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# Quantitative determination of T-2 toxin, HT-2 toxin, deoxynivalenol and deepoxy-deoxynivalenol in animal body fluids using LC–MS/MS detection

S. De Baere*, J. Goossens, A. Osselaere, M. Devreese, V. Vandenbroucke, P. De Backer, S. Croubels

*Dept. of Pharmacology, Toxicology and Biochemistry, Fac. of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium*

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ABSTRACT

A sensitive and specific method for the quantitative determination of deoxynivalenol (DON), deepoxy-deoxynivalenol (DOM-1), T-2 toxin (T-2) and HT-2 toxin (HT-2) in animal body fluids (plasma and bile) using liquid chromatography combined with electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) is presented. The extraction of plasma consisted of a deproteinization step using methanol, followed by a clean-up using an Oasis® HLB solid-phase extraction column. For bile analysis, an extraction using a methanol/water mixture (70/30, v/v), followed by a liquid–liquid extraction using ethyl acetate, was performed. Chromatographic separation was achieved on a reversed-phase Nucleosil (100-5 C18 G100 × 3.0 mm) column. For the analysis of DON and DOM-1, a mixture of 0.1% acetic acid in water and methanol was used as the mobile phase. T-2 and its metabolite HT-2 were separated using 5 mM ammonium acetate in a mixture of water/methanol/acetic acid. The mass spectrometer was operated in the negative or positive ESI selected reaction monitoring mode for DON and T-2 analysis, respectively. Calibration graphs (1–250 ng mL$^{-1}$) were prepared for all matrices and correlation and goodness-of-fit coefficients were between 0.9978–1.000 and 2.96–11.77%, respectively. Limits of quantification were between 1 and 2.5 ng mL$^{-1}$ for all compounds. Limits of detection ranged from 0.01 to 0.63 ng mL$^{-1}$. The results for the within-day precision and accuracy fell within the ranges specified. The method has been successfully used for the quantitative determination of DON, DOM-1, T-2 and HT-2 in plasma and the semi-quantitative determination of the same compounds in bile from broiler chickens and pigs, respectively.



## 1. Introduction

T-2 toxin (T-2), HT-2 toxin (HT-2) and deoxynivalenol (DON) are among the most predominant *Fusarium* mycotoxins in Europe. They belong to the group of the type-A and -B trichothecenes, respectively, and are produced by *Fusarium* moulds that are infecting agricultural crops during growth, drying and subsequent storage [1]. As a consequence, animal feed can be contaminated with these mycotoxins, resulting in impaired livestock productivity due to health problems after the consumption of contaminated feed.

In order to decrease the risk for toxicity due to ingestion of *Fusarium* mycotoxins in farm animals (e.g. pigs, chickens, cattle) many attempts have been made to search for ways to detoxify contaminated feed [2,3]. The addition of mycotoxin binders/modifiers to the animal feed seems a promising possibility, but the positive effects have not yet been clearly proven. Manufacturers have to prove the efficacy of these products, which is generally based on *in vitro* adsorption studies. However, a lot of controversy exists regarding the correlation between the *in vitro* binding capacity and the *in vivo* efficacy of mycotoxin binders/modifiers. Therefore, the European Food Safety Authority (EFSA) has recently proposed guidelines for the performance of efficacy studies with mycotoxin binders/modifiers [4]. Apart from the *in vitro* studies, which can be used for screening purposes, the efficacy of mycotoxin binders/modifiers has to be proven a.o. by *in vivo* absorption, distribution, metabolisation and excretion (ADME)

*Abbreviations:* ADME, absorption, distribution, metabolisation and excretion; CID, collision-induced dissociation; $^{13}C_{15}$-DON, $^{13}C$ labelled deoxynivalenol (internal standard); $^{13}C_{24}$-T-2, $^{13}C$ labelled T-2 toxin (internal standard); DOM-1, deepoxy-deoxynivalenol; DON, deoxynivalenol; EFSA, European Food Safety Authority; ESI, electrospray ionisation; *g*, goodness-of-fit coefficient; HPLC, high performance liquid chromatograpy; HT-2, HT-2 toxin; IAC, immuno affinity column; IS, internal standard; IV, intravenous administration; LC–ESI-MS/MS:, liquid chromatography combined with electrospray ionization tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; *m/z*, mass to charge ratio; $N_2$, nitrogen; OR, oral administration; *r*, correlation coefficient; $R_A$, apparent extraction recovery; $R_E$, recovery of the extraction step; RSD, relative standard deviation; S/N, signal-to-noise ratio; SPE, solid-phase extraction; SRM, selected reaction monitoring; SSE, signal suppression/enhancement; T-2, T-2 toxin.

* Corresponding author. Tel.: +32 09 264 73 48; fax: +32 09 264 74 97.
*E-mail addresses:* siegrid.debaere@ugent.be (S. De Baere), joline.goossens@ugent.be (J. Goossens), ann.osselaere@ugent.be (A. Osselaere), mathias.devreese@ugent.be (M. Devreese), virginie.vandenbroucke@ugent.be (V. Vandenbroucke), patrick.debacker@ugent.be (P. De Backer), siska.croubels@ugent.be (S. Croubels).

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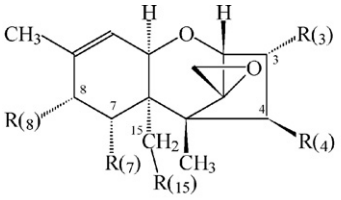
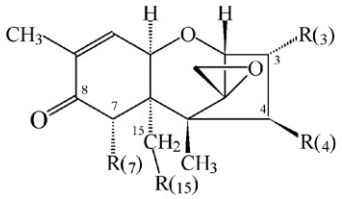


Type A Trichothecenes



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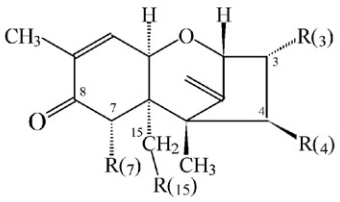

DOM-1

| Type-A Trichothecenes | R(3) | R(4) | R(7) | R(8) | R(15) |
|---|---|---|---|---|---|
| T-2 | OH | OAc | H | i-Val | OAc |
| HT-2 | OH | OH | H | i-Val | OAc |
| **Type-B Trichothecenes** | | | | | |
| DON | OH | H | OH | | OH |
| DOM-1 | OH | H | OH | | OH |

**Fig. 1.** Chemical structures of DON, DOM-1, T-2 and HT-2.

studies (e.g. by performing toxicokinetic studies with relevant mycotoxins, whether or not in combination with the target mycotoxin binders/modifiers). The relevant end-points that have to be evaluated for DON are the concentrations of DON and metabolites in blood/plasma/serum [4]. Since deepoxy-deoxynivalenol (DOM-1) was reported to be the main *in vivo* metabolite of DON, it was also included as a target analyte [5]. For T-2 and HT-2 no end-points were specified in the current guideline [4] and therefore only the parent compounds were included in the presented analysis method. Chemical structures of the compounds investigated are shown in Fig. 1.

It is obvious that for the investigation of the toxicokinetics of trichothecenes in biological fluids (e.g. plasma, bile, urine), the availability of sensitive and validated analytical methods is necessary.

In the literature many methods are reported for the analysis of several mycotoxines, among which trichothecenes in food and feed [6–12]. Analytical methods for the determination of these compounds in biological matrices are, however, scarce (e.g. Razzazi-Fazeli et al., 2003 for the determination of major type-B trichothecenes in pig urine; Bily et al., 2004 for the analysis of DON, 15-acetyl deoxynivalenol and zearalenone in pig serum) [13,14].

An important and critical step in the analysis of mycotoxins is the initial sample preparation and further clean-up. Most type-A and -B trichothecenes are extracted from food and feed with aqueous methanol or acetonitrile at varying ratios [15,16]. For the further sample clean-up of trichothecenes, the use of solid-phase extraction (SPE), immuno-affinity (IAC) and multifunctional clean-up columns are reported in [13,17,18].

SPE columns are available in a variety of stationary phases. The simultaneous extraction of type-A and -B trichothecenes from different food matrices, using reversed phase Oasis® HLB SPE columns has been reported by Lattanzio et al. [19]. Good recoveries were obtained for all studied trichothecenes. Multifunctional MycoSep® columns have the advantage of a rapid handling and a reduction in amount of organic solvents used [13,20,21]. The selection of the right column may sometimes be tricky and the selectivity is substantially lower when compared to IAC columns [22]. The use of IAC columns results generally in cleaner extracts due to the high selectivity and specificity, in a smaller variability between samples and in chromatograms free from matrix interferences. However, sample preparation using IAC columns is rather time consuming and very expensive, which is a disadvantage if a large amount of samples has to be analysed, which is the case when a toxicokinetic study is performed.

