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Quantitative determination of the *Fusarium* mycotoxins beauvericin, enniatin A, A1, B and B1 in pig plasma using high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A sensitive and reliable method was developed for the identification and quantification of beauvericin, enniatin A, A1, B and B1 in pig plasma using liquid chromatography combined with heated electrospray ionization tandem mass spectrometry. Sample clean-up consisted of a deproteinization step using acetonitrile, followed by evaporation of the supernatant and resuspension of the dry residue in acetonitrile/water (80/20, v/v). The method was in-house validated: matrix-matched calibration graphs were prepared for all compounds and correlation and goodness-of-fit coefficients ranged between 0.9980 and 0.9995 and between 5.2% and 11.3%, respectively. The within- and between-run precision and accuracy were evaluated and the results fell within the ranges specified. The limits of quantification were 0.1 ng/mL for enniatin A and A1 and 0.2 ng/mL for beauvericin, enniatin B and B1, whereas limits of detection were $\leq$ 10 pg/mL for all analytes. The method has been applied for the analysis of real plasma samples from one pig that received an oral bolus (0.05 mg/kg BW) of the investigated mycotoxins. At the applied dosage, the results indicated the suitability of the method for use in toxicokinetic studies with enniatins.

1. Introduction

Mycotoxins are secondary metabolites produced by different fungal species contaminating several food and feed commodities. Over 100 mycotoxins have been identified, although only a few of them have been thoroughly investigated because of their distinct toxic effects. The most prevalent mycotoxin producing fungi in moderate climates are *Fusarium* species. Several *Fusarium* spp. are capable of producing well-known mycotoxins like the trichothecenes, fumonisins and/or zearalenone, but also other mycotoxins including beauvericin (BEA) and enniatins (ENNs) such as ENN A, A1, B and B1 [1].

These *Fusarium* mycotoxins are cyclic hexadepsipeptides consisting of alternating D-α-hydroxyisovaleric acids and N-methyl-L-amino acids [2] (Fig. 1). BEA is a common contaminant of grains and has been detected for example in 6 out of 22 Italian maize samples, in concentrations ranging from <1 up to 520 mg/kg, with an average of 102 ± 119 mg/kg. In maize samples from Poland, BEA was detected in 13 of 14 samples, with contamination levels ranging between 5 and 60 mg/kg with an average of 18 ± 11 mg/kg [3,4]. ENNs have been detected for example in Scandinavian barley (ENN A: 21/22 positive samples, range 2–950 μg/kg and mean contamination level of 67 ± 90 μg/kg; ENN A1: 22/22 contaminated samples, contamination range: 10–2000 μg/kg and mean 282 ± 300 μg/kg; ENN B: 22/22 positive samples, range 4–9760 μg/kg, mean 1622 ± 1482 μg/kg and ENN B1 22/22 positive samples, range 11–5720 μg/kg and mean 1514 ± 1691 μg/kg) [5]. More recently, ENNs have been detected in rice from Maroc (35/70 samples contaminated with ENNs and for ENN A: maximum level 120 mg/kg, mean 23 ± 15 mg/kg; ENN A1: maximum level 449 mg/kg, mean 258 ± 135 mg/kg; ENN B: maximum level 26 mg/kg, mean 9 ± 1 mg/kg; ENN B1: maximum level 24 mg/kg and mean 9 ± 2 mg/kg) [6].

ENNs and BEA are of interest because they can exert antibacterial, antifungal and phytotoxic effects [5,7,8]. On the other hand, they can also be toxic for mammalian cells. Their main mode of action is based on their ionophoric properties [9]. They are capable of transporting cations through the cell membrane, leading to toxic actions by an altered membrane potential [9,10]. Next to their ionophoric properties, ENNs exhibit other effects, such as inhibiting acyl-coenzyme A:cholesterol acyl transferase (ACAT) and 30,50-cyclo-nucleotide phosphodiesterase enzymes [11] causing mitochondrial dysfunction [12], and the inhibition of multidrug resistance associated protein-1 (ABCG2) and P-glycoprotein (ABCB1) efflux pumps [13]. Many *in vitro* studies

