adler, W. Edinger, W. Brodacz, E. Kiendler, J. Hinterholzer, M. Oberforster, Mycotoxin Res. 17A (2001) 1.
[6] B.A. Rotter, D.B. Prelusky, J.J. Pestka, J. Toxicol. Environ. Health 48 (1996) 1.
[7] R.A. Canady, R.D. Coker, S.K. Egan, R. Krska, T. Kuiper-Goodman, T.M. Olsen, J. Pestka, S. Resnik, J. Schlatter, The joint FAO/WHO expert committee on food additives (JECFA) deoxynivalenol, 2002 (http://www.inchem.org/documents/jecfa/jecmono/v47je05.htm).
[8] J. Böhm, Übers. Tierernährg. 28 (2000) 95.
[9] D.B. Prelusky, K.E. Hartin, H.L. Trenholm, J.D. Miller, Fundam. Appl. Toxicol. 10 (1988) 276.
[10] R.W. Coppock, S.P. Swanson, H.B. Gelberg, G.D. Koritz, W.E. Hoffman, W.B. Buck, R.F. Vesonder, Am. J. Vet. Res. 46 (1985) 169.
[11] T. Yoshizawa, T. Hiroaki, T. Ohi, Agric. Biol. Chem. 47 (1983) 2133.
[12] R. Hedman, H. Pettersson, Arch. Anim. Nutr. 50 (1997) 312.
[13] B. Kollarczik, M. Gareis, M. Hanelt, Nat. Toxins 2 (1994) 105.
[14] D.B. Prelusky, D.M. Veira, H.L. Trenholm, K.E. Hartin, Fundam. Appl. Toxicol. 6 (1986) 356.
[15] L.M. Côté, J. Nicoleti, S.P. Swanson, W.B. Buck, J. Agric. Food Chem. 34 (1986) 458.
[16] A.M. Dahlem, S.P. Swanson, L.-M. Côté, T. Yoshizawa, W.B. Buck, J. Chromatogr. 378 (1986) 226.
[17] T. Yoshizawa, L.-M. Côté, S.P. Swanson, W.B. Buck, Agric. Biol. Chem. 50 (1986) 227.
[18] S.P. Swanson, J. Nicoleti, H.D. Rood, W.B. Buck, L.-M. Côté, T. Yoshizawa, J. Chromatogr. 414 (1987) 335.
[19] M.W. Trucksess, A.E. Pohland (Eds.), Mycotoxin Protocols, Methods in Molecular Biology, vol. 157, Humana Press, Totowa, 2001, Part III, p. 97.
[20] K.K. Sinha, D. Bhatnagar (Eds.), Mycotoxins in Agriculture and Food Safety, Marcel Dekker, Basel, 1998, Chapter 5, p. 135.
[21] L. Schneider, H. Pichler, R. Krska, Fresenius J. Anal. Chem. 367 (2000) 98.
[22] E. Usleber, V. Renz, E. Märtlbauer, G. Terplan, J. Vet. Med. B 39 (1992) 617.
[23] R. Krska, S. Baumgartner, R. Josephs, Fresenius J. Anal. Chem. 371 (2001) 285.
[24] W. Langseth, T. Rundberget, J. Chromatogr. A 815 (1) (1998) 103.
[25] K.F. Nielsen, U. Thrane, J. Chromatogr. A 929 (1–2) (2001) 75.
[26] M. Schollenberger, U. Lauber, H. Terry Jara, S. Suchy, W. Drochner, H.M. Müller, J. Chromatogr. A 815 (1998) 123.

[27] J. Weingärtner, R. Krska, W. Praznik, M. Grasserbauer, H. Lew, Fresenius J. Anal. Chem. 357 (1997) 1206.
[28] R.J. Krska, Chromatogr. A 815 (1) (1998) 49.
[29] Y. Onji, Y. Aoki, N. Tani, K. Umebayashi, Y. Kitada, Y. Dohi, J. Chromatogr. A 815 (1) (1998) 59.
[30] L.M. Cahill, S.C. Kruger, B.T. McAlice, C.S. Ramsey, R. Prioli, B. Kohn, J. Chromatogr. A 859 (1) (1999) 23.
[31] M. Jiménez, R. Mateo, J. Chromatogr. A 778 (1–2) (1997) 363.
[32] F. Walker, B. Meier, J. AOAC Int. 81 (4) (1998) 741.
[33] J.J. Mateo, A. Llorens, R. Mateo, M. Jiménez, J. Chromatogr. A 918 (1) (2001) 99.
[34] J.J. Mateo, R. Mateo, M.J. Hinojo, A. Llorens, M. Jiménez, J. Chromatogr. A 955 (2) (2002) 245.
[35] R.D. Josephs, R. Krska, M. Grasserbauer, J.A.C. Broekaert, J. Chromatogr. A 795 (2) (1998) 297.
[36] D.B. Prelusky, H.L. Trenholm, J. Agric. Food Chem. 39 (1991) 748.
[37] R.D. Plattner, Nat. Toxins 7 (1999) 365.
[38] E. Razzazi-Fazeli, J. Böhm, W. Luf, J. Chromatogr. A 854 (1999) 45.
[39] U. Berger, M. Oehme, F. Kuhn, J. Agric. Food Chem. 47 (10) (1999) 4240.
[40] F. Berthiller, R. Schuhmacher, M. Freudenschuss, G. Adam, R. Krska, Mycotoxin Res., in press.
[41] Guidelines for validation of analytical methods for non-agricultural pesticide active ingredients and products, 2001 (http://www.hse.gov.uk/hthdir/noframes/validation.pdf).
[42] H. Pettersson, European Commission, BCR information, Report EUR 18214 EN, 1998.