For the separation and detection of trichothecene mycotoxins, HPLC in combination with various detectors (ultraviolet, diode-array, fluorescence and MS/MS) has become the most important analytical technique for the analysis of mycotoxins in various matrices (food, feed, biological samples) [17,22]. Among the different detectors, mass spectrometry is very sensitive, selective and specific, but an appropriate sample clean-up is still

necessary to reduce matrix interferences. Chromatography is generally performed using reversed-phase C18 columns with mobile phases consisting of a mixture of water and methanol or acetonitrile. Due to ion suppression effects in MS, the use of suitable internal standards, ideally $^{13}C$ labelled mycotoxins, is recommended [17,22].

The aim of the present study was to develop a sensitive and reliable LC–MS/MS method for the quantitative determination of type-A and -B trichothecenes which are most affecting pig and poultry husbandry (i.e. DON, T-2 and HT-2) and DOM-1 in animal body fluids (plasma and bile). The optimization of the extraction, clean-up and chromatographic conditions is discussed, as well as the occurrence of matrix effects. Method performance characteristics are presented (linearity, accuracy and precision, limit of quantification and detection, extraction recovery). Following validation, the method was used to investigate the toxicokinetics of DON, T-2, HT-2 and the major metabolite of DON (DOM-1) in plasma and bile of pigs and broiler chickens.

## 2. Experimental

### 2.1. Chemicals, products and reagents

All standards (DON, DOM-1, T-2, HT-2, $^{13}C_{15}$-DON and $^{13}C_{24}$-T-2) were obtained from Sigma–Aldrich (Bornem, Belgium) and stored at ≤−15 °C. Water, methanol and acetonitrile were of HPLC grade, while ammonium acetate, glacial acetic acid and ethyl acetate were of analytical grade. All these reagents were obtained from VWR (Leuven, Belgium).

Oasis® HLB solid-phase extraction (SPE) columns (60 mg/3 cc) were obtained from Waters (Zellik, Belgium).

Millex®-GV PVDF filter units (0.22 μm) were obtained from Millipore (Brussels, Belgium).

Vacutest® Kima Lithium heparin 215 I.U tubes (9 mL) were obtained from Novolab (Geraardsbergen, Belgium).

### 2.2. Preparation of standard solutions

Stock solutions were prepared in acetonitrile (DON – 1 mg mL$^{-1}$) or methanol (T-2 – 0.5 mg mL$^{-1}$, HT-2 – 1 mg mL$^{-1}$). The standards of DOM-1, $^{13}C_{15}$-DON and $^{13}C_{24}$-T-2 were purchased as solutions of 50, 25 and 25 μg mL$^{-1}$ in acetonitrile, respectively. The stock solutions were stored at ≤−15 °C.

Working solutions of 100 μg mL$^{-1}$ of each individual mycotoxin (except DOM-1) were prepared. Combined working solutions of 2.5, 0.25 and 0.025 μg mL$^{-1}$ (plasma analysis) and 10, 1 and 0.1 μg mL$^{-1}$ (bile analysis) of each mycotoxin (except the internal standards) were prepared by mixing an appropriate amount of each working solution of 100 μg mL$^{-1}$ or 50 μg mL$^{-1}$ (DOM-1), followed by further dilution in acetonitrile/water (50/50, v/v). For the internal standards a combined working solution of 1 μg mL$^{-1}$ of $^{13}C_{15}$-DON and $^{13}C_{24}$-T-2 was prepared in acetonitrile/water (50/50, v/v). The working solutions were stored at 2–8 °C.

### 2.3. Biological samples

*Blank samples* (plasma, bile) were obtained from pigs and broiler chickens that received blank feed (DON, T-2 concentrations <LOQ of 1–2.5 ng mL$^{-1}$).

*Incurred plasma samples* were obtained as a part of several preliminary toxicokinetic studies with T-2 and DON in pigs and broiler chickens.

*Preliminary bolus toxicokinetic study with T-2*. Blood samples were obtained in heparinized tubes from 2 broiler chickens that received one intravenous (IV) bolus of T-2 toxin (dose: 0.15 μg/kg BW, corresponding with a theoretical daily intake of 0.15 kg of feed contaminated with 100 μg/kg of T-2). The blood samples were taken before mycotoxin administration and at 2, 5, 10, 15, 20, 30, 40 and 50 min and at 1, 1.25, 1.5, 1.75 and 2 h after T-2 administration.

*Preliminary bolus toxicokinetic study with DON in broiler chickens and pigs. Chickens.* Blood samples were obtained in heparinized tubes from 2 broiler chickens (1 fasted and 1 non-fasted animal) that received one oral bolus of DON (dose: 0.15 mg/kg BW, corresponding with a theoretical daily intake of 0.15 kg of feed contaminated with 1 mg/kg of DON). Plasma samples were taken before and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 h after administration.

*Pigs*. Blood samples were obtained from one fasted pig that received an oral bolus of DON (dose: 0.05 mg/kg BW, corresponding with a theoretical daily intake of 1 kg of feed contaminated with 0.9 mg/kg of DON). Blood samples were taken before and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 h after administration.

*Preliminary steady-state toxicokinetic study with DON*. Blood samples were taken in heparinized tubes from broiler chickens ($n$ = 6 per group) that were given blank feed or feed that was (naturally) contaminated with DON for 21 days. Blood samples were taken before and at 1, 2 and 3 weeks after the start of administration of the (contaminated) feed. After slaughter, the bile of each chicken was sampled. The samples were pooled per treatment group ($n$ = 6 chickens).

Plasma samples were obtained after centrifugation (2054 × $g$, 10 min) of the blood samples. All blank and incurred samples were stored at ≤−15 °C until the moment of analysis.

### 2.4. Sample pretreatment

#### 2.4.1. Plasma

To 250 μL of plasma were added 12.5 μL of the IS working solution and 250 μL of methanol, followed by a vortex mixing (15 s) and centrifugation step (10 min, 7826 × $g$). The supernatant was transferred to another extraction tube and 2 mL of water were added. After vortex mixing, the sample was applied onto an Oasis® HLB SPE column that was conditioned with 2 mL of methanol and 2 mL water/methanol (90/10, v/v). The column was washed with 2 mL of water/methanol (90/10, v/v), followed by a drying step (10 min, vacuum suction). The analytes were eluted using 1 mL of methanol. The eluate was evaporated using a gentle nitrogen ($N_2$) stream (∼50 °C). The dry residue was reconstituted in 125 μL of a 95/5 (v/v) mixture of mobile phase A/B for DON analysis or 70/30 (v/v) for T-2 analysis. After vortex mixing, the sample was transferred to an autosampler vial and an aliquot (10 μL) was injected onto the LC–MS/MS instrument.

#### 2.4.2. Bile

To 1.0 mL of bile were added 25.0 μL of the IS working solution. After vortex mixing, the sample was left at room temperature for 5 min. Thereafter, 4 mL of a water/methanol mixture (30/70, v/v) were added, followed by a vortex mixing step (15 s). The sample was extracted on a vertical rotary apparatus for 10 min, followed by a centrifugation step (10 min, 2054 × $g$). The supernatant was transferred to another extraction tube and 4 mL of water and 8 mL of ethyl acetate were added. After vortex mixing, the sample was extracted again for 20 min, followed by a 10-min centrifugation step (2054 × $g$). The combined supernatants were transferred to another tube and evaporated using a gentle stream of $N_2$ (∼50 °C). The dry residue was reconstituted in 250 μL of mobile phase A/B (95/5 (v/v) for DON analysis and 70/30 (v/v) for T-2 analysis and vortex mixed for 15 s. After vortex mixing, the sample was passed trough a Millex® GV-PVDF filter and transferred to an autosampler vial. An aliquot (10 μL) was injected onto the LC–MS/MS instrument.