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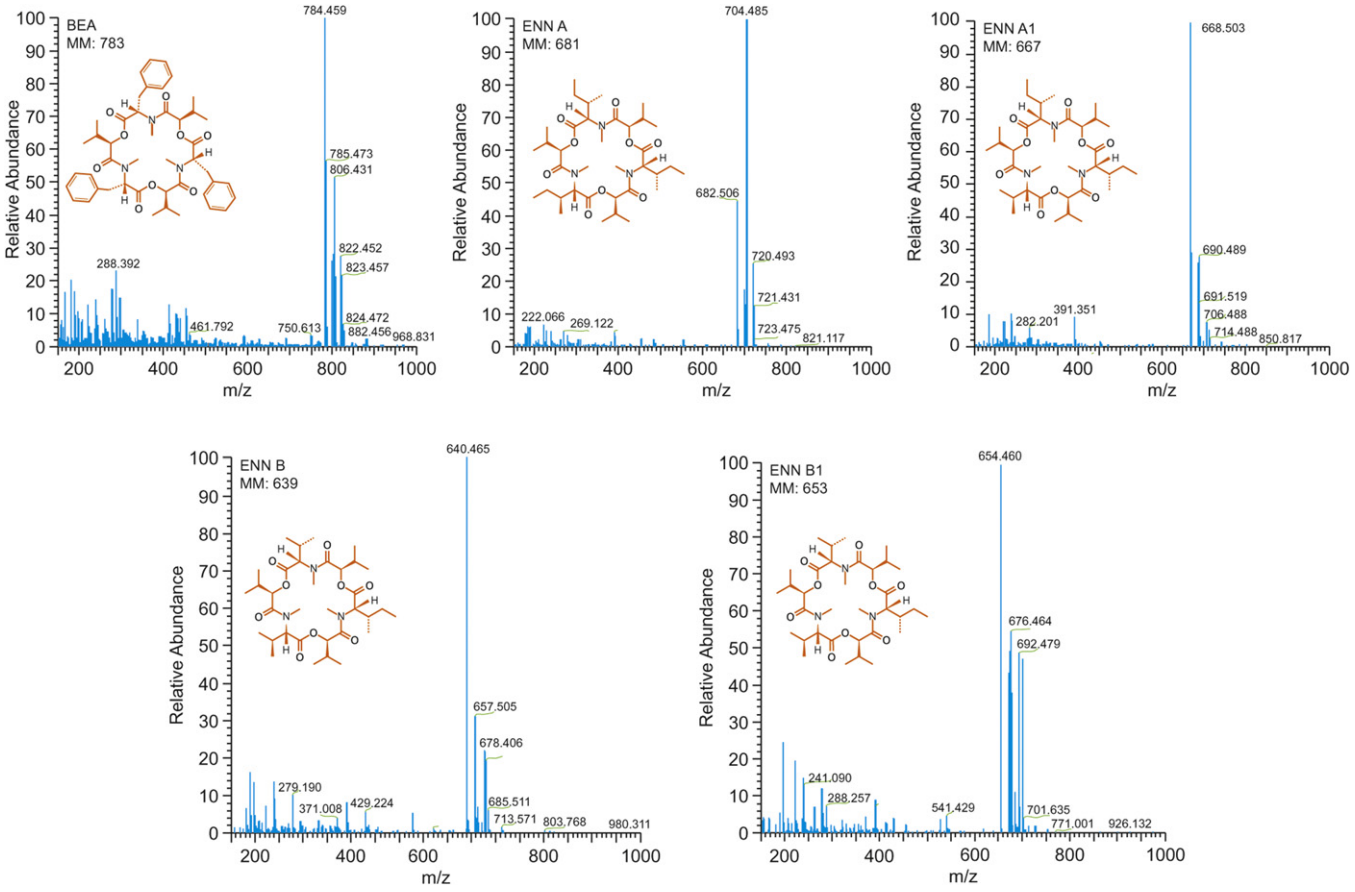

Fig. 1. Full mass spectrum (range 400–1000 m/z), chemical structure and molecular mass (MM) of beauvericin (BEA), enniatin (ENN) A, A1, B and B1.

have reported cytotoxic effects of ENNs and BEA on several cell types, as reviewed by Jestoi [5]. Data on their *in vivo* toxicity are however lacking [5]. Nevertheless, their possible subclinical effects are of greater importance as these may lead to reduced performance parameters in food producing animals and increased susceptibility to infectious disease. Opinions of the European Food Safety Authority (EFSA) on BEA and ENNs are currently being drafted on request by the European Commission. To evaluate their potential *in vivo* toxic effects, knowledge of their toxicokinetic properties, namely absorption, distribution, metabolization and excretion (ADME) in livestock is crucial. As an example, no information is available on their oral absorption and bioavailability in any animal species, including pigs. Jestoi et al. [10,14] detected trace level residues of ENNs and BEA in Finnish eggs, poultry meat and liver samples. Another study [15] reported trace levels of BEA in eggs from local retailers in Spain. To date, no other data are available on the tissue distribution and the presence of these mycotoxins in other animal tissues or on their toxicokinetic properties in general [16]. This lack of data could explain why there are no maximum guidance levels set for animal feed yet, in contrast to other mycotoxins [17].

It is obvious that for assessing animal exposure to BEA and ENNs and to investigate their toxicokinetics, the availability of sensitive and specific validated analytical methods is mandatory. In recent decades, high-performance liquid chromatography (HPLC) has become the most important separation method for the analysis of mycotoxins in food, feed and other matrices [18]. For BEA and ENNs, the most common detection method used is UV-detection. Nowadays mass spectrometry (MS) is in favor over spectrophotometric techniques because of unambiguous identification, detection and accurate quantification with only a single elution procedure. Furthermore, it allows quantification of multiple residues and lowers interferences among them [5,19–21].

Many high-performance liquid chromatography–tandem mass spectrometric (LC–MS/MS) methods for the analysis of ENNs and BEA in food and feed have been described [4,20,22,23]; however, analytical methods for detection of these mycotoxins in biological matrices are scarce [10,14,15]. Until now, no method for the analysis of these compounds in plasma has been reported.

The goal of this study was to develop a method which quantifies the *Fusarium* mycotoxins BEA, ENN A, A1, B and B1 in animal plasma, using liquid chromatography combined with heated electrospray ionization tandem mass spectrometry (LC-h-ESI–MS/MS). The final method should be applicable for evaluation of their toxicokinetic properties and oral bioavailability in different animal species. Since a large amount of samples are generated in such experiments, special attention has been paid to the development of a high-throughput sample preparation procedure and LC–MS/MS analysis, but not at the expense of reliability and sensitivity.

2. Materials and methods

2.1. Chemicals, products and reagents

The analytical standards of ENN A, A1, B and B1 were obtained from Sigma–Aldrich (Bornem, Belgium). Standard of BEA was obtained from Fermentek (Jerusalem, Israel). The internal standard (IS) maduramicin (MAD) was a kind gift from Alpharma (Wilrijk, Belgium). Mycotoxin standards were stored at ≤ -15 °C, maduramicin standard at 2–8 °C. Water, methanol and acetonitrile

(ACN) were of LC–MS grade and obtained from Biosolve (Valkenswaard, the Netherlands). Glacial acetic acid was of analytical grade and obtained from VWR (Leuven, Belgium).

2.2. Preparation of standard solutions

Separate standard stock solutions of all mycotoxins were prepared in ACN (analyte concentration: 1 mg/mL) and were stored at ≤ -15 °C [15,24].