**Table 1**
SRM transitions and MS/MS parameters for the target analytes.

| Analyte | MM[a] (g mol$^{-1}$) | Precursor ion | Product ions | CE[b] (%) | Dwell time (ms) | Retention time (min) |
|---|---|---|---|---|---|---|
| DON | 296 | 355.05 $[M+Ac]^-$ | 265.15 (quantifier) | 15 | 500 | 6.95[c] |
| | | | 295.10 (qualifier) | 15 | 500 | |
| DOM-1 | 280 | 339.00 $[M+Ac]^-$ | 59.10 (qualifier) | 35 | 500 | 7.50[c] |
| | | | 249.00 (quantifier) | 15 | 500 | |
| $^{13}C_{15}$-DON | 311 | 370.05 $[M+Ac]^-$ | 279.2 (quantifier) | 15 | 500 | 7.00[c] |
| | | | 310.1 (qualifier) | 10 | 500 | |
| T-2 | 466 | 484.08 $[M+NH_4]^+$ | 185.05 (qualifier) | 25 | 350 | 12.60[d] |
| | | | 215.07 (quantifier) | 24 | 350 | |
| HT-2 | 424 | 442.02 $[M+NH_4]^+$ | 215.07 (qualifier) | 15 | 350 | 11.50[d] |
| | | | 263.10 (quantifier) | 14 | 350 | |
| $^{13}C_{24}$-T-2 | 490 | 508.18 $[M+NH_4]^+$ | 198.10 (qualifier) | 25 | 350 | 12.60[d] |
| | | | 229.14 (quantifier) | 23 | 350 | |

[a] MM: molecular mass.
[b] CE: collision energy.
[c] LC–MS/MS run for DON.
[d] LC–MS/MS run for T-2.

### 2.5. Liquid chromatography

The LC system consisted of a quaternary, low-pressure mixing pump with vacuum degassing, type Surveyor MSpump plus and an autosampler with temperature controlled tray and column oven, type Autosampler plus, from ThermoFisher Scientific (Breda, The Netherlands). Chromatographic separation was achieved on a Nucleosil (100-5 C18 G100 × 3.0 mm) column in combination with a ChromSep Guard Column (SS 10 × 2 mm), both from Varian/Agilent (Sint-Katelijne-Waver, Belgium).

For the analysis of DON and DOM-1, the mobile phase A consisted of 0.1% acetic acid in water, while the mobile phase B was methanol. A gradient elution was performed: 0–2 min (95% A, 5% B), 6 min (linear gradient to 90% B), 6–10 min (10% A, 90% B), 10.5 min (linear gradient to 95% A), 10.5–15 min (95% A, 5% B). The flow-rate was 300 μL min$^{-1}$.

The mobile phase A for the analysis of T-2 and HT-2 was composed of 5 mM ammonium acetate in water/methanol/acetic acid (94/5/1, v/v/v), while the mobile phase B consisted of 5 mM ammonium acetate in methanol/water/acetic acid (97/2/1, v/v/v). A gradient elution was performed: 0–2 min (70% A, 30% B), 6 min (linear gradient to 30% A), 6–14 min (30% A, 70% B), 15 min (linear gradient to 70% A), 15–20 min (70% A, 30% B). The flow-rate was set at 200 μL min$^{-1}$.

The temperatures of the column oven and autosampler tray were set at 35 °C and 5 °C, respectively.

### 2.6. Mass spectrometry

The LC column effluent was interfaced to a TSQ® Quantum Ultra triple quadrupole mass spectrometer, equipped with a heated electrospray ionisation (h-ESI) probe operating in both the positive and negative ionisation mode (all from ThermoFisher Scientific).

Instrument (or CID) parameters were optimised by syringe infusion of working solutions of 1 μg mL$^{-1}$ of each compound (flow-rate: 10 μL min$^{-1}$) in combination with the mobile phase (50% A, 50% B, flow-rate: 200 μL min$^{-1}$). The resolution for Q1 and Q3 were set at 0.7 peak width half-height.

The following parameters were used for ESI(+) analysis (T-2 and HT-2): spray voltage: 4000 V, vaporizer temperature: 300 °C, sheath gas pressure: 40 au (arbitrary units), ion sweep gas pressure: 2.0 au, auxiliary gas pressure: 15 au, capillary temperature: 300 °C, tube lens offset: 100 V, source CID collision energy: 5, collision pressure: −1.5 mTorr and quad MS/MS bias: 3.0. The parameters for ESI(−) analysis (DON and DOM-1) were set at: spray voltage: −3500 V, vaporizer temperature: 300 °C, sheath gas pressure: 40 au (arbitrary units), ion sweep gas pressure: 2.0 au, auxiliary gas pressure: 15 au, capillary temperature: 300 °C, tube lens offset: −60 V, source CID collision energy: 5, collision pressure: −1.5 mTorr and quad MS/MS bias: 3.0.

Acquisition was performed in the selected reaction monitoring (SRM) mode. For each compound, the two most intense product ions of the precursor ion were monitored in the SRM mode for quantification and identification, respectively (see Table 1).

**Table 2**
Evaluation of the apparent extraction recovery ($R_A$, %), the signal enhancement/suppression (SSE, %) and the recovery of the extraction step ($R_E$, %).

| Analyte | $R_A$ (%) | SSE (%) | $R_E$ (%) |
|---|---|---|---|
| *Chicken plasma* | | | |
| DON | 91.6 | 105.5 | 86.8 |
| DOM-1 | 82.0 | 95.8 | 85.6 |
| T-2 | 58.8 | 96.7 | 60.8 |
| HT-2 | 69.1 | 99.1 | 69.8 |
| *Pig plasma* | | | |
| DON | 114.5 | 130.3 | 87.8 |
| DOM-1 | 39.4 | 45.9 | 85.9 |
| T-2 | 106.8 | 110.4 | 96.7 |
| HT-2 | 97.6 | 93.6 | 104.2 |
| *Chicken bile* | | | |
| DON | 43.7 | 108.8 | 40.1 |
| DOM-1 | 44.1 | 101.7 | 43.4 |
| T-2 | 88.4 | 70.2 | 125.9 |
| HT-2 | 89.2 | 90.2 | 98.9 |

**Table 3**
Results of the linearity evaluation for the analysis of type-A and -B trichothecenes in animal plasma and bile.

| Analyte | Calibration range ($ng\,mL^{-1}$) | Goodness-of-fit coefficient (g, %)[a] | Correlation coefficient ($r$)[a] | Matrix |
|---|---|---|---|---|
| DON | 1.0–250 | 4.26 | 0.9997 | Pig plasma |
| | 1.25–250 | 3.95 | 0.9998 | Chicken plasma |
| | 1.0–250 | 6.09 | 0.9987 | Chicken bile |
| DOM-1 | 2.5–250 | 3.99 | 0.9992 | Pig plasma |
| | 1.25–250 | 5.54 | 0.9985 | Chicken plasma |
| | 2.5–250 | 7.32 | 0.9978 | Chicken bile |
| HT-2 | 1.0–250 | 4.98 | 0.9996 | Pig plasma |
| | 1.25–250 | 11.77 | 0.9993 | Chicken plasma |
| | 2.5–250 | 6.64 | 0.9985 | Chicken bile |
| T-2 | 1.0–250 | 3.25 | 1.0000 | Pig plasma |
| | 1.25–250 | 7.20 | 0.9987 | Chicken plasma |
| | 1.0–250 | 2.96 | 0.9995 | Chicken bile |

[a] Acceptability ranges: $r \geq 0.99$, $g \leq 20\%$.

### 2.7. In house method validation

The developed method was validated by a set of parameters that were in compliance with the recommendations as defined by the European Community [23] and with reference guidelines defined in other EU and FDA documents [24,25].

*Calibration curves*. Calibration curve samples (concentration range 1–250 $ng\,mL^{-1}$) were prepared by applying standard working solutions directly onto the homogenized blank samples, followed by a vortex mixing step. After 5 min of equilibration, the calibration curve samples were treated in a similar way as the unknown samples. The correlation coefficients ($r$) and goodness-of-fit coefficients ($g$) were determined and had to be ≥0.99 and ≤20%, respectively [23].

*Accuracy and precision*. Within-day precision (repeatability) and accuracy were determined by analyzing 6 blank samples

**Table 4**
Results of the within-run precision and accuracy, LOQ and LOD evaluation for the analysis of type-A and -B trichothecenes in animal plasma.

| Analyte | Mean conc. ± SD ($ng\,mL^{-1}$) | Precision, RSD[a] (%) | Accuracy[a] (%) | LOD ($ng\,mL^{-1}$) |
|---|---|---|---|---|
| **DON** | | | | |
| *Pig plasma* | | | | |
| 1.0 $ng\,mL^{-1}$ ($n=5$) | 0.9 ± 0.08 | 9.8 | −14.3 | 0.10 |
| 2.5 $ng\,mL^{-1}$ ($n=6$) | 2.7 ± 0.31 | 11.4 | 8.0 | |
| 25.0 $ng\,mL^{-1}$ ($n=6$) | 25.5 ± 1.46 | 5.7 | 1.9 | |
| 250.0 $ng\,mL^{-1}$ ($n=6$) | 260.6 ± 20.39 | 7.8 | 4.2 | |
| *Chicken plasma* | | | | |
| 1.25 $ng\,mL^{-1}$ ($n=6$) | 1.3 ± 0.11 | 8.5 | 0.9 | 0.20 |
| 5.0 $ng\,mL^{-1}$ ($n=6$) | 5.1 ± 0.26 | 5.2 | 1.3 | |
| 25.0 $ng\,mL^{-1}$ ($n=6$) | 24.2 ± 0.85 | 3.5 | −3.1 | |
| **DOM-1** | | | | |
| *Pig plasma* | | | | |
| 2.5 $ng\,mL^{-1}$ ($n=6$) | 2.7 ± 0.14 | 5.0 | 9.3 | 0.19 |
| 25.0 $ng\,mL^{-1}$ ($n=6$) | 20.4 ± 2.90 | 14.2 | −18.5 | |
| 250.0 $ng\,mL^{-1}$ ($n=6$) | 212.6 ± 15.20 | 7.1 | −14.9 | |
| *Chicken plasma* | | | | |
| 1.25 $ng\,mL^{-1}$ ($n=6$) | 2.4 ± 0.54 | 22.9 | −5.7 | 0.63 |
| 5.0 $ng\,mL^{-1}$ ($n=6$) | 4.2 ± 0.42 | 10.1 | −16.5 | |
| 25.0 $ng\,mL^{-1}$ ($n=6$) | 26.2 ± 2.75 | 10.5 | 4.8 | |
| **HT-2** | | | | |
| *Pig plasma* | | | | |
| 1.0 $ng\,mL^{-1}$ ($n=6$) | 1.1 ± 0.11 | 10.0 | 7.5 | 0.08 |
| 2.5 $ng\,mL^{-1}$ ($n=6$) | 2.4 ± 0.10 | 4.2 | −2.7 | |
| 25.0 $ng\,mL^{-1}$ ($n=6$) | 25.2 ± 0.54 | 2.1 | 0.6 | |
| 250.0 $ng\,mL^{-1}$ ($n=6$) | 257.1 ± 17.60 | 6.8 | 2.8 | |
| *Chicken plasma* | | | | |
| 2.5 $ng\,mL^{-1}$ ($n=6$) | 2.4 ± 0.51 | 20.9 | −2.3 | 0.07 |
| 5.0 $ng\,mL^{-1}$ ($n=6$) | 4.8 ± 0.75 | 15.5 | −3.7 | |
| 25.0 $ng\,mL^{-1}$ ($n=5$) | 21.2 ± 2.33 | 11.0 | −15.2 | |
| **T-2** | | | | |
| *Pig plasma* | | | | |
| 1.0 $ng\,mL^{-1}$ ($n=6$) | 1.1 ± 0.31 | 28.8 | 6.0 | 0.01 |
| 2.5 $ng\,mL^{-1}$ ($n=6$) | 2.4 ± 0.05 | 2.2 | −5.3 | |
| 25.0 $ng\,mL^{-1}$ ($n=6$) | 25.3 ± 0.34 | 1.3 | 1.1 | |
| 250.0 $ng\,mL^{-1}$ ($n=6$) | 253.7 ± 19.75 | 7.8 | 1.5 | |
| *Chicken plasma* | | | | |
| 2.5 $ng\,mL^{-1}$ ($n=6$) | 2.6 ± 0.40 | 15.4 | 2.7 | 0.06 |
| 5.0 $ng\,mL^{-1}$ ($n=5$) | 4.8 ± 0.57 | 12.0 | −4.8 | |
| 25.0 $ng\,mL^{-1}$ ($n=6$) | 25.1 ± 1.75 | 7.0 | 0.2 | |

[a] *Acceptability ranges:* accuracy: conc. 1–10 $ng\,mL^{-1}$: −30 to +10%, conc. >10 $ng\,mL^{-1}$: −20% to +10%, within-run precision ($RSD_{max}$, %): 1.0 $ng\,mL^{-1}$ → 30.2%, 1.25 $ng\,mL^{-1}$ → 29.2%, 2.5 $ng\,mL^{-1}$ → 26.3%, 5.0 $ng\,mL^{-1}$ → 23.7%, 25.0 $ng\,mL^{-1}$ → 18.6%, 250.0 $ng\,mL^{-1}$ → 13.1%, LOD: S/N = 3.

that were spiked at low (2.5 and 5 ng mL$^{-1}$) and high (25 and 250 ng mL$^{-1}$) concentration levels on the same day. The between-day precision and accuracy were determined by analyzing quality control samples (concentration level: 2.5, 5 or 25 ng mL$^{-1}$) together with each analytical batch of samples, run on different days. The acceptance criteria for accuracy were: −30% to +10% and −20% to +10% for concentrations between 1 and 10 ng mL$^{-1}$, and ≥10 ng mL$^{-1}$, respectively. For the precision, the relative standard deviation (RSD) had to be below the $RSD_{max}$ value with $RSD_{max} = 2^{(1-0.5\log Conc)} \times 2/3$ for within-day precision and $2^{(1-0.5\log Conc)}$ for between-day precision [23,24].

*Limit of quantification*. The limit of quantification (LOQ) was the lowest concentration for which the method was validated with an accuracy and precision that fell within the recommended ranges (see section for accuracy and precision). The LOQ was also established as the lowest point of the calibration curve. The LOQ was determined by analyzing 6 spiked samples.

*Limit of detection*. The limit of detection (LOD) was the lowest concentration that could be determined with a signal-to-noise (S/N) ratio of ≥3. The LOD values were calculated using samples spiked at the LOQ level.

*Specificity*. The specificity of the method was evaluated with respect to interferences from endogenous compounds. The S/N ratio of a possible interfering peak in a blank sample ($n = 1$) had to be below to the S/N ratio of the analyte(s) in the same elution zone at the LOD level.

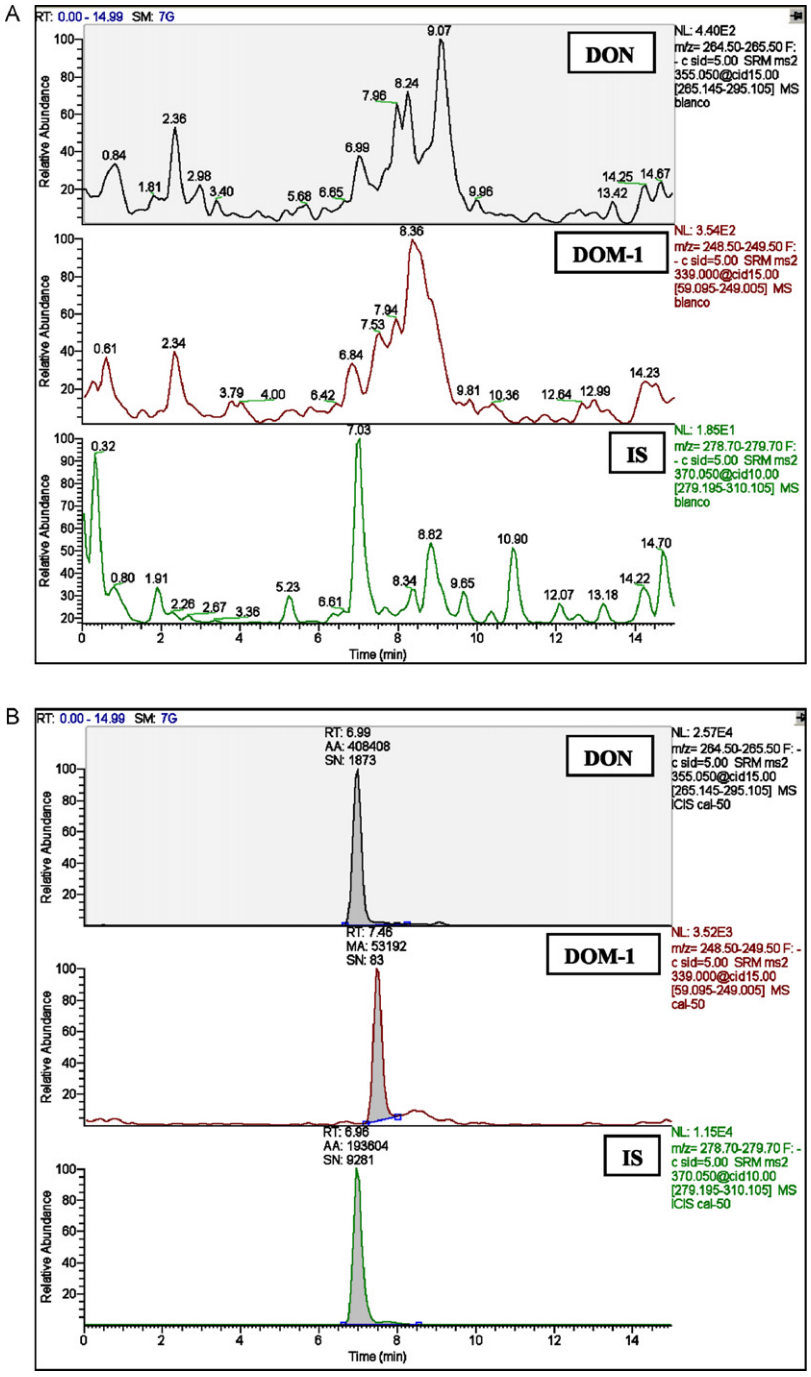


**Fig. 2.** LC–MS/MS chromatograms of DON and DOM-1 in pig plasma: (a) blank plasma sample, (b) plasma sample spiked at a level of 50 ng mL$^{-1}$ of DON and DOM-1, (c) incurred plasma sample (DON concentration: 8.1 ng mL$^{-1}$).