A combined working solution of 10 μg/mL of all analytes (except IS) was prepared by transferring 10 μL of each stock solution of 1 mg/mL into an eppendorf tube, followed by further dilution with ACN/water (50/50, v/v) up to a final volume of 1.0 mL. Combined working solutions of 1, 0.1, 0.01 and 0.001 μg/mL were obtained by diluting 100 μL of the 10 times higher concentrated solution with 900 μL ACN/water (50/50, v/v). This method allows leverage to be kept to a minimum. For the IS, a working solution of 1 μg/mL was prepared in ACN/water (50/50, v/v). All working solutions were stored at 2–8 °C. The decrease in signal intensity after 4 months storage was less than 10% for all compounds and working solutions were therefore considered as stable when stored at 2–8 °C.

2.3. Biological samples

A bolus toxicokinetic study was performed in one piglet (20.2 kg BW). The pig received a single oral intra-gastric bolus of the investigated mycotoxins BEA, ENN A, A1, B and B1 (all 0.05 mg/kg BW). This dose resembled a feed contamination level of 1 mg/kg, since the feed intake of a 20 kg weighing pig is about 1 kg/day. This theoretical feed contamination level was chosen arbitrarily as it lies within the wide range of *Fusarium*-mycotoxins concentration levels that have been detected in feed ($\sim$10 μg/kg to $>$100 mg/kg) [5]. The bolus solution was prepared instantly before administration by dissolving the mycotoxin standards (1 mg of each individual compound) in 1 mL of ACN and further dilution with 50 mL of water. This solution was given by gavage using an intragastric tube. The tube was rinsed sufficiently with water to assure complete delivery in the stomach. Blood samples were drawn before (0 min) and at 10, 20, 30 and 40 min, 1, 1.5, 2, 3, 4, 6, 8, 10 and 12 h post-administration in heparinized tubes and centrifugated (2851*g*, 10 min, 4 °C). Plasma samples (250 μL) were stored at ≤ -15 °C until analysis.

This animal experiment was approved by the Ethical Committee of Ghent University (Case number EC 2012/110).

2.4. Sample pretreatment

To 250 μL of plasma were added 12.5 μL of the IS working solution and 750 μL of ACN, followed by a vortex mixing (15 s) and centrifugation step (8517*g*, 10 min, 4 °C). Next, the supernatant was transferred to another tube and evaporated using a gentle nitrogen (N_2) stream (45 $\pm$ 5 °C). The dry residue was reconstituted in 200 μL of ACN/water (80/20, v/v). After vortex mixing (15 s), the sample was transferred into an autosampler vial. The injection volume was 5 μL. One extraction and LC–MS/MS analysis was performed for each sample.

2.5. Liquid chromatography

The chromatographic system consisted of a quaternary, low-pressure mixing pump with vacuum degassing (Surveyor MSpump Plus, ThermoFischer Scientific, Breda, The Netherlands) connected to an autosampler (Autosampler Plus, ThermoFisher Scientific) with temperature controlled tray and column oven. Chromatographic separation was achieved on a Hypersil Gold column (50 mm $\times$ 2.1 mm i.d., dp: 1.9 μm) in combination with a guard column of the same type (10 mm $\times$ 2.1 mm i.d., dp: 3 μm), both from ThermoFisher Scientific. The temperatures of the column oven and autosampler tray were set at 45 °C and 5 °C, respectively. Mobile phase A consisted of 0.1% glacial acetic acid in water whereas mobile phase B was ACN. Following gradient elution program was run: 0–0.5 min (70% A, 30% B), 0.5–2.5 min (linear gradient to 20% A), 2.5–8.5 min (20% A, 80% B), 8.5–10.0 min (linear gradient to 70% A), 10.0–12.0 min (70% A, 30% B). Flow rate was set at 300 μL/min.

2.6. Mass spectrometry

The LC column effluent was interfaced to a TSQ® Quantum Ultra triple quadrupole mass spectrometer, equipped with a heated electrospray ionization (h-ESI) probe operating in the positive ionization mode (all from ThermoFisher Scientific). Instrument parameters were optimized by syringe infusion of working solutions of 1 μg/mL of each compound (flow rate 10 μL/min) in combination with the mobile phases (50% A, 50% B, flow rate: 200 μL/min).

The following general MS/MS parameters were used: spray voltage: 4000 V, vaporizer temperature: 300 °C, sheath gas pressure: 49 au (arbitrary units), ion sweep gas pressure: 2.0 au, auxilliary gas pressure: 30 au, capillary temperature: 250 °C, source CID collision energy: 10 V, collision pressure: -1.5 mTorr and quad MS/MS bias: 2.9. The resolution for Q1 and Q3 were set at 0.7 full width at half maximum (FWHM).

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).

2.7. In-house method validation

The method was validated according to a validation protocol previously described by De Baere et al. [25]. A set of parameters that were in compliance with the recommendations and guidelines defined by the European Community and with criteria described in the literature, were evaluated [26–29]. This includes evaluation of linearity, within- and between-run accuracy and precision, limit of detection (LOD), limit of quantification (LOQ), specificity, carry-over, extraction recovery (R_E) and signal suppression/enhancement (SSE).

Linearity was evaluated by preparing matrix-matched calibration curves over a concentration range of 0.1–200 ng/mL. This concentration range was chosen based on studies with other mycotoxins [25,30,31]. Calibration curve samples (calibration

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

Analyte	Precursor ion (m/z)	Product ions (m/z)	RT (min)	CE (V)	TLO (V)
BEA	784.46	244.06*	5.12	25	214
	$[M+H]^+$	261.99		24	
ENN A	704.48	350.15*	5.45	55	180
	$[M+Na]^+$	231.99		45	
ENN A1	668.50	210.05*	5.26	29	145
	$[M+H]^+$	228.18		30	
ENN B	640.46	196.15*	4.79	28	137
	$[M+H]^+$	214.01		30	
ENN B1	654.46	196.08*	5.03	35	186
	$[M+H]^+$	214.07		35	
MAD	934.49	629.23*	6.99	24	150
	$[M+H]^+$	393.14		29	

m/z: mass to charge ratio; RT: retention time; CE: collision energy; TLO: tube lens offset; "*" indicates the quantifier ion; BEA: beauvericin; ENN: enniatin; MAD: maduramicin.

levels (0.1), 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100 and 200 ng/mL) were prepared by applying standard working solutions directly onto the blank plasma 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 calculated and had to be $\geq 0.99\%$ and $\leq 20\%$, respectively.