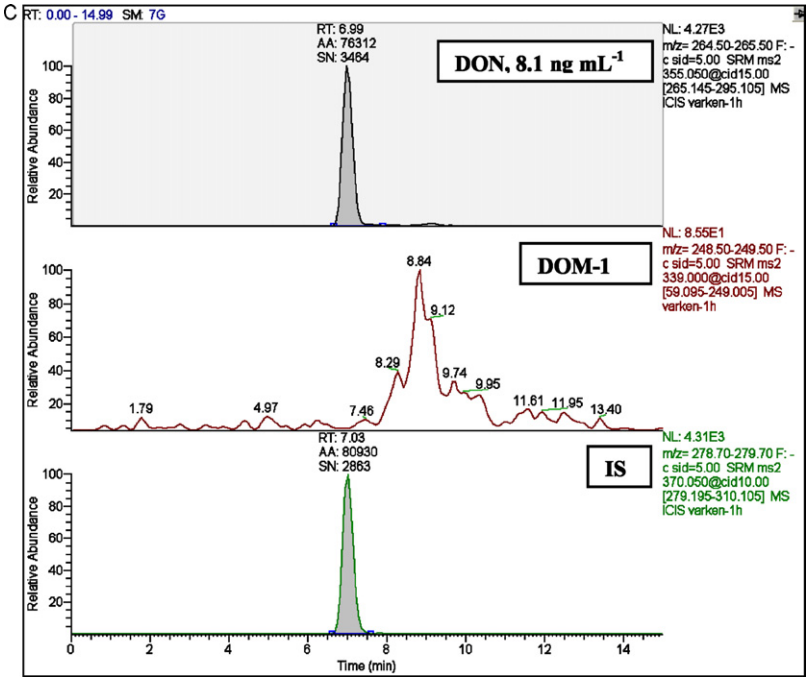


**Fig. 2.** (*continued*)

*Carry-over*. The carry-over was evaluated by analysing a water sample just after the highest calibrator sample. The eventual analyte concentration in the water sample had to be below the LOD.

*Recovery and ion suppression*. Recovery experiments were performed according to Matuszewski et al. [26]. Briefly, two types of matrix-matched calibration curves were prepared for each analyte in each matrix, i.e. by spiking the blank calibrator samples before (=spiked) and after extraction (=spiked extract). One calibration curve was prepared using appropriate working solutions of standards (=liquid standards).

The slopes of the resulting linear, $1/x$ weighted spiked calibration curves (i.e. spiked and spiked extracts) were compared with the related slopes of the standard calibration curves to calculate the apparent recovery ($R_A$ = (100 × slope spiked sample)/slope liquid standards), the signal suppression/enhancement due to matrix effects (SSE = (100 × slope spiked extracts)/slope liquid standards) and the recovery of the extraction step ($R_E$ = (100 × $R_A$)/SSE) [26].

## 3. Results and discussion

The initial aim of this study was to develop a method to analyse type-A and -B trichothecenes in animal plasma and bile by one single clean-up procedure and analytical run. The development of such a method, however, was not straightforward and was hampered mainly by the diversity in chemical and physical properties of the two toxin classes, which include polar (DON and DOM-1) and less polar (T-2 and HT-2) compounds. Therefore, compromises had to be made with respect to the sample preparation (choice of extraction solvents, solid-phase extraction column), chromatographic separation (column, mobile phase) and detection (positive or negative ionisation mode), resulting in conditions that were not always optimal for all analytes of interest [13,22,27].

### 3.1. Sample extraction and clean-up

#### 3.1.1. Plasma analysis

At first, deproteinization with 750 μL of acetonitrile or methanol was tried out, followed by the direct injection of the supernatant onto the LC–MS/MS system. Using acetonitrile, the extraction recovery for DON was only 20%, while for the other analytes recoveries were all above 70%. The recovery of DON could be increased to 80% by using methanol as the deproteinization solvent. However, the sample was diluted by a factor 4.

Because it was the aim of this study to determine mycotoxin concentrations in biological samples at the lower ng mL$^{-1}$ levels, the deproteinized samples were subjected to a further clean-up and concentration step using Oasis® HLB solid-phase extraction (SPE) columns (Waters). This type of SPE column was chosen, because it is filled with a patented hydrophilic/lipophilic balanced polymer, which retains both polar and non-polar compounds [19]. In addition, it was reported by Stecher et al. [28] that these columns provided superior recovery for type-B trichothecenes. All analytes were best retained onto the SPE column if the solvent for loading or washing did not contain >10% of methanol. Hence, it was decided to use a 90/10 (v/v) mixture of water/methanol for both loading the sample and washing the SPE column.

For elution of the analytes from the SPE column similar results were obtained using methanol or acetonitrile. 1 mL was needed to elute all analytes from the SPE column.

#### 3.1.2. Bile analysis

A primary extraction using 4 mL of a methanol/water mixture (70/30, v/v) was performed, followed by a second liquid–liquid partitioning step. In order to obtain an optimal phase separation during the liquid–liquid extraction step, it was necessary to dilute the first extract with an equal volume (i.e. 4 mL) of water. Several organic solvents were tested for liquid–liquid extraction (ethylacetate, diethyl ether, dichloro methane, hexane and hexane/isoamyl alcohol (95/5, v/v)) and the best results (extraction recovery, specificity) were obtained using ethylacetate. This solvent was also used by Bily et al. [14] for the extraction of DON and zearalenone from pig serum and by several other authors [10,11,29]. Extractions were tested in neutral, acidic and alkaline medium. By performing the extraction in neutral or alkaline conditions, many matrix-related interferences (such as bile acids and bilirubin) could be removed.

After evaporation of the organic phase, the dry residue was redissolved in 250 μL of the mobile phase, resulting in a concentration of the initial sample by a factor of 4.

Filtering of the reconstituted samples using a Millex®-GV filter was necessary before injection onto the LC–MS/MS instrument. Since chromatograms were free of interfering peaks, no further purification using an Oasis® HLB solid-phase extraction column was performed.

Both sample preparation procedures could be performed within a reasonable time (i.e. 1 h), which is comparable to other methods in the literature (e.g. Bily et al., liquid–liquid extraction using ethylacetate, 2 times for DON in pig serum [14]; Razzazi-Fazeli et al., extraction using Mycosep 277 columns for DON and DOM-1 in pig urine [13]). The advantage of our method, however, is that not only type-B (DON and DOM-1), but also type-A trichothecenes (T-2 and HT-2) can be determined using the same extraction procedure.

### 3.2. Liquid chromatography–mass spectrometry

*Chromatography*. Several reversed-phase C18 columns (Nucleodur, Macherey-Nagel; Alltima HP, Alltech; Hypersil Gold, Thermo Scientific; Nucleosil, Varian/Agilent) and one polymeric column (PLRP-S, Varian/Agilent) were tested in order to optimize the chromatographic separation of the different analytes of interest. The best results were obtained with the Nucleosil column.

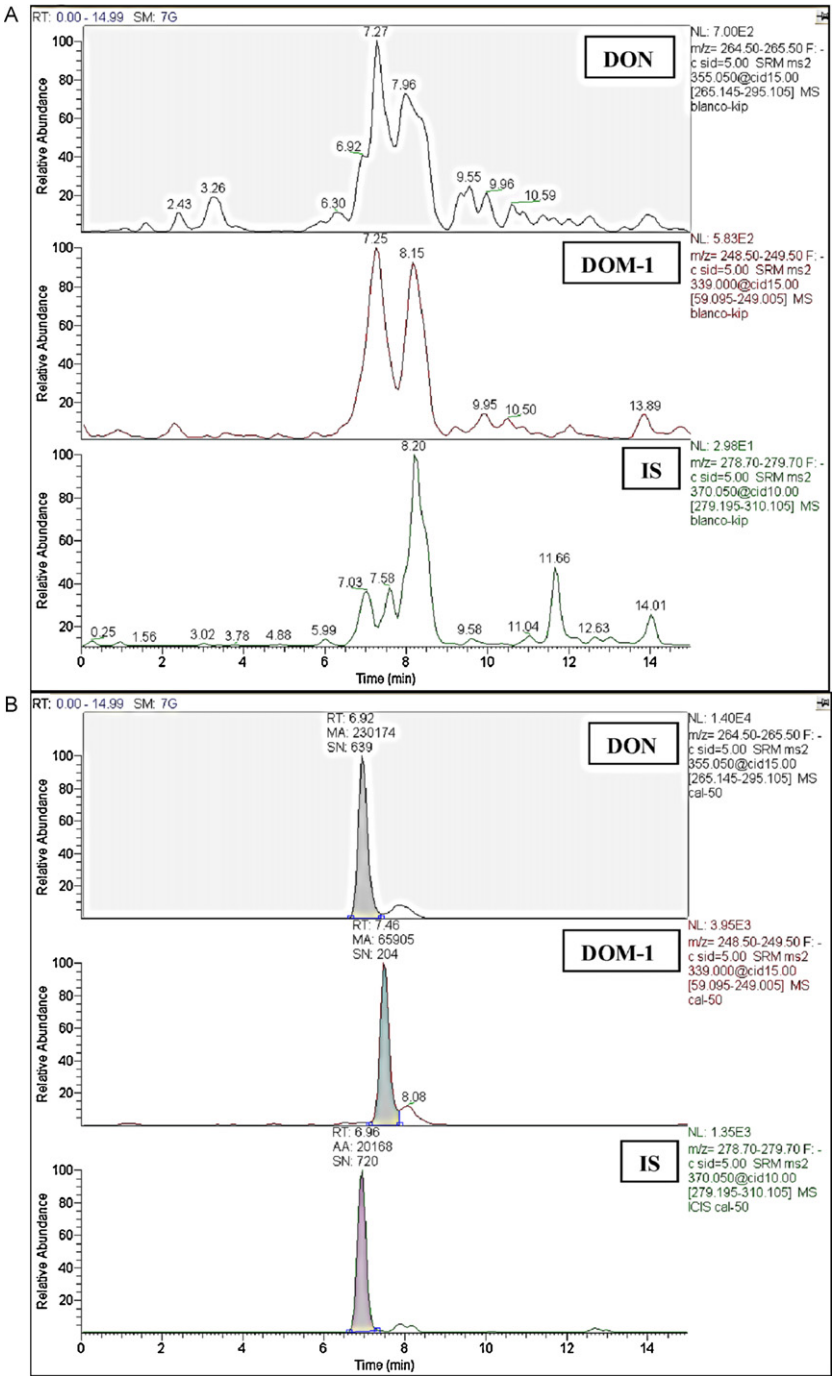


**Fig. 3.** LC–MS/MS chromatograms of DON and DOM-1 in pooled chicken bile: (a) blank bile sample, (b) pooled bile sample spiked at a level of 50 ng mL$^{-1}$ of DON and DOM-1, (c) incurred pooled bile sample (DON concentration: 16.2 ng mL$^{-1}$, DOM-1 concentration: 103.9 ng mL$^{-1}$).

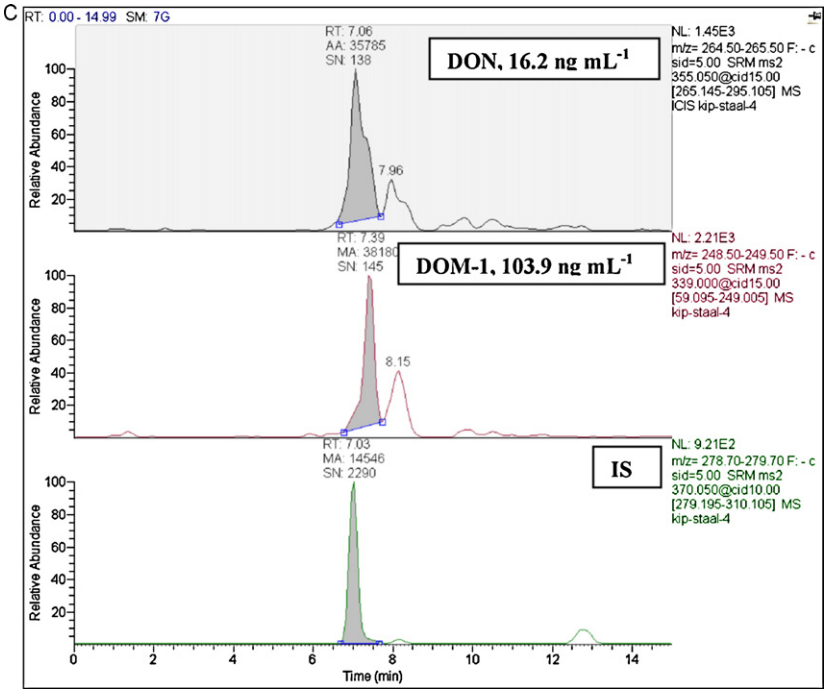


**Fig. 3.** (*continued*)

A second issue of concern was the choice of the mobile phase. In the literature most mobile phases consist of a combination of water and acetonitrile or methanol to which some additives are added to improve chromatography and detection [22]. A better sensitivity was observed for most compounds when methanol was used instead of acetonitrile. The addition of a 5 mM ammonium acetate buffer resulted in a better chromatography and ionization efficiency for T-2, HT-2 and $^{13}C_{24}$-T-2 due to the formation of stable ammonium adducts. With the same mobile phase, acetate adducts were formed for DON, $^{13}C_{15}$-DON and DOM-1. However, the MS sensitivity of these compounds could be increased if only acetic acid was added as additive to the mobile phase.

Because it was the aim of our study to reach a sensitivity of about 1 ng mL$^{-1}$ for all compounds, it was decided to perform separate chromatographic runs at the optimal conditions for the analysis of type-A and -B trichothecenes, respectively.

*Mass spectrometry*. The compounds were tuned in their most sensitive ESI mode (+ or −). A negative ionization mode has been found to be more sensitive for type-B trichothecenes, in contrast to type-A trichothecenes for which the positive ion mode was found to provide higher signal intensities [22].

Since the mobile phase for DON analysis contained acetic acid, acetate adduct ions $[M+CH_3COO]^-$ were observed for DON ($m/z$ = 355.05), $^{13}C_{15}$-DON ($m/z$ = 370.05) and DOM-1 ($m/z$ = 339.00). For T-2, HT-2 and $^{13}C_{24}$-T-2 protonated ammonium adducts $[M+NH_4]^+$ were most prominent in combination with a mobile phase containing 5 mM ammonium acetate.

*Carry-over*. The carry-over of analytes from one sample to another was evaluated by the analysis of a solvent sample after the highest calibrator samples. No peaks were observed in the same elution zone of the analytes of interest, indicating that no carry-over was present.

### 3.3. Internal standard

The method of internal standardization was applied in order to compensate for analyte losses during sample preparation and for matrix effects during LC–MS/MS analysis. Ideally, the structural, physical and chemical properties of an internal standard should be similar to the analyte of interest [13,22]. Stable isotope-labelled compounds have all these characteristics, but they are not always available and are very expensive. For the presented method, it was decided to use two isotope-labelled internal standards, i.e. one for compounds detected in the ESI(−) and in the ESI(+) mode, respectively. During initial experiments $d_1$-DON was used as internal standard for DON and DOM-1. However, no linear calibration curves could be constructed. This could possibly be attributed to the non-complete degree of deuteration of the IS. In addition, the mass increment of one results in a spectral overlap between the SRM transitions for quantification of the IS ($m/z$ 296 → 265, interference with DON: 1.2%) and DON ($m/z$ 295 → 265, interference with IS: 2.5%) [30]. This problem could be overcome by the use of the more expensive $^{13}C_{15}$-DON as internal standard [31]. No unlabeled DON was detected, if a standard solution of $^{13}C_{15}$-DON was analysed by LC–MS/MS, confirming its suitability for use as IS.