Within-run precision (repeatability) and accuracy were determined by analyzing 6 blank samples spiked at low (1 ng/mL) and high (10 and 100 ng/mL) concentration levels in the same run. The between-run precision (reproducibility) and accuracy were determined by analyzing quality control samples (1, 10 and 100 ng/mL) together with each analytical batch of samples, run on 3 different days. The acceptance criteria for accuracy were as follows: -50% to $+20\%$, -30% to $+10\%$ and -20% to $+10\%$ for concentrations <1 ng/mL, between 1 and 10 ng/mL and >10 ng/mL, respectively. For the precision, the relative standard deviation (RSD%) had to fall within 2/3 of the values calculated according to the Horwitz equation: $RSD_{max}=2^{(1-0.5\ \log\ Conc)}\times 2/3$ for within-run precision; and within the values calculated according to the Horwitz equation for between-run precision $RSD_{max}=2^{(1-0.5\ \log\ Conc)}$ [26,27]. RSD_{max} values for within-run precision were as follows: 1 ng/mL: 30.2%, 10 ng/mL: 21.3% and 100 ng/mL: 15.1%. For between-run precision the RSD_{max} values were: 1 ng/mL: 45.3%, 10 ng/mL: 32.0% and 100 ng/mL: 22.6%.

The limit of quantification (LOQ) was the lowest concentration of the analytes for which the method was validated with an accuracy and precision that fell within the recommended ranges (see section accuracy and precision and Table 2). The LOQ was also established as the lowest point of the calibration curve. The LOQ was determined by analyzing 6 samples spiked at 0.1 or 0.2 ng/mL, on the same day.

The limit of detection (LOD) was defined as the lowest concentration of the respective analytes that could be recognized by the detector with a signal-to-noise (S/N) ratio of ≥ 3. The LOD values were calculated using samples spiked at the LOQ level.

The specificity of the method was evaluated with respect to interferences from endogenous compounds. Therefore, one blank sample was analyzed using the above mentioned procedure. The S/N ratio of a possible interfering peak in the blank sample had to be below the S/N ratio of the analytes in the same elution zone at LOD level.

The carry-over on the LC–MS/MS instrument was evaluated by analyzing water/ACN sample (50/50, v/v) just after the highest calibrator sample. The eventual analyte concentration in the sample had to be below the LOD.

Recovery experiments were performed according to Matuszewski et al. [32]. In summary, two types of matrix-matched calibration curves were prepared for each analyte, namely by spiking the blank calibrator samples before (spiked) and after extraction (spiked extract). One calibration curve was prepared using standard solutions. The calibration curves were $1/x$ weighted in order to reduce the risk of leverage. The slopes of the resulting linear, or quadratic for ENN A, $1/x$ weighted, calibration curves with spiked and spiked extracts samples were compared with the related slopes of the calibration curves with spiked extracts and standard solution, in order to calculate the recovery of the extraction step (R_E) and the signal suppression/enhancement (SSE) due to matrix effects, respectively.

3. Results and discussion

3.1. Method development

3.1.1. Sample preparation and extraction

The goal of this study was to develop a sensitive LC–MS/MS method for screening and quantitation of BEA and ENNs in pig plasma. These plasma samples can be obtained during assessment experiments to evaluate animal exposure to these mycotoxins (screening) or as a part of toxicokinetic studies (quantitation). Since these surveys or trials generate a large number of samples, the use of a simple and practical sample preparation procedure is advisable in order to reduce the time and cost of analysis. When using a sensitive and specific analytical technique such as LC–MS/MS, clean-up of raw extracts can be kept to a minimum [23]. However, the most simple procedure, i.e. dilute-and-shoot, was not found suitable for plasma analysis, as this could lead to clogging and serious contamination of the MS instrument. On the other hand, more sophisticated extraction procedures using solid-phase extraction (SPE), for example, are not recommended as they are generally time-consuming and expensive, which is a disadvantage if a large amount of samples have to be analyzed (i.e. for screening or toxicokinetic analysis). Moreover, selecting the right column may sometimes be tricky and when using a silica based SPE column, selectivity is rather low [21]. Such SPE columns have been used as sample preparation technique for BEA and ENNs analysis, although the recoveries are generally lower compared to our sample preparation method [24]. The sample preparation for eggs, meat and liver samples described by Jestoi et al. [10,14] consisted of extraction with ACN followed by SPE (silica). Frenich et al. [15] used a QuEChERS-based extraction procedure followed by SPE (silica or Oasis HLB) for egg samples. As mentioned for SPE, the disadvantage is mainly the labor when a large amount of samples have to be analyzed. To achieve a good compromise between simplicity of extraction and acceptable sample clean-up, a generic extraction procedure was developed which consisted of a combination of protein precipitation with liquid extraction.

Organic solvents, such as methanol and acetonitrile, are commonly used for deproteinization of plasma samples [25,33]. In our method, 750 μL of ACN was used for combined precipitation of plasma proteins and extraction of mycotoxins, as it showed high extraction recoveries (R_E, %) for all compounds (ranging from $\pm 82\%$ to 94%), as can be seen in Table 2. Acetonitrile gave more clear supernatants after centrifugation compared to methanol, indicating improved deproteinization efficiency. Deproteinization with ACN has also been successfully applied for extracting other mycotoxins from plasma [30,31]. In order to

Table 2
Results of the evaluation of linearity (goodness-of-fit coefficient (g), correlation coefficient (r)), extraction recovery (R_E), signal suppression/enhancement (SSE), limit of quantification (LOQ), limit of detection (LOD) for all analytes.