$^{13}C_{24}$-T-2 was used as internal standard for compounds detected in the ESI(+) mode. As for $^{13}C_{15}$-DON, no natural T-2 was detected in the IS solution, when analysed with the above mentioned LC–MS/MS method.

### 3.4. In house method validation

A complete in house method validation was performed for the analysis of the analytes of interest in animal plasma. For practical reasons (i.e. limited availability of blank bile matrix), only the linearity and specificity were evaluated for the analysis of these analytes in bile. Hence, the method can be designated as semi-quantitative for the analysis of DON, DOM-1, T-2 and HT-2 in animal bile (Table 3).

*Linearity*. All calibration curves were constructed using matrix-matched calibrator samples in order to compensate for matrix effects [24]. Linear calibration curves covering a concentration range of 2 to 3 orders of magnitude have been obtained in all matrices and for all analytes (see Table 3).

*Accuracy and precision*. The within-run accuracy and precision was tested at least at two different concentration levels. The accept-

ability ranges were met for all compounds at the specified levels (see Table 4). The between-run precision and accuracy was tested by the analysis of quality control samples and the results fell within the ranges specified (results not shown).

*LOQ and LOD*. The results of the LOQ and LOD determination are shown in Table 4. As can be seen, the LOQ values ranged from 1.0 to 2.5 ng mL$^{-1}$ for all compounds. The calculated LOD values, corresponding with a S/N ratio of 3, ranged from 0.01 to 0.63 ng mL$^{-1}$ for all compounds. These LOQ and LOD values were much lower than those reported by Bily et al. for the analysis of DON in pig serum (i.e. LOQ = 8 ng mL$^{-1}$) [14] and by Razzazi-Fazeli et al. for DON and DOM-1 in pig urine (LOQ = 25 ng mL$^{-1}$) [13]. As can be seen from the results of the analysis of real samples (Fig. 5 and Table 5), it is really important to reach sensitivities in the lower ppb range (~1–2.5 ng mL$^{-1}$). The presented method succeeds in quantitating not only type-B trichothecenes (DON and DOM-1), but also type-A trichothecenes in biological matrices at these low levels.

*Specificity*. For the evaluation of the specificity of the method, blank samples of each matrix were analysed. As can be seen from Figs. 2A–4A, which show the mass chromatograms of a blank chicken plasma and bile sample and a blank pig plasma sample, no peaks could be detected in the elution zone of the analytes of interest, indicating the specificity of the developed method with respect to endogenous interferences.

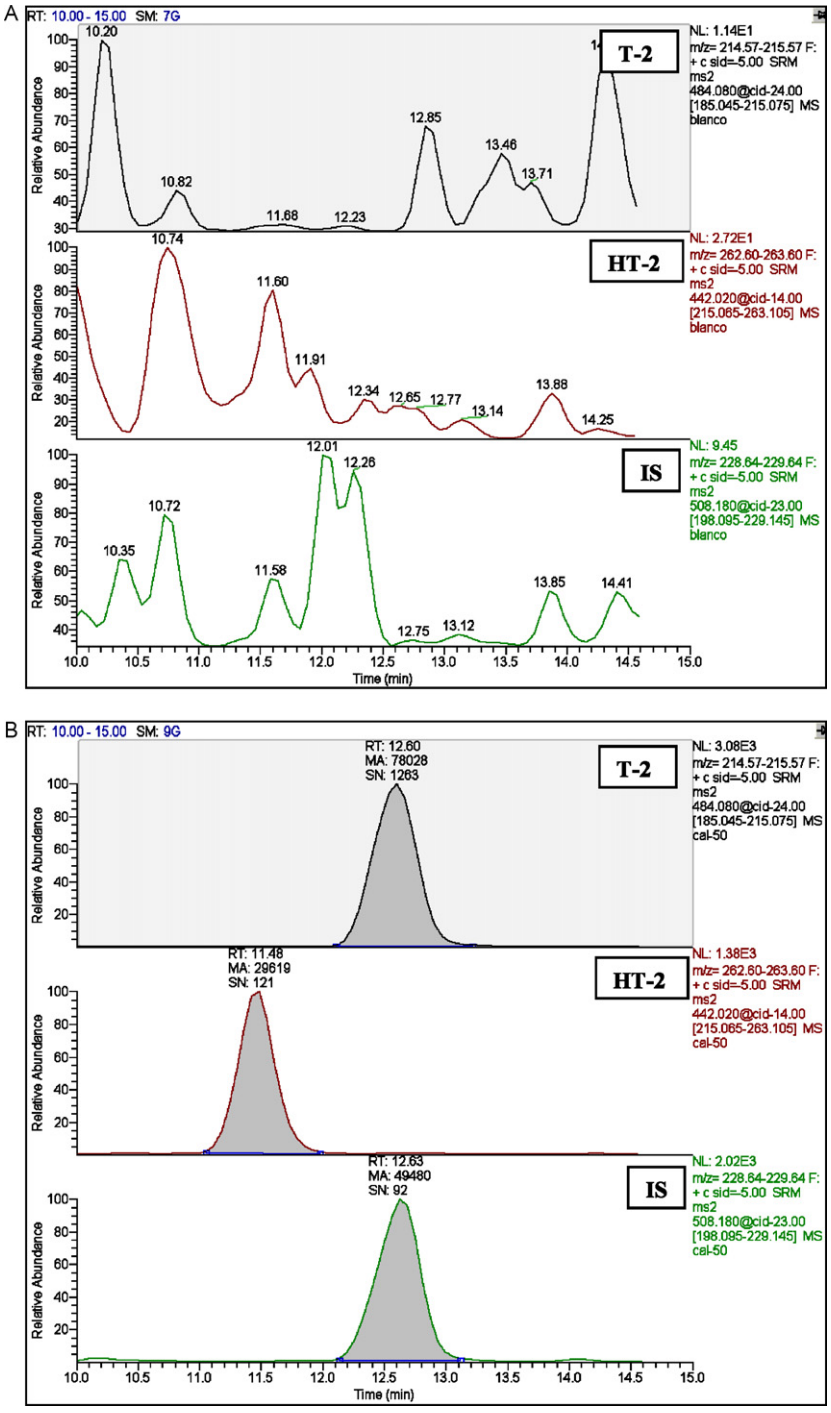


**Fig. 4.** LC–MS/MS chromatograms of T-2 and HT-2 in chicken plasma: (a) blank plasma sample, (b) plasma sample spiked at a level of 50 ng mL$^{-1}$ of T-2 and HT-2, (c) incurred plasma sample (T-2 concentration: 15.6 ng mL$^{-1}$, HT-2 was not detected).

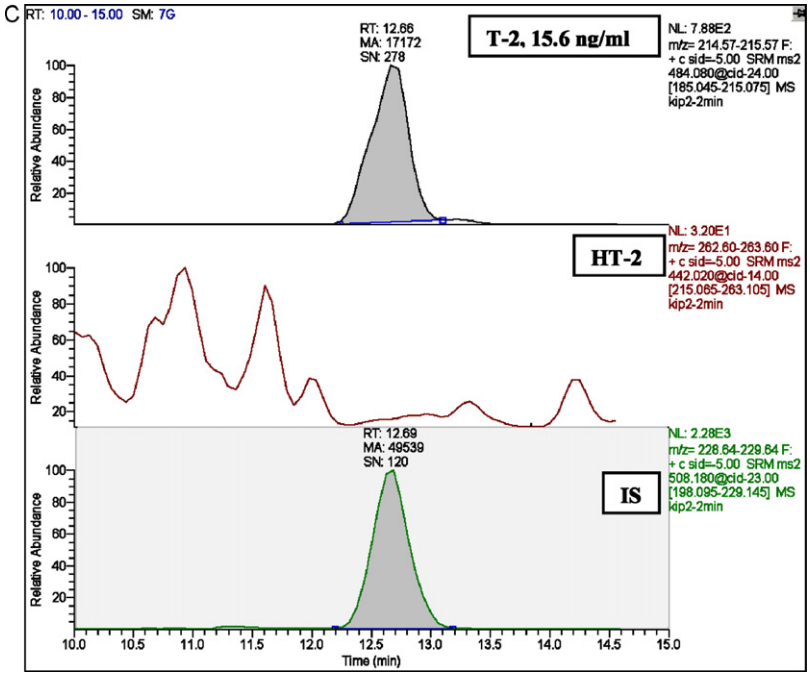


**Fig. 4.** (*continued*)

*Extraction recovery*. The optimized sample clean-up procedures for plasma and bile were evaluated by determining the recovery of the extraction step ($R_E$, %) and the apparent extraction recovery ($R_A$, %, taking into account both $R_E$ and SSE) according to the procedure described by Matuszewski et al. [26]. The results are shown in Table 2. For the different combinations of matrices and analytes, the $R_E$ values were higher than 60% for most compounds, except for DON and DOM-1 in bile (~40%).