Analyte	Calibration range (ng/mL)	Spike levels (ng/mL)	g (%)	r	R_E (%)	SSE (%)	LOQ (ng/mL)	LOD (pg/mL)
BEA	0.2–200	0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200	11.30	0.9980	81.94	90.56	0.2	10
ENN A	0.1–200	0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200	8.48	0.9995	91.03	111.03	0.1	1
ENN A1	0.1–200	0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200	6.19	0.9995	82.28	81.49	0.1	3
ENN B	0.2–200	0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200	5.19	0.9992	94.29	91.60	0.2	4
ENN B1	0.2–200	0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200	11.31	0.9992	88.31	92.26	0.2	8

Note: acceptance criteria: $g \leq 20\%$ [29], $r \geq 0.99$ [27], LOD: S/N >3 [27,28], LOQ: accuracy -50 % to +20%, $RSD_{max} < < < 30.2$ % [26,27] , R_E and SSE [32],

concentrate the samples as much as possible, the supernatants were evaporated under nitrogen and reconstituted in 200 μL acetonitrile/water (80/20, v/v). The addition of organic solvent to dissolve the residue is crucial due to the lipophilicity of the investigated toxins [5].

Signal suppression/enhancement (SSE, %) was evaluated and varied between 90% and 111% for all compounds (Table 2). These results are excellent taking into account that a generic extraction procedure was used. They correlate well with other matrices such as wheat [22] and there is less ion suppression compared to egg samples [10,15]. Hence, it can be stated that a generic extraction procedure was developed for the simultaneous extraction of several emerging *Fusarium* mycotoxins from animal plasma. The extraction procedure was straightforward, cheap and combined a high extraction recovery with limited matrix effects on the LC–MS/MS instrument.

3.1.2. Optimization of LC–MS/MS conditions

The Hypersil Gold column was used as good results were obtained with respect to separation of analytes by HPLC within a short analysis time, i.e. <15 min, comparable to other chromatographic methods used for separation of these compounds [10,15,31].

For the detection of ENNs, the mobile phases reported in the literature generally consist of a combination of water and an organic solvent (methanol or acetonitrile) [5]. To facilitate the ionization process in mass spectrometry, often volatile acids are added (formic acid, acetic acid) [5]. In our experiments the best sensitivity and separation for all investigated compounds was achieved using 0.1% acetic acid in water (A) and ACN (B) as mobile phase (Supplementary Material Fig. 2S). For other mycotoxins, including deoxynivalenol (DON), zearalenone (ZON), T-2 toxin (T-2), ochratoxin A (OTA), fumonisin B1 (FB1) and aflatoxin B1 (AFB1), methanol seems to be a more suitable organic solvent for the separation of those compounds [30].

The MS instrument was operated in the positive ion electrospray mode (ESI +) as this was the most sensitive mode, confirmed by previous studies [4,14,16]. Using ESI, adducts are often formed, i.e. with NH_4^+, Na^+, K^+ and Ca^{2+} [24]. This is, however dependent on type of mobile phases and MS parameters. Uhlig and Ivanova [20] showed that a higher capillary temperature favors protonation ($[M+H]^+$) instead of $[M+NH_4]^+$ adduct formation for ENNs and BEA. Despite adduct formation, the majority of the molecules are protonated and consequently used for detection and quantification by several other authors [10,14,20]. In our experiments sodium adduct formation was also present, but $[M+H]^+$ ions gave the most intense signal except for ENN A where the $[M+Na]^+$ ion was monitored, as did Frenich et al. [15]. The full scan MS spectra of precursor and product ions are shown in Figs. 1 and 2, respectively.

Acquisition was performed in the SRM mode, which means that for each analyte the two most intense precursor ion > product ion transitions were monitored. The two most intense product ions were generated both with an automated procedure and manually by comparing the signal intensity with applying increased collision energy. The same product ions were retrieved by both methods and comparable collision energies were found. The product ion with the highest intensity was selected as quantifier, while the other ion was used as qualifier. In Table 1 the MS/MS conditions for all target analytes are shown.

3.1.3. Internal standard

Other authors [10,14,15] describing methods for quantification of ENNs and BEA in biological matrices did not use an internal standard (IS). However, it is preferable to use an IS, because this