The extraction recoveries for DON and DOM-1 in plasma were also in accordance with those reported by other authors for the analysis of DON and DOM-1 in biological matrices (i.e. Razzazi-Fazeli et al. [13], analysis of swine urine: DON: 83.8%, DOM-1: 96.6%, Bily et al. [14], analysis of pig serum: DON: 72%). However, these methods determined only type-B trichothecenes.

No results for the extraction recoveries of the analysis of T-2 and HT-2 in biological matrices could be found in the literature, indicating the novelty of our method.

*Matrix-effect*. LC–MS/MS has been known for its specificity and selectivity, but it has also been shown that co-eluting matrix components may affect the ionization efficiency. This phenomenon can be reduced by performing an extensive sample clean-up and by optimizing the chromatographic separation. In the present method the influences of matrix effects have been further minimized by preparing matrix-matched calibrator samples and by the use of two isotopic labelled internal standards. Signal suppression/enhancement fell within 75–110% for nearly all compounds in the tested matrices.

As can be seen from Table 2, extraction recoveries and matrix effects for an analyte can differ depending on the origin of the matrix (e.g. chicken or pig plasma). This indicates the importance of preparing matrix-matched calibrator samples in order to allow for a proper quantification of the target analytes in biological matrices originating from different animal species.

**Table 5**
Results of the analysis of DON and DOM-1 in plasma and bile from broiler chickens that received DON with the feed for 21 consecutive days.

| *DON concentration in the feed (μg/kg)* | | |
|---|---|---|
| | Group 1[a] | Group 2[a] |
| | 2438 ± 700 μg/kg | 7540 ± 2200 μ/kg |

| *Mean plasma concentrations (ng mL$^{-1}$)* | | |
|---|---|---|
| Time (days)[b] | Group 1 DON | Group 2 DON |
| 7 | <LOQ[c] ($n$[d] = 8) | 3.87 ± 0.86 ($n$ = 8) |
| 14 | ND ($n$ = 8) | 2.66 ± 2.02 ($n$ = 8) |
| 21 | ND[e] ($n$ = 8) | <LOQ ($n$ = 8) |

*Note:* DOM-1 was not detected.

| *Mean bile concentrations (ng mL$^{-1}$)*[f] | | | | |
|---|---|---|---|---|
| Time (days) | Group 1 | | Group 2 | |
| | DON | DOM-1 | DON | DOM-1 |
| 21 | 4.5 | 22.0 | 16.2 | 103.9 |

[a] Group 1 received chicken feed that was artificially contaminated with DON; Group 2 consisted of mais that was naturally contaminated with DON.
[b] Time: time after start of administration of contaminated feed.
[c] <LOQ: plasma: 1.25 ng mL$^{-1}$, bile: 2.5 ng mL$^{-1}$.
[d] $n$: the number of animals in a group.
[e] ND: not detected.
[f] The bile samples of the animals of a group were pooled.

### 3.5. Analysis of biological samples

*Preliminary bolus toxicokinetic study with T-2 in broiler chickens*. Plasma samples from two broiler chickens that received one IV dose of 0.15 μg/kg BW of T-2 were analysed using the developed method. T-2 concentrations above the LOQ level could only be detected at 2 min after IV administration (i.e. 9.9 and 15.6 ng mL$^{-1}$, see Fig. 4C). This can be attributed to the low dose that was administered to the chickens or to a fast elimination of T-2 from the central compartment. No HT-2 concentrations above the LOQ level could be detected in any of the plasma samples.

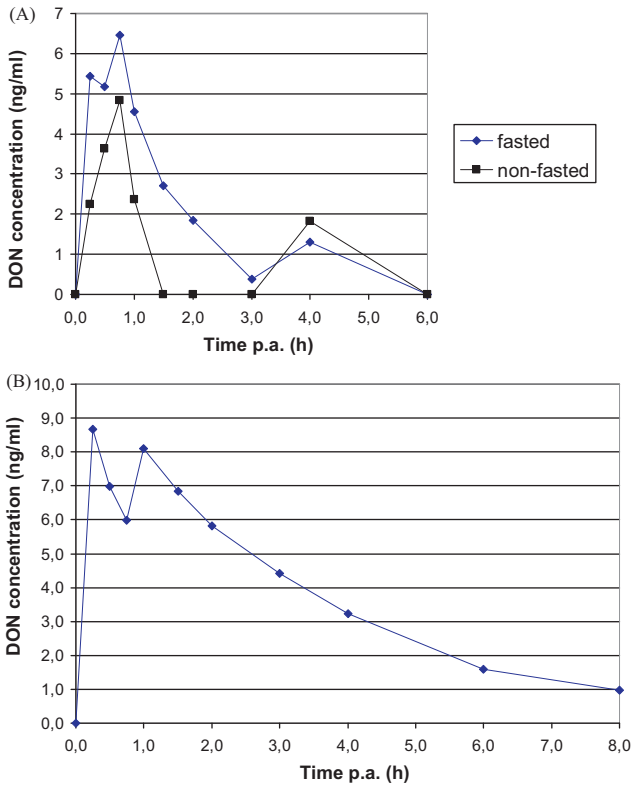


**Fig. 5.** Plasma concentration versus time profiles for DON after administration of (A) one oral bolus of DON (dose: 0.15 mg/kg BW) to one non-fasted and one fasted broiler chicken and of (B) one oral bolus of DON (dose: 0.05 mg/kg BW) to one fasted pig.

*Preliminary toxicokinetic studies with DON in broiler chickens and pigs*. The results of the analysis of the plasma samples that were taken from one fasted and non-fasted broiler chicken and from one pig after the administration of one oral bolus of DON (dose: 0.015 mg/kg BW for chicken and 0.05 mg/kg BW for pig, respectively) are shown in Fig. 5. As can be seen from this figure, maximal plasma concentrations for DON were reached within one hour after administration and ranged between ~5 and 10 ng $mL^{-1}$ in both animal species. DON plasma concentrations declined fast and fell below the LOQ level within 4–8 h after administration. No DOM-1 was detected in any of the plasma samples. These findings were in accordance with those found by other authors [5,32].

*Preliminary steady-state toxicokinetic and residue study with DON in broiler chickens*. The results of the analysis of DON and its main metabolite DOM-1 in plasma and bile samples that were taken from broiler chickens during a preliminary steady-state toxicokinetic and residue study are shown in Table 5. As can be seen from this table, DON could only be detected in plasma samples of chickens that received feed that was naturally contaminated with a high level of DON (i.e. 7540 ± 2200 μg/kg of feed). No DOM-1 was detected in any plasma sample.

In contrast to plasma, DON and its major *in vivo* metabolite DOM-1 could be determined in pooled bile samples that were taken from the same chickens after 3 weeks of continuous administration of (naturally) DON contaminated feed (see Table 5).

The above results show the usefullness of the presented method for the performance of *in vivo* toxicokinetic and residue studies with DON in chickens and pigs after the administration of feed (naturally) contaminated with DON. In addition the application of this method for the performance of ADME studies with DON and T-2 as a part of *in vivo* efficacy studies with mycotoxin binders/modifiers is promising [33,34].

## 4. Conclusions

We succeeded in the development of a highly sensitive and specific LC-ESI-MS/MS method for the quantitative and semi-quantitative determination of type-A (T-2, HT-2) and -B (DON, DOM-1) trichothecenes in animal plasma and bile, respectively. The method was validated for all analytes of interest in animal plasma according to EU regulations (linearity, precision, accuracy, LOQ, LOD, specificity), and good results were obtained. For the analysis of the target analytes in bile, a shortened validation procedure was performed, because not enough blank matrix was available. LOQ levels of 1–2.5 ng $mL^{-1}$ were obtained, which were lower than reported by other authors, but necessary to determine the low trichothecene concentrations in real samples.

Incurred plasma and bile samples that were taken from broiler chickens or pigs during preliminary toxicokinetic and residue studies with DON and T-2 were quantitatively analysed using the described method. The obtained results proved the usefulness of the method for the application in the field of toxicokinetic, residue or exposure assessment with mycotoxins and efficacy testing of mycotoxin binders/modifiers.

In conclusion it is – to our knowledge – the first time that an in house validated LC-ESI-MS/MS method for the quantitative determination of type-A and -B trichothecenes in animal body fluids at the lower ng $mL^{-1}$ levels, has been reported.

## Acknowledgements

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