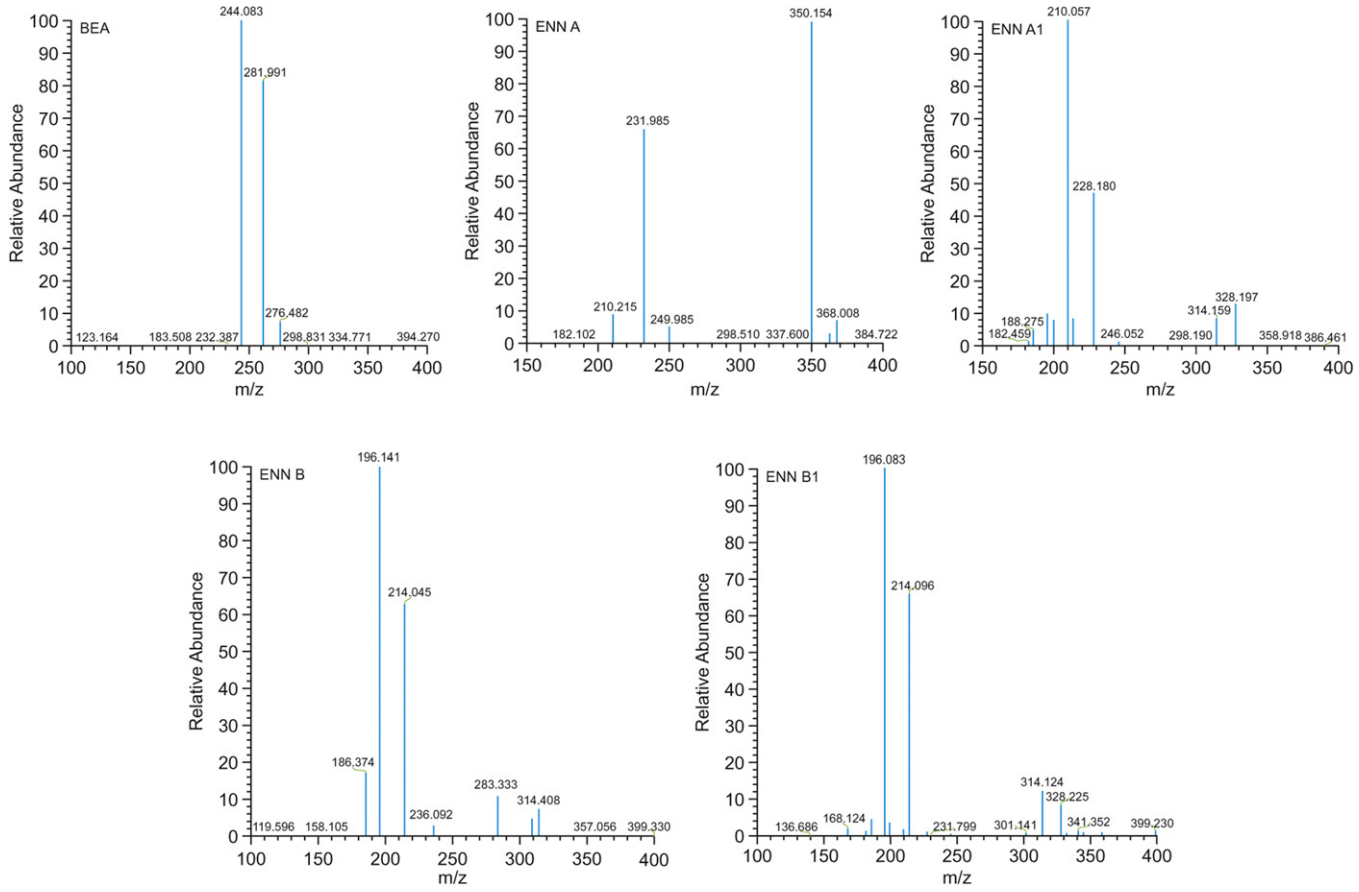

Fig. 2. Full mass spectrum (100–400 m/z) of the product ions of beauvericin (BEA), enniatin (ENN) A, A1, B and B1.

Table 3
Results of the within-run and between-run precision and accuracy evaluation for the analysis of emerging *Fusarium* mycotoxins in pig plasma determined by a multi-mycotoxin LC–MS/MS method.

Analyte	Theoretical concentration (ng/mL)	Mean concentration ± SD (ng/mL)	Precision, RSD (%)	Accuracy (%)
BEA	0.2[a]	0.2 ± 0.01	6.9	1.1
	1[a]	1.0 ± 0.13	12.1	4.4
	1 [b]	1.0 ± 0.11	11.0	0.6
	10 [a]	10.5 ± 0.29	2.8	4.7
	10 [b]	10.0 ± 1.38	13.8	0.1
	100 [a]	100.4 ± 5.03	5.0	0.4
	100 [b]	105.5 ± 11.45	10.9	5.5
ENN A	0.1 [a]	0.1 ± 0.01	10.8	7.6
	1 [a]	1.0 ± 0.02	1.9	2.3
	1 [b]	0.9 ± 0.11	12.0	-6.1
	10 [a]	9.8 ± 0.20	2.1	9.3
	10 [b]	9.0 ± 2.80	30.9	-9.5
	100 [a]	103.3 ± 9.66	9.3	3.3
	100 [b]	105.3 ± 14.4	13.7	5.3
ENN A1	0.1 [a]	0.1 ± 0.01	10.0	13.1
	1 [a]	1.0 ± 0.15	14.5	0.5
	1 [b]	1.1 ± 0.10	9.4	8.9
	10 [a]	10.8 ± 0.73	6.7	8.3
	10 [b]	10.3 ± 0.44	4.2	2.7
	100 [a]	109.5 ± 7.62	7.0	9.5
	100 [b]	100.0 ± 7.49	7.5	0.0
ENN B	0.2 [a]	0.2 ± 0.02	10.8	4.4
	1 [a]	1.1 ± 0.07	6.7	6.9
	1 [b]	1.0 ± 0.09	9.0	1.1
	10 [a]	10.6 ± 0.74	7.0	5.7
	10 [b]	10.5 ± 0.55	5.2	4.7
	100 [a]	101.4 ± 1.69	1.7	1.4
	100 [b]	102.9 ± 6.96	6.8	2.9
ENN B1	0.2 [a]	0.2 ± 0.03	13.6	5.8
	1 [a]	1.0 ± 0.04	3.4	4.7
	1 [b]	1.1 ± 0.08	7.0	6.3
	10 [a]	10.4 ± 0.59	5.7	3.5
	10 [b]	10.2 ± 0.71	7.0	1.9
	100 [a]	97.9 ± 4.12	4.2	−2.1
	100 [b]	105.9 ± 7.83	7.4	5.9

[a] Within-run accuracy and precision ($n=6$).
[b] Between-run accuracy and precision ($n=12$); SD: standard deviation; RSD: relative standard deviation; acceptance criteria: accuracy: < 1 ng/mL: −50% to +20%, 1–10 ng/mL: −30% to +10%, > 10 ng/mL: −20% to +10%, within-run precision (RSD_{max}): 1 ng/mL: 30.2%, 10 ng/mL: 21.3% and 100 ng/mL: 15.1%, between-run precision: 1 ng/mL: 45.3%, 10 ng/mL: 32.0% and 100 ng/mL: 22.6%.

leads to maximal compensation for losses during extraction, signal suppression/enhancement effects during LC–MS/MS analysis, and general analysis errors [21]. Isotopically labeled IS are seen as the ideal IS, because of their similar physico-chemical properties as the target compound [34]. However, these IS are very costly and not commercially available for ENNs nor BEA, in contrast to other mycotoxins [30]. Then compounds having similar physicochemical properties are often used as IS. Ionophoric coccidiostats are closely related to ENNs and BEA, especially based on their chemical structure and properties [14], therefore they can be used as an IS [23]. In our study maduramicin was used since it showed improved fragmentation patterns compared to others such as valinomycin. However, it has to be considered that coccidiostats are frequently used as feed additives in a practical situation for animal production. Therefore, it must be verified before the start of the analysis that MAD is not present in the animal feed, e.g. by analyzing the feed or an animal plasma sample without the addition of the IS (MAD). Otherwise contamination of plasma samples with MAD can occur, which should be avoided when performing toxicokinetical studies or assessing exposure.

3.2. Method validation

The following parameters were evaluated for each mycotoxin: linearity, within- and between-run precision and accuracy, LOQ, LOD and specificity. The results are shown in Table 3.

Matrix-matched calibration graphs were linear over the working concentration range for all tested mycotoxins, with r values between 0.9980 and 0.9995 and g values between 5.2% and 11.3%. For all compounds except ENN A, the best fitting graphs were linear and $1/x$ weighted. For ENN A, the best fitting was obtained by applying quadratic, $1/x$ weighted regression since a small deviation from linearity was observed for concentrations above 50 ng/mL. As can be seen from Table 3, values for r (0.9995) and g (8.48%) fell within the accepted ranges, indicating the reliability of the quadratic calibration model for quantitation of ENN A in real samples up to a concentration level of 200 ng/mL.

The within- and between-run accuracy and precision were tested at 3 different concentration levels and fell within the acceptability ranges. The limits of quantification (LOQ) were 0.1 ng/mL for ENN A and A1 and 0.2 ng/mL for ENN B, B1 and BEA. These limits are comparable [10,14] or 10-fold lower [15] than other LC–MS/MS methods quantifying ENNs and BEA in other biological matrices.

The limits of detection (LOD) were calculated taking into account a S/N ratio of 3 and were 10 pg/mL, for BEA, or lower, for all other analytes. Several studies reported that plasma concentrations of mycotoxins in general fall within the lower parts per billion (ng/mL) range. After bolus administration of DON, ZON, OTA and AFB1 to pigs (all 0.05 mg/kg BW), mean plasma concentrations ranged between 0 and 30.1, 2.1, 132.0 and 2.1 ng/mL, respectively [30]. After feeding a DON contaminated diet (7.5 mg/kg feed) to broiler chickens for 21 consecutive days, mean plasma levels were 3.87 ± 0.86 ng/mL at day 7, 2.66 ± 2.02 ng/mL at day 14 and < LOQ (1.25 ng/mL) after 21 days [35]. In human plasma samples, OTA was detected at very low concentrations, 0.25 ± 0.03 ng/mL [36]. When developing a method for assessing mycotoxin exposure in animals by analyzing plasma, it is therefore a prerequisite to reach these low LOQ and LOD levels. Moreover, for toxicokinetic studies, dosing high mycotoxin concentrations to animals is no longer ethically acceptable as it compromises animal welfare. From the results shown in Table 3, it can be concluded that the developed method succeeded in quantifying the investigated ENNs in spiked matrix-matched samples at concentration levels which can be expected in real pig plasma samples after administration of one oral bolus of 0.05 mg/kg BW (range: 0.1/0.2–200 ng/mL). For BEA, the results for accuracy and precision fell within the specified ranges for the analysis of matrix-matched samples that were spiked in the same concentration range (0.2–200 ng/mL). However, taking into account the low oral bioavailability of BEA (see Section 3.3), it is clear that a higher oral dosing (> 0.05 mg/kg BW) is needed to reach quantifiable plasma levels.

An analyzed blank sample did not demonstrate possible interfering peaks with a S/N ratio above the S/N ratio of the analytes in the same elution zone at the LOD level, testifying the good specificity of the method.

No carry-over was present, as there were no peaks detected in the same retention time zone of the compounds of interest, as can be seen in Fig. 1S (Supplementary Material).

3.3. Biological samples

To demonstrate the applicability of the developed mycotoxin method, one piglet was administered an intra-gastric bolus of BEA, ENN A, A1, B and B1 (dose 0.05 mg/kg BW). Blood samples were drawn before and at different time points post-administration.

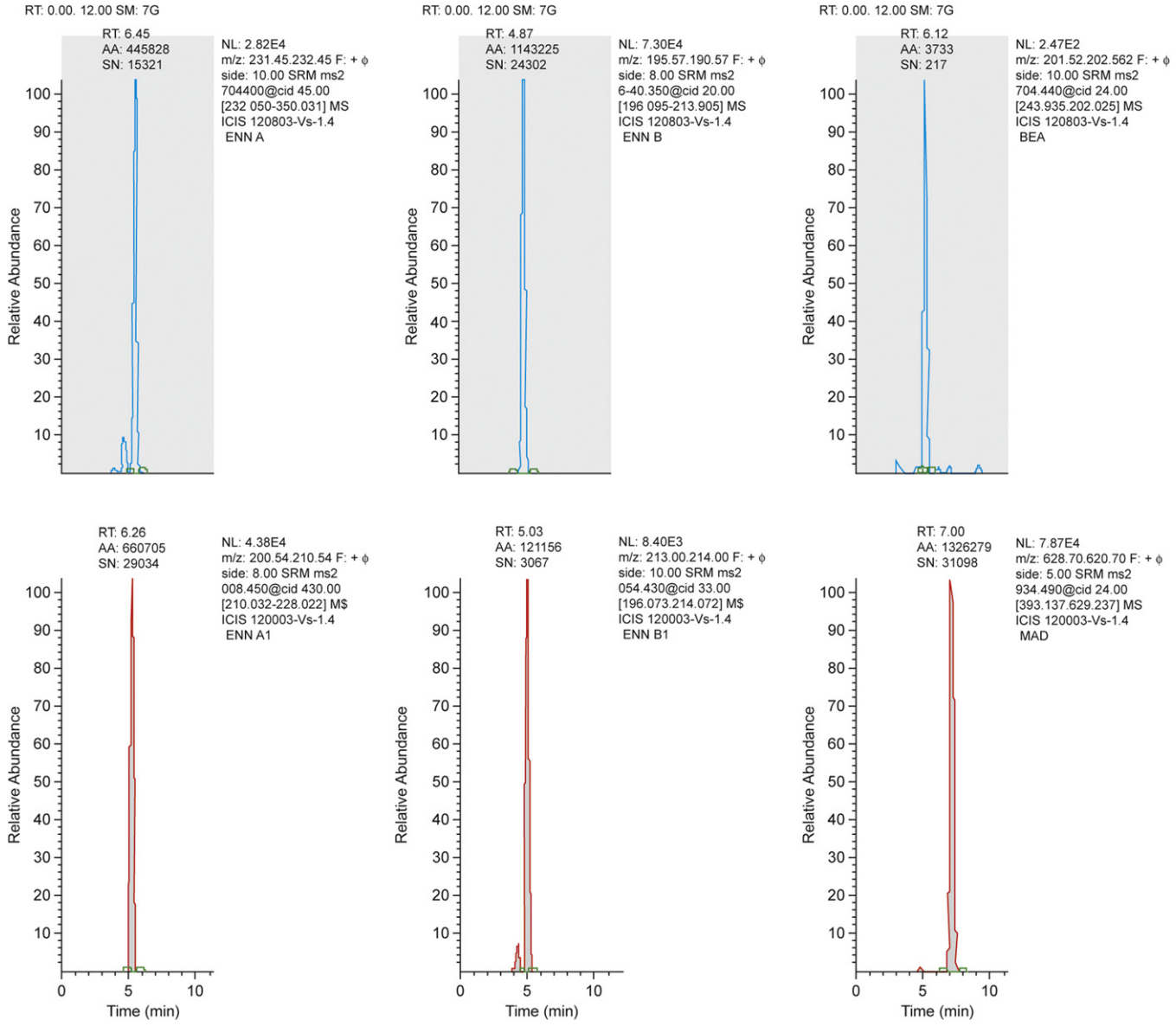

Fig. 3. LC–MS/MS chromatogram showing the SRM traces of the target analytes for the analysis of a pig plasma sample that was taken at 40 min after the oral administration of 0.05 mg/kg BW of the target analytes.

One blood sample was drawn per time-point and analyzed once using the described method. In Fig. 3, a LC–MS/MS chromatogram is demonstrated showing the SRM traces of the target analytes for the analysis of a pig plasma sample that was taken at 40 min after the oral administration of 0.05 mg/kg BW of the target analytes. Following analysis, a plasma concentration–time profile for ENN A, A1, B and B1 was set up and shown in Fig. 4. Interestingly, there is a big difference in oral absorption between the different ENNs although they have a similar chemical structure. ENN B seems to have the highest oral absorption, followed by ENN B1, A1, A and finally BEA. The maximal plasma concentrations (C_{max}) for ENN B, B1, A1 and A were, respectively, 73.4, 35.2, 11.6 and 6.8 ng/mL. The time to maximal plasma concentration (T_{max}) was 20 min after bolus administration for ENN B1, B and A1, whereas the T_{max} for ENN A was 30 min post-administration. For BEA, no plasma concentration–time profile could be designed as the plasma concentration was above the LOQ level at only 2 time points, i.e. 0.51 ng/mL at 30 min and 0.82 ng/mL at 40 min post-administration. The elimination rate of all ENNs is fast and

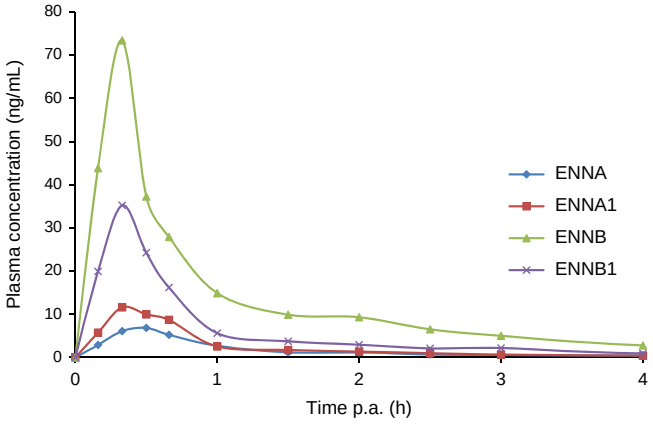

Fig. 4. Plasma concentration time profile of ENN A, A1, B and B1 after single oral bolus administration of the target analytes (dose 0.05 mg/kg BW) to a pig.

comparable to DON [30,37]. With the dose tested, the applicability of the developed method for toxicokinetical analysis of ENNs has been demonstrated. As BEA has now been demonstrated to have a lower oral bioavailability, higher dosing is needed to reach quantifiable plasma levels. These results should be taken into account when a comprehensive animal experiment would be conducted to determine the toxicokinetic parameters of these mycotoxins.

4. Conclusions

This paper describes a sensitive and specific LC–MS/MS method for the analysis of several important *Fusarium* mycotoxins in pig plasma. All analytes were isolated from plasma by performing a generic, fast and low-cost sample preparation procedure. The extracted samples were analyzed using a multi-mycotoxin method which can be applied in the field of animal exposure assessment and toxicokinetic studies. The method was successfully validated and the applicability for toxicokinetical analysis of ENNs was shown by the analysis of real plasma samples that were taken from a pig which received an oral bolus of the investigated mycotoxins.

In conclusion, this is – to our knowledge – the first paper describing the simultaneous detection and quantification of these emerging *Fusarium* mycotoxins in animal plasma.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.11.068.

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